Var gene transcription and clinical disease manifestation in African P. falciparum malaria field isolates

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Abstract

The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) variant surface antigens, encoded by the *var* gene family, play a crucial role in malaria pathogenesis through mediating immunomodulation and host cell adhesion. *Var* genes can be sub-grouped according to genetic or functional features. This thesis examined *var* gene transcription of conserved groups of *var* genes in the context of clinical malaria disease manifestation in African field isolates.

Analysis of *var* gene transcription in 26 *P. falciparum* field isolates from Malian children revealed that field isolates from children with cerebral malaria show significantly higher transcription of group A *var* genes than the field isolates from children with equally high parasite burdens but no symptoms or signs of severe malaria (hyperparasitaemia). These results suggest that group A *var* genes are important determinants of parasite virulence and strengthen the growing body of evidence associating group A *var* expression with severe disease in children.

Analysis of *var* gene transcription in six *P. falciparum* placental malaria field isolates showed that *var2csa* was transcribed in all placental malaria field isolates, but not in 10 childhood isolates examined. This finding, also reported in other recent and subsequent studies, suggests that *var2csa* expression is a critical factor in the onset of clinical malaria disease in pregnant women.

Examination of type 3 *var* gene transcription in laboratory and field isolates established that these *var* genes were commonly transcribed in blood-stage parasites,
and sequence analysis of the transcribed domains confirmed a very high level of conservation across this var gene sub-family.

Finally, rosetting is a property of some group A PfEMP1 and is associated with disease severity in African childhood malaria. Certain glycoconjugate compounds can disrupt rosetting, possibly due to the functional similarities of interactions between rosetting PfEMP1 and host rosetting ligands. A non-toxic compound (curdlan sulfate) was found to be effective at disrupting rosettes in all 18 rosetting field isolates examined, showing potential for use in treatment of severe malaria due to rosetting P. falciparum isolates.

The findings presented in this thesis expand current knowledge of the role and significance of var genes/PfEMP1 in P. falciparum malaria disease pathogenesis. The work demonstrates the importance of continued research on var genes/PfEMP1 in further understanding this complex parasite, and ultimately in combating this severe disease.
Declaration

The following contributions were made to my thesis:

1. Childhood malaria samples were a gift from the Bandiagara Malaria Project case-control study of severe malaria and were cultured by Ahmed Raza. Data for age, parasitaemia, rosette frequency and platelet-mediated clumping were collected by Dr Alex Rowe. The phylogenetic network (Appendix 2) was created by Dr Graham Stone.

2. Dr Stephen Rogerson collected the placental field isolate samples, and Dr Alex Rowe had previously prepared the RNA and cDNA and assessed the number of genotypes present in each isolate. TM180CSA had been selected by Dr Alex Rowe.

3. *P. falciparum* laboratory isolate gDNA and cDNA for type 3 PCR and RT-PCR had previously been prepared by Dr Alex Rowe and Ahmed Raza where stated. Kilifi field isolate gDNA was a gift from Dr Pete Bull.

4. Eurogentec prepared and purified the peptides for antibody production, immunised the two chickens and collected and pooled the egg yolks.

5. Various *P. falciparum* cultures were provided from other members of the Rowe laboratory: Ahmed Raza, Jean-Philippe Semblat, Ruth Corrigan and Antoine Claessens, as stated.

Subject to the above exceptions, I declare that the material presented in this thesis is my own work and has not been submitted to any other university or for any other degree or qualification.

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Finally, thanks to my parents for their continual love and support.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATS</td>
<td>Amino terminal sequence</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CIDR</td>
<td>Cysteine-rich interdomain region</td>
</tr>
<tr>
<td>CRDS</td>
<td>Curdlan sulfate</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin sulfate A</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBL</td>
<td>Duffy-like binding domain</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>IE</td>
<td>Infected erythrocyte</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IgY</td>
<td>Immunoglobulin Y</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo daltons</td>
</tr>
<tr>
<td>KDH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>P</td>
<td><em>Plasmodium</em></td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PfEMP1</td>
<td><em>P. falciparum</em> erythrocyte protein 1</td>
</tr>
<tr>
<td>PMC</td>
<td>Platelet-mediated clumping</td>
</tr>
<tr>
<td>Pt</td>
<td>Parasitaemia</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>R+</td>
<td>Rosetting</td>
</tr>
<tr>
<td>R-</td>
<td>Non-rosetting</td>
</tr>
<tr>
<td>RF</td>
<td>Rosette frequency</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
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</table>
TI  Triton-X100 insoluble
TS  Triton-X100 soluble
TM  Transmembrane
Ups Upstream sequence
UV  Ultra Violet
VSA Variant surface antigen
WHO World Health Organisation
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Chapter 1

Introduction
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1.1.1 Global burden of *P. falciparum* malaria

*Plasmodia* are members of the apicomplexan parasite family of eukaryotic protozoa. Four *Plasmodia* infect humans, *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*, the last of which causes the majority of malaria cases worldwide (Fig. 1.1). Of these at least 90% occur in sub-Saharan Africa, where there are an estimated 200 million clinical events and 1-5 million deaths per year, predominantly in children under 5 years of age (Snow *et al.* 1999; Breman 2001; Snow *et al.* 2005). Clinical immunity to malaria disease symptoms in malaria endemic areas is achieved by adulthood, though an asymptomatic infection can persist throughout life.

![Global burden of malaria map.](image)

Fig.1.1. Global burden of malaria map. *P. falciparum* endemicity map taken from Snow *et al.* (2005). Childhood infection prevalence: light green, <10%; medium green, 11% to 50%; dark green, >50%; yellow, unclassified; grey areas are outside the limit of transmission.
1.1.2 *P. falciparum* lifecycle

*P. falciparum* has a complex lifecycle, dependent on transmission via an invertebrate host, the anopheles mosquito. An infection starts during a mosquito bite as sporozoite-stage parasites from the mosquito salivary gland are delivered into the blood. The free sporozoites travel in the blood circulation until they reach the liver to which they adhere using surface adhesive proteins circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) (Robson et al. 1995; Pradel et al. 2004). The sporozoites then glide across the liver sinusoidal cells, passing through Kupffer cells, the macrophages of the liver (Frevert et al. 2005). They traverse several hepatocytes, before settling within a particular hepatocyte for differentiation and mitotic replication (Tarun et al. 2006). After approximately 6 days, thousands of new parasites, merozoites, have been created. These are released into the circulation as the hepatocyte ruptures (Frevert et al. 2005; Tarun et al. 2006).

The erythrocytic cycle of asexual development (reviewed in(Bannister et al. 2000) now begins. The free merozoites attach to the surface of an erythrocyte, usually to separate erythrocytes, though multiply-infected cells are also seen (Deans et al. 2006). The attached merozoite reorientates so that the apical end can form a tight junction with the erythrocyte. An actin-myosin motor complex then pulls the merozoite inside the erythrocyte (Keeley and Soldati 2004).

The merozoite is now contained within the parasitophorous vacuole, which is then released into the erythrocyte cytoplasm through the proteolytic shedding of surface proteins by subtilase and transmembrane rhomboid proteases (Harris et al. 2005; O'Donnell et al. 2006). Invasion processes are complex with a high level of
redundancy in the pathways and the exact combination of parasite receptors and host ligands used varies considerably between different laboratory and field isolates (Cowman and Crabb 2006; Nery et al. 2006; Cortes et al. 2007; Deans et al. 2007). Merozoite surface protein 1 (MSP-1) and apical membrane protein 1 (AMA-1) appear to be essential (O'Donnell et al. 2000; Triglia et al. 2000). Antibodies to MSP-1 and AMA-1 interfere with invasion, and both proteins are vaccine candidates (Cavanagh et al. 2004; Malkin et al. 2005; Malkin et al. 2007). Proteins containing Duffy binding-like (DBL) domains, including EBA-175, EBA-140 and EBA-181, are also involved, and are linked to the actin-myosin motor via a TRAP homolog (Baum et al. 2006). These bind erythrocyte ligands glycophorin A, glycophorin C and sialic acid on the erythrocyte (Orlandi et al. 1992; Duraisingh et al. 2003; Maier et al. 2003; Cowman and Crabb 2006). The interaction between EBA-175 and glycophorin A is the dominant chymotrypsin-resistant invasion pathway in P. falciparum (Duraisingh et al. 2003).

The newly internalised parasite adopts a cup-shaped ring-like morphology (Aikawa et al. 1967), and is thus known as a ring stage trophozoite, commonly referred to simply as a ring. As the ring stage parasite matures it becomes larger, more rounded and increasingly complex. The endoplasmic reticulum and Golgi increase in size reflecting the high level of protein production. Approximately 16 hours post-invasion, parasite-derived proteins begin to be exported to the erythrocyte surface, often via Maurer’s clefts, membranous structures in the erythrocyte cytosol (Atkinson et al. 1988; Haeggstrom et al. 2004; Knuepfer et al. 2005). Many proteins destined for the erythrocyte membrane, including PfEMP1, are deposited in knobs (Baruch et al. 1995; Horrocks et al. 2005). These are protrusions of the erythrocyte surface, several
thousand of which appear 16-18 hours post invasion, and are structurally maintained by internal parasite-derived proteins such as knob-associated histidine-rich protein (Gruenberg et al. 1983; Rug et al. 2006). Once on the erythrocyte membrane, the surface proteins perform the dual function of adhesion and sequestration of infected erythrocytes, and antigenic variation for immune evasion. There is also an increase in the rigidity of the erythrocyte containing the mature trophozoite (Suwanarusk et al. 2004).

Repetitive cycles of nuclear division and parasite multiplication commence in the trophozoite stage, resulting in the creation of 16-20 new merozoites encased within the parasitophorous vacuole membrane of the parasite, now referred to as a schizont. These nonmotile merozoites are disseminated by an explosive rupture of the schizont, and are released for a new cycle of invasion (Glushakova et al. 2005).

The whole erythrocytic cycle takes approximately 48 hours. This stage is responsible for the disease pathology of malaria. Repeated cycles of erythrocyte invasion, intracellular maturation, schizogeny, erythrocyte rupture and merozoite release take their toll, causing anaemia and inflammatory responses. In addition, the adhesive properties of infected erythrocytes including sequestration within the body and other adhesion-mediated phenomena are associated with disease pathogenesis (discussed in section 1.3).

To complete the life cycle, a proportion of parasites must be taken up into a mosquito, and for this purpose a small proportion of merozoites differentiate into sexual stage parasites called gametocytes upon erythrocyte invasion, rather than into further
asexual trophozoites. Commitment to gametocytogenesis is thought to occur at the previous invasion cycle as all gametocytes from a particular schizont are male or female (Smith et al. 2000c). A stressed environment within the host is thought to trigger gametocytogenesis, which can be stimulated by culturing in partially spent media after a sudden increase in haematocrit in vitro (Carter et al. 1993; Sharp et al. 2006). These gametocytes can be readily taken up into the mosquito through the blood meal during feeding. Once inside the mosquito midgut, female gametocytes are fertilised by male gametocytes after DNA replication and exflagellation, forming a zygote. Triggers for gametocyte activation include a drop in temperature, or presence of xanthurenic acid (Muhia et al. 2001). Differentiation of the zygote occurs, resulting in a motile ookinete, which migrates through the midgut wall to settle on the basal lamina and form an oocyst. Here extensive replication occurs, producing 2000-8000 haploid nuclei after 12-18 days, which are released as sporozoites into the haemocoel as the oocyst bursts (Sinden 2002). The sporozoites migrate to the salivary glands of the mosquito, where they are prepared for invasion the next time the mosquito bites. The *P. falciparum* life cycle is summarised in Fig. 1.2.
Fig. 1.2. *Plasmodium falciparum* life cycle. Reproduced from Center for Disease Control & Prevention Division of Parasitic Diseases Parasite Image Library [http://www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm](http://www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm). A human host is inoculated with sporozoites during an *Anopheles* mosquito bite (1). The sporozoites reach the liver (2) where they mature into liver stage schizonts (3). Rupture of this schizont results in the release of merozoites into the bloodstream (4), completing the exo-erythrocytic cycle (A). These infect new erythrocytes (5), entering the erythrocytic cycle (B). Ring stage trophozoite parasites mature to pigmented trophozoites, and then to schizonts, which rupture (6) releasing merozoites for another round of asexual propagation. A proportion of the merozoites enter the sexual stage, forming gametocytes (7) which can be taken up by the mosquito (8). Within the mosquito the sporogenic cycle (C) occurs. Male gametocytes exflagellate and enter female gametocytes (9) forming a zygote. The zygote become motile and elongated, now an
ookinete (10), and invades the midwall gut of the mosquito for maturation into an oocyst (11). Growth and rupture of the oocyst results in release of sporozoites (12) which are deposited in the salivary gland of the mosquito.

1.1.3 Antigenic variation

The ability to evade the host immune system is crucial for the survival of the parasites. Certain parasite-derived proteins must be inserted into the erythrocyte membrane to enable processes such as sequestration to avoid clearance by the spleen. This causes parasite-derived proteins to be exposed to the extracellular environment and thus to be vulnerable to antibody-mediated immunity. To avoid providing consistent targets for the host immune system, the P. falciparum genome encodes several large hypervariable polymorphic gene families called the variant surface antigens (VSA). Each member of a VSA family can be expressed individually and function independently but can become replaced by a novel VSA variant before a successful antibody response can be elicited. Cross-reactivity of antibodies between members of the hypervariable gene families is low to avoid recognition by any previously induced antibodies within the host, and exposed proteins are under a high level of divergent evolution (Hughes and Hughes 1995; Bockhorst et al. 2007). However, certain functional or structural constraints result in a number of shared epitopes (Joergensen et al. 2006) which eventually allows the host to develop protective immunity to clinical disease. Expression of these polymorphic gene families can be simplified as waves of expression of different members of the family over the course of an infection. Switching occurs before an immune response builds up, each time keeping one step ahead of the immune system (Fig. 1.3), although in a
natural infection the dynamics of immune response and VSA expression may become more complex.

Fig. 1.3. Simplified schematic diagram of the theory of antigenic variation. VSA are expressed for a limited period and then switched to a new variant prior to a successful immune response. Waves of different VSA variants are indicated by colour. Different switching rates result in different duration of expression for the different variants.
1.2 PfEMP1 and var genes

1.2.1 PfEMP1 repertoire

The most well-studied *P. falciparum* VSA family involved in antigenic variation is the polymorphic var gene family which encodes the *Plasmodium falciparum* erythrocyte membrane protein 1 family (PfEMP1) (Biggs *et al.* 1991; Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995). Each parasite has a diverse repertoire of 50-60 var genes, and a correspondingly diverse array of PfEMP1 molecules (Gardner *et al.* 2002; Kraemer *et al.* 2007). Only one PfEMP1 variant is displayed on the host erythrocyte surface in a single cell cycle, and this is controlled at the level of var gene transcription (Smith *et al.* 1995; Scherf *et al.* 1998). However, PfEMP1 expression can be switched at each reinvasion cycle during an infection to avoid host recognition (Roberts *et al.* 1992; Chen *et al.* 1998b; Gatton *et al.* 2003). The genomic var gene repertoire of different isolates is thought to be largely non-overlapping, apart from a small number of well-conserved subfamilies, var1csa, var2csa and the type 3 var genes. However, estimations of the extent of global var gene diversity are confused by different levels of conservation and recombination in var genes in different geographic areas (Fowler *et al.* 2002; Tami *et al.* 2003; Albrecht *et al.* 2006; Barry *et al.* 2007). Other multigene families involved in antigenic variation in *P. falciparum* include rifins (Petter *et al.* 2007) and the smaller gene families of stevors and Pfmc-2TM (Lavazec *et al.* 2007).
1.2.2 Var gene structure

Var genes have 2 exons separated by a 0.8-1.2 kb intron (Fig. 1.4) (Su et al. 1995). The extracellular portion of PfEMP1 molecules is encoded by the highly polymorphic exon 1 (4-10 kb). This extracellular domain comprises several domains: an N-terminal segment, cysteine-rich interdomain region (CIDR), constant 2 (C2) domains and Duffy-binding-like (DBL) domains, named because of their homology to the duffy binding protein of *P. vivax*. DBL domains can be subclassified by consensus motifs into 5 well-defined groups (DBLα-ε), plus a sixth heterogeneous group, DBL-X (Smith et al. 2000; Gardner et al. 2002).

Var genes sequenced to date have between 2 and 7 DBL domains and up to 2 CIDR domains (Gardner et al. 2002; Kraemer et al. 2007). DBLα domains are always N-terminal, and occur in most known var gene types. There are two forms of DBLα domain, DBLα1 and DBLα0 (also sometimes just called DBLα). These are defined by presence/absence of three conserved cysteines, and conserved hydrophobic residues summarised in Fig. 1.5. (Kraemer and Smith 2003; Lavstsen et al. 2003; Robinson et al. 2003). There are three main CIDR types, α, β, and γ. CIDR α domains are further classified as CIDRα, which always occur in tandem with DBLα0 domains, or CIDRα1, which always occur with DBLα1 domains, though a DBLα1 domain can occur without a CIDRα1 domain (Robinson et al. 2003; Kraemer et al. 2007). The tandem pairing of DBLβ2-C2 is also well conserved.

The central region of the intron is AT-rich and contains a bi-directional promoter which produces a ‘sterile’ (untranslated) transcript (Calderwood et al. 2003). The conserved intracellular acidic terminal sequence and the transmembrane domain are
encoded by the highly conserved exon 2 (1.5 kb). The relationship between var genes and PfEMP1 is summarised in Fig. 1.4.

Fig. 1.4. Var gene/PfEMP1 structure. Var gene exon 1 encodes DBL and CIDR domains; exon 2 encodes transmembrane region (TM) and acidic terminal sequence (ATS). The var gene promoter (grey) and intronic promoter (diagonal stripes) are indicated.
Fig. 1.5. Consensus residue features of the DBL_0 and DBLα1 subtype, adapted from Robinson et al. (2003), showing results from a multiple alignment of 57 DBL_0 and nine DBL_1 subtype sequences from the 3D7 genome and other parasite genotypes. The entire DBLα domain is included. The 80% consensus for each type is shown with conserved residues indicated by capital letters of the single amino acid code. Residues that were conserved for similar amino acid character are indicated as: c, charge: D, E, H, K, R; +, positive: H, K, R; h, hydrophobic: A, C, F, I, L, M, V, W, Y; p, polar: C, D, E, H, K, N, Q, R, S, T; s, small: A, C, D, G, N, P, S, T, V; u, tiny: A, G, S; b, big: E, K, R, I, L, N, S, Y, W. Within the alignment, periods represent positions that were not conserved and dashes (–) indicate gaps that were introduced to maintain the alignment. Shaded residues were highly discriminatory and present in at least 80% of one type of sequences but absent or present in less than 5% of the other type. Previously defined DBL homology blocks defined by Smith et al., 2000 are overlined and labelled A to J. Block arrows show the tag section of DBLα amplified by the Taylor primers discussed in chapter 3 (Taylor et al. 2000a; Bull et al. 2005a). Stars show the two cysteines within this block that can be used to classify amplified sequences as DBLα0 or DBLα1 within the amplified tag.
**1.2.3 Var gene groups**

Many of the *var* genes of sequenced laboratory isolates are situated close to the telomeres, often in tandem with another *var* gene or with a *rif* gene, the rest being centromerically located (Gardner *et al.* 2002; Kraemer *et al.* 2007). *Var* genes have been classified into three major groups (A, B and C) and two intermediate groups (B/A and B/C), defined by the gene’s position and orientation within the chromosome (Voss *et al.* 2000; Gardner *et al.* 2002; Vazquez-Macias *et al.* 2002; Kraemer and Smith 2003; Lavstsen *et al.* 2003), summarised in Table 1.1. The groupings are based on the 3D7 *var* gene repertoire, but the same groupings are observed in *var* genes from the recently sequenced *P. falciparum* isolates HB3 and IT (Kraemer *et al.* 2007) and the group classification is applicable to all *var* genes. Group A, B and C *var* genes can be found in *P. reichenowi*, which diverged from *P. falciparum* approximately 5-7 million years ago (Escalante *et al.* 1995; Trimnell *et al.* 2006), suggesting an ancient origin for the *var* gene groups.

Group A *var* genes are subtelomeric and transcribed towards the telomere. They have a semi-conserved upstream sequence (UpsA) and typically encode large PfEMP1 with four or more DBL domains. They contain an N-terminal DBLα1 domain (Kraemer and Smith 2003; Lavstsen *et al.* 2003; Robinson *et al.* 2003). The group A *var* gene group includes members of the conserved *var* gene family *var1csa* (PFE1640w in 3D7). *Var1csa var* genes have an “UpsA2” upstream sequence, which is a variation on the UspA sequence with unique 60 bp and 150 bp repeated motifs, and was formerly referred to as UpsD (Vazquez-Macias *et al.* 2002; Kraemer and Smith 2003; Lavstsen *et al.* 2003; Kraemer *et al.* 2007). The UpsA2 upstream sequence has only been seen in one non-*var1csa var* gene, found in HB3 (Kraemer *et al.* 2007). A
**var1csa** homologue is present in most strains of *P. falciparum* (Rowe et al. 2002a; Salanti et al. 2002; Kyes et al. 2003; Winter et al. 2003), and forms a highly conserved var gene family. In laboratory strain 3D7 var1csa is a pseudogene due to a truncation, and it lacks the exon 2 (Gardner et al. 2002; Rowe et al. 2002a). The type 3 var genes are also group A var genes, see section 1.2.4.

Group B var genes are also mostly telomERICALLY located, but have a different upstream sequence type (UpsB) and are transcribed towards the centromere. Group B/A genes have an UpsB upstream region with an N-terminal DBLα0 domain, or a hybrid DBLα0/α1 domain that is DBLα0-like at the N-terminus, but DBLα1-like in the expressed sequence tag region (such as PF08_0140 and MAL6P1.316 in 3D7) and have a group A-type complex domain structure. Essentially they are group B var genes that have group A var gene-like characteristics. The precise definition of these B/A var genes is currently under debate, and as more var gene sequences are available for examination (Kraemer et al. 2007).

Group C var genes are centromERIC and have a third upstream sequence type (UpsC). Group B and C var genes tend to be shorter, with two DBL domains and two CIDR domains, and their DBLα domain is of the DBLα0 type. Group B and C var genes are similar in architecture, mostly type 1 (DBLα-CIDRα-DBLδ-CIDR“not-α” (usually β or γ)-ATS) in HB3 and 3D7 (Gardner et al. 2002; Kraemer et al. 2007). Group B/C var genes resemble group C genes in chromosomal location, but have an UpsB upstream region.
A conserved var gene family, \textit{var2csa} (PFL0030c in 3D7), has a distinct upstream region (UpsE) which has with little similarity to Ups A, B or C sequences (Kraemer and Smith 2003; Lavstsen \textit{et al.} 2003; Kraemer \textit{et al.} 2007). Most strains have a var2csa homologue (Trimnell \textit{et al.} 2006), which is central to sequestration in the placenta during infection of pregnant women (more detail in section 1.4.5).

<table>
<thead>
<tr>
<th>\textit{Var} gene group</th>
<th>Upstream Sequence</th>
<th>No. of genes in 3D7</th>
<th>No. of DBL domains</th>
<th>Position</th>
<th>Orientation</th>
<th>DBL\text{α}0/\text{α}1</th>
<th>No. of cysteines in amplified tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>UpsA</td>
<td>10</td>
<td>2-7</td>
<td>Subtelomeric</td>
<td>Telomeric</td>
<td>DBL\text{α}1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>UpsA2</td>
<td>1</td>
<td>7</td>
<td></td>
<td></td>
<td>DBL\text{α}_{var1csa}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UpsA</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td>DBL\text{α}_{type3}</td>
<td></td>
</tr>
<tr>
<td>B/A</td>
<td>UpsB</td>
<td>4</td>
<td>4-7</td>
<td>Subtelomeric</td>
<td>Centromeric</td>
<td>DBL\text{α}1 or DBL\text{α}0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>UpsB</td>
<td>21</td>
<td>2-3</td>
<td>Subtelomeric</td>
<td>Centromeric</td>
<td>DBL\text{α}0</td>
<td>4</td>
</tr>
<tr>
<td>B/C</td>
<td>UpsB</td>
<td>10</td>
<td>2-3</td>
<td>Central</td>
<td>Telomeric</td>
<td>DBL\text{α}0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>UpsC</td>
<td>13</td>
<td>2-3</td>
<td>Central</td>
<td>Telomeric</td>
<td>DBL\text{α}0</td>
<td>4</td>
</tr>
<tr>
<td>var2csa</td>
<td>UpsE</td>
<td>1</td>
<td>6</td>
<td>Subtelomeric</td>
<td>Telomeric</td>
<td>None</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.1. Characteristics of \textit{P. falciparum} var gene groups. Type 3 \textit{var} gene family and the \textit{var1csa} \textit{var} gene homologues are indicated as conserved subgroups of group A \textit{var} genes. DBL\text{α}_{var1csa}: \textit{var1csa} homologues have a conserved DBL\text{α}1 domain (Rowe \textit{et al.} 2002a). DBL\text{α}_{type3}: type 3 \textit{var} genes have a semi-conserved DBL\text{α}1 domain (see chapter 5).
1.2.4 Type 3 var genes

The type 3 genes are the smallest var genes and are highly conserved (Gardner et al. 2002; Trimnell et al. 2006). The extracellular portion has only two DBL domains: N-terminal DBLα domain, followed by a DBLε domain (Smith et al. 2000; Gardner et al. 2002). They are the only genes in 3D7 apart from PFL0030c (var2csa) not to have a CIDR domain. Their high sequence conservation indicates that recombination is restricted to within the type 3 var gene family. Of the few P. falciparum strains studied so far, most have 1-2 type 3 var gene homologues, though HB3 has none and 3D7 has 3 (Gardner et al. 2002; Trimnell et al. 2006). The type 3 var genes in 3D7, pfa0015c, pfi1820w and mal6p1.314 (renamed pff0020c), are telomERICally located and transcribed towards the telomere in 3D7. The upstream region is UpsA-type and there are no distinguishing features to the type 3 upstream region (Trimnell et al. 2006). They also have group A type ATS domain and 3’ downstream untranslated region. They are thus classified as group A var genes even though other group A var genes are longer with a more complex domain structure (Gardner et al. 2002; Kraemer and Smith 2003) as shown in Fig. 1.6. More detail on the type 3 var genes and PfEMP1 is given in chapter 5.
Fig. 1.6. Domain structure of Group A var genes of 3D7 (adapted from Lavstsen et al. 2003). The three type 3 var from 3D7 are indicated. DBLζ are a subset of DBLX.

The DBLα of the type 3 var genes is DBLα1-type, consistent with other group A var genes. The first portion of type 3 DBLα1 domains is indistinguishable from that of other DBLα1 domains. However, the type 3 DBLα1 domain sequence then diverges after around 480 nucleotides from other group A DBLα1 domains, and all known type 3 DBLα domains end in a highly conserved sequence. The DBLε domains of type 3 var genes are also very highly conserved (further details in chapter 5).

The type 3 var genes probably originated after the split from P. reichenowi, which does not appear to contain a type 3 var gene homologue (Trimnell et al. 2006). This may indicate specificity for infection of humans rather than other primates. Little has previously been reported about type 3 var gene transcription or type 3 PfEMP1
protein expression timing or prevalence in *P. falciparum* laboratory strains or field isolates.

### 1.2.5 *Var* gene recombination

Recombination of *var* genes occurs within and between isolates. It is thought to occur during sexual stage development within the mosquito, and probably also during mitosis within the asexual cell cycle (Freitas-Junior *et al.* 2000; Taylor *et al.* 2000b). Small or large recombination events can occur, which contribute to the mosaic-nature of *var* genes, and play a role in *var* gene evolution (Ward *et al.* 1999; Freitas-Junior *et al.* 2000; Kaestli *et al.* 2004; Bull *et al.* 2005a; Trimnell *et al.* 2006; Barry *et al.* 2007; Kraemer *et al.* 2007). Telomeric clustering is believed to facilitate recombination between *var* genes, which preferentially occurs between *var* genes with a similar upstream region and orientation (Kraemer and Smith 2003). Thus, group B and C *var* genes appear to recombine separately from the group A *var* genes. The three laboratory strains sequenced to date indicate that the distinct *var* gene groups are of similar proportions in all in *P. falciparum* isolates (Gardner *et al.* 2002; Kraemer *et al.* 2007). As with the type 3 *var* gene family, conserved gene families *var1csa* and *var2csa* also recombine separately from other *var* genes (Kraemer *et al.* 2007). This has allowed them to be maintained in most *P. falciparum* genomes since evolutionary separation from *P. reichenowi*, which also contained a *var1csa* and a *var2csa* homologue (Trimnell *et al.* 2006).

Genes arise through gene duplication and diversifying selection. There are two sets of duplicated genes in 3D7, PFD1235w and MAL8P1.207.
1.2.6 Timing of var gene expression

Var genes cluster in telomeric “bouquets”, caused by crosslinking of telomeres at the nuclear periphery (Freitas-Junior et al. 2000; Marty et al. 2006). Expression is controlled by a mutually exclusive expression, so that only one PfEMP1 is expressed during each asexual growth cycle (Smith et al. 1995; Scherf et al. 1998; Dzikowski et al. 2006).

Var gene transcripts are detectable by RT-PCR during ring and trophozoite stage, though the single full length var gene transcript is often only detectable by Northern blot at ring stage, up to 20 hr post invasion (Chen et al. 1998b; Scherf et al. 1998; Kyes et al. 2000; Kyes et al. 2003; Dahlback et al. 2007). The same var gene transcripts can be detected by RT-PCR at ring and trophozoite stage of parasite growth in vitro and in infected human volunteers (Peters et al. 2002; Dahlback et al. 2007). The var1csa gene is transcribed later, present only at trophozoite stage (Kyes et al. 2003) and may not be counted in the mutually exclusive expression system, as it appears to be constitutively expressed in many field isolates (Winter et al. 2003). Var1csa can be transcribed in 3D7 even though it is a pseudogene and so cannot produce functional PfEMP1 (Kyes et al. 2003; Kyes et al. 2007). Some studies have reported multiple var gene transcripts within a single parasite (Duffy et al. 2002), though it is hard to distinguish between var genes from different parasites, and this phenomenon is controversial. No data on timing of transcription or protein expression of the type 3 var genes has been published to date.

Gametocytes also express PfEMP1 early in development (stages I and II) and commonly express UpsC var genes (Sharp et al. 2006). Whether this is a switch on
commitment to gametocytogenesis, or a reflection of UpsC var-expressing parasites being more likely to undergo gametocytogenesis is unclear. Gametocytes sequester specifically in spleen and bone marrow (Smalley 1976; Rogers et al. 2000), but it is unclear whether this sequestration is PfEMP1-mediated. As gametocytes are the transmitted form of the parasite, the molecules causing sequestration and gametocytogenesis may be involved in determining transmission rates and parasite virulence.

1.2.7 Activation and silencing of var genes

DNA rearrangement is not required for var gene activation, which is controlled by an in situ gene activation, causing transcription initiation via RNA polymerase II (Kyes et al. 2007). Physical movement of var genes to a distinct location along the nuclear periphery may be required for expression (Ralph et al. 2005).

It appears to be crucial for the parasite to only express one var gene at a time, thus requiring the remaining var genes to be transcriptionally silent. For example, silencing of the remaining var genes is crucial for maintaining the reserve var repertoire for immune evasion. Perhaps surprisingly, the activity of the intronic promoter located between the two var gene exons (Fig. 1.4) has been shown to be required for a var gene to remain silenced, though the precise role of the promoter or “sterile” transcript is not yet understood (Deitsch 2001; Calderwood et al. 2003; Frank et al. 2006). The late transcription pattern seen for var1csa, may be due to lack of a regulatory intronic promoter (Gardner et al. 2002; Winter et al. 2003).
Chromatin modifications, including methylation and acetylation of histones, appear to be involved in var gene silencing, though this is not yet fully understood. Histone acetylation is controlled by a homologue of the yeast Sir2 (silent information regulator), called PfSir2 (Duraisingh et al. 2005; Freitas-Junior et al. 2005). PfSir2 co-localises with telomeric bouquets, binding to transcriptionally repressed chromosomes, and disruption of PfSir2 results in aberrant var gene transcription of certain var genes (Duraisingh et al. 2005). The methylation state of particular histones, such as histone H3 at lysine K9, is also involved in transcription control (Chookajorn et al. 2007). Recently, histone methylation has also been shown to be involved in the “memory” process by which new ring stage parasites are able to re-express the var gene which was expressed by the parent parasite at the previous invasion cycle (Lopez-Rubio et al. 2007).

However, var genes can be active in an otherwise transcriptionally silent region of the chromosome, and in tandem with silent var or rif genes (Ralph et al. 2005; Marty et al. 2006). Silencing of var genes thus appears to be controlled independently for each individual gene rather than simply packaging vast swathes of chromatin into a generally repressed state.

1.2.8 Var gene switching

Var genes need to switch fast enough to evade host immune response without running out of novel PfEMP1 variants before uptake by mosquito. The order of var gene expression is complex; there is no predetermined order of var gene expression, but the expression order does not appear to be entirely random. Var genes have a range of switching rates, though switching rate is an intrinsic property of each particular var
gene (Horrocks et al. 2004b). Estimates of switching rates vary from 2% per generation in vitro (Roberts et al. 1992) up to 16% in vivo (Peters et al. 2002), though the rate differs for different var genes (Horrocks et al. 2004a). In one study, Ups C (centromeric) var showed slower “off rates” than group A var genes and were thus transcribed for longer in culture (Dzikowski et al. 2006). The different var gene groups appear to be controlled separately. For example, silencing of UpsA and E genes is dependent on the histone deacetylase activity of Pfsir2, whereas Ups B and C genes remained silenced after Pfsir2 knock out (Duraisingh et al. 2005). This may be a mechanism directing var gene choice in response to a particular situation, though this is not fully understood.

Recent computer modelling of var gene expression switching in culture has suggested that var genes may use intermediate var genes during switching. This is the “single to many to single” hypothesis” (A. Serazin and C. Newbold personal communication) and comes from observations of cultures expressing a predominant var gene activating transcription of several different var genes which are all rapidly silenced again as the culture as a whole switches to expression of a second predominant var gene. It is possible different switching mechanisms are utilised by different var genes in different situations.
1.3 P. falciparum and adhesion

1.3.1 P. falciparum adhesion phenotypes

P. falciparum has the unique property of adhesion among human-infective Plasmodia. The major role for adhesion in P. falciparum is sequestering to avoid host clearance by the spleen (Chotivanich et al. 2002). This leads to adhesion phenotypes associating with pathology and virulence (Fig. 1.7).

![Diagram showing adhesion phenotypes of P. falciparum](image)

Fig. 1.7. Common host ligands P. falciparum infected erythrocytes and associated adhesion phenotypes.

PfEMP1 mediates adhesion of P. falciparum infected erythrocytes. Export into knobs allows efficient adhesion of the PfEMP1 to its various ligands (Baruch et al. 1995;

1.3.2 PfEMP1-mediated adhesion phenotypes: cytoadhesion

Many host ligands are potential adhesion sites for PfEMP1-mediated sequestration, as detailed in Table 1.2.

CD36 is an endothelial scavenger receptor, present on the peripheral vasculature endothelium, on many components of the blood, including monocytes, platelets and erythrocytes, and in organs such as liver and spleen, but is not present on cerebral endothelium (Turner et al. 1994; Serghides et al. 2003). CD36 can act as a ligand for infected erythrocytes (Handunnetti et al. 1992; Udomsangpetch et al. 1997; Urban et al. 1999; Heddini et al. 2001; Pain et al. 2001; Robinson et al. 2003) and binding to CD36 is the major adhesion phenotype for infected erythrocytes. Affinity for CD36 is a property of group B, C or B/C PfEMP1 via the CIDRα domain and the majority of PfEMP1 (49/60 in 3D7) bind to the CD36 (Robinson et al. 2003). However, CD36-binding appears to mediate sequestration of infected erythrocytes away from vital organs such as the brain, and adhesion to CD36 is inversely associated with severe malaria (Rogerson et al. 1999; Serghides et al. 2003).

CSA is a clinically important ligand for cytoadhesion, in the specific case of placental malaria, where CSA on the placenta offers a novel ligand for sequestration of infected erythrocytes (Fried and Duffy 1996; Reeder et al. 2000). Hyaluronic acid (Beeson and
Brown 2004) and IgM binding (Creasey et al. 2003) have also been associated with placental parasites (see section 1.4.4 and chapter 4 for more detail).

Intracellular adhesion molecule 1 (ICAM-1) is another clinically important adhesion phenotype of *P. falciparum* field isolates. Upregulation of ICAM-1 has been reported during *P. falciparum* cerebral malaria (Turner et al. 1994; Silamut et al. 1999), and as such sequestration via ICAM-1 has been associated with cerebral malaria. However, ICAM-1 upregulation is not seen in all cerebral malaria patients and can occur prior to parasitisation of the cerebral microvasculature, and has even be reported to occur in infected individuals without cerebral sequestration (Brown et al. 1999; Silamut et al. 1999). Adhesion to ICAM-1, via a tandem pairing of DBLβ and C2 domains, can be selected for *in vitro* and has been observed in field isolates (Smith et al. 2000a; Chattopadhyay et al. 2004; Springer et al. 2004). E-selectin has also been reported to be upregulated in cerebral endothelium during parasite sequestration, but affinity for E-selectin in field isolates of laboratory strains has not been commonly reported and consistent link with cerebral malaria is unconfirmed (Turner et al. 1994; Silamut et al. 1999).

Adhesion can be a cooperative process. For example, P-selectin can mediate temporary adhesion, which allows rolling across endothelium and platelets, promoting firm adhesion through other ligands such as CD36 (Roberts et al. 1985; Senczuk et al. 2001). Adhesion to ICAM and CD36 also appears to be synergistic (McCormick et al. 1997).
Other host ligands that may support cytoadhesion include vascular cell adhesion molecule (VCAM), thrombospondin and platelet endothelial cell adhesion molecule (PECAM-1). Affinity to multiple ligands is correlated with malaria severity in *P. falciparum* field isolates (Heddini *et al.* 2001). Adhesion to complement receptor 1 (CR1) (Rowe *et al.* 1997), ABO blood sugars (Carlson and Wahlgren 1992; Barragan *et al.* 2000), and heparan sulfate (Ockenhouse *et al.* 1992) on uninfected erythrocytes can mediate binding to uninfected erythrocytes, in a process called rosetting (see section 1.3.3).

A link between the cytoadhesion properties of the expressed PfEMP1 of an isolate and clinical disease is logical. Parasites from different organs within a patient show differential *var* gene expression (Montgomery *et al.* 2007), despite reports suggesting sequestered masses are genotypically identical (Dembo *et al.* 2006; Montgomery *et al.* 2006) suggesting a link between PfEMP1 (or other VSA) and site of cytoadhesion. In addition, parasites from cerebral malaria field isolates do not tend to be genetically more similar to each other than those causing uncomplicated malaria (Ferreira *et al.* 2002), supporting the hypothesis that it is the expressed VSA that affects disease pathology.
<table>
<thead>
<tr>
<th>Host ligand</th>
<th>Description</th>
<th>Location</th>
<th>PfEMP1 domain</th>
<th>Associated phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>Ig family adhesion molecule 80-110 kDa</td>
<td>Endothelial cells including cerebral endothelium with inducible upregulation.</td>
<td>DBLβ/C2</td>
<td>Expression reported to increase in cerebral malaria and to co-localise with sequestration</td>
<td>Turner et al. 1994; Springer et al. 2004</td>
</tr>
<tr>
<td>CSA</td>
<td>Poly-sulfated matrix protein</td>
<td>Placental syncytiotrophoblasts Endothelial cells including cerebral Various blood cells</td>
<td>DBLγ var1csa DBLX var2csa</td>
<td>Placental malaria major role</td>
<td>Gamain et al. 2004; Duffy et al. 2005; Fried et al. 2006 Gamain et al. 2005</td>
</tr>
<tr>
<td>HA</td>
<td>Non-sulfated glycosaminoglycan</td>
<td>Placental syncytiotrophoblasts</td>
<td></td>
<td>Placental malaria minor role</td>
<td>Beeson et al. 2000; Duffy et al. 2005</td>
</tr>
<tr>
<td>IgG</td>
<td>Monomeric immunoglobulin</td>
<td>Blood plasma</td>
<td></td>
<td>Placental malaria</td>
<td>Flick et al. 2001</td>
</tr>
<tr>
<td>Non-specific IgM</td>
<td>Pentameric immunoglobulin</td>
<td>Blood plasma</td>
<td>DBLε</td>
<td>Placental malaria Rosetting</td>
<td>Creasey et al. 2003; Semblat et al. 2006</td>
</tr>
<tr>
<td>CR1</td>
<td>Immune adherence receptor</td>
<td>Erythrocytes</td>
<td>DBLα1</td>
<td>Rosetting</td>
<td>Rowe et al. 1997</td>
</tr>
<tr>
<td>Blood sugars ABO</td>
<td>O-linked glycoproteins</td>
<td>Erythrocytes</td>
<td></td>
<td>Rosetting</td>
<td>Carlson and Wahlgren 1992; Barragan et al. 2000</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>Sulfated glycoconjugate</td>
<td>Erythrocytes</td>
<td></td>
<td>Rosetting</td>
<td>Ockenhouse et al. 1992</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Selectin family adhesion molecule</td>
<td>Activated endothelial cells</td>
<td></td>
<td>Expression reported to increase in cerebral malaria and to co-localise with sequestration</td>
<td>Springer et al. 2004 Turner et al. 1994</td>
</tr>
<tr>
<td>Host ligand</td>
<td>Description</td>
<td>Location</td>
<td>PfEMP1 domain</td>
<td>Associated phenotype</td>
<td>References</td>
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<td>------------</td>
</tr>
<tr>
<td>VCAM</td>
<td>Ig superfamily adhesion molecule</td>
<td>Activated endothelial cells</td>
<td></td>
<td>Expression reported to increase in cerebral malaria</td>
<td>Ockenhouse et al. 1992</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Glycoprotein</td>
<td>Platelets and secreted</td>
<td></td>
<td></td>
<td>Roberts et al. 1985</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Selectin family adhesion molecule</td>
<td>Endothelial cells Activated platelets</td>
<td></td>
<td>“Rolling” enhanced adhesion via other ligands including CD36</td>
<td>Ho et al. 1998; Senczuk et al. 2001</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Transmembrane glycoprotein</td>
<td>Many blood cells including erythrocytes and platelets</td>
<td></td>
<td></td>
<td>Treutiger et al. 1997</td>
</tr>
</tbody>
</table>

Table 1.2. Host ligands supporting adhesion of *P. falciparum* infected erythrocytes: CD36, intracellular adhesion molecule 1 ICAM-1, chondroitin sulphate A CSA, hyaluronic acid HA, IgG, IgM, complement receptor 1 CR1, ABO Blood sugars, Heparan sulfate, E-selectin, vascular cell adhesion molecule VCAM, thrombospondin, P-selectin and platelet endothelial cell adhesion molecule PECAM-1.
1.3.3 PfEMP1-mediated adhesion phenotypes: rosetting

Rosetting is the spontaneous binding of an infected erythrocyte to uninfected erythrocytes (Fig. 1.8).

Fig. 1.8. Rosetting. A) Photograph showing a rosetting-TM284 infected erythrocyte. B) Schematic diagram representing a pigmented trophozoite-infected erythrocyte adhering to 3 uninfected erythrocytes.

All known human-infective Plasmodia can rosette (Udomsanpetch et al. 1995; Angus et al. 1996; Lowe et al. 1998), and rosettes have also been seen in rodent malaria strains including P. chabaudi (Mackinnon et al. 2002). Rosettes can be seen in the peripheral blood of patients infected with P. vivax (Chotivanich et al. 1998), but only very rarely in the peripheral blood of patients infected with P. falciparum as the mature stages are usually sequestered. In P. falciparum field isolate samples taken from peripheral blood, the ring stage parasites must be cultured in vitro to trophozoite stage to see rosettes form. The rosette frequency (RF) is the percentage of infected erythrocytes binding to 2 or more uninfected erythrocytes. The combination of sequestration and rosetting in P. falciparum field isolates may cause obstruction of the microvasculature (Kaul et al. 1991), which may contribute to the
association between rosetting and severe malaria (Carlson et al. 1990; Rowe et al. 1995; Rowe et al. 2002c).

Rosetting is a strain-specific property (Wahlgren et al. 1990). Rosetting isolates are relatively uncommon, though an estimated 25-50% of severe malaria isolates have a high rosette frequency (RF > 30%) (Rowe 2005). PfEMP1 is the major ligand for rosetting (Rowe et al. 1997; Chen et al. 1998a). A well-characterised rosetting interaction involves adhesion between the DBLα1 domain from R29R+ var1 of parasite clone R29, and CR1 on the uninfected erythrocyte surface (Rowe et al. 1997; Rowe et al. 2000; Mayor et al. 2005). Other erythrocyte ligands can also support rosetting including heparan sulfate-like molecules (Carlson et al. 1992) and ABO blood group antigens (Carlson and Wahlgren 1992; Chen et al. 1998a; Barragan et al. 2000). CD36 binding parasites do not usually support rosetting, except in the case of Malayan Camp (Handunnetti et al. 1992). Many diverse PfEMP1 support rosetting, and rosetting PfEMP1 can be considered a functional subgroup of PfEMP1, but no single consensus ‘rosetting motif’ has so far been identified by comparisons of different rosetting PfEMP1 (Normark et al. 2007). Rosetting PfEMP1 have been reported that include a DBLα1 domain (e.g. Rowe et al. 1997), or a DBLα0 domain (Chen et al. 1998a), though recent evidence from numerous laboratory isolates strongly suggests that most rosetting PfEMP1 gene are group A PfEMP1, and that group B and C PfEMP1 rarely support rosetting (J.A. Rowe, unpublished data).
The physical properties of the rosetting interactions allow certain molecules to effectively disrupt rosetting on a wide range of field and laboratory *P. falciparum* isolates. These molecules include glycoconjugate compounds such as heparin and fucoidan (Carlson *et al.* 1992; Rowe *et al.* 1994). However, their high anticoagulant activity induces bleeding (Munir *et al.* 1980; Rampengan 1991; Carlson *et al.* 1992), which prevents their use as rosetting-inhibiting drugs in the treatment of severe malaria (WHO 2000). Identification of effective rosette-inhibiting drugs without harmful side effects such as high anticoagulant activity would offer potential therapeutic benefit for severe clinical symptoms caused by rosette *P. falciparum* isolates.

It remains unclear what the advantage (if any) of rosetting is for the parasite. It does not appear to be linked with increased merozoite invasion (Deans and Rowe 2006). It may be a form of immune evasion, for example protecting the infected erythrocyte from the innate immune systems by preventing contact-dependent activation of natural killer cells (Artavanis-Tsakonas *et al.* 2003), or acting as a cloak to reduce phagocytosis by macrophages or neutrophils. It may be a by-product of evolution of adhesive phenotypes “intended” for sequestration, for example to endothelium. It remains possible that rosetting is an *in vitro* artefact.

**1.3.4 PfEMP1-mediated adhesion phenotypes: platelet mediated clumping**

CD36 binding also mediates platelet-mediated clumping (PMC), whereby platelets adhere to infected erythrocytes, bridging them together and causing them to clump
(Pain et al. 2001). Not all CD36-binding parasites support PMC in culture, and any additional interactions between the infected erythrocyte and the platelets are unknown at present. PMC is associated with high parasitaemia (Arman et al. 2007). Higher PMC levels are seen in patients with severe malaria (Pain et al. 2001), though as this category often has a high parasitaemia, PMC may not be associated with virulence per se (Arman et al. 2007). PMC is not usually seen in rosetting parasites, as the two binding mechanisms are both PfEMP1-mediated and appear to be mutually exclusive. The relevance and function of PMC in vivo remains unclear.

1.3.5 PfEMP1-mediated immunomodulation

Sequestration and associated adhesion phenotypes may not be the only active reason for affinity for host molecules; PfEMP1-mediated adhesion to cells of the immune system has been reported to cause modulation of the immune response. Binding of infected erythrocytes to dendritic cells halts dendritic cell maturation, prevents up-regulation of MHC class II molecules, inhibits T cell activation, and causes a switch in cytokine production towards immunosuppressive cytokine release (Urban et al. 1999; Urban et al. 2001). This interaction appears to be via CD36 (McGilvray et al. 2000; Urban et al. 2001), and is probably mediated by the CIDR domains of group B or C PfEMP1. Whether other domains from group A PfEMP1 can also fulfil this function is unclear. Binding to natural killer NK cells is contact dependent and can be mediated through PfEMP1 (Artavanis-Tsakonas et al. 2003; Baratin et al. 2007). Binding of infected cells to immune cells thus appears to be a parasite-driven attempt to halt an inflammatory immune response. However, in some studies, activation of dendritic cells due to *P. falciparum* infection, for example during schizont rupture, is
reported to stimulate an immune response (Pichyangkul et al. 2004). The stimulation and suppression of the immune system during a malaria infection is complex, and is probably dependent on previous malaria exposure, host genotypic factors and the infective parasite strain.

**P. falciparum malaria disease manifestation**

1.4.1 *P. falciparum* malaria disease manifestation in African children

*P. falciparum* malaria is the major cause of malaria disease in young children in endemic areas. Malaria disease is characterised by periodic fevers, with an asexual parasitaemia detectable by Giemsa staining of the peripheral blood. *P. falciparum* disease is heterogeneous, differing markedly from infection to infection and a variety of other symptoms commonly accompanies this classic fever. The range of clinical manifestations, from fever to increasingly severe complications, is outlined in Fig. 1.9 and Table 1.3. “Severe malaria” broadly refers to fever with at least one severe clinical complication due to *P. falciparum* infection. In Africa, severe anaemia is common in the youngest children (under 2 years old), with seizures and cerebral malaria predominating in slightly older children (Newton et al. 2000). In areas of lower transmission such as South East Asia, severe disease is commonly seen in adults, and multi-organ failure is the most common severe disease manifestation.

In malaria-endemic high transmission areas such as across sub-Saharan Africa, immunity to clinical disease is achieved by 5 years of age, followed by increased immunity to infection (Snow 1999; Breman 2001; Snow et al. 2005). Total immunity is not usually achieved, and an asymptomatic low parasitaemia is prevalent in
malaria-endemic communities, and is required to maintain the immunity to clinical
disease. Severe malaria occurs in those with little previous exposure to malaria, and
thus predominantly in young children in malaria endemic areas, but also in travellers,
and in returning migrants. At present, development of these severe complications
remains hard to predict, and the contribution of parasite genetic factors or virulence
factors in the onset of severe complications is an area of great interest.

![Diagram showing increasing severity of symptoms due to P. falciparum malaria.]

**Defining criteria of severe disease**

1. Cerebral Malaria
   - Unrousable coma
2. Severe normocytic anaemia
   - Hæmatocrit < 15% or Hb < 5 g/dl
3. Renal failure
   - Urine output < 400 ml in 24 hr
4. Pulmonary failure/oedema
5. Hypoglycaemia
   - Blood glucose < 40 mg/dl
6. Circulatory collapse, shock
   - Systolic blood pressure < 50 mm Hg
7. Spontaneous bleeding
   - E.g. from gums, nose, GI tract
8. Repeated generalized convulsions
   - > 2 convulsions within 24 hr
9. Acidaemia
   - Arterial pH < 7.25
10. Acidosis
    - Plasma bicarbonate < 15 mmol/litre
11. Malaria haemoglobinuria

**Other manifestations**

1. Impaired consciousness but rousable
2. Prostration, extreme weakness
3. Hyperparasitaemia
   - > 10% erythrocytes infected
4. Jaundice
   - Serum bilirubin > 3.0 mg/dl
5. Hyperpyrexia
   - Rectal temperature > 40°C.

Table 1.3. Severe malaria manifestations and complications of *P. falciparum* malaria
1.4.2 Definitions of clinical childhood disease used in this analysis

i) Cerebral malaria

An unrousable coma (Blantyre coma score ≤ 2) in the presence of asexual parasitaemia, where all other causes of encephalopathy are excluded, defines cerebral malaria (WHO 2000). There are approximately half a million cases of cerebral malaria in African children every year, of which 10-30% are fatal and the pathology is associated with sequestration of parasitized erythrocytes in the cerebral microvasculature (MacPherson et al. 1985; White 1987; Brewster et al. 1990; Berendt et al. 1994a; Newton et al. 1998; Silamut et al. 1999). Sequestration is due to adhesion of infected erythrocytes to the cerebral microvasculature and mature trophozoites and schizonts are often seen at high concentration within the brain capillaries (MacPherson et al. 1985). In addition, due to the tightly packed nature of infected erythrocytes (in some areas of brain capillaries 100% erythrocytes can be infected), some young ring stage parasites can also become trapped within some vessels (Silamut et al. 1999). The coma is rapidly reversible, and only an estimated 7-12% patients are left with permanent neurological impairments. In these cases neural damage is possibly due to relatively rare development of focal lesions, or ring haemorrhages, within the cerebral hemisphere (Brewster et al. 1990; Newton et al. 1998). Widespread ischaemic damage appears to be avoided as the “sequestered” erythrocytes are not entirely static; the microvasculature obstruction is caused by “sludging” of a mass of infected cells which allows very slight oxygenation of the cerebral endothelium (White 1987). There is usually an absence of platelets and minimal fibrin deposition associated with the coma and large cerebral haemorrhages are uncommon (White 1987). Levels of inflammatory cytokines such as TNFα are
higher in cerebral malaria cases than uncomplicated cases in some studies, though
the involvement of cytokines is inconsistent between reports, and immune damage is
probably not significant in disease pathology in all cases (Kwiatkowski et al. 1990;
Day et al. 1999; Newton et al. 2000). However, an increase in permeability of the
blood brain barrier, due to activation of endothelial cells and activation of
perivascular macrophages, may contribute to disease pathology in some cerebral
malaria patients (Brown et al. 1999).

ii) Uncomplicated malaria

*P. falciparum* parasitaemia and fever with no signs or symptoms of severe disease
(such as prostration, impaired consciousness or repeated convulsions, see Table 1.3),
is defined as uncomplicated malaria. This condition is rarely fatal and recovery is
rapid. This is a commonly used non-severe disease category in disease-association
studies (Kirchgatter and Portillo Hdel 2002; Bull et al. 2005a; Kaestli et al. 2006;
Rottmann et al. 2006).

iii) Hyperparasitaemia

If the parasite density of uncomplicated malaria (parasitaemia and fever with no
signs or symptoms of severe disease) exceeds 500,000 parasites per microlitre of
blood, the patient is classed as having hyperparasitaemia. This parasite density is
equivalent to approximately 10% parasitaemia. Such an enormous parasite burden
may be expected to cause severe disease manifestation, as severe malaria
complications are associated with a high parasitaemia (WHO 2000; Rowe et al.
2002b). In Asia parasitaemia is directly correlated with severity and risk of death.
However, in Africa the prognosis for patients with hyperparasitaemia but no other symptoms of severe malaria is good (Lyke et al. 2003). Thus while high parasitaemia is associated with severe disease, hyperparasitaemia alone in Africa can be regarded as a form of uncomplicated malaria; perhaps such a high parasitaemia without other signs of severe disease indicates particularly non-virulent parasites. A comparison of cerebral and hyperparasitaemia patients is a more accurate comparison of parasite virulence than cerebral versus uncomplicated malaria, as there is no significant difference in parasitaemia. In this study uncomplicated malaria was thus defined as parasitaemia < 500,000 parasites/µl blood.

1.4.3 PfEMP1 and severe malaria in children

As PfEMP1 family proteins are surface exposed, and involved in immunomodulation, sequestration, and other adhesive phenotypes such as rosetting, they are prime candidates for involvement in disease manifestation.

Severe malaria has previously been associated with expression of a restricted and antigenically conserved subset of variant surface antigens (VSA) through field isolate agglutination studies (Bull et al. 1999; Bull et al. 2000; Nielsen et al. 2002). These reveal that VSA from isolates from patients with severe malaria (VSA severe malaria; VSA<sub>SM</sub>) are commonly recognised by sera from semi-immune individuals. PfEMP1 associated with severe disease thus appear to be preferentially expressed in a naïve individual, thus being common targets for protective immunity. Increasing levels of VSA-specific antibodies are thought to then modulate the VSA repertoire available for expression in a semi-immune host.
This VSA$_{SM}$ hypothesis was used to model severe malaria-phenotype parasites in a laboratory strain \textit{in vitro}, by using bead-selection of parasites with sera from semi-immune children, and thus selecting for parasites expressing VSA that are commonly recognised (Jensen \textit{et al.} 2004). Selecting 3D7 parasites in this fashion resulted in upregulation of many group A \textit{var} genes, and downregulation of certain group C genes, suggesting an association of group A \textit{var} genes and severe disease (Jensen \textit{et al.} 2004). The pattern of \textit{var} gene upregulation after selection on sera was similar to the level of upregulation seen after infection of volunteers with NF54 parasites (genotypically identical to 3D7), such that group A and B/A \textit{var} genes expanded relatively quickly, and groups B, C, and B/C tended to be downregulated during the infection (Lavstsen \textit{et al.} 2005).

Recent field isolate studies have so far failed to show a consistent link between \textit{var} gene group and disease outcome, and data from different studies are conflicting, in part due to differences in disease classification and methods of analysis.

In Brazil, DBL$\alpha$ domains lacking 1-2 conserved cysteine residues (DBL$\alpha$1 domains; probably group A PfEMP1) were more frequently expressed in patients with severe malaria than with uncomplicated malaria (Kirchgatter and Portillo Hdel 2002). Field isolates from Tanzania showed an upregulation of group A and B \textit{var} gene transcription in severe malaria field isolates (Rottmann \textit{et al.} 2006), while in Papua New Guinea, expression of group B \textit{var} genes were associated with clinical malaria, both severe and mild (Kaestli \textit{et al.} 2006). However, in Kenya no significant
association was found between *var* gene group and disease category, using DBLα domain sequence type as a marker of *var* gene group (Bull *et al.*, 2005a). Thus there is a trend for group A PfEMP1 to be associated with severe malaria, and group C with asymptomatic parasitaemia. However, differences in the parasitaemia of the asymptomatic parasitaemia or “mild” disease field isolates with the severe malaria category make true associations with parasite virulence hard to determine. The diverse range of symptoms grouped in the different “severe malaria” categories confound the data making comparisons between studies difficult. In addition, disease manifestation differs between countries. For example, in South East Asia, multi-organ failure is relatively common, whereas cerebral malaria is not, and rosetting is not associated with severe disease (al-Yaman *et al.*, 1995), possibly due to high prevalence of CR1 deficiency (Cockburn *et al.*, 2004). PfEMP1 involvement in Asia may therefore be different from in Africa, where the association between rosetting and severe malaria is significant, and cerebral malaria is a common severe complication (Carlson *et al.*, 1990; Rowe *et al.*, 1995; Rowe *et al.*, 2002c).

A consistent and compelling link between *var* group expression between *in vivo* *P. falciparum* infection and clinical disease manifestation has not previously been shown.

### 1.4.4 *P. falciparum* placental malaria

In malaria-endemic areas adults are usually clinically immune to malaria. However, pregnant women once again become susceptible to the disease, which is characterised by an accumulation of mature-stage erythrocytes in the placenta.
(Beeson and Brown 2002). 50 million pregnancies are exposed to malaria each year (Steketee et al. 2001) and placental malaria is a major cause of infant morbidity and mortality, reduced birth weight and severe maternal anaemia, especially common in first pregnancies (Brabin et al. 1993). Pregnancy-associated immunosuppression was originally blamed for the susceptibility of pregnant women to the disease. However, it is now believed that the placenta provides a new niche for parasitic survival, which may be exploited by parasites that can display particular surface antigens. This is thought to be possible due to the presence of the glycosaminoglycan chondroitin sulfate A (CSA) displayed via the proteoglycan thrombomodulin on the syncytiotrophoblast cells of the placenta, which can act as a receptor for infected erythrocytes (Fried and Duffy 1996; Reeder et al. 2000). Infected placenta were observed to have higher CSA levels than uninfected, suggesting CSA-upregulation may occur, or that placenta with higher CSA levels are more likely to become infected (Muthusamy et al. 2007).

Resistance to placental malaria increases with gravidity alongside an increase in IgG which recognise erythrocytes displaying CSA-binding surface antigens (Fried et al. 1998; Ricke et al. 2000; Staalsoe et al. 2004). These antibodies also correlate with improved birth outcome (Duffy and Fried 2003; Staalsoe et al. 2004). Moreover, these antibodies recognise infected erythrocytes from different continents, suggesting there is a common mechanism for protection against placental malaria worldwide (Fried et al. 1998).
There has also been some evidence to suggest that other host molecules such as hyaluronic acid (HA) binding may also be an important placental receptor (Beeson and Brown 2004), though CSA is thought to be the major placental sequestration receptor (Fried et al. 2006; Muthusamy et al. 2007). Malaria during pregnancy thus comes about due to the placenta providing a new niche for parasitic survival, which may be exploited by the parasite by using a distinct repertoire of placenta-specific surface antigens.

1.4.5 PfEMP1 and placental malaria

The main candidates for binding to CSA are PfEMP1. Various DBL\(\gamma\) domains have been associated with CSA binding in placental isolates, and common DBL\(\gamma\) domain sequences have previously been reported within populations of pregnant women in Cameroon, Gabon and Kenya (Khattab et al. 2001; Fried and Duffy 2002; Khattab et al. 2003). Three main subfamilies of potential placenta-specific CSA-binding PfEMP1 have been identified: var\(1\)csa family (Buffet et al. 1999) (also called FCR3varCSA), var\(2\)csa (Salanti et al. 2003), and also the CS2var gene (Reeder et al. 1999), (reviewed in Rowe et al. 2004). Var\(1\)csa, is highly conserved (Rowe et al. 2002a; Salanti et al. 2002; Winter et al. 2003), and was initially a promising candidate for mediating CSA-binding, as antibodies raised to the DBL\(3\)\(\gamma\) domain were able to recognise many infected erythrocytes from CSA-binding parasite lines (Lekana Douki et al. 2002; Costa et al. 2003). However, transcription of var\(1\)csa (Fried and Duffy 2002; Winter et al. 2003) and CS2var (Duffy et al. 2006a) were not found to be consistently up-regulated in pregnancy-associated malaria, and CS2var is not well conserved between strains.
There is now strong evidence for \textit{var2csa} playing a major role in placental malaria. \textit{Var2csa} is expressed on the surface of CSA-selected parasites (Salanti \textit{et al.} 2004) and directly binds CSA though its DBL3X domain, which is closely related to the 67 amino acid minimal CSA-binding region portion of FCR3-CSA DBL3-\(\gamma\) (Gamain \textit{et al.} 2004; Gamain \textit{et al.} 2005). VAR2CSA is consistently upregulated in laboratory isolates after selection for CSA-affinity, and VAR2CSA knock-out disrupts CSA-affinity (Salanti \textit{et al.} 2003; Viebig \textit{et al.} 2007). Interestingly, VAR2CSA does not contain a DBL\(\gamma\) domain (Gardner \textit{et al.} 2002) which was surprising given the association of both VAR2CSA and DBL\(\gamma\) domains with placental malaria, though this can partially be explained by cross-reacting epitopes between VAR1CSA DBL3\(\gamma\) and VAR2CSA DBL3X (Bir \textit{et al.} 2006).

A \textit{var2csa} homologue has been identified in most strains and field isolates of \textit{P. falciparum}; two \textit{var2csa} homologues were found in HB3 (Trimnell \textit{et al.} 2006; Kraemer \textit{et al.} 2007).

The DBL6\(\varepsilon\) domain of VAR2CSA also binds non-specific IgM (Semblat \textit{et al.} 2006), consistent with nonspecific IgM binding having a role in placental malaria (Flick \textit{et al.} 2001; Creasey \textit{et al.} 2003).

VAR2CSA is recognised by endemic plasma in a sex-specific and parity-dependent manner (Salanti \textit{et al.} 2004) and \textit{var2csa} transcription was detected in infected erythrocytes specifically from pregnant women from Senegal (Tuikue Ndam \textit{et al.} 2005).
Anti-VAR2CSA antibodies are present in pregnant women from East and West Africa (Salanti et al. 2004; Tuikue Ndam et al. 2006). Despite the antigenic variation between VAR2CSA from different isolates, these antibodies appear to be cross-reactive to different polymorphic versions of VAR2CSA, though are not always able to inhibit binding of diverse VAR2CSA to CSA (Beeson et al. 2006).

Literature to date thus consistently indicates an important role for VAR2CSA in placental malaria. However, there remains a lack of evidence of var2csa expression directly from field isolates, rather than from CSA-binding laboratory isolates. It is also unclear to what extent other PfEMP1 play a role in placental malaria. Field isolate data on relevance of var2csa in placental malaria is very important as var2csa is of great interest as a vaccine candidate against placental malaria.

1.5 Aims of the thesis

The general aim of the thesis was to investigate the role of conserved PfEMP1 subgroups as virulence factors in clinical disease in African field isolates from children (Mali and Kenya) and pregnant women (Malawi).

In the case of childhood disease, two approaches were taken to investigate PfEMP1 as a virulence factor. Firstly, transcribed var genes in childhood malaria field isolates from Mali were examined. Var gene groups were determined using DBLα domain transcript sequences as an indicator of var gene group of the transcribed var genes. The hypothesis was that there would be a difference in the pattern of transcription of var genes between the three clinical groups. It was hypothesised that there would be
a relatively higher level of group A var gene transcription in the cerebral malaria isolates compared to the hyperparasitaemia and uncomplicated malaria isolates, extrapolating from the in vitro work of Jensen et al (2004). The type 3 var gene family were not included in this analysis due to the primers used, and so were investigated separately in laboratory strains and field isolates. As they are also group A var, albeit atypically short and conserved, expression of type 3 var genes was also hypothesised to be different in the three clinical categories, with an expected association with cerebral malaria.

Secondly, a major contribution of PfEMP1 in severe disease in Africa is the binding of uninfected erythrocytes causing rosettes. Rosetting PfEMP1 form a functional subgroup of PfEMP1; they are predominantly group A (Rowe, JA, personal communication), and are possibly structurally conserved due to similar interactions with rosetting ligands. I spent two months in Kilifi, Kenya, examining rosetting field isolates. Unfortunately, there was insufficient material available to examine the expressed var genes responsible for rosetting in these field isolates. However, as well as understanding the molecular mechanism of rosetting, development of non-toxic compounds for rosette disruption is an important area of research. Attempts to identify rosette-disrupting compounds for auxiliary treatment of severe malaria complications caused by rosetting P. falciparum isolates have so far been troubled by the high anti-coagulant activity of candidate molecules. I was able to test the ability of a sulfated glycoconjugate compound, curdlan sulfate, to disrupt rosettes in a group rosetting P. falciparum field isolates.
In addition to work on childhood malaria isolates from Mali and Kenya, I also examined \textit{var} gene transcription in a group of placental field isolates from Malawi. Specifically, I investigated transcription of DBL\textgreek{y} domains, and a well conserved \textit{var} gene, \textit{var2csa}. The hypothesis was that \textit{var2csa} would be specifically and consistently transcribed in the placental isolates, but not in a comparison group of childhood malaria isolates, and that there might also be some differences in the sequence of DBL\textgreek{y} domain transcripts from placental and childhood malaria isolates.

My four results chapters address the following specific aims:

1. To compare \textit{var} gene transcription in childhood malaria field isolates in three strictly defined clinical manifestation groups. DBL\textgreek{a} domain transcripts, as indicators of \textit{var} gene group, were analysed to test the hypothesis that a subset of \textit{var} may influence clinical disease.

2. To analyse \textit{var} gene transcription in placental malaria field isolates, investigating the involvement of a specific \textit{var} gene, \textit{var2csa}, and of conserved DBL\textgreek{y} domains in malaria during pregnancy.

3. To study \textit{var} gene transcription and protein expression of the unusual, small and highly conserved type 3 \textit{var} gene family.

4. To assess the anti-rosetting potential of a sulfated glycoconjugate compound, curdlan sulfate, on rosetting \textit{P. falciparum} field isolates, as a potential therapeutic agent against severe malaria caused by rosetting \textit{P. falciparum} strains.
Chapter 2

Materials and methods
2.1. Aim of chapter

This chapter describes general materials and methods that are relevant to more than one chapter of the whole thesis. Further detail of specific materials and methods used within each individual chapter is given within that chapter.

2.2 List of suppliers

All of the suppliers mentioned in this chapter and in the individual chapter specific material and methods sections are listed below.

Abcam, Cambridge, UK
Alpha Labs, Hampshire, UK
Ambion, Warrington, UK
Amersham, Buckinghamshire, UK
BDH, Poole, UK
Bio-Rad, Hertfordshire, UK
DNAstar, Inc, Madison, WI, USA.
Eurogentec, Seraing, Belgium
Gibco, now a division of Invitrogen, Paisley, UK
Invitrogen, Paisley, UK
MJ Research, Waltham, MA, USA
Pierce, Pierce Biotechnology Inc, Rockford, IL, USA
Promega, Southampton, UK
Qiagen, Sussex, UK
Roche, Hertfordshire, UK
Sigma, Dorset, UK
2.3 Field isolate collection

2.3.1 Mali childhood malaria field isolates

Childhood malaria samples were collected as part of, and a gift from, the Bandiagara Malaria Project case-control study of severe malaria that has been described in detail previously (Lyke et al. 2003; Lyke et al. 2004). Blood samples were collected from children with malaria after informed consent from parents or guardians, and all protocols received institutional review board approval. Isolates were obtained from 26 patients with strictly defined disease criteria from the following 3 disease categories:

i) **Cerebral malaria** (9 isolates): Unrousable coma (Blantyre coma score ≤ 2) in the presence of asexual parasitaemia where other obvious causes of encephalopathy are excluded (WHO 2000).

ii) **Uncomplicated malaria** (9 isolates): *P. falciparum* parasitaemia and fever with no signs or symptoms of severe disease (such as prostration, impaired consciousness or repeated convulsions, see Table 1.3, introduction). In this study uncomplicated malaria patients were defined as having <500,000 parasites per microlitre of blood (approximately 10% parasitaemia), due to the hyperparasitaemia category (see below).

iii) **Hyperparasitaemia** (8 isolates): As uncomplicated but malaria parasite density exceeds 500,000 parasites per microlitre of blood.

The original isolate numbers were changed in the thesis for clarity. Appendix 1 provides a key for isolate reference numbers, and other isolate details.
2.3.2. Parasite cryopreservation of Mali field isolates

Parasites were prepared for freezing in Mali for long term storage in liquid nitrogen (Rowe, JA, Edinburgh). 1 volume glycerolyte (42.25% w/v glycerol, 0.1 M sodium lactate, 4 mM potassium chloride, 0.1 M sodium dihydrogen phosphate, pH 6.8) was added dropwise to 3 volumes packed cells, allowing cells to equilibrate osmotically for 5 minutes (min) at room temperature (RT) before dropwise addition of a further 4 volumes glycerolyte. Samples were incubated at –70°C overnight (o/n) before storage in liquid nitrogen. A blood spot from each isolate was made on 3MM Whatman paper for genomic DNA extraction (see section 2.3.11). Frozen isolate samples and blood spots were then shipped to Edinburgh. Subsequent thawing of cryopreserved field isolate cultures was carried out by A. Raza, Edinburgh. Cells were thawed at 37°C in a water bath, and 200 µl 12% NaCl was added dropwise over 5 min. After a further 5 min, 5 ml 1.8% NaCl was added dropwise, with gentle agitation of the cells, followed by 5 ml 0.9% NaCl with 0.2% glucose solution. Cells were then washed twice in incomplete RMPI: RPMI1640 medium (Invitrogen), supplemented with 25 mM HEPES, 20 mM glucose, 2 mM glutamine, 25 µg/ml gentamycin and 750 µl NaOH.

2.3.3 Mali field isolate maturation to pigmented-trophozoite stage

Field isolate ring stage parasite were cultured for 8–24 hours (hr) in complete RPMI: RPMI1640 medium (Invitrogen), supplemented with 25 mM HEPES, 20 mM glucose, 2 mM glutamine, 25 µg/ml gentamycin and 750 µl NaOH with 10% pooled normal human serum (Scottish Blood Transfusion Service, Edinburgh, UK). Flasks were gassed with 96% nitrogen, 3% carbon dioxide and 1% oxygen. The maturity of
the culture was regularly assessed by Giesma staining, until morphologically equivalent to 16-20 hr post-invasion in a laboratory strain.

**2.3.4 Giemsa staining of thin blood smears**

Approximately 10 µl of the culture at 20% haematocrit was placed onto a microscope slide and spread thinly with a clean microscope slide. Thin blood smears were air-dried, fixed with 100% methanol for 20 sec and air-dried again. Slides were immersed in 10% Giemsa stain solution (BDH) diluted in Giemsa buffer (prepared using Giemsa buffer tablets; BDH) for 15 min. Slides were then washed thoroughly with tap water and air-dried for light microscopy using a 40x and 100x objective lens under immersion oil (BDH).

**2.3.5 Trizol treatment of parasites for RNA extraction**

Parasites were pelleted (2000 rpm, 4 min), and as much supernatant as possible was aspirated away. Trizol reagent (Gibco) was warmed to room temperature, and 10 volumes of Trizol reagent was added to 1 volume packed cells. Cells were left at RT for 5 min, then stored at -70°C.

**2.3.6 Malawi placental field isolates**

Mature sequestered placental parasites from pregnant women from Malawi were collected directly from *P. falciparum*-infected placenta on delivery, and stored immediately in Trizol before shipment to UK (gift from Stephen J. Rogerson). These were isolates P132, P134, P136, P143, P154 and CS294.
2.4 Molecular methods

2.4.1 RNA extraction

Parasites in Trizol reagent were thawed on ice. Two volumes chloroform were added per volume packed cells. The tube was shaken vigorously for 15 sec then left at RT for 3 min. The aqueous layer was separated from the phenol-chloroform phase by centrifugation (4000 rpm, 45 min, 4°C). This upper aqueous layer was removed into a fresh tube and the RNA was precipitated by incubating with 0.5 ml isopropanol per ml of Trizol (RT, 10 min) before centrifugation to pellet the RNA (4000 rpm, 30 min, 4°C). RNA was washed in 1 ml 70% ethanol prepared with DEPC-H$_2$O and precipitated again (4000 rpm, 10 min, 4°C). The RNA pellet was air dried at RT for 10 min, redissolved in DEPC-H$_2$O (60°C, 10 min) and stored at −70°C. DEPC-H$_2$O was prepared by addition of 0.1% DEPC (diethyl pyrocarbonate) to high grade Millipore water overnight (o/n) at 37°C then autoclaved to destroy the DEPC. Gloves were always worn and filter tips used when handling RNA.

2.4.2 DNase treatment

Any contaminating genomic (g) DNA in the RNA preparation was removed by treatment with 1.5 units DNase (Gibco) for 30 min at RT. 1.5 µl of 25 mM EDTA (10 min, 65°C) was added to inactivate the DNase enzyme.

2.4.3 cDNA preparation

cDNA was then prepared using Superscript III First Strand Synthesis (Invitrogen) according to the manufacturer’s instructions. 50 ng random hexamers, 1 nmol dNTP and 2 µl DEPC-H$_2$O was added to approximately 1 µg RNA. This was mixed gently,
and heated to 65°C for 5 min. The mixture was left on ice for 2 min to cool. The mixture was split into two tubes: “Plus RT” (RT: reverse transcriptase) and “RT Contol” (to detect gDNA contamination in the RT-PCR reaction). 4 µl of 5x first strand synthesis buffer, 0.4 µmol DTT, 100 nmol MgCl₂ and 1 µl DEPC-H₂O was added to each, mixed gently and incubated (25°C, 2 min). 0.5 µl Superscript III reverse transcriptase (RT) was added to each plus RT tube, or 0.5µl DEPC-H₂O added to control tubes. These were incubated at 25°C for 10 min, 50°C for 50 min and 85°C for 15 min. The cDNA was then stored at –20°C until use. cDNA from laboratory isolates (except 3D7) was a gift from JA Rowe.

2.4.4 PCR (polymerase chain reaction)

All PCR and RT-PCR reactions were carried out using amplitaq gold with 10x PCR Gold buffer (Promega) or Platinum taq and Platinum taq buffer (Invitrogen), with separate addition of MgCl₂ and dNTP as detailed in appropriate chapters. All PCR and RT-PCR reactions were performed in sterile 0.5 ml eppendorf tubes on a PCT-100 Peltier thermal cycler PCR machine (MJ Research). Primer sequences and amplification conditions are detailed in each chapter. For RT-PCR, samples without RT were used in all reactions to exclude gDNA contamination. A positive control (3D7 cDNA for RT-PCR and 3D7 gDNA for PCR, unless stated otherwise) and negative control (distilled water) were used in all PCR and RT-PCR reactions.

2.4.5 Agarose gel electrophoresis

All PCR and RT-PCR were examined by agarose gel electrophoresis. Unless otherwise stated, 5 µl of PCR or RT-PCR products was loaded onto a 1.5% agarose
gel, prepared using 1.5 g electrophoresis grade agarose (Invitrogen), 10 ml UltraPure 10xTBE (1M Tris, 0.9 M Boric acid and 0.01M EDTA, Invitrogen) and 90 ml distilled water. DNA ladder, φX174 RF DNA/Hae fragments (Invitrogen), λ DNA/HinDIII fragments (Invitrogen), or 100 bp DNA ladder (Invitrogen), was also loaded. Gels were run at 80 V for 45-60 min. After 25 min running time the gel was removed and stained in 0.5 µg/ml Ethidium Bromide (EtBr) for 5 min, before replacing in the gel tray for the remaining 20-40 min electrophoresis. DNA products were visualised under UV.

2.4.6 PCR/RT-PCR product purification

If only one band of the correct size was seen after agarose gel electrophoresis of a sample of the PCR/RT-PCR products, the rest of the reaction was purified using a QIACquick PCR Purification Kit (Qiagen). 5 volumes buffer PB was added to the PCR/RT-PCR products to promote binding to a QIACquick spin column. Non-bound liquid, salts and primers were washed through the column by spinning on a bench-top centrifuge (13,000 rpm, 1 min) and discarded. 750 µl wash buffer was added to the column to wash remaining impurities from the column upon spinning (13,000 rpm, 1 min). The flow-through was discarded and the column spun again to remove any residual ethanol. PCR/RT-PCR products were then collected in a fresh tube after addition of 50 µl elution buffer (10 mM Tris-Cl, pH 8.5) to the column and a final spin (13,000 rpm, 1 min).

If more than one band was seen, or primer dimers were also seen, PCR/RT-PCR products were purified using a QIagen Gel Extraction Kit (Qiagen). A section of the
gel containing the correct PCR product was excised and melted in 3 volumes buffer QG Melted agarose, and then added to a spin column. Non-bound liquid, salts and primers were washed through the column by spinning on a bench-top centrifuge (13,000 rpm, 1 min) and discarded. Washing with wash buffer and elution was as for QIAquick PCR Purification Kit (Qiagen), described above.

2.4.7 Ligation and cloning

7 μl purified PCR product was ligated into 1 μl PCR II TA cloning vector (TA cloning kit, Invitrogen) using 4 units T4 DNA ligase and 1 μl ligation buffer (all from TA cloning kit, Invitrogen) o/n at 14°C. The resulting plasmids were used to transform One Shot TOP10F competent cells (Invitrogen). 2 μl ligated plasmid was mixed slowly into one vial of One Shot TOP10F competent cells, and the cells were incubated on ice for 30 min. Cells were heat shocked (30 sec, 42°C) followed by 2 min incubation on ice. Transformed cells were recovered in SOC media (Invitrogen), at 37°C for 1 hr with shaking. Plates prepared with Luria Bertani (LB) agar (Sigma) with 50 μl/ml ampicillin were covered in 40 μl each of 20 μg/ml X-gal and 20 μg/ml IPTG for blue/white screening. Transformed cells were plated out onto the LB agar plates and incubated o/n at 37°C. White colonies indicate an insert has disrupted the lacZ locus, whereas blue colonies indicate a re-ligated non-recombinant plasmid as the lacZ gene is active. Individual white colonies were selected for overnight culture in LB (Sigma) with 50 μl/ml ampicillin at 37°C.
2.4.8 Plasmid extraction

Plasmids were extracted from overnight cultures using a miniprep kit (Qiagen). Bacterial cells from overnight growth from individual plate colonies were pelleted (13,000 rpm, 3 min) and resuspended in 250 µl buffer P1. Cells were lysed 250 µl P2 buffer, and neutralised in 350 µl N3 buffer. Bacterial cell debris was removed by centrifugation (13,000 rpm, 10 min), and the supernatant containing the plasmid applied to a spin column. Spinning (13,000 rpm, 1 min) allowed non-bound liquid or remaining salts to flow through the column while the plasmid remained bound. Plasmids were washed in 750 µl wash buffer (13,000 rpm, 1 min). The flow-through was discarded and the column spun again to remove any residual ethanol. Plasmids were then collected in a fresh tube after addition of 50 µl elution buffer (10 mM Tris-Cl, pH 8.5) to the column and a final spin (13,000 rpm, 1 min).

2.4.9 EcoRI restriction enzyme digestion of plasmids

5 µl of the plasmid extraction in elution buffer was digested with 5 unit EcoRI (Promega) in a 10 µl reaction, containing 1 µl buffer H (Promega) and 0.2 5 µl BSA (Promega), which was incubated at 37°C for 1.5-2 hr. 5 µl of the digested plasmid preparation was visualised on a 1.5% agarose gel, prepared using 1.5 g electrophoresis grade agarose (Invitrogen), 10 ml UltraPure 10xTBE (1M Tris, 0.9 M Boric acid and 0.01M EDTA, Invitrogen) and 90 ml distilled water. Gels were run at 80 V for 45-60 min. After 25 min running time the gel was removed and stained in EtBr (0.5 µg/ml) for 5 min, before replacing in the gel tray for the remaining 20-40 min electrophoresis. DNA products were visualised under UV.
2.4.10 Sequencing

Plasmids containing an insert were sequenced. 5 µl of BigDye terminator reaction mix (Applied Biosciences) and 1.6 pmol sequencing vector VR1 (AGATGCATGCTCGAG CGG) or VF1 (ACGTCGGATCCACTAGTA) was added to 5 µl of the miniprep plasmid preparation containing an insert. The mix was incubated for 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min in sterile 0.2 ml eppendorf strips on a PCT-100 Peltier thermal cycler PCR machine (MJ Research). Sequencing was carried out by the University of Edinburgh School of Biological Sciences Sequencing Service.

2.4.11 DNA extraction

For genotyping, parasite DNA was extracted from blood spots on filter paper using chelex-100 extraction (Plowe et al. 1995). A small square of blood spot filter paper was soaked in 0.5x saponin in PBS o/n at 37 °C. This was centrifuged (6000 rpm, 3 min) and the liquid removed. The filter paper was washed with fresh 1x PBS and re-pelleted (6000 rpm, 3 min). The liquid was removed as this contains haem and other debris. 50 µl chelex suspension and 100 µl DEPC-H₂O was added. This was boiled (100°C) for 8 min. The upper layer, above the chelex suspension contains the gDNA. Genomic DNA is also present in the RNA extract before DNase1 treatment. An aliquot of RNA was used for PCR of gDNA for PCR of upstream regions.

2.4.12 Genotyping

The minimum number of genotypes per isolate was estimated by genotyping PCR with primers to the size-variant genes MSP1 and MSP2 (Ranford-Cartwright et al.)
Each PCR was nested. Primer sequences for MSP1 outer PCR: O1 (CACATGAAAGTTATCAAGAACCTTGTC) and O2 (GTACGTCTAATT CATTGCACG). Primer sequences for MSP1 inner PCR: N1 (GCAGTATTGACAGGTTATGG) and N2 (GATTGAAGGTATTTGAC).

Primer sequences for MSP2 outer PCR: S2 (GAGGGATGTTGCTGCTCCACAG) and S3 (GAAGGTAAATTTAAGTTGTC). Primer sequences for MSP2 inner PCR: S1 (GAGTATAAGGAGAAGTATG) and S4 (CTAGAACCATGCATATGTCC). All PCR reactions included 5 µl 10x PCR Gold buffer, 25 pmol each of forward and reverse primers and 0.2 µl Amplitaq Gold polymerase with a final concentration 1.875 mM MgCl₂, and 1.25 mM of each dNTP, and the volume adjusted to 50 µl after addition of template DNA. For outer PCRs, the DNA template was 5 µl chelex extracted gDNA. For inner (nested) PCRs, the DNA template was 3 µl of the outer PCR product. MSP1 outer and inner PCR cycle conditions were 94ºC 5 min, then 30 cycles of 94ºC for 25 sec, 50ºC for 35 sec, 68ºC for 90 sec, followed by a 10 min extension at 68ºC and cooling to 4ºC. MSP2 outer and inner PCR cycle conditions were 94ºC 5 min, then 30 cycles of 94ºC for 5 sec, 50ºC for 15 sec, 65ºC for 1 min, followed by a 10 min extension at 65ºC and cooling to 4ºC. PCR products were run 8% on Novex Pre-cast TBE Polyacrylamide gels (Invitrogen) at 200 V, 1 hr. with Novex TBE Running buffer (Invitrogen). Gels were soaked in EtBr (0.5 µg/ml) and visualised under UV.

2.5 Sequence analysis

2.5.1 Grouping of sequences into contigs

Sequences were analysed using Lasergene software (DNASTAR, Inc). Contigs were
created with a minimum percentage match of 95% to classify sequences for each isolate using SeqMan (DNAnstar, Inc). Nucleotide sequences were translated into amino acid sequence using EditSeq (DNAnstar, Inc). Protein sequences were aligned using the ClustalW method within MegAlign (DNAnstar, Inc) and the alignment converted into a nucleotide alignment with Tranalign (http://bioweb.pasteur.fr/docs/EMBOSS/tranalign.html). Phenograms were then created from nucleotide or amino acids sequences within MegAlign (DNAnstar, Inc). The sequences were also aligned using Muscle (Edgar, et al, 2004) for creation of the network (Graham Stone, Appendix 2). All phenograms are intended to give a measure of sequence similarity rather than to suggest evolutionary descent.

2.5.2 Homology searches

Homology searches were performed using nucleotide-nucleotide BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Default parameters were used, apart from restricting the taxon to P. falciparum (taxon 5833). The Sanger P. falciparum Blast server http://www.sanger.ac.uk/cgibin/blast/submitblast/p_falciparum, Plasmodb Plasmodium genome resource http://www.plasmodb.org/plasmo/home.jsp and the Broad Institute HB3 and Dd2 sequencing data http://www.broad.mit.edu were also used.

2.5.3 Amino acid sequence identity analysis

Each set of sequenced domains (DBLα in chapter 3, DBLγ/var2csa DBL3X in chapter 4 and type 3 DBLα and DBLe in chapter 5) were aligned at the amino acids level using clustal W within Megalign (DNAnstar, Inc). The percentage sequence
identity was recorded into a table. The sequence identity figures within the table were colour-coded into the following groups: 0-30, 30-39.9, 40-49.9, 50-59.9, 60-69.9 70-99.9 and 100% amino acid identity. Sequences were split into various sub-categories, such as disease manifestation, or DBLα0 versus DBLα1, and the figures were repeated. This provided a pictorial illustration of general trends of the level sequence similarities between and within the different groups of var gene domains. Histograms and box plots of percentage sequence identity were then created in Statview, using the amino acid identity data from MegAlign (2.5.3).

2.6 Long term culture and manipulation of P. falciparum

2.6.1 Culture procedures

All processes were carried out in a laminar flow hood, which was regularly cleaned with detergent and 70% ethanol. Aseptic techniques were used throughout, and all plastics and slides used in the handling of parasites were decontaminated with 1% virkon (Alpha Labs). Parasites were cultured in complete RPMI: RPMI1640 medium (Invitrogen) containing 25 mM HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 25 μg/ml gentamycin and 750 μl 1M NaOH with 10% pooled normal human serum (Scottish blood Transfusion service, Edinburgh, UK). Flasks were gassed with 96% nitrogen, 3% carbon dioxide and 1% oxygen. Cultures were maintained at 1-10% parasitaemia by addition of fresh group O blood cells approximately every 48 hours as appropriate. Blood was prepared firstly through removal of white blood cells and buffy coat by centrifugation (4000 rpm, 15 min) and washing twice in incomplete RPMI: RPMI1640 medium (Invitrogen) containing 25 mM HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 25 μg/ml
gentamycin and 750 µl 1M NaOH, before resuspension at 50% haematocrit in incomplete RMPI. Blood was stored at 37°C until required and was used for up to 10 days. Cultures were maintained at 2% haematocrit at 37°C in complete RPMI and were regularly assessed by Giemsa stained thin films (section 2.2.4). This culture method is standard (Trager and Jensen 1976). Preparation for long-term storage in liquid nitrogen was carried out as outlined in section 2.2.2 on cryopreservation.

2.6.2 Sorbitol synchronization of *P. falciparum* infected erythrocytes

Cultures were regularly synchronised using sorbitol (Sigma) to lyse the mature stages by osmotic lysis (Lambros and Vanderberg 1979). Parasites younger than 20 hr post invasion are resistant to sorbitol lysis as they are impermeable to sorbitol. Thus sorbitol treatment leaves only ring stage parasites in the culture. Parasite cultures were pelleted (2000 rpm, 4 min). Culture medium was removed and the cell pellet resuspended in 5 ml of 5% (w/v) D-sorbitol (Sigma) and incubated at 37°C for 15 min. Cells were then pelleted again (2000 rpm, 4 min), and washed twice with 10 ml of RPMI 1640 medium and returned to standard culture conditions.

2.6.3 Enrichment of pigmented trophozoite and schizont infected erythrocytes

Pigmented trophozoite and schizont stages were enriched by gradient separation using using Percoll (colloidal silica particles coated with polyvinylpyrrolidone) which creates smooth, isometric gradients (Sigma, UK). Cell cultures were pelleted (2000 rpm, 4 min) and resuspended in a 5 ml of RPMI 1640 medium without serum, and carefully layered on top of 5 ml 30% percoll in a 15 ml falcon tube. The tube
was centrifuged (4000 rpm, 15 min). Pigmented trophozoite and schizont infected erythrocytes form a clear layer above the sediment of uninfected and ring stage parasite infected erythrocytes, which fall to the bottom of the tube. The upper layer containing pigmented trophozoite and schizont infected erythrocytes was transferred to a fresh tube, washed twice with 10 ml of RPMI-1640 and re-pelleted (2000 rpm, 4 min), and returned to standard culture condition with fresh complete RPMI and washed group O erythrocytes.

2.6.4 Enrichment of rosetting pigmented trophozoite and schizont infected erythrocytes

Pigmented trophozoite infected erythrocyte cultures were selected for high rosetting parasites by gradient separation using Percoll (Sigma, UK). Cell cultures were pelleted (15 min, 4000 rpm) and resuspended in a 5 ml of RPMI 1640 medium without serum, and carefully layered on top of 5 ml 30% percoll in a 15 ml falcon tube. The tube was centrifuged (5 min, 1800 rpm). Non-rosetting pigmented trophozoite infected erythrocytes form a clear layer above the sediment of uninfected and rosetting pigmented trophozoite stage infected erythrocytes, which fall to the bottom of the tube. The upper layer containing pigmented trophozoite and schizont infected erythrocytes was removed by aspiration. The lower layer of rosetting and non-infected erythrocytes was washed twice with 10 ml of RPMI-1640, re-pelleted (2000 rpm, 4 min), and returned to standard culture condition with fresh complete RPMI and washed group O erythrocytes.

2.6.5 Gametocyte preparation
Gametocytogenesis was induced in a culture of 6% rings, by increasing the haematocrit to 5% and incubating in partially spent complete RPMI media (2:3 ratio fresh:used complete RPMI) followed by incubating at 37 °C for 32 hr. After 32 hr the media was changed for fresh complete RPMI. The media was changed again after a further 48 hr. After a further 24 hr, gametocytes were present at 0.5-1% at stage II-IV by microscopy. Gametocytes were purified using a gradient of percoll (52.5%, 45%, and 30% percoll in incomplete RPMI) in a 15 ml corex glass tube, which was spun at 10,000 rpm, 10 min, RT. Gametocytes form a separate layer above the uninfected cells and ring stage-infected erythrocytes, and the fraction containing the gametocytes was collected. Most schizont and pigmented trophozoite-infected erythrocytes also form a separate layer from the gametocytes. Remaining schizont and pigmented trophozoite-infected erythrocytes in the gametocyte fraction were destroyed by sorbitol lysis. Trizol was added to the gametocyte preparation and RNA extracted as section 2.2.5.

2.6.6 Laboratory isolates used in this study

Laboratory strains are single genotype parasite clonal populations, which have been adapted to culture in vitro. For the majority of P. falciparum laboratory strain analysis, strain 3D7 was used as it has been fully sequenced and annotated, available from http://www.plasmodb.org. All other P. falciparum strains used are listed (Table 2.2). Isogenic strains are indicated.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF54a</td>
<td>Schipol Airport</td>
</tr>
<tr>
<td>3D7a</td>
<td>NF54</td>
</tr>
<tr>
<td>FCR3b</td>
<td>SE Asia</td>
</tr>
<tr>
<td>ITb</td>
<td>SE Asia</td>
</tr>
<tr>
<td>R29b</td>
<td>SE Asia</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>A1R^b</td>
<td>SE Asia</td>
</tr>
<tr>
<td>Palo Alto^b</td>
<td>SE Asia</td>
</tr>
<tr>
<td>TM267^b</td>
<td>SE Asia</td>
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<td>Thailand</td>
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</tr>
<tr>
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<td>Thai-Malaysian border</td>
</tr>
<tr>
<td>Dd2</td>
<td>Indochina</td>
</tr>
<tr>
<td>7G8</td>
<td>Brazil</td>
</tr>
<tr>
<td>Muz12</td>
<td>Papua New Guinea</td>
</tr>
</tbody>
</table>

Table 2.2. Laboratory strains used in this thesis and worldwide origin (Mu et al. 2005). *3D7 and NF45 are isogenic. ^FCR3, IT, R29, A1R, Palo Alto and TM267 are also isogenic.

2.7 Statistical methods

Statistical analysis was done with StatView 5.0.1 software. Student’s t-tests were used to compare normally distributed data from two groups with roughly equal variance, including parasitaemia of isolates and age of patients at time of the infection. ANOVA were also used to back up t-test results when multiple t-tests were used to compare different data sets from a single experiment. Chi-squared tests were used to compare distribution of observed data compared to a null hypothesis, such as DBLα1/DBLα0 expression. Kruskal Wallis (non-parametric) tests were used to compare non-normal data such as rosette frequency. Correlations were assessed by Spearman Rank correlation.
Chapter 3

Var gene transcription and childhood malaria disease manifestation

Some of the results presented in this chapter were published as

3.1 Abstract

*P. falciparum* field isolates from Mali were used to test the hypothesis that transcription of a subset of the total var gene repertoire was associated with severe clinical disease outcome. Using DBLα domain as an indicator of var gene group, an association was found between group A var gene transcription and disease manifestation. Children with cerebral malaria disease (coma) showed significantly higher levels of group A var gene transcription than a control group of children with equally high parasite burden but no signs or symptoms of severe disease. This provides evidence for the importance of expressed PfEMP1 in disease pathogenesis. The link demonstrated here between DBLα1-type DBLα domains and severe disease also has potential implications in future drug and vaccine design, for example suggesting that DBLα1 rather than DBLα0 may provide a better target for vaccine development to tackle severe malaria disease.
3.2 Introduction

3.2.1 *P. falciparum* severe disease in Africa

Children under 5 years old in sub-Saharan Africa bear the majority of disease and mortality due to *P. falciparum* malaria infection (WHO 2000), and many distinct severe disease syndromes can develop upon infection (discussed in chapter 1). Cerebral malaria, coma due exclusively to *P. falciparum* infection associated with sequestration of parasitized erythrocytes in the cerebral microvasculature, is a relatively common severe disease manifestation in Africa, occurring in approximately half a million children per year with a 10-30% mortality (MacPherson *et al.* 1985; White 1987; Brewster *et al.* 1990; Berendt *et al.* 1994a; Newton *et al.* 1998; Silamut *et al.* 1999). During the rainy season in malaria-endemic areas, over 70% of children can be infected with an asymptomatic parasitaemia (Bejon *et al.* 2007). Malaria fever without severe complications is defined as “uncomplicated” malaria disease, and is commonly used as a control population for severe disease-association studies (Kirchgatter and Portillo Hdel 2002; Bull *et al.* 2005a; Kaestli *et al.* 2006; Rottmann *et al.* 2006). In addition, some children in Africa have been observed to withstand a surprisingly high parasitaemia (10% erythrocytes infected), with no severe disease complications. This condition is rare in low transmission areas such as Asia or South America, where disease severity is linked more directly with parasitaemia. Parasite isolates from these children provide a good control group for a study of parasite virulence in severe clinical disease isolates, with any effect of parasitaemia being removed.
3.2.2 PfEMP1 and severe disease

The surface-exposed multigene polymorphic PfEMP1 protein family, encoded by var genes mediate immunomodulation, sequestration, and other adhesive phenotypes, and are key candidates for modulating pathology of an infection. PfEMP1 are classified into three main groups A-C and two intermediate groups B/A and B/C (see chapter 1). This classification is based on chromosomal position, direction of transcription, and the upstream region of the var genes, and so potentially separates genes on the basis of different promoter regions. The classification also splits genes into two broad groups. Group A and B/A tend to have a complex domain structure with 4-7 DBL domains and a CIDRα1 domain which does not have affinity for CD36 (Robinson et al. 2003). Group B, C or B/C PfEMP1 in general are shorter commonly with only 2 DBL domains and 2 CIDR domains, including a CIDRα which typically has affinity for CD36 (Gardner et al. 2002; Robinson et al. 2003; Kraemer et al. 2007).

Restricted and antigenically conserved subsets of variant surface antigens (VSA) have previously been associated with severe disease through field isolate agglutination studies, termed VSA “severe malaria”, VSA_{SM} (Bull et al. 1999; Bull et al. 2000; Nielsen et al. 2002). PfEMP1 family variants are obvious targets for investigation of this observation.

A link between group A PfEMP1 and severe disease was suggested by Jensen et al (2004) through transcription analysis of severe malaria-phenotype parasites produced by bead selection of parasites on sera from semi-immune children. A link between
group A var gene expression and severe disease was also indicated by a small field isolate study in Brazil (Kirchgatter and Portillo Hdel 2002), though other studies in Papua New Guinea, Tanzania and Kenya have shown conflicting results (Bull et al. 2005a; Kaestli et al. 2006; Rottmann et al. 2006). These differences may be partly explained by differences in disease classification and methods of analysis, as well as differences in disease manifestation (see introduction for more detail). This study set out to examine var gene transcription in three carefully defined disease manifestation groups.

3.2.3 Var gene DBLα domain analysis

The PfEMP1 DBLα domain is the most well conserved DBL domain type. Almost all sequenced var genes to date contain a DBLα domain, including 59 of the 60 3D7 var genes, the exception being members of the var2csa family (Gardner et al. 2002). Two relatively conserved sections flanking a 400 bp highly polymorphic stretch allowed primers to be designed to recognise and amplify most DBLα domains through polymerase chain reaction (PCR) (Taylor et al. 2000a; Bull et al. 2005a). In 3D7, 56/59 DBLα domains are recognised by these primers with an unbiased distribution. Unfortunately, type 3 var genes cannot be amplified by these primers as the reverse primer site is not recognised (see chapter 5). Reverse transcriptase (RT)-PCR of these primers on RNA from an isolate should amplify all of the non-type 3 DBLα domain transcripts present.

PfEMP1 domain sequences can be aligned and compared phylogenetically, using a phylogenetic tree building program (Smith et al. 2000b; Kraemer and Smith 2003;
Lavstsen et al. 2003). DBLα sequences can also be split into the two groups DBLα0 and DBLα1, on the basis of 2 conserved cysteines in the amplified tag (Table 1.1, introduction). In addition, domains can be further sub-classified into 6 DBLα groups (Table 3.1, Fig. 3.1) (Bull et al. 2005a; Bull et al. 2007).

<table>
<thead>
<tr>
<th>Group</th>
<th>Cysteines in tag</th>
<th>PoLV1</th>
<th>PoLV2</th>
<th>DBLα type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>MFK*</td>
<td>Non-*REY</td>
<td>DBLα1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Non-MFK*</td>
<td>*REY</td>
<td>DBLα1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>Non-MFK*</td>
<td>Non-*REY</td>
<td>DBLα1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>ND</td>
<td>Non-*REY</td>
<td>DBLα0</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>ND</td>
<td>*REY</td>
<td>DBLα0</td>
</tr>
<tr>
<td>6</td>
<td>≠ 2 or 4</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1. DBLα group definitions from Bull et al. (2005). PoLV; position of limited variation. *Any amino acid. ND: Not defined at this position. Group 6 includes DBLα0 and DBLα1 domains.

In addition, the upstream region sequence can be used to identify var gene group (see section 3.5.5). The three methods of var gene analysis/classification used in this chapter are represented in Fig. 3.1.
Fig. 3.1. The three methods of var gene analysis/classification. Green: DBLα sequence analysis from PCR using degenerate primers DBLαAF' and DBLαBR (Taylor et al. 2000a; Bull et al. 2005a) to amplify a variable section of the DBLα domain. Blue: DBLα sequence grouping into DBLα groups 1-6 using positions of limited variation (PoLV) (Bull et al. 2005a; Bull et al. 2007). PoLV1-4 are indicated within the DBLαAF' and DBLαBR amplicon; adapted from Bull et al. (2007). Red: Upstream sequence analysis from PCR of upstream region (UpsA, UpsB or UpsC) from PCR using a generic upstream region forward primer and DBLα domain-specific reverse primer; more detail given in section 3.5.5.
3.2.4 Var gene analysis: DBLα domains as indicator of var gene group

The 3D7 genome and the recently sequenced HB3 and IT genomes provide data on common var gene structures, allowing assumptions to be made about the architecture of a var gene on the basis of DBL domains present (Gardner et al. 2002; Kraemer et al. 2007). In this chapter, analysis of DBLα domain sequence, and subsequent classification of the domains into DBLα1 domain or DBLα0 domain, allows assumptions to be made on the group of var gene transcribed, and thus PfEMP1 group expressed. Presence of a DBLα1 domain indicates a group A or B/A PfEMP1. Conversely, presence of a DBLα0 domain indicates a group B, C or B/C PfEMP1, see chapter 1 for more details.

3.3 Aim of chapter

Variable surface antigens are prime candidates for influencing the pathogenesis of infection, as they mediate immuno-modulation and sequestering within the host. This chapter focuses on a var gene transcription profiling study of P. falciparum childhood malaria field isolates, with the aim of indentifying if there was a link between expressed var gene group and disease manifestation.

3.4 Materials and methods

Materials and methods that are specific to chapter 3 are listed below. General materials and methods and a list of suppliers are given in chapter 2.
3.4.1 Field isolate collection

Childhood malaria samples from cerebral malaria (9), uncomplicated malaria (9) and hyperparasitaemia (8) were collected as part of, and a gift from, the Bandiagara Malaria Project case-control study of severe malaria (Lyke et al. 2003; Lyke et al. 2004) as described in chapter 2. Cerebral malaria is defined as unrousable coma (Blantyre coma score ≤ 2) in the presence of asexual parasitaemia where all other causes of encephalopathy are excluded (WHO 2000). Uncomplicated malaria and hyperparasitaemia are both defined as *P. falciparum* parasitaemia and fever with no signs or symptoms of severe disease (such as prostration, impaired consciousness or repeated convulsions, see Table 1.3, introduction). However, hyperparasitaemia is additionally defined as parasite density exceeding 500,000 parasites per microlitre of blood (approximately 10% parasitaemia), thus in this study uncomplicated malaria patients were defined as having <500,000 parasites per microlitre of blood.

Parasites were cryopreserved in Mali, thawed in Edinburgh, and cultured for 8-24 hr, to early pigmented trophozoite stage as described in chapter 2. RNA was extracted and cDNA prepared as described in chapter 2.

3.4.2 RT-PCR conditions

Reverse-transcriptase (RT)-PCR was used to amplify a region of 300-400 bp of the *var* DBLα domain using unbiased degenerate primers DBLα AF’ GCACG(A/C)AGTTT(C/T)GC and DBLαBR GCCCATTC(G/C)TCGAACCA (Taylor et al. 2000a; Bull et al. 2005a). These primers are capable of amplifying the entire *var* gene repertoire of 3D7 apart from the strain-transcending *var2CSA* gene
implicated in malaria in pregnancy as this gene has no DBLα domain (detailed discussion in chapter 4), and the ‘type 3’ var gene family members, which have atypical DBLα1 domains, of which there are three in 3D7 (detailed discussion in chapter 5). Amplification used 5 µl 10x PCR Gold buffer, 25 pmol each of DBLαAF’ and DBLαBR primers and 0.2 µl Amplitaq Gold polymerase with a final concentration 2 mM MgCl₂, and 1.25 mM of each dNTP, and the volume adjusted to 50 µl after addition of 2 µl cDNA (or distilled water). Amplification conditions were as described (Taylor et al. 2000a; Bull et al. 2005a) with a hot start (95°C, 5 min) followed by 35 cycles of 95°C, 20 sec; 42°C, 20 sec; 60°C, 1 min. Samples without RT were used in all reactions to exclude gDNA contamination. 2 µl 3D7 cDNA was used as a positive control, and distilled water as a negative control.

See chapter 2 for details of ligation, cloning, transformation, and sequencing of RT-PCR products. In brief, RT-PCR products were visualised by electrophoresis on 1.5% agarose gels. RT-PCR products were purified and ligated into PCR II TA cloning vector (Invitrogen) and ligated plasmids were used to transform E. coli. Transform E. coli was grown overnight under ampicillin selection and blue/white colony screening. Individual colonies were selected for overnight growth and the plasmids extracted for sequencing. Presence of an insert in the extracted plasmid preparations was verified by EcoRI restriction digestion prior to sequencing. Sequencing was carried out by the University of Edinburgh School of Biological Sciences Sequencing Service.
3.4.3 Transcript sequence analysis

Transcript sequences from each isolate were grouped into contigs of >95% nucleotide identity using the SeqMan programme within the DNASTar software package. Consensus sequences from each isolate were defined as sequence A, B, C etc from that isolate as EditSeq files (DNASTar software package), and protein sequences were obtained by translating the DNA sequences using the Editseq translation function. Nucleotide and protein sequences were then compared within and between isolates using MegAlign (DNASTar software package). Alignments and phenograms were produced using the clustal W alignment programme within MegAlign, using default settings. Sequence distances were calculated using MegAlign, and colour-coded in Excel into the following groups; 0-30, 30-39.9, 40-49.9, 50-59.9, 60-69.9, 70-99.9 and 100% amino acid identity for production of sequence distance figures. Homology searches were performed using nucleotide-nucleotide BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Pie charts were created in Excel. Phenograms were laid out in Adobe Illustrator. Histograms and boxplots were created in Statview.

3.4.4 Var gene upstream region PCR

Upstream PCRs were carried out on gDNA with each of 4 upstream primers, UpsA TATTYHATKTATTAYATTTGGTA, UpsB GTTAGAACATTTAAAATTATA, UpsC1 AVAGAWATATGRTAGATAYAG and UpsC2 ACAAAACATAGTGAC TACC based on sequences from 3D7, and a gene-specific reverse primer (Table 3.2) for each isolate dominant var gene DBLα domain, and for laboratory isolates Palo Alto, TM180 and A1R. Amplification used 5 µl 10x PCR Gold buffer, 25 pmol of an
upstream primer, 25 pmol of a downstream primer and 0.2 µl Amplitaq Gold polymerase with a final concentration 2 mM MgCl₂, and 1.25 mM of each dNTP, and the volume adjusted to 50 µl after addition of 0.5 µl gDNA from the RNA preparation (or distilled water). Amplification conditions were 35 cycles of 94°C, 5 sec; 46°C, 15 sec; 60°C, 2 min. 0.25 µl gDNA (3D7 unless otherwise stated) was used as a positive control, and distilled water as a negative control. RT-PCR products were obtained for 22 isolates. The remaining 4 isolates (Hyp6, HYP8, U1 and U4) were not studied due to lack of parasite material. PCR product were purified, ligated into PCR II vector (Invotrogen) and used to transform E. coli for colony picking, plasmid extraction and sequencing as described chapter 2. The upstream sequence of the predominant var gene from 9 isolates was studied. The remaining upstream sequences were not obtained due to problems with cross-reactivity of the “specific” primer with other DBLα domains (13 isolates).

<table>
<thead>
<tr>
<th>Isolate/strain</th>
<th>Isolate predominant domain</th>
<th>Upstream PCR domain specific reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM1</td>
<td>CM1A</td>
<td>CTT ACT TTA GGT AGT GG</td>
</tr>
<tr>
<td>CM2</td>
<td>CM2A</td>
<td>AAC AAT CGT TGT ATT ACT CGC</td>
</tr>
<tr>
<td>CM3</td>
<td>CM3A</td>
<td>TTT GAA TGG GCT TGA TTT</td>
</tr>
<tr>
<td>CM4</td>
<td>CM4A</td>
<td>TGT ATT AGT CGA AGA TGT TCC</td>
</tr>
<tr>
<td>CM5</td>
<td>CM5A</td>
<td>ATG AAT TCC CCT GTT GTT</td>
</tr>
<tr>
<td>CM6</td>
<td>CM6A</td>
<td>ACC ATC ATA ATC ACT TAT TTG</td>
</tr>
<tr>
<td>CM7</td>
<td>CM7A</td>
<td>AAC TCC ATT TGG TTT ATA TAC</td>
</tr>
<tr>
<td>CM8</td>
<td>CM8A</td>
<td>CTT GAC TTG TAA AAA CAT CAC</td>
</tr>
<tr>
<td>CM9</td>
<td>CM9A</td>
<td>CAC CAT TTT TAT CTT GGT</td>
</tr>
<tr>
<td>HYP1</td>
<td>HYP1A</td>
<td>TTC TTC AGT CCA ACT ATC GTT</td>
</tr>
<tr>
<td>HYP2</td>
<td>HYP2A</td>
<td>TTT CCA CCA TTT TTA TCT TGG</td>
</tr>
<tr>
<td>HYP3</td>
<td>HYP3A</td>
<td>TTT CAC ATA TTT AGC TAC TCC</td>
</tr>
<tr>
<td>HYP4</td>
<td>HYP4A</td>
<td>TTG GTC TCT ATT AGC GTT</td>
</tr>
<tr>
<td>HYP5</td>
<td>HYP5A</td>
<td>GTC CTT CGT CAA ATC ACC</td>
</tr>
<tr>
<td>HYP6</td>
<td>HYP6A</td>
<td>ND</td>
</tr>
<tr>
<td>HYP7</td>
<td>HYP7A</td>
<td>TTC ATC CTG AAT CTT CTG</td>
</tr>
<tr>
<td>HYP8</td>
<td>HYP8A</td>
<td>ND</td>
</tr>
<tr>
<td>------</td>
<td>-------</td>
<td>----</td>
</tr>
<tr>
<td>U1</td>
<td>U1A</td>
<td>ND</td>
</tr>
<tr>
<td>U2</td>
<td>U2A</td>
<td>TCT AGC CAT AGA TGG ACT TTC</td>
</tr>
<tr>
<td>U3</td>
<td>U3A</td>
<td>ACC ATC ATA ATC ACT TAT TTG</td>
</tr>
<tr>
<td>U4</td>
<td>U4A</td>
<td>ND</td>
</tr>
<tr>
<td>U5</td>
<td>U5A</td>
<td>GAA TAT ATA TCA CCT TGC TCT</td>
</tr>
<tr>
<td>U6</td>
<td>U6A</td>
<td>CCT TTG GAG CTI TG</td>
</tr>
<tr>
<td>U7</td>
<td>U7A</td>
<td>TTT TTG TCA TTG TCA TTG TAG</td>
</tr>
<tr>
<td>U8</td>
<td>U8A</td>
<td>CTG CTA GCC TGT TGT CGT</td>
</tr>
<tr>
<td>U9</td>
<td>U9A</td>
<td>TTG AGC ATC TTT TAG CTT GTT</td>
</tr>
<tr>
<td>Palo Alto</td>
<td>PARvar1</td>
<td>GTT CTT GTA CTT TAG GTG A</td>
</tr>
<tr>
<td>TM180</td>
<td>TM180var1</td>
<td>TAA ATT GGT AAG AAC GGC TCC TTG</td>
</tr>
<tr>
<td>A1R</td>
<td>A1Rvar1</td>
<td>CAT TCT TCC TCT CTC TG T</td>
</tr>
</tbody>
</table>

Table 3.2. Reverse primer sequences for upstream regions PCR for the predominant gene from each isolate. ND: Not designed due to lack of parasite material.

### 3.4.5 Statistical analysis

Statistical analysis was done with StatView 5.0.1 software. Student’s t-tests were used to compare normally distributed data from two groups with roughly equal variance (patient age, isolate parasitaemia, number of genotypes in each isolate). Kruskal Wallis (non-parametric) tests were used to compare non-normal data (isolate rosette frequency and transcription frequency of the six DBLα groups in the different clinical categories). Chi-squared tests were used to compare distribution of observed data with a null hypothesis, such as DBLα1/DBLα0 expression levels. Fisher’s Exact Test was used to compare the predominant gene per isolate for DBLα1/DBLα0 type. A 3x6 contingency table was used to compare the number of transcripts classified into the 6 DBLα domains groups proposed by Bull et al. (2005) in each clinical manifestation group. Correlations between DBLα1% and age, parasitaemia and rosette frequency were assessed by Spearman Rank correlation.
3.5. Results 1: DBLα domain transcription analysis in Malian childhood field isolates

3.5.1 Malian childhood malaria field isolate details

Field isolates were obtained from 26 children with strictly defined conditions of uncomplicated (9) or cerebral malaria (9), or hyperparasitaemia (8) from Bandiagara, Mali, as part of a case-control trial study (Lyke et al. 2003; Lyke et al. 2004). The aim of the study was to examine var gene transcription within these isolates, using degenerate primers to DBLα domains to amplify DBLα domain transcripts by RT-PCR.

Before this was done it was important to assess the 26 field isolates to check that there were no significant differences in age and parasitaemia between the groups which may influence the data. No significant difference in patient age was seen across the three clinical categories (Fig. 3.2A, Table 3.3; t-test, p > 0.45), probably due to age matching in the study group from which these isolates were taken. Cerebral and hyperparasitaemia isolates did not differ significantly in parasitaemia (t-test, p = 0.83). Both had significantly higher parasitaemia than the uncomplicated isolates (Fig. 3.2B, Table 3.3; p > 0.001).

In addition to age and parasitaemia, data on the rosette frequency and level of platelet-mediated clumping within each isolate was also available. Cerebral isolates showed significantly higher rosette frequency than both other categories (Fig. 3.2C, Table 3.3; p = 0.03), as expected in isolates from Africa (Carlson et al. 1990; Rowe et al. 1995; Rowe et al. 2002b). Only 4 isolates showed platelet-mediated clumping
(PMC; Fig. 3.2D), including the only cerebral isolate in which there were no rosettes, CM8 (PMC 53%, RF 0). Low PMC levels meant that comparison of the disease categories was not significant, though the data has been analysed elsewhere, showing an association between parasitaemia and PMC levels, though not with disease severity per se (Arman et al, 2007).

Furthermore, field isolates can comprise multiple infections. To assess the number of genotypically distinct infecting parasite strain within each isolate, the number of distinct genotypes in each isolate was estimated by PCR of size-variant genotype markers MSP1 and MSP2 (see materials and methods). Multiple distinct genotypes were present in many isolates (range 1-5, Table 3.4). There was no significant difference in number of genotypes between the three clinical categories (t-tests, p > 0.48, Table 3.3).

This data is also shown in Appendix 1, which shows a table of all data regarding the Mali childhood isolates discussed in the thesis.
Fig. 3.2. Individual isolate details for the 26 Malian childhood *P. falciparum* malaria field isolates studied showing A) age, B) parasitaemia, C) rosette frequency (RF; percentage of infected erythrocytes bound by ≥ 2 uninfected erythrocytes) and D) platelet-mediated clumping frequency (PMC; percentage of infected erythrocytes bound by ≥ 2 uninfected erythrocytes upon addition of platelets).
<table>
<thead>
<tr>
<th>Malaria disease category</th>
<th>Age (months) (mean ± SD)</th>
<th>Parasitaemia (%) (mean ± SD)</th>
<th>Rosette frequency&lt;sup&gt;a&lt;/sup&gt; (%) (median: 25th, 75th percentiles)</th>
<th>Genotypes (mean:range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncomplicated</td>
<td>45.8 ± 28.0</td>
<td>3.3 ± 1.5</td>
<td>8.0 : 0, 15.8</td>
<td>2.4 (1-4)</td>
</tr>
<tr>
<td>Hyperparasitaemia</td>
<td>42.5 ± 16.9</td>
<td>11.9 ± 3.9</td>
<td>9.5 : 0, 18.0</td>
<td>2.6 (1-4)</td>
</tr>
<tr>
<td>Cerebral</td>
<td>35.2 ± 22.0</td>
<td>12.3 ± 6.3</td>
<td>22.0 : 19.8, 46.8</td>
<td>2.2 (1-5)</td>
</tr>
<tr>
<td>P values</td>
<td>P &gt; 0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P = 0.83 H:C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>P &lt; 0.001 U:C/H</td>
<td>P = 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.3. Combined data from all isolates within each disease category

<sup>a</sup> % of mature-stage infected erythrocytes that bind 2 or more uninfected erythrocytes.

<sup>b</sup> Uncomplicated:cerebral p = 0.46, uncomplicated:hyperparasitaemia p = 0.72, cerebral:hyperparasitaemia p = 0.68, t-test.

<sup>c</sup> H: hyperparasitaemia; C: cerebral malaria; U: uncomplicated malaria. t-test.

<sup>d</sup> Kruskal Wallis test.

<sup>e</sup> Uncomplicated:cerebral p = 0.72, uncomplicated:hyperparasitaemia p = 0.72, cerebral:hyperparasitaemia p = 0.49, t-test.
<table>
<thead>
<tr>
<th>Uncomplicated isolates</th>
<th>Cerebral isolates</th>
<th>Hyperparasitaemia isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>Genotypes</td>
<td>Isolate</td>
</tr>
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</tr>
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<table>
<thead>
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<td>1 to 5</td>
<td>2.2</td>
<td>1 to 4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Table 3.4. Estimated number of distinct genotypes present per isolate for each isolate on the basis of PCR of size-variant markers MSP1 and MSP2.

### 3.5.2 DBLα RT-PCR technique

The aim of this chapter was to investigate the transcribed \textit{var} genes in Malian \textit{P. falciparum} malaria field isolate by examining the sequence and relative frequency of transcribed DBLα domains by RT-PCR. There was not enough parasite material to perform real-time RT-PCR for quantitative analysis of the DBLα domain transcripts in each isolate, so RT-PCR was quantified by colony analysis.

RNA was extracted from the 26 field isolates, and cDNA was prepared. DBLα transcripts were amplified from cDNA by RT-PCR using degenerate primers to DBLα domains (Taylor \textit{et al.} 2000a; Bull \textit{et al.} 2005a). An aliquot of products from
each RT-PCR reaction was visualised on a 1.5% agarose gel. Fig. 3.3 shows a representative gel; the amplified DBLα RT-PCR products from isolates HYP1, HYP3 and HYP5 are seen as bands of approximately 400 bp. Multiple bands can be seen, representing multiple DBLα domain transcripts of slightly different length.

![Representative gel of DBLα domain RT-PCR products from Mali field isolates.](image)

Fig. 3.3. Representative gel of DBLα domain RT-PCR products from Mali field isolates. RT-PCR products from amplification of DBLα domains from cDNA from Mali field isolates HYP1, HYP3 and HYP5, with primers DBLαAF' and DBLαBR, were visualised by electrophoretic separation on a 1.5% agarose gel. Reverse transcriptase negative cDNA preparation control (CTL) for each isolate reveal PCR products from gDNA contamination, and are next to the cDNA amplification for each isolate. These lanes remain blank indicating there is no gDNA contamination in the cDNA preparation. 3D7 cDNA was used for a positive control, and distilled water as a negative control for the PCR reaction. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).
These DBLα RT-PCR products were then purified and ligated into plasmids. These recombinant plasmids were used to transform *E. coli*, which were plated onto LB agar/ampicillin plates and grown up overnight at 37°C. Ampicillin in the plates selected for growth of transformed bacteria, and blue-white screening allowed bacterial colonies containing a recombinant plasmid to be selected. 14-19 white bacterial colonies were selected for expansion in LB broth at 37°C, and after approximately 18 hours a number of recombinant plasmids from colonies from each RT-PCR were extracted using a QIAgen miniprep kit. The presence of an insert of the correct length was confirmed by enzymatic digestion of the multiple cloning site using restriction enzyme *Eco*RI of a sample of each extracted plasmid. Digested plasmids were visualised on 1.5% agarose gels, and plasmids containing an insert were marked by presence of an approximately 400 bp band. Fig. 3.4 shows a typical gel.
Fig. 3.4. A typical 1.5% agarose gel of EcoRI digests products of plasmids extracted from a set of minipreps. Plasmid preparations were digested in EcoRI for 1.5-2 hr at 37°C. In this case 8 minipreps from 3 isolates HYP1, HYP2 and HYP3 are shown, all of which had inserts of approximately 400 bp. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).
The inserted DBLα domain tags in purified plasmids from each isolate RT-PCR transformation were sequenced, using a primer site just upstream of the multiple cloning site on the vector.

The RT-PCR colony picking analysis technique described above is summarised in Fig. 3.5.

Fig. 3.5. Schematic diagram of RT-PCR colony picking analysis technique.
3.5.3 DBLα RT-PCR validity

In the first instance, I investigated the validity of my approach by sequencing inserts in 16-17 “blue” colonies derived from each of 3 field isolate. To determine if sampling a larger number of plasmids would influence the results of the study, colony picking was repeated, picking 50 further colonies for two of the isolates, CM7 and CM9 (Fig. 3.6, colony pick repeat column). In addition, to determine whether the RT-PCR results were representative and repeatable, RT-PCR, cloning and sequencing was repeated from a separate RT-PCR reaction from the same RNA extraction from three isolates, CM4, CM7 and CM9 (Fig. 3.6, RT-PCR repeat column).

In all cases, the same predominant sequence was obtained from the second amplification, supporting RT-PCR analysis from a lower number of sequences. For CM9, the same two sequences were obtained in similar ratio in all three analyses (15:1, 42:8 and 14:1 respectively). For CM4, the dominant domain was seen at a similar frequency in both RT-PCR (15:1:1 and 12:1), but a different minor sequence was seen in the second RT-PCR reaction. For CM7, a more complex pattern of gene expression was seen, with a total of 10 different minor domains obtained, though the dominant sequence remained consistent, and there was some overlap in the minor domain transcripts (Fig. 3.6; colours represent the same sequence).
Fig. 3.6. RT-PCR validation. The pie charts represent the frequency of transcripts sequenced from miniprep plasmids from three isolates, CM9, CM7 and CM4. For each isolate the same colour represents the same unique sequence. Left: 16-17 plasmids sequenced from bacteria transformed with ligation products from an RT-PCR reaction. Middle: a further 50 plasmids were sequenced from the same plate of bacteria transformed with the ligation products from the original RT-PCR reaction. Right: 12-15 plasmids were sequenced from bacteria transformed with ligated plasmids from a new RT-PCR reaction from the original cDNA preparation.
3.5.4 Transcribed DBLα domains in childhood malaria field isolates

Having established that the technique was valid, 14-19 minipreps from plates of transformed bacteria carrying plasmids representing DBLα var gene transcript from each of the remaining 23 Malian childhood field isolates were sequenced. Sequences from each isolate were compared using SeqMan software. Identical or almost identical sequences from the same isolate (>95% identity) were defined as isolate sequence A, B, C etc in order of abundance (Genbank accession numbers DQ367086-DQ367226).

The relative abundance of the different DBLα sequences for each isolate is shown in Fig. 3.7. Here, pie charts represent the total repertoire of DBLα transcripts detected by RT-PCR, with the area of the pie slices representing the relative levels of abundance of each DBLα sequence from colony picking analysis. The number of colonies picked for plasmid sequencing is also shown.

In addition, DBLα domains can be defined as either DBLα1 or DBLα0 on the basis of conserved cysteines (DBLα1-like genes have 2 cysteines in the amplified tag, and DBLα0-like genes have 4 cysteines in the amplified tag; Table 1.1). Sequences are coloured as DBLα1 (white) or DBLα0 (black) in Fig. 3.7.
<table>
<thead>
<tr>
<th>UNCOMP LICATED</th>
<th>CEREBRAL</th>
<th>HYPERPA RAS ITAEMIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1 (15)</td>
<td>CM1 (18)</td>
<td>HYP1 (15)</td>
</tr>
<tr>
<td>U2 (15)</td>
<td>CM2 (14)</td>
<td>HYP2 (15)</td>
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<td>U3 (17)</td>
<td>CM3 (14)</td>
<td>HYP3 (18)</td>
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<td>U4 (15)</td>
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<td>U5 (19)</td>
<td>CM5 (18)</td>
<td>HYP5 (15)</td>
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<td>U6 (19)</td>
<td>CM6 (17)</td>
<td>HYP6 (16)</td>
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<td>U7 (16)</td>
<td>CM7 (17)</td>
<td>HYP7 (16)</td>
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<tr>
<td>U8 (18)</td>
<td>CM8 (19)</td>
<td>HYP8 (17)</td>
</tr>
<tr>
<td>U9 (14)</td>
<td>CM9 (16)</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.7. Frequencies of distinct DBLα sequences in each isolate. The pie charts represent the relative number of different DBLα sequences detected in each isolate by RT-PCR, cloning and analysis of 14-19 mini-prep clones per isolate. For each isolate, each pie-slice represents a different sequence, and the size of each pie-slice represents relative abundance of transcript. Isolate name is in the top left corner of each box. Bracketed numbers indicate the exact number of mini-prep clones sequenced for each isolate. Shading indicates DBLα0-like (grey) or DBLα1-like (white) sequences.

The number of sequences per isolate varied between 1 and 14 (Fig. 3.7). There was no significant difference in the number of distinct DBLα sequences per isolate detected in each clinical category (cerebral malaria: median 5, range 1-9; hyperparasitaemia: median 6.5, range 2-14; uncomplicated malaria: median 4, range 2-10, p = 0.72, Kruskal-Wallis test).

The frequency of DBLα1-like var gene transcripts within each clinical manifestation group was analysed (Fig. 3.8). There was a highly significant difference in the frequency of DBLα1-like var gene transcripts within the cerebral malaria isolates (80.1%) compared to the hyperparasitaemia isolates (25.7%; p < 0.001, Chi-squared test). 40.5% of the sequences from the uncomplicated malaria patients were DBLα1-like. The frequency of DBLα1-like var gene transcripts in both cerebral and hyperparasitaemia was significantly different from in uncomplicated isolates (P < 0.005, Chi-squared test) which showed intermediate DBLα1-like var gene transcription.
Fig. 3.8. Percentage of DBLα0 (grey) and DBLα1 (white) transcripts detected across the 26 Malian field isolates separated by disease category cerebral (C), hyperparasitaemia (HYP) and uncomplicated malaria (U). *There is a significant difference in DBLα1 proportion between cerebral and uncomplicated isolates, and between hyperparasitaemia and uncomplicated isolates (both p < 0.005, Chi-squared test).  ** There is also a significant difference in DBLα1 proportion between cerebral and hyperparasitaemia isolates (P < 0.001, Chi-squared test).
If only the predominant gene from each isolate was examined, a marked difference remained between the cerebral malaria and hyperparasitaemia isolates. The predominant gene in 8/9 isolates from cerebral malaria patients was DBLα1-like, whereas the predominant gene in 6/8 hyperparasitaemia isolates was DBLα0-like (Fig. 3.9, DBLα0-specific cysteines boxed). This difference is significant (Fisher’s Exact test, p = 0.013). For uncomplicated malaria isolates, the predominant gene was DBLα1-like in 5/9 isolates and DBLα0-like type in 4/9 isolates, not significantly different from the cerebral or hyperparasitaemia isolates (U:HYP, P=0.29; U:CM P=0.13, Fisher’s exact test).

Fig. 3.9. Clustal W alignment of the predominant DBLα domains gene transcript in *P. falciparum* isolates CM1-9 from cerebral malaria patients and HYP 1-8 from hyperparasitaemia patients. The second half of the expressed sequence tag is shown and the position of the residues that distinguish DBLα0-like domains (cysteines present) from DBLα1-like domains (cysteines missing) are arrowed, and the cysteine residues are boxed. Symbols: * indicates conserved residues; : indicates conservative substitution; . indicates semi-conservative substitution.
DBLα domains can be separated into 6 groups according to sequence motifs in the expressed sequence at positions of limited variation as well as the number of cysteine residues (Bull et al. 2005a), as described in Table 3.1. Group 1 domains have 2 cysteines in the amplified tag and the sequence MFK* at a particular locus defined as PoLV1. Group 2 domains also have 2 cysteines in the amplified tag and have *REY at a second locus defined as PoLV2. Group 3 domains contain neither MFK* or *REY, but also have 2 cysteines in the amplified tag. Group 4 and 5 domains have 4 cysteines in the amplified tag. Group 4 domains do not contain *REY at PoLV2 whereas group 5 domains do. Group 6 domains have 1,3,5 or more cysteines in the amplified tag.

Under these definitions, DBLα1 domains are Bull groups 1-3 and DBLα0 domains are groups 4 and 5. Group 6 are atypical domains with 1, 3, or >5 cysteines in the amplified tag and can be DBLα1 or DBLα0 domains. Examination of the Mali sequences classified into these six DBLα domain sequence groups did not reveal any further differences between the three disease categories (Fig. 3.10). As expected, most of the genes transcribed in the isolates from cerebral malaria patients are in groups 1-3 (DBLα1), and isolates from hyperparasitaemia patients mostly transcribe var genes in groups 4 and 5 (DBLα0). The isolates from uncomplicated malaria patients show an intermediate pattern with 4 isolates mostly transcribing groups 1-3 (DBLα1) and 5 isolates mostly transcribing groups 4 and 5 (DBLα0). Transcription frequencies of the six different DBLα groups in the different clinical categories are significant (P = 0.049, Kruskal Wallis test). A 3x6 contingency table was also used.
to compared the number of transcripts classified into the 6 DBLα groups. For data in the three clinical groups combined, the distribution of reads in each group is significantly different (P<0.001). Seven group 6 sequences described by Bull et al (number of cysteines ≠ 2 or 4) were detected, none of which were transcribed at high level, and all of which could be classified as either DBLα1 or DBLα0. Neither of the extra 2 cysteines found in DBLα0 was ever present alone.

A
Fig. 3.10. The transcribed var genes domain sequences from each isolate, and for all cerebral, hyperparasitaemia and uncomplicated malaria isolate sequences grouped, were separated into 6 DBLα sequence types (Bull et al. 2005a). Group 1-3 domains have 2 cysteines in the amplified tag (DBLα1). In addition, group 1 domains have MFK* at a particular locus defined as PoLV1, group 2 domains have *REY at a second locus defined as PoLV2 and group 3 domains contain neither MFK* or *REY. Group 4 and 5 domains have 4 cysteines in the amplified tag (DBLα0). Group 4 domains do not contain *REY at PoLV2 whereas group 5 domains do. Group 6 domains have 1,3,5 or more cysteines in the amplified tag. A) Proportion of transcript in all isolates in the 6 DBLα sequence groups, and combined data for the three clinical manifestation groups as a bar chart. B) Data for three disease manifestation groups combined as pie charts.
3.5.5 Upstream sequence analysis for predominant DBLα domain transcripts

In the sequenced laboratory strains 3D7, IT and HB3 and in other published var genes, DBLα1 domains are exclusively seen in group A or B/A var genes while DBLα0 domains are found in group B, C or B/C var genes (Gardner et al. 2002; Lavstsen et al. 2003; Kraemer et al. 2007). Thus, DBLα(0/1) domain sequences are indicators of var gene group. The association of DBLα1 domain transcription with cerebral malaria described above could be simplified as an association between transcription of group A or B/A var with cerebral malaria. To validate using DBLα(0/1) domain to denote groups of transcribed var genes, the different var gene group upstream region sequences were examined.

Degenerate primers were designed to recognise type A, B or C upstream regions, using an alignment of published upstream sequences, to allow classification of the upstream regions of the detected DBLα domains sequences by PCR. These were designed to amplify the specific upstream regions from laboratory isolate gDNA (gift from A Raza and JA Rowe) when paired with a gene-specific reverse primer (Fig. 3.11). These primers successfully amplified the upstream region of a specific var gene from gDNA from three parasite strains PAR+, TM180 and AIR only when the appropriate upstream group primer (Ups A, B or C) was used (Fig. 3.12).
Fig. 3.11. Strategy for specific amplification of transcribed DBLα domain for gDNA. Degenerate primers recognising Ups A, B and C regions were used in PCR reactions with a specific primer to the domain of interest. Only the reaction with the appropriate upstream region primer resulted in PCR product, which was then sequenced to verify amplification of the correct DBLα domain and to obtain upstream sequence data.
Fig. 3.12. PCR gel showing domain amplification of Ups A, B and C upstream regions. Specific primers to var genes PARvar1 (Strain: It/Palo Alto [PAR+]; UpsA), TM180var1 (Strain TM180; UpsB) and A1Rvar1 (Strain A1R; UpsC) were used with specific primers for UpsA, UpsB and two different UpsC primers C1 and C2, and with the appropriate gDNA. PCR product is only seen with the expected upstream primer. DNA ladder was λ DNA/HindIII fragments (Invitrogen).

Specific primers to the dominant DBLα domain sequence tag from the field isolate were designed (Table 3.2). These specific primers were used with each of the upstream primers to amplify the upstream region of the predominant domains in each isolate. PCR product was obtained and fully sequenced from nine isolates. This gave the expected result that all of the examined field isolate var genes with DBLα1 domains had UpsA-type or UpsB-type upstream regions whereas all of the field isolate var genes with DBLα0 domains had UpsB-type or UpsC-type upstream regions as is seen in 3D7 laboratory strain (Lavstsen et al. 2003). Upstream sequence group and predicted var gene groups are shown in Table 3.5. Other domains could
not be sequenced due to cross recognition of other DBL domains with the “specific” primer, or lack of parasite material.

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>Domain</th>
<th>DBL(\alpha)/0</th>
<th>Ups sequence</th>
<th>Predicted Var group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral Malaria</td>
<td>CM2A</td>
<td>DBL(\alpha)1</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>CM3A</td>
<td>DBL(\alpha)1</td>
<td>A</td>
<td>A</td>
</tr>
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<td>A</td>
</tr>
<tr>
<td></td>
<td>CM8A</td>
<td>DBL(\alpha)1</td>
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<td>A</td>
</tr>
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<td>Hyperparasitaemia</td>
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<td>B</td>
</tr>
<tr>
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<td>B</td>
<td>B/A</td>
</tr>
<tr>
<td></td>
<td>HYP5A</td>
<td>DBL(\alpha)0</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Uncomplicated Malaria</td>
<td>U2A</td>
<td>DBL(\alpha)0</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>U8A</td>
<td>DBL(\alpha)0</td>
<td>B</td>
<td>B</td>
</tr>
</tbody>
</table>

Table 3.5. Ups sequence and predicted var groups for the dominant domain in 9 isolates with successful upstream region. Upstream regions were amplified with specific primers for UpsA, UpsB and UpsC upstream regions and a reverse gene specific primer for the predominant DBL\(\alpha\) gene for each isolate.
3.5.6 Phylogenic analysis of DBLα domain transcripts

The association between disease phenotype and var gene expression was explored by mapping symptoms across a phylogeny of the DBLα sequences. A phenogram was created from a clustal W alignment of protein sequences. Phenograms depict genetic relationships without assumptions of evolutionary history. EBA175 was added as a root, as it is a common ancestor of DBL domains, and forms a clear outgroup (Adams et al. 1992). For reference, a selection of DBLα sequences from the three major groups A, B and C from the fully sequenced P. falciparum laboratory clone 3D7 (Gardner et al. 2002) were included, plus four 3D7 genes with intermediate characteristics (B/A or B/C) (Fig. 3.13).

There is a pronounced split as DBLα1-like form a separate clade from DBLα0-like sequences in the phenogram. All sequences above the node where the two clades split (marked *, Fig. 3.13) are DBLα0-like and all below are DBLα1-like. The DBLα0-like upper clade is comb-like, indicating a high level of recombination or an explosive gene duplication event of these sequences. The DBLα1-like lower clade has a more complex branch structure, suggesting more directed evolution.

The phenogram was also coloured according to the 6 DBLα groups classified by Bull et al (2005), Fig. 3.14. Group 4 and 5 sequences cluster separately from groups 1-3. Group 2 sequences form an outgroup of DBLα1 sequences, in general clustering separately from group 1 and 3 sequences. There is no obvious pattern to the position of group 6 sequences.
Clinical manifestation Groups
CEREBRAL - RED
HYPERPARASITAEMIA - BLUE
UNCOMPPLICATED - GREEN

3D7 reference domains - BLACK
Fig. 3.13. Phenogram produced from an amino acid alignment using Clustal W, and the tree-building program in MegAlign (DNA star lasergene software). Sequences transcribed by isolates from African children with cerebral malaria (CM, red), hyperparasitaemia (HYP, blue), and uncomplicated malaria (U, green) are compared to a selection of var genes from the laboratory clone 3D7. For the 3D7 genes, the gene name is preceded by A, B, C, BA or BC to indicate the group to which the gene belongs. The sequences fall into 2 major clades, which split at the asterisked node (*) with the DBLα1-like sequences in the higher clade and the DBLα0-like sequences lie in the lower clade. For the predominant gene from 9 of the Malian isolates, the var gene upstream region was determined by PCR and is indicated after the appropriate domain (UpsA, UpsB or UpsC). Bootstrap values were calculated by Graham Stone through 1000 repeat iterations of the tree and superimposed onto the phenogram.
DBLα groups
1 (DBLα1)
2 (DBLα1)
3 (DBLα1)
4 (DBLα0)
5 (DBLα0)
6 (DBLα1/α0)
Fig. 3.14. Phenogram was produced as for Fig. 3.13. Amino acid sequences were aligned in Clustal W, and the phenogram was created tree-building program in MegAlign (DNA star lasergene software). Sequences transcribed by isolates from African children with cerebral malaria (CM), hyperparasitaemia (HYP), and uncomplicated malaria (U) are compared to a selection of var genes from the laboratory clone 3D7. For the 3D7 genes, the gene name is preceded by A, B, C, BA or BC to indicate the group to which the gene belongs. The sequences fall into 2 major clades, which split at the asterisked node (*) with the DBLα1-like sequences in the higher clade and the DBLα0-like sequences lie in the lower clade. For the predominant gene from 9 of the Malian isolates, the var gene upstream region was determined by PCR and is indicated after the appropriate domain (UpsA, UpsB or UpsC). Sequences are coloured according to the DBLα group classification of Bull et al. 2005: DBLα1 groups: group 1: red, group 2: purple, group 3: pink. DBLα0 groups: group 4: dark blue, group 2: light blue. Group 6 sequences are in grey. Bootstrap values were calculated by Graham Stone through 1000 repeat iterations of the tree and superimposed onto the phenogram.
The phenogram successfully separates the domains on the basis of sequence. However, recombination can generate conflicting signals during the process of tree-building, potentially causing a significant source of error (Huson and Bryant 2006). Phylogenetic networks take recombination into account better than traditional phenogram building algorithms. As var genes are subject to a high level of recombination (Freitas-Junior et al. 2000; Taylor et al. 2000b; Kraemer et al. 2007) a network was considered more accurate. A network (Appendix 2) was created by Dr Graham Stone (University of Edinburgh) to replace the phenogram. The network was produced from an initial Clustal W alignment of the amino acid sequences, converted to a nucleotide alignment. The network (Appendix 2) recapitulates the split of DBLα1 versus DBLα0 sequences seen in the phylogenetic tree. Sequences from the cerebral malaria patients (red in network; Appendix 2) are significantly concentrated into a ‘cerebral malaria clade’ (P < 0.001, chi-squared test), while sequences from the hyperparasitaemia patients (blue) are significantly concentrated in a ‘hyperparasitaemia clade’ (P < 0.025, chi-squared test). Sequences from the uncomplicated malaria patients (green) showed no bias in distribution between the two main clades of the network.
3.6 Results 2: Sequence conservation between var gene DBLα domain transcripts

3.6.1 Sequence identity between DBLα domain transcripts

Identical sequences from different isolates (branch length zero in Fig. 3.13, or parallel branches in network, Appendix 2) were rare, reflecting minimal overlap in var gene repertoires between isolates. Only 12 of the 142 unique sequence tags were present as identical or almost identical (>95% nucleotide identity) in more than 1 isolate. The mean amino acid sequence identity between sequences was 42.2% (SE 0.2%), SD 8.6% (Fig. 3.15).

![Box Plot and Histogram](image)

Fig. 3.15. The percentage amino acid identity between all DBLα transcripts from the Mali field isolates is displayed as a box plot (showing 10th, 25th, 50th, 75th and 90th percentiles; values <10th percentile and >90th plotted separately) and as a histogram. Amino acid sequences were created from nucleotide sequences in EditSeq, and percentage identity was calculated using MegAlign. Histograms and box plots of sequence identity were then created in Statview.
3.6.2 Sequence identity between var gene DBLα domain transcripts in disease categories

The level of amino acid identity between different Mali DBLα sequence domain transcripts in each clinical category was also determined. The mean amino acid identity between transcripts was surprisingly similar between the three disease categories. Cerebral isolate transcripts showed mean 42.9% (SE 0.26%), and hyperparasitaemia isolate transcripts showed mean 42.8% (SE 0.27%). The mean percentage sequence identity uncomplicated isolate transcripts, 40.9% (SE 0.27%), was slightly lower from that of hyperparasitaemia and cerebral isolate transcripts, and this difference was significant (t-test, p < 0.005). There was no significant difference in transcript identity between hyperparasitaemia and cerebral isolate sequences (t-test, p = 0.89). The variance of amino acid identity in the three categories was similar (SD: cerebral 8.6%, hyperparasiteamia 8.8%, uncomplicated, 8.1%). Fig. 3.16 shows histograms and a box plot of the data.

The level of amino acid identity between different Mali DBLα sequence domain transcripts in each clinical category was also illustrated pictorially (Fig. 3.17). Amino acid identity between 2 domains was colour coded such that darker shading reflects higher amino acid identity.
Fig. 3.16. Histograms and box plots (showing 10th, 25th, 50th, 75th and 90th percentiles; values <10th percentile and >90th plotted separately) of percentage amino acid identity between cerebral, hyperparasitaemia and uncomplicated malaria DBLa transcripts. Sequences transcribed in cerebral, hyperparasitaemia or uncomplicated malaria isolates were considered separately. Amino acid sequences were created from nucleotide sequences in EditSeq, and percentage identity was calculated using MegAlign. Histograms and box plots of identity were then created in Statview.
Fig. 3.17. Amino acid sequence distance charts showing percentage amino acid identity between domains separated by disease classification. Sequences transcribed in cerebral, hyperparasitaemia or uncomplicated malaria isolates were considered
separately. Amino acid sequences were created from nucleotide sequences in EditSeq, and percentage identity was calculated using MegAlign. Darkness of shading indicates level of identity between domains.

3.6.3 Sequence identity between var gene DBLα domain transcripts in DBLα(1/0) groups

The sequence identity of the DBLα1-like and DBLα0-like transcripts separately was also calculated. The mean amino acids identity between DBLα0-like transcripts was 46.0% (SE 0.2%) slightly higher than that for DBLα1-like transcripts 45.6% (SE 0.1%). Though a small difference, because of the large sample size this difference is significant (t-test, p < 0.005). The standard deviation of the DBLα1 sequences (10.5%) is much greater than for DBLα0 sequences (5.5%), seen as a broader curve in the histogram (Fig. 3.18). Both DBLα1 sequences and DBLα0 sequences are more similar to each other than sequences separated by disease category, due to the definitions of DBLα1 and DBLα0 sequences. This is equivalent to DBLα1 and DBLα0 sequences clustering separately on the phenogram (Fig. 3.13) and network (Appendix 2). Fig. 3.19 shows the results pictorially.
Fig. 3.18. Histograms and box plot (showing 10th, 25th, 50th, 75th and 90th percentiles; values <10th percentile and >90th plotted separately) of percentage amino acid identity between all DBLα0 domain and DBLα1 domain transcripts. Amino acid sequences were created from nucleotide sequences in EditSeq, and percentage identity was calculated using MegAlign. Histograms and box plots of identity were then created in Statview.
Fig. 3.19. Amino acid sequence distance charts showing percentage amino acid identity between domains separated by $\text{DBL}\alpha 0/1$ classification. Darkness of shading indicates level of identity between domains. $\text{DBL}\alpha 1$ and $\text{DBL}\alpha 0$ transcript sequences were considered separately. Amino acid sequences were created from nucleotide sequences in EditSeq, and percentage identity was calculated using MegAlign.
3.6.4 Evidence for conserved var genes

To examine the extent of conservation of these genes to other published global var genes, sequences were entered in to NCBI BLAST homology search. Database matches (> 95% nucleotide identity and > 95% sequence coverage) to the Mali sequence described in this study as of August 2007 are given in Table 3.6.

The most common sequence found in the Mali sequences (CM6A) was present in 5 isolates (CM6, U3, U5, U9 and HYP2), and was the only var transcript detected in isolate CM6. Nine further non-var1csa Mali sequences were common to 2 isolates (>95% identity): CM9A:CM8B (100% identity), CM7A:CM2D (100%), CM9B:CM7D (100%), U7A:HYP2D (100%), CM2E:CM7F (99%), HYP8E:U2A (99%), U1B:HYP8G (98%), CM2A:HYP3A (98%), U3D:CM7B (96%). One of these (CM7D/CM9B) was identical to a DBLα domain from IT/FCR3 family laboratory strain family (Accession number DQ408000). This domain belongs to the PfEMP1 PARvar1, which is responsible for rosetting in the Palo Alto form of this strain (J.A. Rowe, unpublished data). None of the other 8 sequences found in multiple isolates in Mali (CM6A group, or the 8 transcripts found in 2 isolates) showed any highly homologous database matches.

Of the non-var1csa sequences found in multiple isolates, most (7/10) involved cerebral isolate transcripts, and almost half (4/10) were exclusive to cerebral isolates. No transcript was exclusive to uncomplicated or hyperparasitaemia isolates. In addition, of the non-var1csa sequences found in multiple isolates, most (7/10) were DBLα1-like. The group 4 var (Jensen et al. 2004) were not amplified in any of the
isolates. A similar proportion of DBLα1/0 sequences had no database matches (35% DBLα1, 28% DBLα0 sequences).

Seven var1csa homologues were also found; CM8D, HYP3B, U5D, U7B were 3D7 var1csa homologues and CM1G, CM4C, CM5B were FCR3 var1csa homologues, as shown in Table 3.7.

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## Mali field isolate DBLα sequences

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<th>Group</th>
<th>Accession Number (Origin)</th>
<th>Nucleotide identities (%)</th>
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<td>AY462831 (PNG)²</td>
<td>337/344 (97%)</td>
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<td>DQ367115 (CM6a, Mali)³</td>
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<td>2</td>
<td>PFE1640w (Var1CSA, 3D7)¹⁸</td>
<td>331/331 (100%)</td>
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<td>2</td>
<td>AM116429 (Kenya)¹</td>
<td>315/325 (96%)</td>
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</table>
### Table 3.6. NCBI database homologues for Mali isolate DBLα transcripts described in this study. Accession number, frequency (number of clones sequenced per isolate with identical sequence), DBLα0/1 classification and group (Bull et al. 2007) are given. Up to 10 database matches (>95% identity, >95% sequence coverage, http://www.ncbi.nlm.nih.gov/BLAST) are also described (accession number, country of origin, and% nucleotide identity). Multiple database matches from the same strain or isolate but entered multiply on the NCBI database multiple times are not repeated in the table.

… more than 10 database matches were found.

- no database matches were found.
(Bull et al. 2005a), (Fowler et al. 2002), (Su et al. 1995), Barry et al. 2005 (unpublished), (Trimnell et al. 2006), (Barry et al. 2007), (Rowe et al. 2002a), (Kaestli et al. 2004), (Taylor et al. 2000b), (Winter et al. 2003), (Albrecht et al. 2006), (Tami et al. 2003), (Salanti et al. 2002), (Horrocks et al. 2002), (Duffy et al. 2002), (Fernandez et al. 2002), Ozarkar, et al unpublished, (Hall et al. 2002), (Khattab et al. 2003), (Kirchgatter et al. 2000), (Kraemer et al. 2007), *this study/(Kyriacou et al. 2006).

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<th>Nucleotide identities (%)</th>
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<td>331/331 (100%)</td>
</tr>
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<td>3D7 var1csa</td>
<td>331/331 (100%)</td>
</tr>
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<td>3D7 var1csa</td>
<td>326/331 (98%)</td>
</tr>
<tr>
<td>CM1G</td>
<td>FCR3 var1csa</td>
<td>321/357 (89%)</td>
</tr>
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<td>CM4C</td>
<td>FCR3 var1csa</td>
<td>322/353 (91%)</td>
</tr>
<tr>
<td>CM5B</td>
<td>FCR3 var1csa</td>
<td>325/353 (92%)</td>
</tr>
</tbody>
</table>

Table 3.7. Details of homology of the Mali isolate DBLα transcripts described in this study to the two forms of var1csa described in Rowe et al. (2002a). Nucleotide identity to 3D7 var1csa, PFE1640w, (Hall et al. 2002) or FCR3 var1csa, AJ133811, (Scherf et al. 1998) is shown.
3.7 Results 3: Correlations of DBLα1 domain transcript frequency and isolate characteristics

3.7.1 DBLα1 domain transcription correlates with high rosetting

Rosette frequency (RF) was significantly higher in the cerebral malaria isolates than in the hyperparasitaemia or uncomplicated malaria isolates (Table 3.3; \( p = 0.035 \)), in agreement with consistent observations that rosetting is a virulence factor in Africa (Carlson et al. 1990; Rowe et al. 1995; Rowe et al. 2002b). Group A var genes are associated with rosetting, and var genes responsible for rosetting in 8 rosetting laboratory isolates (unpublished data, Rowe JA) are all group A with DBLα1 domains that cluster within the ‘cerebral’/DBLα1-like portion of the tree (data not shown).

In my isolates, DBLα1-like gene expression was also associated with rosetting (Spearman Rank correlation Rho = 0.52, \( p = 0.009 \); Fig. 3.20). This is in accordance with the association of group A var genes and rosetting (unpublished data, Rowe JA), and of rosette frequency and malaria severe/cerebral malaria in Africa (Carlson et al. 1990; Rowe et al. 1995; Rowe et al. 2002b). Colouring the points according to disease manifestation shows that the correlation is driven by high RF levels in the cerebral malaria isolates which also have high DBLα1% transcription levels (red in Fig. 3.20), and by low RF levels in the hyperparasitaemia malaria isolates which showed low DBLα1% transcription levels (blue in Fig. 3.20).
Fig. 3.20. Correlation between isolate rosette frequency (RF) and proportion of DBLα1-like domain transcripts detected. Spearman Rank correlation: Rho = 0.52, p = 0.009. Red circles: cerebral malaria isolates, green squares: uncomplicated malaria isolates, blue diamonds: hyperparasitaemia isolates.

Splitting the sequences into the DBLα groups 1-5 (Bull et al. 2005a) (there were not enough group 6 domains to examine) reveals that DBLα1 groups 1 and 3 positively correlate with rosetting, but only group 1 reaches significance (P < 0.05) (Fig. 3.21). This is consistent with laboratory rosetting lines that are often groups 1 or 3 (Alex Rowe, personal communication) although Bull et al. (2005) found group 2 to be most strongly correlated with rosetting.
Fig. 3.21. Spearman Rank correlations between isolate rosette frequency (RF) and proportion of DBLα group 1-5 domain transcripts detected (Bull et al. 2005a). Groups 1 and 3 show positive Rho (Spearman Rank correlation) for association with rosette frequency. *Only group 1 association with rosette frequency is significant (Rho = 0.394; p = 0.049). There was a tendency for group 4 to be negatively associated with rosette frequency, but this was not significant (Rho = -0.365; p = 0.068).

*
3.7.2 DBLα1 domain transcription does not correlate with age

There was no significant correlation of any of the groups with age (Fig. 3.22), though there was a tendency for Bull group 4 transcripts to be associated with age (Rho = 0.385; p = 0.055, Fig. 3.23). All other groups showed (not significant) negative association with age (all p > 0.1) (Fig. 3.23).

Fig. 3.22. No significant correlation was found between age (months) and proportion of DBLα1-like domain transcripts detected in the isolate. Spearman Rank correlation: Rho = -0.30, p = 0.14.
Fig. 3.23. Spearman Rank correlations between age and proportion of DBLα group 1-5 domain transcripts detected in the isolate (Bull et al. 2005a). No correlation produced a significant p value (all p > 0.1).
3.7.3 DBLα1 domain transcription does not correlate with parasitaemia

There was also no significant correlation of any of the groups with parasitaemia (in all groups p > 0.1) (Figs 3.24 and 3.25).

![Graph showing no significant correlation between DBLα1 and parasitaemia](image)

Fig. 3.24. No significant correlation was found between parasitaemia (%) and proportion of DBLα1-like domain transcripts detected in the isolate. Spearman Rank correlation: Rho = 0.35, p = 0.18.
Fig. 3.25. Spearman Rank correlations between parasitaemia and proportion of DBLα groups 1-5 transcripts detected in the isolate (Bull et al. 2005a). No correlation produced a significant p value (all p > 0.1).
3.8 Discussion

The major finding presented in this chapter was that a higher level of DBLα1 domain transcription was observed in cerebral malaria isolates compared to hyperparasitaemia isolates. This indicates an association of group A var transcription with cerebral malaria, and therefore an association of group A PfEMP1 and malaria severity. This study of clinical isolates provides evidence to support *in vitro* work reporting a link between group A var gene transcription and “severe malaria phenotype parasites,” parasites selected on semi-immune plasma pools (Jensen *et al.* 2004). I will recap my findings and then discuss my data in the context of other relevant studies.

Transcription profiling of 26 Malian field isolates revealed a marked difference in DBLα(0/1) transcription level in the three disease manifestation groups, with significantly higher frequency of DBLα1-like domain transcripts detected in cerebral malaria isolates compared to hyperparasitaemia isolates (P < 0.001, Chi-squared test). In the majority of cerebral malaria isolates the predominant transcript was DBLα1-like, significantly different to the hyperparasitaemia group, where the predominant transcript in the majority of isolates was DBLα0-like (P = 0.013, Fisher’s Exact test). The majority of isolates possessed multiple genotypes (18/26), and thus different subpopulations of parasites had different genetic repertoires of var gene available to express. Expression of different var genes from subpopulations of parasites from the same genotype was also expected, due to switching of the expressed var gene. Most isolates (17/26) displayed expression of DBLα1-like and DBLα0-like domains. Of the remaining isolates, 6 exhibited 100% DBLα1-like
domain expression (U3, U6, CM6, CM7, CM9 and HYP4) and 3 had 100% DBL\(\alpha\)0-like domain expression (U4, HYP1 and HYP8). No cerebral isolate exhibited 100% DBL\(\alpha\)0-like domain expression. It is tempting to speculate that CM4 may be particularly susceptible to cerebral malaria, only having 1/17 DBL\(\alpha\)1-like sequences. It is also possible that HYP4 may be protected from the cerebral malaria which might be expected from 100% DBL\(\alpha\)1-like domain expression, for instance by having a protecting genetic polymorphism.

Younger host age and increased severity of disease has previously been associated with parasite expression of var sequences which are more frequently recognised by plasma from semi-immune children (Bull et al. 1999; Bull et al. 2000; Nielsen et al. 2002; Lindenthal et al. 2003). This suggested that var genes associated with severe disease, and young host age, are more common within a parasite population. This may suggest certain var genes being more commonly found in the genome of distinct parasites. Alternatively, certain PfEMP1 may be more frequently expressed, or commonly elicit a particularly successful immune response. The data in this chapter show that group A var genes are linked with severe disease, and group A var genes appear to be more ‘common’ in terms of sequence homology as most transcripts appearing in multiple isolates were group A. This fits with the hypothesis that surface antigens involved in severe disease can be recognised by plasma from high numbers of people within a population (Bull et al. 1999; Bull et al. 2000; Nielsen et al. 2002; Lindenthal et al. 2003).
A number of previous studies support the findings reported here. A small Brazilian study reported a link between severe malaria and expression of genes lacking 1-2 cysteines (those subsequently defined as missing in DBLα1 domains). However, only eight such sequences in total were found, across only six severe malaria patients (Kirchgatter and Portillo Hdel 2002). The association of group C var and non-severe disease was also seen in Tanzania (Rottmann et al. 2006) and Papua New Guinea (Kaestli et al. 2006), consistent with the data presented in this study. However, in Tanzania both group A and B were increased in the severe malaria group, and in Papua New Guinea, only group B was associated with clinical disease. Both studies used upstream region PCR to define var gene group (Kaestli et al. 2006; Rottmann et al. 2006). It is possible that some of the group B transcripts associated with severe malaria are group B/A (UpsB region with group A-like domain structure).

Previous studies used a broad definition of severe disease, grouping patients with very different severe complications, and presumably very different underlying pathologies and molecular mechanisms (Kirchgatter and Portillo Hdel 2002; Bull et al. 2005a; Kaestli et al. 2006; Rottmann et al. 2006). Uncomplicated malaria was commonly used as the comparison to severe malaria, resulting in studies comparing groups with disease severity and also highly different parasitaemia. Controlling for parasite burden by using hyperparasitaemia as the comparative non-severe malaria group was an important contributing factor to allow a clear result to be found from this study. Transcription from the uncomplicated malaria isolates showed an intermediate distribution across the DBLα(0/1) spectrum. However, analysis of the parasites causing the “uncomplicated” low parasitaemia disease must be treated with
caution. On presentation at hospital, these patients received treatment and thus it was not possible to follow disease progression to the natural end point of severe clinical symptoms, hyperparasitaemia or parasite clearance. The data from this study is consistent with the uncomplicated isolates representing an earlier time point in disease pathogenesis, where disease progression may be predestined but not yet clinically evident.

The success of this study relied on a precise and accurate disease definition for cerebral malaria of Blantyre score ≤ 2 with an asexual *P. falciparum* parasitaemia and no other possible cause of coma. Due to the high prevalence of low level asymptomatic parasitaemia in the general population, post-autopsy investigation revealed that *P. falciparum* was not the genuine cause of coma and/or death in up to 23% of patients diagnosed with cerebral malaria in Malawi (Taylor et al. 2004; Milner et al. 2005). In this study it was not possible to confirm cerebral diagnosis by autopsy, and as such it is theoretically possible that some of the cerebral malaria cases were incorrectly diagnosed. This could explain isolate CM4, unique in the cerebral malaria group with such a high frequency of DBLα0-like domain transcription (16/17 sequences). A strict definition of cerebral malaria was used in diagnosis of the isolates obtained for this study and the clear pattern of gene expression that emerged suggests that the diagnoses were as accurate as possible, although problems of misdiagnoses in a small proportion of cases remains a possibility in studies of field isolate disease manifestation and should be taken into consideration. This study and other reports (Kurtzhals et al. 2001; Nacher et al. 2001) emphasise the need for strictly defined disease criteria and carefully chosen
control groups in studies examining the molecular basis for malaria disease outcome. It is likely that this study was successful due to use of a strictly defined severe disease category with a precise pathology, and an appropriate control group.

In *P. falciparum* malaria, mature parasites sequester to avoid splenic clearance (Chotivanich *et al.* 2002) and cerebral malaria is characterised by sequestration of infected erythrocytes in the microvasculature of the brain (Berendt *et al.* 1994b; Silamut *et al.* 1999). Ideally, sequestered parasites, for example from postmortem brain, would be examined (Taylor *et al.* 2004; Milner *et al.* 2005). These parasites would be mature trophozoites, in contrast to the younger rings found in peripheral blood. Although once controversial (Chen *et al.* 1998b; Noviyanti *et al.* 2001; Duffy *et al.* 2002), the recent data strongly indicates that a single *var* gene is transcribed at ring stage and this corresponds to the PfEMP1 protein on the surface at trophozoite stage (Dahlback *et al.* 2007). This *var* gene transcript appears to remain present at high enough concentration for RNA extraction and RT-PCR in the mature trophozoite, and studies comparing *var* gene transcription across a cell cycle report the same *var* gene transcripts are present in ring and trophozoite parasites for a given culture (Peters *et al.* 2002; Montgomery *et al.* 2007). However, taking the RNA too late in the cell cycle may result in gaining relatively increased proportions of *var1csa*, which is transcribed throughout trophozoite stages (Kyes *et al.* 2003). A detailed study of *var* gene transcription in laboratory isolate NF54 suggests collection of parasites for *var* gene transcription studies at ring stage and pigmented trophozoite stage provides a good representation of *var* gene transcription for that invasion cycle (Dahlback *et al.* 2007). The report also confirmed that *var* gene
transcripts collected at ring stage are reflective of PfMEP1-mediated adhesion properties of the mature parasite. In this work and other similar studies (e.g. (Bull et al. 2005b), ring stage parasites were obtained from peripheral blood, carrying the assumption that the population of peripheral ring parasites directly reflects the sequestered mature population. Genotypically, peripheral parasites reflect the sequestered parasites (Dembo et al. 2006; Montgomery et al. 2006).

Sequestered parasite populations in different organs of the same patient show differential var gene expression, supporting a link between cytoadhesion and pathology (Montgomery et al. 2007). Although peripheral rings were not directly examined in this study, the assumption from previous studies is that the peripheral ring population reflects the sequestered parasite mass, which is then sequestered upon PfEMP1 expression at maturity in whichever organ best suits its particular cytoadhesive properties (Dembo et al. 2006; Montgomery et al. 2006). However, an absolute correlation between var gene transcript range in peripheral rings and sequestered erythrocyte PfEMP1 remains an assumption.

In addition, within an infection, var gene transcription is highly dynamic (Peters et al. 2002; Kaestli et al. 2004). Each var gene has its own intrinsic switching rate (Horrocks et al. 2004b), ranging from <0.025% per generation to at least 16% in experimentally infected individuals (Peters et al. 2002; Horrocks et al. 2004b). Var gene switching and silencing is very complex and is controlled at multiple levels. This involves tight transcriptional control with a silencing role of internal promoters and epigenetic chromatin modifications (Horrocks et al. 2004b; Duraisingh et al. 2004b).
2005; Frank et al. 2006). At present it is not possible to predict the order of var gene expression as switching is not completely understood, but it is clear that the order of var gene transcription is far from random. In vitro, telomeric var genes (group A, B or A/B) have faster “off” rates than centromeric var genes (group C) (Frank et al. 2007). Upon adaptation to culture, parasites from infected individuals rapidly reduce the relative level of group A var gene transcription compared to group B or C (Peters et al. 2007). Laboratory strains tend to revert to group C var gene expression during long term culture (Frank et al. 2007). In contrast to fast “off” rates in vitro, group A and B genes expand more rapidly than group C genes in experimentally infected human volunteers (Lavstsen et al. 2005). It is possible that the activation of group A or B/A requires an additional stimulus from an infection, such as a detection of a signal from cerebral endothelium. Little is known about transcription factors which promote var gene activation, but it is possible that differences in the upstream region of the group A var genes may be involved in selection for group A var gene expression. For example, the mechanism of silencing appears to be different between the group A and B or C var genes, as silencing of group A, but not B or C, var genes is dependent on PfSir2 (Duraisingh et al. 2005).

In many studies (e.g. Kirchgatter et al. 2002), low parasitaemia isolates are cultured for a few cycles before RNA extraction. Combining the data showing that group A var genes are more likely to be switched off, and the fact that it is most often the uncomplicated malaria isolates that show low parasitaemia, suggests an unfair bias in the reporting of lower group A transcription levels in uncomplicated isolates. In this study, RNA was collected from all isolates at ring stage and was processed and
stored immediately. Isolates were not cultured through to the next cell cycle, and so no var gene switching could occur. However, even taking this extra precaution, the var gene expressed at collection may have been switched on since development of severe disease symptoms, and thus the currently expressed var gene may not be responsible for the clinical condition. Collecting field isolates as close as possible to onset of clinical symptoms, and avoiding culturing where possible, will increase confidence of a direct correlation between detected var gene transcription and disease outcome.

In addition to parasite isolates, the choice of DBLα domain to investigate was advantageous. Good primers were available (Taylor et al. 2000a; Bull et al. 2005a), and the vast majority of var genes contain a DBLα domain (Gardner et al. 2002). Though there was not enough parasite material for quantitative real-time RT-PCR, the RT-PCR colony picking technique used proved accurate and reproducible and has been used in many previous studies (e.g. Kirchgatter et al. 2002; Bull et al, 2005). A recent study comparing RT-PCR and real-time RT-PCR of var gene transcripts found RT-PCR gave very similar results to a real-time RT-PCR, with the same dominant transcript being detected with both approaches in the majority of isolates (Gatton et al. 2006). Though a clear distinction in DBLα(0/1) domain transcription levels between cerebral malaria and hyperparasitaemia isolates was discovered, the study could be reinforced and strengthened by sequencing more transcripts per isolate, the inclusion of more isolates from each group, and the addition of further clinical disease categories.
The disparity in DBLα (0/1) domain transcription levels between the two isolate groups suggests there might be a functional difference between DBLα0 and DBLα1 domains. The only known adhesive and well-characterised property of DBLα domains is rosetting, for example mediated via interaction of a PfEMP1 DBLα1 domain with CR1 on non-infected erythrocytes (Rowe et al. 1997). The association of rosetting with severe malaria in Mali and group A var genes in this present study was expected, as rosetting has been repeatedly linked to severe malaria in Africa (Carlson et al. 1990; Rowe et al. 1995; Bull et al. 2005a; Rowe 2005; Kaestli et al. 2006). In these Malian isolates, DBLα groups 1 and 3 correlated with rosette frequency (RF), in contrast to the correlation between DBLα group 2 and RF reported in a recent Kenyan study (Bull et al. 2005a). This could be a genuine difference between the Mali and Kenya isolates. Unlike DBLα groups 1 and 3, the proportion of DBLα group 2 sequences is not significantly different between cerebral and hyperparasitaemia isolates in this study. Interestingly, a link between DBLα1 domains in general and rosetting, but not group 2 DBLα domains and rosetting was also reported in a recent study of Ugandan field isolates (Normark et al. 2007). The functional significance of this DBLα grouping (1-6) compared to the broader grouping of DBLα(0/1) remains to be confirmed, though the classification is a useful tool for DBLα domain analysis. Although results associating sequences separated into the DBLα groups with RF are conflicting, the association of DBLα1 in general with rosetting is supported in both geographic locations and in laboratory isolates (Rowe, JA, unpublished data). The association of rosetting and severe malaria demonstrated in these field isolates, and in many other studies, highlights rosetting as a key target of clinical intervention to control parasite-mediated
phenomena associated with severe disease complications (see chapter 6 for discussion of rosette inhibition in severe malaria).

Though DBLα0 domains have been reported to support rosetting (Chen et al. 1998a), rosetting in laboratory strains appears to be predominantly via DBLα1 domains (Rowe, JA, unpublished data), suggesting that there is a steric restriction on the conformation of DBLα1 domain that can retain affinity for CR1 or other rosetting ligands. Perhaps the two extra cysteines found in DBLα0 domains form a disulfide bond that fundamentally alters the DBLα domain structure. Recent structural predictions of DBLα domain structure based on the known structures of EBA175 and P. knowlesi DBLα, suggest that DBLα0 domains have a GAG-binding motifs, which mimic the carbohydrate-binding pocket of EBA-175 (Tolia et al. 2005; Singh et al. 2006; Normark et al. 2007). In the DBLα1 domains, some of these GAG motifs disappear with the loss of the two cysteines, supporting this hypothesis. Motifs and predicted structural features present in rosetting PfEMP1 include an alpha helix that appears to be conserved between rosetting PfEMP1 DBLα domains (Normark et al. 2007).

Conversely, it may not be the DBLα1 domains per se, but other features of the group A var genes that is involved in cerebral malaria pathogenesis. Group A and B/A PfEMP1 are in general larger, with a more complex domain structure than PfEMP1 from group B, C and B/C. The plethora of other domains available in the group A and B/A PfEMP1 may have clinically relevant binding potential. For example, the majority of DBLγ domains, some of which were originally implicated in CSA
binding (see chapter 4) are found in group A or B/A var (12/14 in 3D7), as are the majority of DBLε domains (11/12 in 3D7), some of which enable binding to naturally occurring IgM antibodies (Semblat et al. 2006). There is also evidence that certain PfEMP1 have affinity for molecules on the cerebral endothelium such as ICAM1, mediated by a tandem array of DBLβ and C2 domain (Smith et al. 2000a; Springer et al. 2004). Perhaps surprisingly, ICAM-binding DBLβ/C2 tandem arrays do not appear to be associated with particular PfEMP1 groups (Smith, JD, personal communication).

Whilst DBLα1 domain transcripts are indicative of a group A, B/A PfEMP1, identification of DBLα0 domains is indicative of a group B, C or B/C PfEMP1. These typically possess a DBLα0-CIDRα type semi-conserved head structure (Gardner et al. 2002; Kraemer et al. 2007) and are capable of CD36-binding (Robinson et al. 2003). Though the DBLα0 domain itself does not have affinity for CD36, its presence suggests expression of PfEMP1 with affinity for CD36.

CD36-binding is the most common known adhesive property of malaria isolates, with the majority of field isolates having affinity for CD36 (Udomsangpetch et al. 1997). The role of CD36 in malaria pathogenesis is complex (reviewed in Serghides et al. 2003) and not fully understood. CD36 is a scavenger receptor expressed on erythrocytes, platelets, and many other cellular components of the blood, in many tissues including endothelium and muscle, and on organs such as liver and spleen, but not on the cerebral endothelium (Turner et al. 1994; Serghides et al. 2003). CD36-binding is thus associated with sequestration of infected erythrocytes in areas
such as skin and muscle away from vital organs such as the brain (Serghides et al. 2003). The lack of CD36-binding in infected erythrocytes coated in group A PfEMP1 may access cerebral endothelium, as they are less likely to be sequestered elsewhere. Earlier studies reported CD36 binding being associated with non-severe malaria without reference to PfEMP1 (Rogerson et al. 1999; Traore et al. 2000). It is tempting to speculate these results came about because of expression of group B, C or B/C PfEMP1 in these mild malaria patients.

CD36 binding also mediates platelet mediated clumping, a phenomenon originally associated with severe malaria (Pain et al. 2001). The only isolate with high PMC was a cerebral malaria isolate (CM8), with high parasitaemia (20%), but with low group B/C expression (only 1/19 DBLα0-like transcripts). The in vivo relevance of PMC remains unclear and the phenomenon is now thought to be associated with high parasitaemia but not severe malaria per se (Arman et al, in press).

Finally, CD36 promotes non-opsonic (non-inflammatory) phagocytic clearance (McGilvray et al. 2000) and phagocytosis of Plasmodium infected erythrocytes by human macrophages can be reduced by 50% by blocking CD36-dependent interactions (Smith et al. 2003). However, CD36-binding parasites are also able to cause inhibition of dendritic cells (Urban et al. 1999). These data suggest a complex relationship between CD36-binding and sequestration and immune response. In general, the data indicate a low immunopathological burden where CD36-binding parasites dominate. This is a good strategy for a parasite that does not benefit from host death, and is consistent with the association presented here between group B, C

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or B/C var genes and hyperparasitaemia. However, the extent of CD36-binding in parasites not expressing a CD36 binding PfEMP1, through other proposed ligands such as modified band 3, sequestrin and CLAG9, is unclear (Serghides et al. 2003).

There was also no significant correlation of any of the DBLα groups with parasitaemia. The lack of association of any group with parasitaemia supports the idea that a particular var gene group does not encourage an expansion of parasitaemia, but the var gene group expressed plays a role in determining whether a severe clinical manifestation will develop. The lack of association of DBLα group with parasitaemia is also reflected in the sequences from uncomplicated malaria patients being distributed across the phenogram and network.

The relative proportion of DBLα1 var genes detected in this study is similar to the relative proportion of DBLα1 var genes in parasite genomes seen in published strains and other reports, at around 20-30% (Fowler et al. 2002; Gardner et al. 2002; Albrecht et al. 2006; Kraemer et al. 2007). If the proportion of DBLα1 var genes in an isolate is relative stable, as appears to be the case from 3D7, IT and HB3 genomes, this suggests that there is no general bias of expression towards DBLα1 or DBLα0 var genes, though the gDNA repertoire of DBLα var gene sequences within these particular isolates would have to be examined to confirm this.

Roughly one third of the transcript sequences were unique with no database matches, while others showed a high level of conservation. Interestingly, a non-var1csa highly conserved sequence, CM6A, was found in 5 isolates. Extended sequencing of the
CM6A domain from the var genes from which these sequences belong would reveal whether the entire gene is conserved between these isolates, or just the DBLα domain. It would be interesting to screen for the presence of this domain in other isolates to identify to what extent this is a strain transcending var gene in addition to the var1csa, var2csa and type 3 var gene family. Expression of this domain in vitro would be a useful starting point for the examination of any particular roles for which this sequence has been conserved, for example through a series of binding studies. The identification of a domain identical to the DBLα1 of Palo Alto rosetting var gene PARvar1 in 2 rosetting cerebral malaria isolates (CM7, RF 20% and CM9, RF 41%) is also intriguing, suggesting this may be a conserved rosetting var gene family. It would be very interesting to examine the prevalence of this gene in other isolates, and investigate whether the homology to PARvar1 extends throughout the entire var gene or is confined to the DBLα domain. These domains suggest that var1csa, var2csa and the type 3 var genes may not be the only var genes which are common to many different isolates.

The immunity achieved against P. falciparum infection by adulthood in malaria-endemic areas suggests a finite global diversity of var genes and other VSA, or at least a high enough level of cross-reactive epitopes for antibody-mediated immunity (Bull et al. 2000). Certain geographic locations appear have a limited var gene repertoire with a relatively stable var gene repertoire and considerable interstrain var gene overlap, including Brazil (Afonso Nogueira et al. 2002; Albrecht et al. 2006) and West Pacific (Fowler et al. 2006), while data from other areas suggests a lower sequence overlap globally (Fowler et al. 2002; Bull et al. 2005a; Trimnell et al.
Though a study of the domains of 3D7 predicts that cross-reactive epitopes are relatively uncommon, antibodies directed at group A PfEMP1 show higher levels of cross-reactivity between domains than those directed at group B or C PfEMP1 (Joergensen et al. 2006). This is consistent with my findings that group A var genes tend to be more common in terms of conserved sequences between isolates and database matches, and with the original agglutination studies showing high frequency of recognition of parasites causing severe disease (e.g. Bull et al. 1999). The high level of diversifying selection on PfEMP1 (Lavstsen et al. 2003), and recombination between var genes within and between isolates, will continue to drive expansion of the global var gene repertoire.

The data presented in this chapter is of relevance to the exploration of PfEMP1 as a potential vaccine target. For example, as the majority of var genes contain DBLα0 domains these might be considered to provide a more comprehensive or universal target than DBLα1 domains. However, a DBLα0 domain vaccine, even if it was very effective at protecting against parasites expressing DBLα0 domains, may select for parasites expressing DBLα1 domains. In this study, DBLα1 domains are associated with cerebral malaria, and thus a DBLα0 domain vaccine may have the potential to select for cerebral malaria-causing PfEMP1-expressing parasites. Thus, a general DBLα0 domain vaccine would not necessarily be useful in protecting against severe disease. Analysis of this kind is critical to avoid creation of a vaccine which may actually be detrimental to those at risk of infection. Secondly, database analysis of the domains sequenced here highlight the huge global range of DBLα domains. Even from parasites collected from a small geographic location at a particular time
relatively little overlap between domains was seen. However, the presence of some very common DBLα1 domains such as the three described in this chapter could be exploited. Of course, sequence data is not enough to provide evidence for cross-reactive epitopes, as cryptic (conformational) epitopes are also possible (Joergensen et al. 2006).

In conclusion, the data presented in this study show a strong association of group A var gene transcription and cerebral malaria. This is consistent with previous work, and provides further evidence for the importance of PfEMP1 in clinical complications due to *P. falciparum* infection. The study was successful due to carefully chosen severe disease criteria and a suitable control group. As with any multifactorial disease, other factors such as household factors (Mackinnon et al. 2005) as well as polymorphisms in human genes such as CR1 (Cockburn et al. 2004) or blood group (Rowe et al. 2007) may also affect or modulate parasite virulence. However, the data strongly suggest that group A PfEMP1 play a critical role in cerebral malaria pathogenesis. As described, these findings have implications for future work on vaccine or drug design against PfEMP1, and increase our understanding of the molecular pathogenesis of parasite genetics contributing to cerebral malaria.
Chapter 4

Var gene transcription in placental malaria field isolates

Some of the results presented in this chapter were published as

4.1 Abstract

*P. falciparum* placental malaria field isolates from Malawi were used to test the hypothesis that *var2csa* and/or conserved DBLγ domains are commonly transcribed in placental malaria field isolates. *Var2csa* transcription was detected in all placental isolates examined by RT-PCR, but not in a control group of childhood malaria isolates also analysed. Many DBLγ domain transcripts were also detected in these placental and childhood isolates, but no DBLγ domain features were found to be specific to placental isolates compared to the childhood isolates through phylogenetic analysis. This data strengthens the growing body of evidence pointing to *var2csa* as crucial for placental malaria pathogenesis, and confirms non-*var2csa* var genes are also expressed in placental field isolates.

4.2 Introduction

Malaria during pregnancy is the most common manifestation of clinical malaria in adults in malaria-endemic countries, where adults are typically immune to clinical disease (Brabin 1993; Brabin *et al.* 2004). Immunity to clinical disease increases through childhood with increasing episodes of disease, probably through cross-reactive immune responses developed from previously employed VSA variants. Parasite-infected erythrocytes depend on sequestration to avoid clearance in the spleen (Chotivanich *et al.* 2002), and once immunity is achieved, parasites have effectively exhausted the supply of novel sequestration receptors. However, upon pregnancy, the placenta becomes a novel niche for parasitic sequestration. As placental sequestration is a novel process in terms of disease episodes for an individual, adhesion is thought to require fresh VSA variants, which are not
expressed in malaria in childhood, and to which there is thus no protective immunity in the first pregnancy. These infected erythrocytes are unusual in their high affinity for chondroitin sulfate A (CSA). CSA is a sulfated glycosaminoglycan, present on the intervillous spaces and on syncitiotrophoblasts, which is where placental adhesion occurs (Achur et al. 2000; Valiyaveettil et al. 2004). Though CSA is also present on other cell types including cerebral endothelium and erythrocytes, CSA is not commonly a ligand for infected erythrocytes in non-placental field isolates. Affinity for CSA has thus provided a starting point for studies on placental malaria-phenotype parasites, and CSA-binding infected erythrocytes have become synonymous with placental malaria-type parasites. Though there is evidence that other adhesion phenotypes also play a minor role in placental sequestration, for example adhesion to hyaluronic acid (HA) (Beeson et al. 2000), CSA appears to be the major sequestration receptor in the placenta (Duffy et al. 2006b; Muthusamy et al. 2007). Some data regarding HA-mediated adhesion has been confused due to the possibility of contaminating CSA in the HA preparations used (Valiyaveettil et al. 2004; Fried et al. 2006).

As with childhood malaria, the PfEMP1 family are key suspects for mediating adhesion during pregnancy. PfEMP1 mediating placental adhesion would be expected to bind CSA, to be transcribed in the majority of placental field isolates and to be expressed exclusively in placental malaria field isolates (not for example in childhood malaria isolates). At the time of the study design, the conserved features which define var gene DBLγ domains were the only var gene sequences associated with expression in placental malaria (Khattab et al. 2001; Lekana Douki et al. 2002;
Costa et al. 2003; Khattab et al. 2003). In particular, transcription of well-conserved gene var1csa which contains 2 DBLγ domains was associated with placental malaria field isolates and with adhesion to CSA (Buffet et al. 1999; Rowe et al. 2002a; Gamain et al. 2004). Other DBLγ-containing var genes have been implicated in placental malaria: CS2var (Reeder et al. 1999) and varPAM732 (Khattab et al. 2001; Badaut et al. 2007). The pan-reactivity of antibodies to DBLγ-containing var genes to placental malaria isolate worldwide suggested a small set of epitopes common to DBLγ domains was responsible for placental sequestration (Lekana Douki et al. 2002; Costa et al. 2003).

It was not logical to suspect all DBLγ domains would be involved in placental malaria, as DBLγ domain variants are common in the group A PfEMP1 which can be expressed in childhood malaria. Involvement of var genes in placental malaria which are also common in childhood infection is inconsistent with the novel placental-specific var gene hypothesis. It was hypothesised that only a subset of DBLγ domains may be involved in placental adhesion. The study thus was originally designed to amplify transcribed DBLγ domains from parasites taken directly from infected placenta, to explore any association of particular sequences with placental sequestration in field isolates. However, since the outset of the study two major advances altered the direction of the chapter.

Firstly, evidence for var1csa involvement in placental malaria was losing momentum. In 2003, Winter et al. reported that var1csa is commonly expressed in childhood isolates, and since then many other field isolate studies (including chapter
3 RT-PCR data) have supported this observation of \textit{var1csa} expression in children and men. If \textit{var1csa} plays a role in placental isolates, it is certainly not the major contributor.

Secondly, there was increasingly convincing evidence for involvement of another strain transcending \textit{var} gene, \textit{var2csa}, in placental malaria (Salanti \textit{et al.} 2003; Salanti \textit{et al.} 2004). VAR2CSA has since been shown to support adhesion to CSA through adhesion and knock out studies (Salanti \textit{et al.} 2003; Salanti \textit{et al.} 2004; Gamain \textit{et al.} 2005; Viebig \textit{et al.} 2005; Viebig \textit{et al.} 2007) and to be expressed in infected erythrocytes in pregnant women from Senegal (Tuikue Ndam \textit{et al.} 2005). Conservation of \textit{var2csa} in the majority of field isolates examined, and in laboratory strains 3D7, HB3 and IT also suggests a strong selective pressure to retain a \textit{var2csa} copy in the genome (Trimnell \textit{et al.} 2006; Kraemer \textit{et al.} 2007). The high conservation of this gene also indicated that it may provide a promising vaccine candidate, to mimic the natural immunity to placental malaria which increases with subsequent pregnancies (Fried \textit{et al.} 1998; Duffy and Fried 2003), if it was proved that \textit{var2csa} was essential for, or at least a common contributor to, placental sequestration.

As it became apparent that VAR2CSA may play a crucial role in placental malaria, the RT-PCR strategy adopted in this chapter was enhanced to also amplify the DBL3X domain of \textit{var2csa} family \textit{var} genes homologues. The primers still retained the ability to also amplify any transcribed DBL\(\gamma\) domains, as the primer sites were designed to recognise an area of homology between \textit{var2csa} and DBL\(\gamma\) domains in
general. It was considered important to also detect DBLγ domain-containing transcribed var genes in the placental isolates firstly as a comparative set of var gene transcripts to var2csa, and secondly as the extent to which other var genes contribute to the placental malaria phenotype is still unclear. The panel of field isolates was also increased to include a range of childhood isolates for comparison.

4.3 Aim of chapter

The aim of this chapter was to test the hypothesis that a conserved var gene, var2csa, and/or conserved DBLγ domains are commonly and specifically transcribed in placental malaria field isolates.

4.4 Materials and methods

Materials and methods that are specific to chapter 4 are listed below. General materials and methods and a list of suppliers are given in chapter 2.

4.4.1 Field isolates

Placental malaria field isolates were obtained from Stephen J. Rogerson, from Blantyre, Malawi (Chapter 2).

4.4.2 RT-PCR conditions

RT-PCR was used to amplify approximately 500 bp of the var gene DBLγ domains, using unbiased primers; 1st set (DBLγ amplification only, designed by S.J. Rogerson, Melbourne, Australia): D3F (CCTCCWAGRAGAMAAAAATTAT) and D3R2 (CCATCKNAR AAATTGNGGY), and second set (DBLγ/var2csaDBL3X
amplification) D3F as above and D3R1.2 (ACAAWANTSNTCDBMCCATTC, designed by J.A. Rowe). Neither of these pairs were nested. Amplification conditions were a hot start, 95°C, 5 min, followed by 40 cycles of 95°C, 20 sec; 42°C, 30 sec; 60°C, 30 sec finishing with 60°C, 10 min. 25 pmol of each primer, 2.5 mM MgCl₂ and 0.125 units Platinum taq (Invitrogen) were used in each 50 µl reaction. 2 µl cDNA or H₂O, or 0.5 µl gDNA were used in the amplification reaction. Samples without RT were used in all reactions to exclude DNA contamination.

4.4.3 Primer bias calculation

The intrinsic bias for D3F and D3R1.2 primer pair had previously been calculated as < 2% for the most frequently detected sequence (S.J. Rogerson, Melbourne). The intrinsic bias for D3F and D3R1.2 primer pair was calculated using excel BIOMONDIST as < 2% for the most frequently detected sequence. The most frequently detected sequence was counted 9 times when only 5 counts would be expected in 37 minipreps. This is a bias of (9/5)^((1/35)) over 35 cycles = 1.017, i.e. a 1.7% bias per cycle.

Details of ligation, cloning, transformation, and sequencing of RT-PCR products are given in chapter 2. In brief, RT-PCR products were visualised by electrophoresis on 1.5% agarose gels. RT-PCR products were purified and ligated into PCR II TA cloning vector (Invitrogen) and ligated plasmids were used to transform TOP10F E. coli. Transformed E. coli was grown overnight under ampicillin selection and blue/white colony screening. Individual white colonies were selected for overnight growth and the plasmids extracted for sequencing. Presence of an insert in the
extracted was verified by EcoRI restriction digestion prior to sequencing. Sequencing was carried out by the University of Edinburgh School of Biological Sciences Sequencing Service (details in chapter 2).

4.4.4 Transcript sequence analysis

Transcripts sequences from each isolate were grouped into contigs of >95% nucleotide identity using the SeqMan programme within DNASTar software package. Consensus sequences from each isolate were saved as sequence A, B, C etc from that isolate as EditSeq files (DNASTar software package), and protein sequences were obtained by translating the DNA sequences using EditSeq translation function. Nucleotide and protein sequences were then compared within and between isolates using MegAlign (DNASTar software package). Alignments and phenograms were produced using the clustal W alignment programme within MegAlign, using default settings. Sequence distances were calculated using MegAlign, and colour-coded into the following groups; 0-30, 30-39.9, 40-49.9, 50-59.9, 60-69.9 70-99.9 and 100% amino acid identity for production of sequence distance figures. Where nucleotide identity levels were not normally distributed, a non-parametric test (Mann-Whitney Test) as used rather than a t-test for comparisons between groups. Homology searches were performed using nucleotide-nucleotide BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Pie charts were created in Excel. Phenograms were laid out in Adobe Illustrator.
4.5 Results 1: Preliminary DBLγ transcription data with primer pair D3F and D3R2

4.5.1 RT-PCR to amplify transcribed DBLγ domains in placental malaria isolates

Six placental malaria field isolates (CS294, P132, P134, P136, P143, P154) were obtained from Queen Elizabeth Central Hospital in Blantyre (gift from Stephen J. Rogerson). These were mature pigmented trophozoites which had been extracted directly from placenta and stored immediately in Trizol. Isolates P132, P136, P143 and P154 had previously been genotyped using PCR of size-variant markers (MSP1, MSP2 and GLURP allelic forms), which revealed that each isolate showed multiple infections; six different parasite genotypes were detected in isolate P132 and three were detected in P136, P143 and P154 (Rowe et al. 2002a). The number of genotypes for P134 and CS294 was not determined.

RNA extraction from each isolate and cDNA preparation had previously been performed by Dr JA Rowe (Rowe et al. 2002a). In addition, cDNA from 2 parasite strains that had previously been panned for affinity to CSA, TM180CSA and TM284CSA was obtained (gift from Dr JA Rowe).

Degenerate primers to amplify the expressed DBLγ domains by RT-PCR were designed by Stephen J. Rogerson; Primers: D3F and D3R2. This primer pair amplifies an approximately 500 bp region of DBLγ domains (Fig. 4.1) and had previously been tested for unbiased recognition of DBLγ domains (Stephen J. Rogerson, personal communication). At this stage the importance of VAR2CSA was
not known, and the focus of research was on involvement of DBLγ domains. Thus these primers did not recognise the DBLX domain, and so could not amplify var2csa (Fig. 4.1; see section 4.7 for primers to also amplify var2csa).

![Primer pairs to amplify DBLγ domains. A Clustal W alignment of 3D7 DBLγ domains and 3D7 var2csa DBL3X (PFL0030c3X) created in MegAlign is shown.](image)
Primers D3F (green), D3R2 (blue) and D3R1.2 (red) are shown. Primer pair D3F/D3R2 recognises DBLγ domains only. Primer pair D3F/D3R1.2 also recognises var2csa (PFL0030c3X) see section 4.7.

### 4.5.2 RT-PCR to amplify transcribed DBLγ domains in placental malaria isolates: primer pair D3F and D3R2

DBLγ domain RT-PCR with primers D3F and D3R2 was carried out on the 6 placental isolates, and also the two parasite strains selected for CSA-affinity, TM180CSA and TM284CSA (Fig. 4.2). CS294, P132, P136, P143, P154 and TM180CSA gave RT-PCR product. P134 and TM284CSA did not give a RT-PCR product.

![Fig. 4.2. 1.5% agarose gel of RT-PCR products from amplification of DBLγ domains from cDNA from Malawi placental field isolates CS294, P132, P143, P136, P143, P154, and laboratory strains TM180CSA and TM284CSA with primers D3F and D3R2. gDNA control (CTL) for each isolate, where the cDNA synthesis reaction is](image)

500 bp
performed without reverse transcriptase, is next to the cDNA amplification. These lanes remain blank indicating there is no gDNA contamination in the cDNA preparation. A4 gDNA was used for a positive control, and distilled water as a negative control. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).

4.5.3 DBLγ domain transcripts detected by RT-PCR with primers D3F and D3R2

DBLγ domain RT-PCR products were purified by gel extraction and PCR products were used to transform E. coli, resulting in plates of colonies carrying plasmids with inserted copies of the DBLγ domains. A random selection of 12-17 of these colonies were picked and grown over night in LB broth at 37°C to allow growth and replication of the bacteria carrying the plasmid. After approximately 18 hours the recombinant plasmids were extracted. Presence of an insert of the correct length was confirmed by enzymatic digestion of the multiple cloning site using restriction enzyme EcoRI of a sample of each extracted plasmid. Digested plasmids were run out on 1.5% agarose gels, and plasmids containing an insert were marked by presence of an approximately 500 bp band. Fig. 4.3 shows a typical gel. Two bands were seen when the insert contained an EcoRI restriction site (GTAAAC, e.g. P143, digests 2, 4 and 7, Fig. 4.3).
Fig. 4.3. A typical agarose gel of EcoRI digests products of plasmids extracted from a set of minipreps. In this case 8 plasmid extractions each from isolates P143 and P154 are shown, all of which had inserts of approximately 500 bp. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).

The sequences obtained from each parasite isolate were compared for multiple copies of the same sequence (over 95% sequence identity), and defined as placental (Pl) var gene A, B, C… from each isolate. Up to five sequences were detected per isolate though the presence of more var gene transcripts cannot be excluded. Results were displayed as pie charts to indicate the relative abundance of distinct transcribed DBLγ domains (Fig. 4.4).
Fig. 4.4. Frequencies of distinct DBLγ sequences in each isolate. The pie charts represent the relative number of different DBLα sequences detected in each isolate by RT-PCR, cloning and analysis of 12-17 mini-prep clones per isolate. Each pie represents a different isolate, and the size of each pie-slice represents relative abundance of transcript. Isolate name given in bold and bracketed numbers indicate the exact number of plasmids sequenced for each isolate. Domains with homology to var1csa are shaded (3D7 var1csa DBL3γ; yellow. 3D7 var1csa DBL5γ; purple. FCR3 var1csa DBL3γ; blue).

All of the placental field isolates showed a predominant var gene. The predominant gene in three of the five isolates (CS294, P132 and P136) was almost identical (>97% homology). Nucleotide sequence comparisons revealed these sequences were highly homologous (>92% identity) to the DBL3γ of 3D7 var1csa (yellow in Fig. 4.4). Sequences homologues to the DBL5γ of 3D7 var1csa were also seen (CS294P1varD, P143P1varB and P132P1varB, > 87% homology to 3D7 var1csa.
DBL5γ, purple in Fig. 4.4). The DBL3γ of FCR3 var1csa is slightly different to that of 3D7 (Rowe et al. 2002a), and the predominant gene of P143 was a FCR3-like var1csa homologue (97% homology, blue in Fig. 4.4).

Var1csa is a highly conserved sequence (Khattab et al. 2001), and has previously been reported in many placental isolates worldwide, including from Cameroon (Fried and Duffy 2002), Kenya (Khattab et al. 2003) and from Gabon (Kyes et al. 2003), but is also seen in childhood malaria field isolates (Winter et al. 2003, and discussed in chapter 3). The finding of var1csa in these placental isolates, collected as trophozoites and schizonts, is unsurprising as var1csa has an unusual transcription profile, being expressed at trophozoite stage (Kyes et al. 2003), rather than at ring stage, as for other var genes (Khattab et al. 2001; Salanti et al. 2004).

4.5.4 Sequence similarities between transcripts detected by RT-PCR with primers D3F and D3R2

Sequence identity between domains was 27.8-100% (mean 47.7%, s.d. 13.6%). Two sequences P154PlvarA and CS294PlvarB were identical, as were CS294PlvarA and P132PlvarB the var1csa DBL3γ homologues discussed above.

4.5.5 Phylogenetic analysis of transcript sequences detected by RT-PCR with primers D3F and D3R2

A phenogram was constructed to compare the DNA sequences from Malawi (red) and from laboratory CSA-selected strain TM180CSA (blue), with published DBLγ mRNA sequences from pregnancy-associated malaria in Gabon, Cameroon and
Kenya, and also with var1csa gene DBL3γ and DBL5γ domains from 3D7 and FCR3 (Beeson et al. 1999; Buffet et al. 1999; Reeder et al. 1999; Reeder et al. 2000; Khattab et al. 2001; Fried and Duffy 2002; Khattab et al. 2003; Salanti et al. 2003; Gamain et al. 2005). This was intended to examine the extent of the similarities between the DNA sequences, rather than to suggest phylogenetic evolution, and to display how the Malawian sequences were distributed in relation to DBLγ domains from other placental isolates. The predominantly expressed var gene from each of the isolates was marked with an arrow (←) (Fig. 4.5).

The Malawian sequences were distributed throughout the extent of the tree. There is no marked distinction between the different groups of sequences dependent on country of origin (Fig. 4.5).
Fig. 4.5. Phenogram displaying transcribed DBLγ domains in *P. falciparum* placental field isolates from Malawi (this study; red) amplified with primers D3F and D3R2, with transcribed DBLγ domains in *P. falciparum* placental field isolates from Cameroon (498, 482, 732 and 720/734), Kenya (Plcx) and Gabon (Gbx). Transcribed DBLγ domains from CSA-binding laboratory strain TM180CSA (this study: blue) and the DBLγ domains of *var1csa* (FCR3 and 3D7; shaded in yellow) and *CS2var* (shaded in blue) are also included. The predominant transcript from each Malawi placental isolate and TM180CSA is marked with an arrow (↩).
4.5.6 Limitation of transcription analysis using D3F and D3R2

Literature at the time of this study was emerging which strongly implicated var2csa as a (if not “the”) major CSA-binding PfEMP1 (Salanti et al. 2003; Salanti et al. 2004; Tuikue Ndam et al. 2005; Viebig et al. 2005; Beeson et al. 2006). Unfortunately, var2csa could not be amplified with this primer pair, which became an obvious limitation of the study. The DBL3X domain of var2csa, which has been shown to support CSA binding (Gamain et al. 2005; Dahlback et al. 2006) has similarity to a DBLγ domain, and it was possible to design a primer pair to extend the range of DBL domains amplified to include the var2csa DBL3-X domain, without losing specificity for PfEMP1 DBLγ domains (section 4.6). It was decided to proceed with a second primer pair which would include var2csa in the RT-PCR analysis, rather than to extend the analysis with primer pair D3F and D3R2. Unfortunately, this meant that DBLγ domains from non-placental malaria isolates were not studied with D3F and D3R2.

4.6 Results 2: DBLγ/var2csa transcription analysis using a second set of primers D3F and D3R1.2

4.6.1 PCR on 3D7 genomic DNA to determine bias of a second set of primers D3F and D3R1.2

A second set of degenerate primers was designed (Dr JA Rowe) to amplify var2csa DBL3X domains as well as PfEMP1 DBLγ domains (D3F and D3R1.2; Fig. 4.1 and 4.6).
Fig. 4.6. Primer sites of D3F and D3R1.2 on a clustal W alignment of 3D7 DBLγ domains and var2csa DBL3X (PFL0030c 3X).

The degree of bias in this primer pair (D3F and D3R1.2) was determined by amplification of 3D7 genomic DNA. 3D7 genomic DNA was amplified with D3F and D3R1.2. The PCR products were cloned and 38 recombinant plasmids were sequenced. 12/16 gamma domains were represented in the sequences from the plasmids from 38 minipreps (Fig. 4.7). The primers showed acceptable primer bias (<2%) of amplification of 3D7 DBLγ domains (Gardner et al. 2002), in line with other published degenerate primers (Taylor et al. 2000a; Duffy et al. 2006a). Var2csa (PFL0030c 3X) was amplified with average frequency (Fig. 4.6.)
Fig. 4.7. Amplification of 3D7 gDNA using primer pair D3F and D3R1.2. Bars indicated frequency of amplification of the DBLγ domain/var2csaDBL3X transcripts. Black line shows expected binomial distribution of frequencies as a polynomial trendline. This shows a bias of <2% for the most frequently detected sequence.

4.6.2 DBLγ/var2csa DBL3X transcript sequences detected in Malawian placental malaria field isolates by RT-PCR with primers D3F and D3R1.2

RT-PCR was carried out with the new primer pair D3F and D3R1.2 for the six placental isolates CS294, P132, P134, P136, P143, P154. Transcripts from TM180CSA and TM284 were not amplified as the focus of the study was transcription in placental isolates not laboratory strains. RT-PCR products were obtained for all six isolates and were purified, ligated and cloned as before. 11-19 colonies were picked at random for growth overnight in LB broth, and the plasmids were extracted as before for sequencing.
The sequences obtained with primer pair D3F and D3R1.2 from parasite isolates CS294, P132, P134, P136, P143, P154 were compared for multiple copies of the same sequence (over 95% sequence identity), and again defined as placental (Pl) var gene A-H from each isolate. Pie charts were then created to show frequency of different domains for the D3F/D3R1.2 primer pair (Fig. 4.8). *Var1csa* was present in 5/6 isolates with these primers (shaded in yellow, Fig. 4.8). All of the parasite isolates expressed at least one *var* gene with strong homology to *var2csa* (shaded in grey, Fig. 4.8).

Fig. 4.8. Proportions of DBLγ/var2csa domains in 6 placental malaria isolates. Pie charts represent the relative number of different DBLγ/X sequences as detected by RT-PCR. Bracketed number is the number of cloned sequences for the isolate, and the isolate name is shown in bold. Sequences with homology to *var2csa* are shaded in grey, and with homology to *var1csa* in yellow.
Some sequences amplified with D3F and D3R2 were amplified again D3F and D3R1.2 (Fig. 4.9). Where a sequence was amplified that had already been identified from the first amplification, the original name was kept, e.g. P136PlvarA was amplified with both primer sets. Though some of the same sequences were amplified, the predominant transcript with D3F/D3R2 was not predominant when amplified with D3F/D3R1.2 in any isolate. With these primers, a wider range of domains was amplified, and a predominant gene was less clear.
Fig. 4.9. DBLγ/var2csa RT-PCR on placental isolates P132, P134, P136, P143, P154 and CS294. Both amplifications used forward primer D3F. Y-axis shows frequency of RT-PCR products relating to transcripts A, B, C etc for each isolate with products with reverse primer D3R2 (striped) and D3R1.2 (black). There was no RT-PCR product for P134 with primer pair D3F/D3R2.
4.6.3 Phylogenetic analysis of transcript sequences detected by RT-PCR with primers D3F and D3R1.2

A phenogram was constructed to give an indication of sequence similarity of the var genes expressed from the difference isolates from the second set of primers, D3F and D3R1.2 (Fig. 4.10), and together sequences amplified from D3F and D3R2 primer pair (Fig. 4.11) with the published sequences from Gabon, Cameroon and Kenya, and also with var1csa DBL3γ and DBL5γ domains from 3D7 and FCR3 and var2csa DBL3X domain from 3D7 (Beeson et al. 1999; Buffet et al. 1999; Reeder et al. 1999; Reeder et al. 2000; Khattab et al. 2001; Fried and Duffy 2002; Khattab et al. 2003; Salanti et al. 2003; Gamain et al. 2005).

Fig. 4.10 and Fig. 4.11 both highlight the striking clustering of some transcripts from the placental malaria isolates discussed in this chapter around var2csa DBL3X. Only sequences from the second set of primers, D3F and D3R1.2 cluster here, due to primer specificity. The non-var2csa transcripts amplified with D3F and D3R1.2 are spread evenly throughout the rest of the tree. There was no obvious separation of transcripts from the two different primer sets, or from the Malawian isolates examined here compared to the published sequences.
Fig. 4.10. Phenogram showing expressed DBLγ sequences from *P. falciparum* isolates of placental malaria from females in Malawi (red), alongside the DBLγ domains of *var1csa* (FCR3varCSA 3/5γ and 3D7Chr5var 3/5γ, yellow), and the DBL-3X domain of *var2csa* (pink). The phenogram was created using a clustal W alignment of amino acid sequences.
Fig. 4.11. Phenogram displaying transcribed DBL\(\gamma\) domains in *P. falciparum* placental field isolates from Malawi (this study) in relation to other DBL\(\gamma\) domains. Red: transcribed DBL\(\gamma\) domains in *P. falciparum* placental field isolates from Malawi amplified with D3F and D3R1.2. Blue: transcribed DBL\(\gamma\) domains in *P. falciparum* placental field isolates from Malawi amplified with primers D3F and D3R2 (some of these were also amplified with D3F and D3R1.2) and transcribed *Var2csa* DBL3X homologues.
DBLγ domains from CSA-binding laboratory strain TM180CSA with primers D3F and D3R2. Black: transcribed DBLγ domains in *P. falciparum* placental field isolates from Cameroon (498,482,732 and 720/734), Kenya (Plcx) and Gabon (Gbx). The DBLγ domains of var1csa (FCR3 and 3D7; shaded in yellow), the DBLγ domain of CS2var (shaded in blue) and the DBL3X domain of var2csa (shaded in pink) are also included.

### 4.6.4 DBLγ domain RT-PCR of childhood isolates using primers D3F and D3R1.2

It is not possible to assess whether var2csa transcription is specific for placental malaria field isolates without comparing the placental transcription pattern to that of non-pregnant individuals, such as non-pregnant women, men or children. The childhood malaria samples described in chapter 3 were available and so these were used for a comparison. RT-PCR, cloning and sequencing were then used to create transcription profiles for a subset of the childhood isolates examined for DBLα expression (Chapter 3). Five cerebral and five uncomplicated isolates were chosen at random.

As with the DBLα domains, a range of transcripts were detected per isolate. There was no significant difference in the number of sequences detected within the three disease categories (Kruskal Wallis test, P=0.48), and a predominant DBLγ domain was seen in most isolates (Fig. 4.12). Var1csa transcripts were detected in 8/10 childhood isolates; 4/5 cerebral isolates and 4/5 uncomplicated malaria isolates (yellow in Fig. 4.12). Var2csa transcripts were not detected in any childhood isolate
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Fig. 4.12. Proportions of DBLγ/var2csa domains in 5 isolates from 3 disease categories; uncomplicated childhood malaria, cerebral childhood malaria, and placental malaria. Pie charts represent the relative number of different DBLγ/X sequences as detected by RT-PCR with D3F and D3R1.2. Bracketed number is the number of cloned sequences for the isolate, and the isolate name is shown in bold. Sequences with homology to var2csa are shaded in grey, and with homology to var1csa in yellow.
A phenogram was constructed, incorporating all sequences amplified from both the primer pairs from all 16 isolates (Fig. 4.13). There was no difference in distribution of the cerebral versus uncomplicated malaria DBLγ sequences. However, there was a very marked clustering of placental sequences around var2csa DBL3X domain, as var2csa is present at a high abundance in placental isolates from Malawi, but is not present in any of the childhood isolates from Mali. Using only sequences amplified from the second primer pair did not alter the overall shape of the tree (data not shown).
Var2csa DBL3X homologues
Fig. 4.13. Phenogram displaying transcribed DBLγ/var2csa transcripts in *P. falciparum* placental field isolates from *P. falciparum* isolates of childhood cerebral (purple) and uncomplicated (green) malaria from Mali and from placental isolates from Malawi (red) amplified with primers D3F and D3R21.2. Transcribed DBLγ domains in *P. falciparum* placental field isolates from Cameroon (498,482,732 and 720/734), Kenya (Plcx) and Gabon (Gbx), and from CSA-binding laboratory strain TM180CSA (blue), the DBLγ domains of var1csa (FCR3 and 3D7; yellow) and CS2var (blue) and the DBL3X domain of var2csa (pink) are also included. Var2csa DBL1X and DBL2X are also included for reference, but are in grey as they could not be amplified by these primers. Phenogram was constructed from a clustal W alignment of amino acid sequences.

A phenogram only including the childhood DBLγ transcripts amplified from the second primer pair from all 16 isolates (Fig. 4.14) also shows clearly there was no difference in distribution of the cerebral versus uncomplicated DBLγ sequences.

Unfortunately there was not enough parasite material to perform real-time RT-PCR for these samples, which would have been useful to quantify relative amounts of *var* gene transcripts detected in these isolates.
Fig. 4.14. Phenogram displaying transcribed DBLγ/var2csa transcripts in *P. falciparum* placental field isolates from *P. falciparum* isolates of childhood cerebral (purple) and uncomplicated (green) malaria from Mali amplified with primers D3F and D3R1.2. Phenogram constructed from a clustal W alignment of amino acid sequences.
4.7 DBL\(\gamma\) transcript analysis

4.7.1 Sequence diversity of DBL\(\gamma\) domain transcripts

The level of amino acid identity between different DBL\(\gamma\) sequence domain tags was calculated. Amino acid identity of the DBL\(\gamma\) transcripts separated by disease category indicates a significantly higher level of amino acid in the placental malaria group (median 33.1\%) than in childhood malaria (median 30.1\% Mann-Whitney Test <0.001) as shown by box plots and histograms (Fig. 4.15). The level of amino acid identity between different Malawi DBL\(\gamma\) sequence domain tags was calculated and also illustrated pictorially (Fig. 4.16). Percentage amino acid identity between 2 DBL\(\gamma\)/\textit{var2csa} domains is colour coded such that darker shading reflects higher amino acid identity.

\textit{Var2csa} homologues are comparatively highly conserved and the \textit{var2csa} transcripts amplified in these isolates shared 64-96\% nucleotide identity, median 88.3\% (Fig. 4.15 and 4.16). Removing the \textit{var2csa} homologues decreased the similarity between placental isolates sequences (median 29.5\%) though this act did not significantly change the distribution of sequence identities (Mann-Whitney test, placental “all sequences” versus placental “non-\textit{var2csa} sequences”, P=0.358). The was still a significant difference between placental DBL\(\gamma\) (non-\textit{var2csa}) and childhood DBL\(\gamma\) sequences (Mann-Whitney test P=0.019).

\textit{Var2csa} homologues were significantly more similar to each other than the DBL\(\gamma\) transcripts from childhood of placental malaria isolates both Mann-Whitney Test P<0.001).
Fig. 4.15. Histograms and box plots of percentage amino acid identity between all DBLγ domain transcripts from placental, childhood, placental (var2csa only) and placental (non-var2csa only) DBLγ transcripts. Box plots show 10th, 25th, 50th, 75th and 90th percentiles; values <10th percentile and >90th plotted separately.
Fig. 4.16. Amino acid sequence distance charts showing percentage amino acid identity between domains for DBLγ/var2csa domains. A) Placental malaria, cerebral malaria, uncomplicated malaria, and all childhood isolate DBLγ/var2csa sequences. B) Placental var2csa homologues and non-var2csa homologues separately. Darkness of shading indicates level of identity between domains.
Although relatively highly conserved, the var2csa homologues retain some polymorphicity. Variability between the var2csa homologues is concentrated towards the end of the amplified tag, with a relatively more conserved region in the middle (alignment amino acids 78-122; Fig. 4.17).

Fig. 4.17. Alignment of var2csa in this study and var2csa DBL3X from FCR3 and 3D7, produced from a clustal W alignment of protein sequences.
4.7.2 Conservation of DBLγ domain transcripts detected

A BLAST homology search performed for sequences not homologous to *var1csa* or *var2csa* on the NCBI database, indicated no other highly conserved DBLγ were present (Table 4.1). There is much less similarity between DBLγ domains than DBLα domains (Table 3.3, chapter 3) though this may reflect the fewer DBLγ domains on NCBI database, see discussion.
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Table 4.1. Homology of Malawi isolate non-<i>vass</i>csa or var<i>2</i>csa DBLγ transcripts described in this study compared to sequences on NCBI database. Database matches > 85% identity, >85% sequence coverage, http://www.ncbi.nlm.nih.gov/BLAST are also described (accession number, origin, and % nucleotide identity). - no database matches were found. <sup>1</sup>Khattab <i>et al.</i> 2003, <sup>2</sup>Kraemer <i>et al.</i> 2007, <sup>3</sup>Gardner <i>et al.</i> 2002, *This study/(Duffy <i>et al.</i> 2006a).
4.8 Analysis of DBLα domain transcripts detected in placental isolates

P132, P136, P143, P154 and CS294, from Rowe et al. (2002a)

As most DBLγ are from Group A or B/A var (12/15 in 3D7, 12/18 in HB3 and 12/25 in IT) amplification of DBLγ domains in this study was expected to result in detection predominantly of group A or B/A var gene transcripts. If the 3D7 gene repertoire is typical, DBLγ domain amplification should detect 91% of non-type 3 group A or B/A var genes, but only 7% of group B, C or B/C var genes. If the HB3 or IT genomes are more typical, more group B,C or B/C var genes would be amplified by DBLγ domain RT-PCR.

To examine the relative proportions of group A and B/C var expressed in these isolates, DBLα(0/1) RTPCR transcription data on isolates P132, P136, P143, P154 and CS294, which already been published (Rowe et al. 2002a) was reanalysed. This paper had used the primers from Taylor et al. (2000a) and the method described in chapter 3. The DBLα domain transcripts were downloaded from NCBI database and classified as DBLα0 or DBLα1, on the basis of number of cysteines in the amplified tag (Table 1.1). Pie charts were created to show the relative proportions of the two subtypes of DBLα domain (DBLα0 shaded black, DBLα1 in white; Fig. 4.18). Var1csa homologues are indicated (yellow). This revealed higher DBLα0 than DBLα1 transcription in these placental isolates. Only 3 non-var1csa DBLα1 transcripts were detected.
Fig. 4.18. DBLα domain RT-PCR data on isolates P132, P136, P143, P154 and CS294 taken from Rowe et al. (2002). Pie charts indicate relative abundance of DBLα domains (DBLα0 grey, DBLα1 white, var1csa homologues in yellow).

4.9 Discussion

The results presented in this chapter highlight the exclusive nature of transcription of a particular var gene, var2csa, in placental malaria isolates. This study of clinical isolates supports the growing body of evidence from studies of field and laboratory strains of P. falciparum advocating VAR2CSA as a major contributor to the opportunistic placental sequestration that commonly occurs during pregnancy in malaria-endemic areas. The data obtained will be discussed alongside current developments concerning the role of VAR2CSA in placental malaria.

In this study, transcription of at least one var2csa homologue was detected by RT-PCR in all six Malawian placental isolates available, but in none of the 10 Malian childhood isolates also examined. Other recent studies have also found var2CSA transcripts to be present in the majority of placental isolates but rarely detected in childhood or non-placental isolates, confirming this strong association specifically
with placental malaria (Salanti et al. 2004; Tuikue Ndam et al. 2005; Duffy et al. 2006a; Francis et al. 2007; Fried et al. 2007). Furthermore, VAR2CSA PfEMP1 protein is recognised by natural antibodies from endemic areas in a sex-specific and parity-dependent manner (Salanti et al. 2003; Salanti et al. 2004; Tuikue Ndam et al. 2006), again emphasising the link between var2csa and pregnancy reported in this study.

Placental isolates possess a unique binding phenotype with a consistently high affinity for CSA (Fried and Duffy 1996; Fried et al. 2006; Fried et al. 2007; Muthusamy et al. 2007). This is consistent with expression of VAR2CSA as VAR2CSA binds to CSA when expressed on the surface of an infected erythrocyte and is consistently upregulated in laboratory isolates after selection for CSA-affinity (Salanti et al. 2003; Avril et al. 2004; Salanti et al. 2004; Gamain et al. 2005; Duffy et al. 2006b; Beeson et al. 2007a; Francis et al. 2007; Fried et al. 2007). In certain strains var2csa disruption irreversibly ablates CSA-affinity, suggesting this protein mediates the majority of CSA-binding (Gamain et al. 2005; Viebig et al. 2005; Duffy et al. 2006b). Most field isolates are predicted to contain a var2csa homologue (Trimnell et al. 2006). If all var2csa homologues retain affinity for CSA, then it is likely that all parasite isolates have the potential to cause placental malaria.

The relative sequence homology between the VAR2CSA DBL3X domains identified in the placental field isolates in this study was 64-96%, similar to the range of homology published in Trimnell et al. 2006 (54-94%). Var2csa homologues are conserved by var gene standards, composed of blocks of highly conserved stretches,
separated by regions of high interclonal diversity (Beeson et al. 2006; Duffy et al. 2006a; Trimmell et al. 2006; Barfod et al. 2007; Bockhorst et al. 2007). Presence of a var2csa homologue in *P. reichenowii* suggests a strong evolutionary advantage for CSA-binding ability (Trimmell et al. 2006; Bockhorst et al. 2007). Var2CSA is under a high level of diversifying selection which appears to be driven by immune pressure (Dahlback et al. 2006; Barfod et al. 2007; Bockhorst et al. 2007). Whether all of the VAR2CSA homologues retain CSA-affinity is not obvious from the sequence.

None of the childhood isolates in this study showed evidence of *var2csa* transcription, consistent with a role for *var2csa* specifically in placental malaria. In other studies, *var2csa* homologues have infrequently been detected in non-pregnant individuals (Duffy et al. 2006a; Francis et al. 2007), though these do not always appear to have the characteristic CSA-binding ability (Fried et al. 2007). Low levels of antibodies with reactivity to VAR2CSA have also recently been detected in plasma from some men and children from various malaria-endemic areas (Beeson et al. 2007b). It may be that these VAR2CSA have a subtly different structure resulting in a different binding phenotype.

CSA is present in other parts of the body, including on the cerebral vasculature (Robert et al. 1995). However, VAR2CSA does not appear to be involved in any other sequestration, such as during cerebral malaria, and *var2csa* was not seen in these cerebral malaria isolates. CSA is hugely heterogeneous, composed of tandem repeats of glucuronic acid and N-acetylgalactosamine-4-sulfate, and subtleties in the structure of CSA in the placenta may be critical for adhesion here. Infected
erythrocytes adhere most efficiently to CSA in a low sulfation state, and this form is common in the intervillous spaces of the placenta (Achur et al. 2000). The origin of CSA in the placenta is foetal, not maternal (Muthusamy et al. 2007), possibly presenting a slightly different binding site for PfEMP1 interaction than maternal-derived CSA, for example on host erythrocytes, and providing the specificity for CSA-adhesion only in the placenta.

Upregulation of CSA upon infection may also be important; Muthusamy et al. (2007) observed infected placentas had higher CSA levels than uninfected placentas. It is also possible that CSA-binding has another function other than as a receptor for sequestration. For example CSA-binding placental parasites have recently been shown to escape non-opsonic phagocytosis compared to CD36-binding parasites (Serghides et al. 2006), though this may be due to loss of CD36-binding rather than gain of CSA-affinity. As placental CSA may be unique in structure, using placental cryosections (Avril et al. 2004) rather than bovine CSA (Fried and Duffy 1996; Ricke et al. 2000) for in vitro adhesion studies may give a more accurate reflection of clinically important in vivo interactions.

The childhood isolates used for comparison in this study were from Mali not Malawi and so have a different geographic origin from the placental isolates. However, the var gene repertoire of isolates in Mali appears to be typical of isolates across Africa and the world (chapter 3), and var2csa expression is seen in placental malaria isolates worldwide (Salanti et al. 2003; Avril et al. 2004; Salanti et al. 2004; Gamain et al. 2005; Duffy et al. 2006a; Beeson et al. 2007a; Francis et al. 2007). Ideally,
isolates from men or non-pregnant women, matched for age, geographic location and socioeconomic group would have been used for comparison. It would also have been interesting to also examine DBLγ transcription in the hyperparasitaemia isolates described in chapter 3 for comparison.

As var2csa has been associated with placental malaria, this study reveals expression of a perhaps surprisingly high number of non-var2csa var genes in placental isolates. The parasites studied were expelled directly from the placenta, raising the question whether these PfEMP1 were also involved in placental sequestration, alongside VAR2CSA. If this were the case it might be possible to detect a particularly “placental” sequence or motif. Antibodies to the DBL3γ domain of FCR3 VAR1CSA recognise 40-60% of placental isolates and block CSA adhesion, suggesting cross-reacting DBLγ domain epitopes are commonly expressed in placental malaria isolates (Lekana Douki et al. 2002; Costa et al. 2003). However, there was no particular clustering in the phenogram of placental isolate DBLγ transcript sequences compared to those from childhood malaria isolates. Furthermore, comparison to sequences on the NCBI database suggested there is not anything particularly “placental” about the DBLγ transcripts detected in the placental isolates in this study compared to the overall var gene repertoire. Of the 9 transcripts with highly homologous database matches, 4 matches were DBLγ transcripts, all from placental malaria isolates. No database matches to childhood malaria isolate transcripts were found. However, this may be misleading as DBLγ domains are most commonly studied in relation to placental malaria, and this may be reflected in the DBLγ RNA sequences represented in the NCBI database. Alternatively, some var
gene transcripts detected in these placental isolates may simply be from an incidental sub-symptomatic chronic infection that had expanded due to pregnancy-associated immunomodulation (reviewed in Ndam and Deloron 2007), and have no direct role in placental sequestration.

The only DBLγ domain transcript seen in multiple isolates was var1csa, detected in 5 of the 6 placental isolates examined here. Var1CSA is commonly detected in placental isolates including those from Cameroon (Khattab et al. 2001), Kenya (Fried and Duffy 2002) and Gabon (Khattab et al. 2003), and VAR1CSA can mediate CSA binding (Buffet et al. 1999; Rowe et al. 2002a; Gamain et al. 2004; Badaut et al. 2007), though the in vivo relevance of this interaction in infection is unclear. Var1csa disruption in laboratory strain FCR3 was reported to knock out CSA-binding (Andrews et al. 2003), but CSA-affinity was regained as the parasite switched on var2csa (Gamain et al. 2005). The mechanism of VAR1CSA-mediated binding to CSA appears to be very similar to that of VAR2CSA. The minimal (67 amino acid) region of FCR3 VAR1CSA required for CSA binding has high homology to the CSA-binding domain of VAR2CSA (Gamain et al. 2004; Gamain et al. 2005), with cross-reacting epitopes (Bir et al. 2006), suggesting a common mechanism of adhesion, and overlapping binding sites. It might thus be expected that var1csa expression in an infection would elicit an antibody response which could be protective against VAR2CSA subsequent infections. However, as var1csa is commonly expressed in non-pregnant and childhood isolates (Fried and Duffy 2002; Rowe et al. 2002a; Winter et al. 2003), including 8/10 childhood isolates in this study, and incidents of placental malaria are high with estimates of infection in up
two-thirds of pregnancies in malaria-endemic sub-Saharan countries (Broen et al. 2007), it seems unlikely a protective antibody repertoire is created and maintained.

It is possible the association of var1csa, transcribed at trophozoite stage (Kyes et al. 2003), with placental isolates is a result of studying mature parasites extracted directly from the placental at delivery in this and many other studies (Khattab et al. 2001; Fried and Duffy 2002; Khattab et al. 2003; Duffy et al. 2006a). To avoid this, trophozoite parasites could be cultured to ring stage before RNA extraction. However, culturing risks var gene switching, as discussed in chapter 3. Alternatively, peripheral ring stage parasites could be studied. Peripheral ring stage parasites matured in vitro to pigmented-trophozoite stage tend to show the same binding phenotype as mature trophozoite parasites collected directly from the placenta from the same isolate (Francis et al. 2007). This suggests the parasites are from the same population, as observed in comparisons of sequestered and peripheral parasite populations in childhood isolates (Dembo et al. 2006; Montgomery et al. 2006). Whichever parasites are studied, it is clear var1csa expression is neither necessary nor sufficient for placental sequestration of infected erythrocytes.

In addition to var1csa and var2csa, other PfEMP1 are capable of binding to CSA and may prove to be conserved mediators of placental sequestration. Distinct DBLγ domains with affinity for CSA have been identified from placental isolates (Khattab et al. 2001; Khattab et al. 2003), including the semi-conserved varPAM732 which specifically binds to immobilised CSA (Chia et al. 2005), and to CSA on placental cryosections (Badaut et al. 2007). CS2var also has affinity for CSA (Reeder et al. 2006).
Expression of CS2var rescued the CSA-binding phenotype of a var2csa knock-out parasite (Duffy et al. 2006b) and a placental isolate from Malawi expressed CS2var as the major transcript (Duffy et al. 2006a). However, CS2var is not commonly conserved between isolates nor expressed at higher levels in placental isolates compared to non-pregnant hosts, so a crucial role in placental malaria is unlikely (reviewed in (Rowe and Kyes 2004)). Neither CS2var nor varPAM732 DBLγ domains transcripts were seen in these placental isolates.

As most DBLγ are from group A or B/A var, amplification of DBLγ domains in this study was expected to result in detection predominantly of group A or B/A var gene transcripts. Examining mainly group A and B/A var genes may be a limitation of the study, as most group B and C var genes would have been overlooked. This prompted analysis of DBLα(0/1) RTPCR data using the primers described in chapter 3 (data from Rowe et al. 2002) revealed higher DBLα0 than DBLα1 transcription in these placental isolates. The low level of transcription of DBLα1 domains in these isolates is consistent with the association of DBLα1 domains/group A PfEMP1 with severe childhood disease, and young immunologically naive hosts.

As syncitiotrophoblasts do not express CD36 (Sartelet et al. 2000), and placental isolates do not tend to show affinity for CD36 (Fried et al. 2007; Muthusamy et al. 2007), group B, C or B/C PfEMP1 expression was not expected. The high frequency of DBLα0 domains in Rowe et al. 2002 is thus surprising. If the association of DBLγ and group A (DBLα1 domains) is true for field isolates, the DBLα0 domains amplified here are unlikely to ‘belong’ to the DBLγ domain transcripts detected in
this chapter. The high presence of DBLα0 domains suggests even more non-\textit{var2csa} \textit{var} genes are being transcribed in these field isolates than would be predicted from the DBLγ analysis alone. Unfortunately, these primers cannot recognise \textit{var2csa}, and so comparisons of abundance of DBLα(0/1) and \textit{var2csa} transcription would not be possible unless quantitative real-time RT-PCR analysis was performed.

Results in chapter 3 showed cerebral isolates expressed a higher proportion of group A or B/A \textit{var} genes than uncomplicated isolates. In this analysis, no difference was seen in the number or clustering of DBLγ/\textit{var2csa} domain transcripts in the cerebral and uncomplicated childhood malaria groups. However, there is no data on the relative levels of transcription in the different isolates. Though a similar level of diversity of DBLγ transcripts per isolate was detected, this does not necessarily equate to a similar level of overall DBLγ transcription as non DBLγ-containing \textit{var} genes are neglected. This data does not therefore conflict with the results of chapter 3.

Non-\textit{var2csa} transcripts were found in each placental isolate, with no detectably “placental” features. There is no reason to assume they have affinity for CSA. However, they may bring about placental sequestration through other interactions. Binding to hyaluronic acid has previously been implicated in placental malaria (Beeson \textit{et al.} 2000). Although subsequent studies have shown this interaction is not as important as CSA interaction (Muthusamy \textit{et al.} 2007), it may still have a minor role. Binding to IgM is a characteristic of many placental isolates (Creasey \textit{et al.} 2003), and can be mediated by PfEMP1 including \textit{var1CSA} and \textit{var2csa} (Semblat \textit{et
IgG binding may also be involved (Flick et al. 2001). ICAM1 also appears to be upregulated in placenta upon *P. falciparum* infection, (Sartelet et al. 2000) which provide a target for adhesion via certain PfEMP1 (Smith et al. 2000a; Springer et al. 2004). The complete binding phenotype of placental parasites is probably still not fully understood.

The clear association of *var2csa* with placental malaria have inevitably led research towards the development of a vaccine against placental malaria, though unfortunately this challenge has so far proved difficult. Assessments of the cross-reactivity of anti-*var2csa* protection are conflicting (Elliott et al. 2005; Beeson et al. 2006; Barfod et al. 2007) and the true level of involvement of other PfEMP1, or other proteins, is unclear. Recently a number of non-PfEMP1 proteins have been associated with placental malaria (Francis et al. 2007; Fried et al. 2007; Gowda et al. 2007; Mok et al. 2007). If other proteins have the potential to cause placental sequestration then a placental malaria vaccine directed exclusively at *var2csa* is unlikely to be successful. Seeking to inhibit parasite protein-mediated adhesion to host cells is not a problem confined to placental malaria. Rosetting, a phenomenon association with malaria severity in Africa (Carlson et al. 1990; Rowe et al. 1995; Rowe et al. 2002b) is brought about through interactions via many different PfEMP1 with uninfected erythrocytes. In the case of rosetting, interactions with host erythrocyte ligands via many different PfEMP1 can be universally inhibited by certain glycosaminoglycan drugs, rather than trying to target each interaction independently (Havlik et al. 2005; Kyriacou et al. 2007), see chapter 6 for details of curdlan sulfate as a rosette inhibiting drug. A similarly broad approach could be
useful in the case of placental malaria of trying to protect the placenta against a wide range of different mechanisms of placental-sequestration, rather than focussing on a single interaction, although this may not be feasible.

In conclusion, the data presented in this chapter are indicative of var2csa being important in placental malaria, an association which has become increasingly convincing in the light of recent supporting studies. In the case of placental malaria a clearer picture of var gene involvement has emerged than in childhood cerebral malaria, with one specific var gene rather than a whole subgroup being involved. This reflects a simpler mechanism of sequestration, whereby a single var gene has high affinity for a novel ligand. It follows that defining conserved sequences that contribute specifically to placental malaria is pivotal in the development of effective drugs and vaccines to combat this disease.
Chapter 5

Type 3 var genes and PfEMP1
5.1 Abstract

This chapter examined type 3 var gene transcription in laboratory strains and field isolates. Type 3 var genes are group A var genes, and it was hypothesised that their transcription would be associated with cerebral malaria, following the link between other group A var genes and cerebral malaria discussed in chapter 3. Type 3 var gene transcripts were detected in the majority (23/26) of field isolates examined, and were highly conserved. Transcription of the type 3 var genes was not associated with cerebral malaria, however, there was an increased diversity of transcripts in the hyperparasitaemia isolates compared to cerebral malaria isolates, possibly suggesting a higher frequency of type 3 var gene transcription. Phylogenetic analysis of the type 3 var gene sequences obtained revealed 6 highly conserved subgroups of type 3 DBLα domain. Type 3 DBLε domains were highly homologous across the entire length of the amplified tag. Transcription of type 3 var genes in P. falciparum laboratory isolates revealed transcription occurs in asexual parasites, in ring stage, pigmented trophozoite stage and schizont stages, of laboratory isolate 3D7. No association with binding phenotype in laboratory isolates (rosetting or IgM affinity) was observed. An attempt was also made to raise a type 3-specific polyclonal antibody.
5.2 Introduction

5.2.1 Type 3 var gene/ PfEMP1 structure

The type 3 var genes are the shortest of the var family, being approximately 4 kb in length (3.948, 3.984 and 3.978 kb in 3D7). The type 3 var genes thus encode the smallest PfEMP1 (approximately 153 kDa). Type 3 PfEMP1 have no CIDR domains and only two extracellular DBL domains; an N-terminal DBLα domain (further referred to as DBLα<sub>type3</sub>), and a membrane-proximal DBLε domain (DBLε<sub>type3</sub>) (Fig. 5.1, Fig. 5.2). The DBL domains are followed by a transmembrane region and ATS, typical for PfEMP1 (Fig. 5.1, Fig. 5.2).

Fig. 5.1. Diagram of type 3 PfEMP1s showing DBLα<sub>type3</sub>, DBLε<sub>type3</sub>, transmembrane region (TM) and ATS region. Hatched and striped N-terminal region of DBLα<sub>type3</sub> domains are variable, light grey shaded area of DBLα<sub>type3</sub> and DBLε<sub>type3</sub>domains, as well as TM and ATS regions, are conserved between different type 3 variants.
Fig. 5.2. Annotated clustal W alignment of the three 3D7 type 3 PfEMP1 amino acid sequences showing NTS region, DBLα domain, conserved cysteine-rich region, DBLe domain, transmembrane (TM) domain and ATS region. Arrows indicate beginning of each domain type.
5.2.2 Type 3 var genes are atypical group A var genes

Type 3 var genes are interesting due to their unusual domain structure, containing only two well-conserved extracellular DBL domains. The data presented in chapter 3 revealed an association between group A var gene transcription and cerebral malaria. However, transcription of type 3 var genes could not be analysed with the primers used in chapter 3. Type 3 var genes are group A genes; as well as having a DBLα1-type DBLα domain, the type 3 var genes have UpsA-type upstream regions and are telomerically located with transcription towards the telomere in 3D7 (Smith et al. 2000b; Gardner et al. 2002; Kraemer and Smith 2003; Lavstsen et al. 2003). However, the other group A var genes are characterised by having many extracellular domains. The type 3 var genes encode atypical group A PfEMP1, without the complex domain architecture of the other group A PfEMP1. It is possible that they have different properties to classic group A PfEMP1, and their transcription may not be associated with severe disease.

5.2.3 Type 3 var gene DBLα1 domains

The nucleotide sequence of first half of the DBLα_type3 domains is variable, and DBLα_type3 domains are indistinguishable from other DBLα1 domains (Fig. 5.3). However, the DBLα_type3 domains become increasingly similar until the final ≈ 336 nucleotides of each DBLα_type3 domain, which form a highly conserved DBLα_type3-specific region, and DBLα_type3 cluster separately in a phenogram from other DBLα1 domains (Fig. 5.4).
Fig. 5.3. Clustal W amino acid alignment of DBLα1 domains from 3D7. The consensus sequence from an alignment of the three DBLα_{type3} domains in 3D7 is shown and identical residues shaded. DBLα_{type3} domains are indicated ( ). Red line (amino acid 191 in this alignment) indicates the start of the type 3-specific portion.

Fig. 5.4. Phenogram of 3D7 group A DBLα1 domains; DBLα_{type3} domains (circled) cluster separately.
5.2.4 Type 3 var gene DBLε domains

Similarly, Type 3 epsilon domains (DBLε<sub>type3</sub>) are extremely conserved; the three DBLε<sub>type3</sub> in 3D7 have only 4 nucleotide differences, and they cluster separately from other epsilon domains after phylogenetic analysis (Fig. 5.5 and Fig. 5.6).

Fig. 5.5. Clustal W amino acid alignment of all 3D7 epsilon domains; type 3 epsilon domains indicated ({}). Shading indicates DBLε<sub>type3</sub> domain residues.
Fig. 5.6. Phenogram of 3D7 epsilon domains; DBLε-type3 domains (circled) cluster separately.

5.2.5 Type 3 var gene intron

Retrieval of data from plasmboDB database revealed Pfa0015c, Pfi1820w and Mal6P1.314 have introns of 158, 171 and 150 bp respectively. These introns are much shorter than those of other var genes, which are usually 0.8-1.2 kb (Su et al. 1995). They do not contain the conserved repeat elements which make up the promoter initiator element, described by Calderwood et al. (2003). Pfi1820w and Mal6P1.314 do not contain the Inr-like initiator sequence (TCATA). Pfa0015c does contain this sequence, but as it is not in the correct context within the initiator element it is unlikely to act as a promoter (Calderwood et al. 2003). This has implications for regulation, as the intron promoter regulates var gene silencing (Deitsch 2001; Calderwood et al. 2003; Frank et al. 2006).
5.2.6 Type 3 var gene ATS region

The ATS region of the type 3 var genes in 3D7 cluster with the ATS region of the other 3D7 group A var genes in a distance tree of the ATS domains by Lavstsen et al. 2003. Mal6P1.314 and Pfi1820w ATS regions are almost identical and form a slight outgroup from other group A ATS regions (Lavstsen et al. 2003).

5.2.7 Type 3 var gene transcription

At present there is little published data on type 3 var gene expression in laboratory or field isolates. Two papers discuss type 3 var gene transcription in relation to disease severity. Firstly, Jensen et al. (2004) report type 3 var gene transcription (pfa0015c) at relatively high levels in unselected 3D7, and a decrease in expression after selection on semi-immune sera. This suggests type 3 PfEMP1 are not associated with severe disease and are not common targets for an immune response. Secondly, Rottman et al. (2006) reported a small (non-significant) upregulation of type 3 var gene transcription in severe malaria compared to uncomplicated or asymptomatic malaria isolates, but in this study there was an increase in all var gene groups in severe malaria compared to the control groups, suggesting an increase in overall var gene transcription in the severe malaria cohort, possibly due to the higher parasitaemia.

5.2.8 Type 3 var gene/PfEMP1 hypotheses

The intention of the chapter was to examine type 3 var gene transcription in a range of laboratory and field isolates. Due to the association of group A var gene transcription with cerebral malaria isolates discussed in chapter 3, it was
hypothesised that type 3 var gene transcription may be more common in cerebral malaria isolates than in hyperparasitaemia isolates. However, as type 3 var genes are not typical group A var genes, the association found between group A var genes and cerebral malaria disease in chapter 3 could not be extended to the type 3 var genes without specifically examining this link.

It was thought possible that the timing of expression could be regulated differently to other var genes due to the small intron and therefore lack of regulatory promoter (Deitsch 2001; Calderwood et al. 2003; Frank et al. 2006). It was also thought possible that the lack of the regulatory promoter may promote type 3 var gene expression outside the mutually exclusive var gene expression system (Smith et al. 1995; Scherf et al. 1998; Dzikowski et al. 2006), and that type 3 var gene transcripts could be constitutively expressed.

The high conservation of type 3 PfEMP1 led me to doubt prolonged exposure of type 3 PfEMP1 on the erythrocyte surface, and so an antibody was designed to specifically recognise type 3 PfEMP1, to allow protease experiments with immunofluorescence and Western blot to investigate type 3 PfEMP1 expression and determine whether it is surface expressed.
5.3 Aim of chapter

In this chapter, transcription of type 3 var genes in *P. falciparum* laboratory and field isolates was examined. Data was obtained on the frequency of expression, timing of expression, and sequence conservation of the type 3 var genes in laboratory strains and field isolates. In addition, to study protein expression of the type 3 PfEMP1, a type-3 specific anti-peptide polyclonal antibody was designed and raised in chicken. This antibody was then used to examine protein expression of type 3 PfEMP1 in *P. falciparum* laboratory strain 3D7.

5.4 Materials and methods

Materials and methods that are specific to chapter 5 are listed below. General materials and methods and a list of suppliers are given in chapter 2.

5.4.1 Type 3 var gene PCR/RT-PCR

Field isolate cDNA prepared as detailed for DBLα analysis was used (see chapter 2 and 3). Laboratory strains used were 3D7, NF54, Dd2, HB3, TM180, TM284, FCR3/It, 7G8, Muz12, TM267 and Malayan Camp all from JA Rowe/A Raza except 3D7. Type 3 var gene sequence taken from 3D7 were pfa0015c, pfi1820w and mal6p1.314 (also renamed pff0020c) (Gardner *et al.* 2002; Hall *et al.* 2002). Type 3 var gene sequences DQ408041-DQ408070 were taken from Trimnell *et al.* 2006.
5.4.2 Type 3 DBLα domain amplification

PCR/RT-PCR was used to amplify a region of the type 3 var gene DBLα domain using forward primer αAF3_type3F; GCAMGAAGTTTTGCG modification of DBLαAF’ (GCACGMAGTTTYGC) (Bull et al. 2005a) and reverse primer Type3alphaR1; CAGAAATATTCAG (PCR product 420 bp) or Type3alphaR2 ATATTCATTGATTGTTCATC (PCR product 580 bp). Amplification used 5 µl 10x PCR Gold buffer, 25 pmol each of DBLαAF’ and DBLαBR primers and 0.2 µl Amplitaq Gold polymerase with a final concentration 2 mM MgCl₂, and 1.25 mM of each dNTP, and the volume adjusted to 50 µl after addition of 2 µl cDNA, 0.25 µl gDNA, or distilled water. Amplification conditions were as described for DBLα RT-PCR (Taylor et al. 2000a; Bull et al. 2005a) with a hot start (95°C, 5 min) followed by 35 cycles of 95°C, 20 sec; 42°C, 20 sec; 60°C, 1 min. Samples without RT were used in all reactions to exclude gDNA contamination.

5.4.3 Type 3 DBLε domain amplification

PCR/RT-PCR was used to amplify a region of 726 bp of the type 3 var gene DBLε domain using forward primer Type3epsF GACAATAATGGTGTTGATTCTCCTC and reverse primer Type3epsR GTATGATGCAGCATTCCCTCA. This pair was unbiased on 3D7 gDNA. Amplification conditions were as for DBLα domain RT-PCR; hot start (95°C, 5 min) followed by 35 cycles of 95°C, 20 sec; 42°C, 20 sec; 60°C, 1 min. Samples without RT were used in all reactions to exclude gDNA contamination. Amplification conditions and mix were as for type 3 var gene DBLα domain RT-PCR.
5.4.4 Tubulin gene amplification

PCR/RT-PCR was used to amplify 389 bp of the 3D7 *P. falciparum* β-tubulin gene using forward primer Tub3’ GATCCAAGTGGTACCTAT and reverse primer Tub5’ GGATACTCCTCTCTTTAT. Amplification used 5 µl 10x PCR Gold buffer, 25 pmol each of Tub3’ and Tub5’ primers and 0.2 µl Amplitaq Gold polymerase with a final concentration of 2.25 mM MgCl₂, and 1.25 mM of each dNTP, and the volume adjusted to 50 µl after addition of 1 µl cDNA, 0.25 µl gDNA, or distilled water. Amplification conditions were a hot start (95°C, 5 min) followed by 35 cycles of 95°C, 10 sec; 50°C, 15 sec; 62°C, 1 min. Samples without RT were used in all reactions to exclude gDNA contamination.

5.4.5 Sequence analysis

See chapter 2 for details of ligation, cloning, transformation, and sequencing of RT-PCR products. In brief, RT-PCR products were visualised by electrophoresis on 1.5% agarose gels. RT-PCR products were purified and ligated into PCR II TA cloning vector (Invitrogen) and ligated plasmids were used to transform TOP10F *E. coli*. Transformed *E. coli* was grown overnight under ampicillin selection and blue/white colony screening. Individual white colonies were selected for overnight growth and the plasmids extracted for sequencing. Presence of an insert in the extracted plasmids was verified by EcoRI restriction digestion prior to sequencing. For Mali isolate gDNA type 3 *var* gene PCR only, plasmids were sequenced directly from transformed *E. coli* colonies. Colonies were grown at 37°C o/n in LB broth with 8% glycerol. Sequencing was then carried out by the University of Edinburgh School of Biological Sciences Sequencing Service.
Transcript sequences from each isolate were grouped into contigs of >95% nucleotide identity using the SeqMan programme within the DNASTar software package. Consensus sequences from each isolate were saved as sequence A, B, C etc from that isolate as EditSeq files (DNASTar software package), and protein sequences were obtained by translating the DNA sequences using the EditSeq translation function.

Nucleotide and protein sequences were then compared within and between isolates using MegAlign (DNASTar software package). Alignments and phenograms were produced using the clustal W alignment programme within MegAlign, using default settings. Sequence distances were calculated using MegAlign, and colour-coded into the following groups; 0-30, 30-39.9, 40-49.9, 50-59.9, 60-69.9 70-99.9 and 100% amino acid identity for production of sequence distance figures. Homology searches were performed using nucleotide-nucleotide BLAST search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Phenograms were laid out in Adobe Illustrator.

5.4.6 Northern blot methods

1.2 g of agarose was dissolved in 80 ml of distilled water with 10 ml of 10x MOPS (110 mM MOPS, 25 mM Na-Acetate, 5 mM EDTA pH 7.0) and 3 ml of 37% formaldehyde. 1 μg RNA, 9 μl formamide, 3 μl formaldehyde (37%), 2 μl 10x MOPS was heated to 65°C for 5 min and loaded immediately onto the gel with 2 μl of RNA gel loading buffer (1x MOPS with 0.01% Bromophenol blue, 10% glycerol,
30% formaldehyde, 3.33% formamide). Millennium Markers (Ambion) were also loaded. Gel was run at 150 volts in 1x MOPS running buffer for 90 min. Gel was stained with final concentration 0.5 µg/ml EtBr in 200 ml of 1x MOPS for 30 min on shaker at RT and destained twice with water for 30 min on shaker at room temperature to visualize RNA and photograph markers. To transfer the RNA to nitrocellulose, the gel was placed on top of a wick of wet MME paper in 10x SSC (1.5 M NaCl, 0.15 M Tri Sodium Citrate) covering the gel tray. Nylon membrane (Roche) prewetted in 10x SSC was placed on top of the gel, followed by 2x MME papers the size of gel prewet in 2x SSC, and then 2 inches of paper towel. A weight (old catalogue) was placed on top of the assembly. Next day the nylon membrane was removed and air-dried before cross-linking the RNA by UV (1.2 sec) in a Stratalinker. PCR for riboprobe used the primers as described in Table 5.1.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Sequence (3D7)</th>
<th>Hybridisation</th>
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<td>Type3epsR</td>
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<td>NI</td>
<td>N2</td>
<td>MSP-1</td>
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</table>

Table 5.1 PCR primers for riboprobe design.

Amplification used 5 µl 10x PCR Gold buffer, 25 pmol each of DBLαAF’ and DBLαBR primers and 0.2 µl Amplitaq Gold polymerase with a final concentration 2 mM MgCl₂, and 1.25 mM of each dNTP, and the volume adjusted to 50 µl after
addition of 2 µl cDNA, 0.25 µl gDNA, or distilled water. Amplification conditions were as described for DBLα RT-PCR (Taylor et al. 2000a; Bull et al. 2005a) with a hot start (95°C, 5 min) followed by 35 cycles of 95°C, 20 sec; 42°C, 20 sec; 60°C, 1 min. Samples without RT were used in all reactions to exclude gDNA contamination. PCR products were purified as described in chapter 2 and were cloned into PCR II vector (Invitrogen) and plasmids obtained by miniprep (QIAgen) and insert size verified by EcoRI digestion (details in chapter 2).

All materials for riboprobe production and Northern blot analysis were from Roche (Northern starter kit). For riboprobe transcription and DIG incorporation, 2µl NTP Mix, 1 µl RNase inhibitor, 2 µl 10xTansription Buffer, 40 units SP6 polymerase and DTT (final concentration 5 µM) was added to 1 µg template plasmid, and water added to give a final volume of 20 µl. Mix was incubated at 37°C for 2 hr. 2 µl of DNaseI was added (37°C, 15 min) to remove any DNA template, and the DNase reaction stopped by the addition of 2 µl of 200 mM EDTA, pH 8.0. The riboprobe was precipitated at –70°C for 2 hr, using 2.5 µl of 4 M LiCl and 75 µl of cold 100% ethanol, pelleted (13,000 rpm, 15 min, at 4°C) and washed with 50 µl 70% cold ethanol. The RNA pellet was air dried for 10 min and resuspended in 50 µl of DEPC-H2O with 1 µl of RNase inhibitor.

Nylon membranes (Roche) were prehybridised in 10 ml hybridisation solution (Maleic acid buffer: 100 mM Maleic acid, 150mM NaCl, pH 7.5 with DIG Easy Hyb granules: Roche) for 1 hr at the hybridisation temperature (DBLαtype3, DBLεtype3 and MSP1 probe at 60 °C, exon 2 probe at 50°C). 2 µl probe was boiled in 100 µl
hybridisation solution for 5 min and added to membrane with fresh hybridisation solution, and incubated at hybridisation temperature, with rotation, o/n. The membrane was then washed twice for 30 min at “hyb temp + 5°C” in 0.2x SSC/0.1% SDS. Membrane was washed for 1 min in Maleic acid buffer (100 mM Maleic acid, 150mM NaCl, pH 7.5) and blocked for 1 hour in 20 ml Northern blocking buffer (18 ml of Maleic acid buffer with 2 ml of 10% Northern block buffer, Roche). Membrane was then incubated for 30 min in 20 ml blocking buffer + 2 µl Anti-DIG antibody (Roche), before two further 15 min washes in Maleic acid buffer with 0.3% Tween 20. Membrane was soaked for 2 min in Detection buffer (100 mM Tris-HCl, 100 mM NaCl pH 9.5) before incubating with 1 ml CDP-Star (Roche) 5 min, RT. Membrane was exposed to X-ray film.

5.4.7 Transcription time course
For the time course RT-PCR and for the Northern blots, RNA was extracted from a fresh culture of laboratory strain 3D7 by chloroform extraction and isopropanol precipitation as detailed in chapter 2. For the time courses, 200 µl packed cell volume was extracted for each time point and immediately stored in Trizol at -70°C, and the rest of the culture re-gassed and incubated at 37°C. A Giemsa slide was also prepared to assess parasite maturity at each time point. RNA extraction and cDNA preparation for each time point was carried out at the same time.
5.4.8 DBL domain structural modelling

The published structures of the EBA175 DBL domain (Tolia et al. 2005) and a *P. knowlesi* DBL domain (Singh et al. 2006) were downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.pdb.org), PDB ID 1zrl and 2c6j, for modelling of type 3 protein structures. The mal6p1.314 DBLα domain amino acid sequence was aligned to the amino acid sequence of EBA175 and *P. knowlesi* DBLα by clustal W. The aligned amino acid sequence was then mapped onto the published structures using Cn3d structural modelling software (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). Secondary structure colouring shortcuts, and worm and spacefill rendering shortcuts, were used. Regions of interest were highlighted in the structure by selection of the corresponding amino acid sequence.

5.4.9 Protean software

Protean/Lasergene software (DNASTAR, Inc) was used to analyse features of the pfa1820w, pfa0015c and mal6P1.314 type 3 var gene DBLα domain protein sequences to aid peptide choice. Alpha helical and beta sheet regions were predicted by Garnier-Robson, Chou-Fasman and Eisenberg plots. These are based on statistical preferences of different amino acids to have particular secondary structure types in known protein structures. Kyte-Doolittle plots were used to predict hydrophobicity. Amino acids are given a hydrophobicity score between -4.6 (hydrophobic) and 4.6 (hydrophilic), and the hydrophobicity plot shows the averaged score over stretches of 9 amino acids. Karplus-Schulz plots show flexible regions based on the characteristics of neighbouring residues. The Emini plot predicts surface exposure
probability, based on values of surface accessibility averages over 6 amino acid stretches. The Jameson-Wolf index predicts antigenicity of a given region, through a complex algorithm combining hydrophobicity, surface probability, flexibility, and alpha/beta regions predictions.

5.4.10 Peptide antibody production

Final peptides were chosen after consultation with peptide.design@eurogentec.com. Peptide synthesis, hen immunization and yolk collection was all performed by Eurogentec, UK. Briefly, peptides DTDPPVVDYIPQC (peptide 1) and CNVLNKEIDEMNNQ (peptide 2) were synthesized by solid phase Fmoc chemistry by Eurogentec, Belgium. Peptides were analysed by MADLI-TOF mass spectrometry and reversed phase analytical HPLC for weight and purity at Eurogentec, Belgium. Peptides alone are not immunogenic, thus conjugation to a carrier is required to elicit an immune response. Keyhole Limpet Hemocyanin (KLH) carrier was linked to the thiol group of the terminal cysteines of each peptide using m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). Hens PO41 and PO42 were immunised under the Eurogentec Double XP protocol. Eggs were collected on days 51-98 after immunisation and pooled as follows: days 51-57, 58-66, 72-78 79-85, 86-92 and 93-98 (Table 5.2). Pooled yolks were sent to Edinburgh at 4°C for IgY extraction and purification.
### Table 5.2. Egg collection and yolk processing schedule.

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**5.4.11 IgY Extraction**

IgY extraction was performed using EggStract kit (Promega, Madison, WI) according to manufacturer’s guidelines. Yolks were warmed to RT. 3 volumes precipitation solution A was added slowly and stirred for 5 min to precipitate the lipids. The mixture was centrifuged (10,000 rpm, 15 min), and the supernatant
collected into a graduated cylinder through gauze. The supernatant was stirred at RT while 1/3 volume solution B was added slowly to precipitate the IgY. The mixture was centrifuged (10,000 rpm, 15 min), and the supernatant discarded. The precipitated IgY was resuspended in PBS. IgY precipitation was repeated to increase the purity of extracted IgY. IgY was stored at -20°C.

5.4.12 Determining IgY concentration
Absorbance at 280 was read on a spectrophotometer. The protein extinction coefficient of IgY is 13.3. This is the absorbance (A280nm) value for 1% (i.e. 1 g/100ml) solution, measured in a 1 cm cuvette. Thus for concentration at mg/ml (0.1 % solution), absorbance at 280 nm must be divided by 1.33.

5.4.13 IgY affinity purification
A sample of the extracted IgY from immunised hen eggs was affinity-purified against the peptide using the SulfoLink Immobilization Kit (Pierce, IL, USA), according to manufacturer instructions. Briefly, 0.5 mg of each peptide was reduced using Tris(2-carboxyethyl)phosphine (TCEP) and both peptides immobilized concurrently on the SulfoLink Resin via the free sulfhydryl groups on the terminal cysteines. The internal methionine of peptide 2 will also stabilise immobilisation on the column. Extracted IgY (4-8 mg) was applied to the column. After washing, bound antibody eluted was in 3 x 2 ml 0.2 M glycine HCl (pH 2.5) and neutralised in 50 µl 1 M Tris HCl (pH 8.5) per ml elution. The full 6 ml elution was then concentrated using a vivaspin column (Vivascience, UK), molecular weight cut off 10 kDa, and buffer exchanged into 500 µl PBS containing 0.05% azide. Affinity-
purified and concentrated antibody was then aliquoted and stored at –20°C. Affinity purification was also carried out with the two peptides separately.

5.4.14 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Microtest III assay plates (Falcon) were coated 150 ml PBS with 10 µg/ml peptide 1, 10 µg/ml peptide 2, 1% BSA or PBS alone, at 4°C, o/n. Plates were washed three times with PBS, blocked for 2 hr at 37°C with 150 µl 2% milk with 0.5% Tween 20 and washed three times with PBS before adding primary IgY 1:100 in blocking solution (150 µl) to each well, for 2 hr at 37°C. Plates were washed a further three times with PBS, before incubation with 150 µl of peroxidase conjugated goat anti-chicken IgG diluted 1:1000, 1hr RT, then washed a final 3 times with PBS. For detection, 100 ml TMB Substrate Reagent (BD OptEIA) was added to each well and the plate was left to develop for 30 min at RT, in the dark, before the reaction was stopped with 50 µl 2M H2SO4 per well. Absorbance measurements were made on an ELISA plate reader at 450 nm. These are shown as mean ± standard error (SE).

5.4.15 Detergent extraction of proteins

Infected erythrocytes (IE) or non-infected erythrocytes were washed twice with PBS. If trypsinisation was required, cells were incubated with 5 volumes 0.1 or 1 mg/ml trypsin (Sigma), or mock treated with PBS, for 5 min at RT. Trypsinisation was stopped by addition of trypsin inhibitor (Sigma) for 5 min at RT. Cells were then pelleted (4000 rpm, 3 min) and washed in RPMI. All cell preparations were then lysed on ice with 5 volumes of NETT buffer (150 mM NaCl, 5 mM EDTA, 50 mM tris pH8.0, 1% triton-X100 (w/v)) plus complete mini protease inhibitor cocktail
Cell lysates were then centrifuged (13,000 rpm, 10 min at 4°C). Supernatants were removed. These are now referred to as triton-X100 soluble (TS) fractions. The triton-X100 insoluble pellet was resuspended in a Tris saline (20 mM tris-HCl pH 8.0, 150 mM HCl, 2% (w/v) SDS) plus complete mini protease inhibitor cocktail (Roche). The solubilised TI fraction was repeatedly pipetted and vortexed to disrupt the parasite DNA. Samples were centrifuged (13,000 rpm, 10 min, RT) to pellet the remaining insoluble fraction, seen as a brown pigment in the infected erythrocyte sample. The soluble TI fractions were removed and transferred to a fresh tube. All samples were stored at –80°C.

5.4.16 SDS-PAGE
SDS-PAGE was performed using the Novex precast gel system (all reagents from Invitrogen). Samples were prepared according to the NuPAGE Novex Tri-acetate mini Gel protocol with NuPAGE LDS sample buffer and NuPAGE reducing agent, and heated to 70°C for 10 min. Samples were then run on 3-8% Tris-acetate gradient gels using Tris-acetate SDS running buffer for 1 hr at 150 V. Pre-stained precision protein molecular weight markers (10-250 kDa; Bio-rad) were used in all gels.

5.4.17 SimplyBlue staining
To visualise all loaded proteins on the acrylamide gel, gels were washed 3x 5 min in distilled water and then soaked in SimplyBlue safe stain (Invitrogen) for 1 hr, and destained 1hr to overnight in distilled water.

5.4.18 Western blotting
Proteins were electrophoretically transferred from SDS-PAGE gels to polyvinylidene difluoride (PVDF) membrane (Invitrogen) using a NuPAGE transfer buffer (Invitrogen) (30 V, 1 hr, RT). PVDF membranes were activated in 100% methanol for 30 sec prior to transfer. Membranes were washed briefly in PBS before incubating in blocking buffer (PBS/5% milk) for 30-60 min RT with rocking, and then o/n at 4°C. Membrane was then incubated for 1 hr in primary antibody or sera was diluted to 1 in 1000 unless otherwise stated in PBS/5% milk with rocking at RT. Blots were washed 3 x 10 min with PBST (PBS with 0.5% Tween-20). Incubation in secondary antibody (1 in 1000 HRP-conjugated goat anti-chicken H&L IgY, Abcam) was for 1 hr. Blots were then washed 2 x 10 min with PBST and 1 x 10 min in PBS alone prior to visualisation with an enhanced chemiluminescence (ECL) detection kit (Amersham). Chemiluminescence was detected using Hyperfilm™ ECL (Amersham).

5.4.19 Immunofluorescence assay (IFA)

For live IFA, cultures at 2% haematocrit were incubated with primary antibody (chicken pre-immune IgY extract (1:10), rabbit preimmune serum (1:10) or immune IgY (1:10) in PBS/1%BSA) for 1 hr on ice. Cells were then washed three times in PBS/1%BSA, and incubated with alexa488-conjugated goat anti-chicken or anti-rabbit secondary antibody (1:1000) in PBS/1%BSA for 1 hr on ice in the dark. Cells were washed a further three times in PBS/DAPI/1%BSA and mounted onto a glass slide with-fluromount. Live cells were viewed under fluorescence at 100x magnification.
For fixed IFA, *P. falciparum* cultures were smeared onto slides and fixed with acetone. Slides were incubated for 1 hour in preimmune IgY, immune IgY extract affinity purified against peptides 1 and 2 together (both 1:10 in PBS/1% BSA), or PBS/1%BSA alone. The culture was then washed and the slides were incubated in alexa 488 conjugated secondary antibody 1:1000 and PBS/DAPI/BSA1% for 1 hour in the dark. Secondary incubation was washed off and the fixed cells were viewed under fluorescence at 100x magnification.
5.5 Results 1: Type 3 var gene analysis

5.5.1 Type 3 var gene DBLα domain amplification: DBLα primer design 1

To examine whether type 3 var genes were transcribed in laboratory and field isolates, I designed a type 3-specific primer pair for use in PCR and RT-PCR. The primers were designed to specifically recognise all type 3 DBLα domains, but not recognise other DBLα domains or other sequences. The DBLαAF’ forward primer from the Taylor primer pair described in section 1.3 recognises DBLα_{type3} domains, but the DBLαBR reverse primer does not amplify type 3 var genes. To amplify the type 3 DBLα domains a new primer pair was designed.

The forward primer was a modification of the DBLαAF’ forward primer from Taylor et al. (2000a), which had increased specificity for the type 3 group A DBLα domains (αAF3_{type3}; GCAMGAAGTTTTGC modification of DBLαAF’; GCACGMAGTTTYGC, Fig. 5.7); degeneracy at positions 6 (M) and 12 (Y) is unnecessary as all known type 3 DBLα domains have an A and T at these positions. However, degeneracy at position 4 was an improvement, as some type 3 DBLα domains (4/17 published sequences) have an A rather than a C here.

A reverse primer was designed to specifically amplify the DBLα_{type3} domains (primer: Type3alphaR1; CAGAAATATTTCAG). This recognises a conserved section just before the end of the DBLα_{type3} domain, and results in a 420 bp PCR product (Fig. 5.8).
Both primer sequences recognised all published type 3 domains, and neither primer sequence showed homology to any genes other than type 3 var genes (NCBI Blast database search).

Fig. 5.7. Forward primers for amplification of type 3 DBLα domains. The forward primer GCAMGAAAGTTTTGC (αAF3_type3) is a modification of the generic forward primer used for DBLα domain amplification in chapter 3 (DBLαAF’ GCACGMAGTTTTYG C) Taylor et al. 2000a; Bull et al. 2005a) which is better suited to amplification of type 3 DBLα domains. Forward primers αAF3_type3 and DBLαAF’ are shown with the primer binding sites from published type 3 DBLα domains from IT, MC, 3D7 and other P. falciparum isolates 713, 7G8, D10, Ecu, Fab6, M2, M5, V15. Altered residues in αAF3_type3 compared to DBLαAF’ are boxed.
Fig. 5.8. Alignment of published type 3 var genes from strains 3D7 (mal6p1.314, pfa0015c and pfi1820w), FCR3 (it4var3), Malayan Camp (mc var5), 713, 7G8, D10, Ecu, Fab6, M2, M5, V1S, showing primer sites for AF3_type3F (F), Type3alphaR1 (R1) and Type3alphaR2 (R2; (see section 5.5.3).

5.5.2 Testing primer pair AF3_type3F and Type3alphaR1 on 3D7 gDNA

The primer pair AF3_type3F and Type3alphaR1 were tested on 3D7 gDNA. Amplification resulted in a PCR product of the correct size for type 3 DBLα domain amplification with this primer pair (420 bp) (Fig. 5.9).

```
3D7  H₂O
gDNA
```

Fig. 5.9. Amplification of type 3 DBLα domains from 3D7 gDNA using primer pair AF3_type3F and Type3alphaR1. An aliquot (5 µl) of PCR product was run on a 1.5% agarose gel. Distilled water was used as a negative control for the PCR reaction. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).
Products from the PCR of type 3 DBLα domains from 3D7 gDNA using primer pair AF3_type3F and Type3alphaR1 were purified and ligated into the TA cloning vector for transformation of *E. coli*, which were then grown overnight (details in chapter 2). 30 colonies were selected for overnight growth, and the plasmids extracted and an aliquot digested with *Eco*RI to verify an insert of the correct length was present (Fig. 5.10).

![Image](image_url)

**Fig. 5.10.** 1.5% agarose gel of 5 µl of *Eco*RI digests products of plasmids extracted from a set of 14 minipreps from RT-PCR of 3D7 gDNA using primer pair AF3_type3F and Type3alphaR1. DNA ladder was φX174 RF DNA/*Hae* fragments (Invitrogen).

Plasmids containing an insert were sequenced to assess the frequency of the three 3D7 DBLα domain amplified by RT-PCR, which was expected to be approximately even. However, primer validation on 3D7 gDNA showed a bias in recognition of Pfi1820w and Pfa0015c DBLα domains compared to Mal6P1.314 (Fig. 5.11). This
bias was repeated with a further 21 colonies. This was surprising as the primer sites showed 100% identity to all 3D7 sequences, and products would be the same length.

The DBLα<sub>type3</sub> domain from the single type 3 var gene from R29/IT strain laboratory isolate was also successfully amplified using the primer pair AF3_type3F and Type3alphaR1. No other genes, such as non-type 3 var genes were amplified with this primer pair.

**AF3_type3F and type3alphaR1 PCR**

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<th>3D7 gDNA (30)</th>
<th>3D7 gDNA(21)</th>
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<tr>
<td>Pfi1820w</td>
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<td>Mal6P1.314</td>
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Fig. 5.11. Pie charts showing relative proportions of DBLα domains from the 3D7 type 3 var genes Pfi1820w, Pfa0015c and Mal6P1.314 after PCR on 3D7 genomic gDNA with primer pair AF3_type3F and Type3alphaR1.
5.5.3 Type 3 var gene DBLα domain amplification: DBLα primer design 2

A new reverse primer was designed to specifically amplify the DBLα\textsubscript{type3} domains in order to achieve a more even amplification of the 3D7 type 3 DBLα domains. Primer Type3alphaR2 (ATATTTCATTGATTGTTTATC) recognises the end of the DBLα\textsubscript{type3} domain, giving a 580 bp PCR product (Fig. 5.12). Type3alphaR2 recognises all published type 3 domains, and does not show homology to any genes other than type 3 var genes (NCBI Blast database search).

PCR products were obtained from PCR of 3D7 gDNA (Fig. 5.12A). These were purified, ligated and a random selection of 27 and 22 transformed plasmids sequenced as for the previous primer pair. Plasmids were digested by \textit{Eco}RI to confirm presence of an insert prior to sequencing (Fig. 5.12B).

![Fig. 5.12. Amplification of type 3 DBLα domains from 3D7 gDNA using primer pair AF3\_type3F and Type3alphaR2. A) An aliquot (5 μl) of PCR product was run](image)

A PCR B Plasmid digestion

3D7 H\textsubscript{0} 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

580 →
on a 1.5% agarose gel. Distilled water was used as a negative control for the PCR reaction. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen). B) EcoRI digests products of plasmids extracted from a set of 18 minipreps from RT-PCR of 3D7 gDNA using primer pair AF3_type3F and Type3alphaR2. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).

This pair was less biased on 3D7 gDNA, recognising all three domains in both sets of sequencing (Fig. 5.13). The primers were also used to amplify type 3 domains from NF54, and 18 plasmids were also sequenced from transformed E. coli. Mal6P1.314 was still amplified less frequently than the other 3D7 type 3 var genes, however the distribution of the three type 3 DBLα domains was not significantly different from expected in the two 3D7 amplifications or the NF54 amplification (Chi-squared P>0.3).

The DBLα<sub>type3</sub> domain from the single type 3 var gene from R29/IT strain laboratory isolate was also successfully amplified with primer pair AF3_type3F and Type3alphaR2. No other genes, such as non-type 3 var genes were amplified from 3D7, NF54 or R29.
AF3_type3F and type3alphaR2 PCR

3D7 gDNA (27)  3D7 gDNA(22)  NF54 gDNA (18)

- Pfi1820w
- Pfa0015c
- Mal6P1.314

Fig. 5.13. Pie charts showing relative proportions of DBLα domains from the 3D7 type 3 var genes Pfi1820w, Pfa0015c and Mal6P1.314 after PCR on 3D7 genomic gDNA with primer pair AF3_type3F and Type3alphaR2.

5.5.4 Type 3 var gene DBLε domain amplification: DBLε primer design

A primer pair was also designed to amplify DBLε_type3 domains, Type3epsF GACAATAATGGTGTGTTAGTTCCTC and type3epsR GTATGATGCAGC ACATTCCTCA (Fig. 5.14). These primers recognise the published type 3 epsilon domains and do not recognise any other genes (NCBI database homology search).
Fig. 5.14. Alignment of published type 3 var genes showing primer sites for Type3epsF and Type3epsR.
The primer pair Type3epsF and Type3epsR gave a PCR product of 726 bp when used with 3D7 gDNA (Fig. 5.15A). The PCR products were purified and ligated into the topo cloning vector for transformation of *E. coli*, which were grown overnight. 10 colonies were selected for overnight growth, and the plasmids extracted and an aliquot digested with *Eco*RI to verify an insert of the correct length was present (Fig. 5.15B). Type 3 epsilon domains contain an *Eco*RI site, the PCR product (726 bp) is seen as two smaller products (399 and 327 bp) after digestion (Fig. 5.15B).

![A PCR](image1.png)

![B Plasmid digestion](image2.png)

Fig. 5.15. Amplification of type 3 DBLα domains from 3D7 gDNA using primer pair Type3epsF, and Type3epsR. A) An aliquot (5 µl) of PCR product was run on a 1.5% agarose gel. Distilled water was used as a negative control for the PCR reaction. B) *Eco*RI digests products of plasmids extracted from a set of 10 minipreps from RT-PCR of 3D7 gDNA using primer pair Type3epsF and Type3epsR. DNA ladders were φX174 RF DNA/*Hae* fragments (Invitrogen).
Cloning and sequencing showed they specifically amplified the three DBLε domains from 3D7, and all three domains could be amplified (ratio 3,3,4, Fig. 5.16). This distribution of sequences was not significantly different from expected: $p > 0.99$, Chi-squared test. The DBLε_{type3} domain from the single type 3 var gene from R29/IT strain laboratory isolate was also successfully amplified using the epsilon primers Type3epsF and Type3epsR.

![3D7 gDNA (10)](image)

Fig. 5.16. Pie charts showing relative proportions of DBLε domains from the 3D7 type 3 var genes Pfi1820w, Pfa0015c and Mal6P1.314 after PCR on 3D7 genomic gDNA with primer pair Type3epsF and Type3epsR.

**5.5.5 Type 3 var gene PCR from laboratory strains gDNA**

Little data on the frequency of type 3 var gene occurring in *P. falciparum* genomes is available; at the time of the start of the study only 3D7 had been shown to contain a type 3 var gene, though subsequent sequencing has revealed a type 3 var gene in IT, but not in Dd2 or HB3. To assess the prevalence of type 3 var genes in strains other than 3D7, gDNA from 10 further strains was examined. Genomic DNA was
available from FCR3, TM267, TM284, Malayan Camp, Dd2, TM180, 7G8, Muz12 and HB3 (gift from JA Rowe/A Raza). Type 3 DBLα domains were amplified by primer pair AF3_type3F and Type3alphaR2. Type 3 DBLε domain was amplified by primer pair Type3epsF and Type3epsF. All PCR products were cloned and sequenced to verify type 3 domains had been amplified. Type 3 alpha and epsilon domains were amplified from 3D7, TM284, FCR3/IT, TM267 and Malayan Camp, but not from Dd2, HB3, TM180, 7G8 or Muz12 (Fig. 5.17 and Table 5.3). 7G8 gave a PCR product with the DBLα primer pair (AF3_type3F and Type3alphaR2), however, this was longer than expected for a type 3 var gene DBLα domain and sequencing proved that this was not a type 3 var gene DBLα domain. A summary of the type 3 domains detected in the laboratory isolates is given in Table 5.3.
Fig. 5.17. PCR of type3 domains from lab strains 3D7, Dd2, HB3, TM180, TM284, FCR3/IT, 7G8, Muz12, TM267, Malayan Camp (MC). Left) Type 3 DBLα domains were amplified by primer pair AF3_type3F and Type3alphaR2. Right) Type 3 DBLε domain was amplified by primer pair Type3epsF and Type3epsF. A 5µl aliquot of all PCR products was run on 1.5% agarose gels. Distilled water was used as a negative control for the PCR reaction. The DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).
Table 5.3. PCR for type 3 DBLα and DBLε domains was carried out on laboratory strains 3D7, FCR3/IT, TM267, TM284, Malayan Camp, Dd2, TM180, 7G8, Muz12 and HB3. Tick (✓) indicates a PCR product was obtained, and the sequence was confirmed as a type 3 domain. Cross (×) indicates no type 3 PCR product was obtained, or was not the correct domain (7G8 DBLα).

Therefore 4/9 genetically distinct laboratory strains have a single detectable type 3 var gene (5/10 tested laboratory strains, but TM267 is isogenic with FCR3/IT). The three type 3 var genes from 3D7 and the one type 3 var gene from FCR3/IT/TM267 have been published and discussed previously, and the sequences amplified here match these sequences perfectly. The TM284 DBLα domain had not previously been published. The type 3 DBLε domain amplified from TM284 is identical to the TM284 type 3 DBLε domain sequence submitted by Trimnell et al. (2006) over the length of the previously submitted sequence, but the sequence is longer as the PCR primers used in this chapter are further apart than those used by Trimnell et al.
Type 3 var gene domains were not found in Dd2 by PCR using these conditions. Though Trimnell et al. found a type 3 var gene in Dd2 (epsilon domain only), subsequent publishing of the Dd2 genome sequence suggests Dd2 does not contain a type 3 var gene. The epsilon domain published as from Dd2 does not correspond with any contigs from the Dd2 genome published so far by the Broad Institute Sequencing Program. Trimnell et al. also found an epsilon domain, but not alpha domain, in TM180. Neither was found in my experiments by PCR. The negative PCR result for type 3 var gene domains in HB3 here are in agreement with those of Trimnell et al. and the Broad Institute Sequencing Program,

A type 3 var gene DBLα domain from 7G8 has been published by Trimnell et al. (2006), accession number DQ408045. A PCR product was detected from amplification of 7G8 gDNA with primers AF3_type3F and Type3alphaR2 but agarose gel electrophoresis (Fig. 5.17) suggested this was too long to be a type 3 DBLα domain and sequencing proved this PCR product was not a type 3 var gene, and was an epsilon domain with 57% identity to the published 7G8 type 3 DBLα domain. No PCR product was obtained from 7G8 gDNA with the epsilon primer pair Type3epsF/Type3epsR. Amplification of the DBLα domain was also attempted with the other primer pair AF3_type3F/Type3alphaR1, but no PCR product was obtained. These PCR suggest 7G8 does not have a type 3 var gene. The 7G8 sequence
published in Trimnell et al 2006 is identical to that of another *P. falciparum* strain examined in that paper, Fab6.

### 5.5.6 Type 3 var gene transcription: laboratory strains

Little data is currently available on the transcription of type 3 var genes in laboratory strains or field isolates. The aim of this section was to assess the frequency of type 3 var gene transcription in the laboratory strains available, and investigate any association with rosetting and IgM binding. In addition, it was necessary to identify a laboratory strain which was transcribing a type 3 var gene in order to investigate the timing of type 3 var gene transcription more precisely.

Early pigmented trophozoite culture cDNA from 7 laboratory strains (3D7, Palo Alto, TM267, A1R, A4R, R29 and TM284) was available for RT-PCR (gift from JA Rowe/A Raza). For 5 of the above strains (Palo Alto, TM267, A1R, A4R and R29), cDNA was also available from cultures that had been selected for high rosette frequency (>70% RF). 3D7 could not be selected for high rosetting, but cDNA was available from parasites selected for IgM binding (Gift from JP Semblat). The transcription patterns of the above strains are presented below in sections 5.5.5a-d and summarised in section 5.5.5e.
5.5.6a Type 3 var gene transcripts are present in rosetting and non-rosetting TM267

RT-PCR amplified DBLα and DBLε domain transcripts from rosetting (R+) and non-rosetting (R-) TM267 (Fig. 5.18). This suggests type 3 var gene transcription does not influence rosetting in TM267.

![TM267 RT-PCR](image)

Fig. 5.18. Type 3 transcripts are present in rosetting and non-rosetting TM267 by RT-PCR. Type 3 DBLα domain transcripts were amplified by primer pair AF3_type3F and Type3alphaR2. Type 3 DBLε domain transcripts were amplified by primer pair Type3epsF and Type3epsF. PCR show gDNA, rosetting (R+) and non-rosetting (R-) cDNA, and distilled water as a negative control. A 5 µl aliquot of all RT-PCR products was run on a 1.5% agarose gel. CTL lanes (without reverse transcriptase, to control for gDNA contamination) were negative, indicating there was no gDNA contamination. DNA ladder was 100 bp ladder.
5.5.6b Type 3 var gene transcripts are present in rosetting and non-rosetting Palo Alto

The Palo Alto Dbla<sub>type3</sub> domain was detected by RT-PCR from this cDNA but the Dblα<sub>type3</sub> domain could not be amplified with the primer pair AF3<sub>type3F</sub> and Type3alphaR2 (Fig. 5.19A). The Dblα primers recognise perfectly the Palo Alto Dblα<sub>type3</sub> domain, and amplification of gDNA from the same Palo Alto culture shows that the alpha domain primers can still bind in this strain. To resolve this, amplification was carried out using the forward primer alpha primer and reverse epsilon primer, to span both the alpha and epsilon domain (1672 bp). This amplification gave PCR product in Palo Alto gDNA and cDNA (R+ and R-). Sequencing of these PCR/RT-PCR products gave the Palo Alto type 3 Dblα and Dbla domains separated by the published Palo Alto type 3 interdomain region (Broad Institute), confirming both DBL domains were linked as expected and were transcribed (Fig. 5.19B).

The RT-PCR amplification of the type 3 var gene in rosetting and non-rosetting Palo Alto suggests that type 3 var gene transcription does not influence rosetting in Palo Alto, as was seen with TM267. Palo Alto and TM267 are both clones of IT and are thus genetically identical. In addition, Palo Alto and TM267 express the same predominant var gene, and are thus essentially identical strains (JA Rowe, unpublished data). The detection of type 3 var gene transcripts in these two clones of IT, is consistent with these two strains being transcriptionally very similar.
Fig. 5.19. Type 3 transcripts are present in rosetting and non-rosetting Palo Alto by RT-PCR. A) Type 3 DBLα/ε domain amplification from Palo Alto gDNA, and
cDNA from rosetting (R+) and non-rosetting (R-) Palo Alto using primer pair AF3_type3F and Type3alphaR2, or Type3epsF/R. B) Type 3 DBLα−ε domain transcripts were amplified from Palo Alto gDNA, and cDNA from rosetting (R+) and non-rosetting (R-) Palo Alto using primer pair AF3_type3F and Type3epsR. Distilled water was used as a negative control. A 5 µl aliquot of all RT-PCR products was run on a 1.5% agarose gel. CTL lanes (without reverse transcriptase, to control for gDNA contamination) were clean, indicating there was no gDNA contamination. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).

5.5.6c Type 3 var gene transcripts are not present in rosetting or non-rosetting strains of A1R, A4R, R29 or in TM284

Type 3 var gene transcripts were not detected in either in the rosetting or non-rosetting subpopulations of the remaining 3 laboratory strains tested (R29, A1R, A4R; all IT-derived clones). Genomic DNA controls were positive for PCR product in all cases indicating PCR was successful. A representative gel is given in Fig. 5.20 for R29. The cDNA was a gift from JA Rowe/A Raza and had been used successfully for non-type 3 var gene RT-PCR (data not shown).
Fig. 5.20. Type 3 transcripts are not present in rosetting or non-rosetting R29. No type 3 DBLα/ε domain transcripts were amplified using primer pair AF3_type3F and Type3alphaR2 or Type3epsF and Type3epsF. Templates used were R29 gDNA, and cDNA from rosetting (R+) and non-rosetting (R-) R29, each with control (CTL) for gDNA contamination. A 5 µl sample of all RT-PCR products was run on a 1.5% agarose gel. Genomic DNA controls were positive for PCR product in all cases indicating PCR was successful. No cDNA lanes were positive indicating type 3 var genes were not being transcribed. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).
5.5.6d Type 3 var gene transcripts are present in IgM-binding and non-IgM binding 3D7

RT-PCR amplified DBLα domain transcripts from IgM-binding and non-IgM binding 3D7; RT-PCR reaction was carried out by JP Semblat (Fig. 5.21).

Fig. 5.21. Type 3 transcripts are present in IgM-binding and non-IgM binding 3D7 by RT-PCR. Type 3 DBLα domain transcripts were amplified by primer pair AF3_type3F and Type3alphaR2 by JP Semblat. A 5μl aliquot of all RT-PCR products was run on 1.5% agarose gels. Distilled water was used as a negative control for the PCR reaction. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).

RT-PCR products (gift from JP Semblat) were purified, ligated and cloned, and 15 or 16 miniprep plasmid extracts were sequenced. All three versions of the type 3 var
gene (pfa0015c, Pfi1820w and Mal6p1.314) DBLα<sub>type3</sub> and DBLε<sub>type3</sub> domains were amplified by RT-PCR from the 3D7 cDNA. There was a slight bias towards Pfi1820w, and away from Pfa0015c in the unselected parasites but this change in distribution was not significant (Chi-squared test, P=0.93, Fig. 5.22).

Fig. 5.22. Type 3 transcripts are present in IgM-binding and non-IgM binding 3D7 by RT-PCR. Type 3 DBLα domain transcripts were amplified by primer pair AF3<sub>type3</sub>F and Type3alphaR2. Pie charts represent distribution of the three 3D7 type 3 DBLα domain transcripts in IgM-binding and non-IgM binding strains from 16/15 purified plasmid extracts.

5.5.6e Type 3 var gene transcription in laboratory strains: summary

These results, summarised in Table 5.4, demonstrate that some but not all strains express a type 3 var gene, and that this expression is not associated with IgM binding in 3D7 or rosetting in TM267, Palo Alto, A1R, A4R or R29. RT-PCR results were the same in rosetting as for the non-rosetting isogenic strains. Amplification across the whole alpha and epsilon domains together using the forward alpha primer and reverse epsilon primer gave PCR product in the cDNA from rosetting Palo Alto.
Detection of type 3 var gene transcripts in Palo Alto and TM267, but not in other IT-derived clones (R29, A1R and A4R), suggests that type 3 var genes can be switched on and off in genetically identical parasites, and are thus not constitutively on as the lack of an intronic promoter suggested.

<table>
<thead>
<tr>
<th>Non-rosetting/ Non-IgM binding</th>
<th>RT-PCR</th>
<th>Rosetting/ IgM-binding</th>
<th>RT-PCR</th>
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<tr>
<td></td>
<td>DBLα&lt;sub&gt;type3&lt;/sub&gt;</td>
<td>DBLε&lt;sub&gt;type3&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>3D7(IgM-)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Palo Alto&lt;sup&gt;T&lt;/sup&gt; (R-)</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>TM267&lt;sup&gt;T&lt;/sup&gt; (R-)</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>A1R&lt;sup&gt;T&lt;/sup&gt; (R-)</td>
<td>✗</td>
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<td>TM284</td>
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Table 5.4. Summary of type 3 RT-PCR results for 7 laboratory <i>P. falciparum</i> strains, including rosetting (R+) and non-rosetting (R-) subpopulations of Palo Alto, TM267, A1R, A4R, R29 and IgM binding (IgM+) and non-binding (IgM-) subpopulations of 3D7. Palo Alto, TM267, A1R, A4R, R29 are all IT-derived clones as indicated. In addition, rosetting Palo Alto and TM267 also express the same predominant non-type 3 var gene, PARvar1 (JA Rowe, unpublished data).

5.5.7 Type 3 var gene transcription time course in laboratory strain 3D7

The 3D7 laboratory strain consistently expressed type 3 var genes in the above RT-PCR experiments. Thus, 3D7 was used for investigation of the timing of transcription of the type 3 var genes. Little data is currently available on timing of
transcription of type 3 var genes, apart from a report of type 3 var gene transcripts at “ring; 30 hr post invasion” and “trophozoite/schizont; 36-48 post invasion” 3D7 (Jensen et al. 2004), though relative levels of transcription were not measured. To examine the timing of transcription of the type 3 var genes, 3D7 parasite schizonts were purified using a percoll, and were incubated with fresh erythrocytes for 6 hours for invasion. After 6 hours, the remaining schizonts were destroyed by sorbitol lysis. RNA was collected from early rings, late rings, trophozoites, schizonts and gametocytes from a culture of laboratory strain 3D7 (Fig. 5.23). RT-PCR using the type 3 DBLα primers AF3_type3F and Type3alphaR2, and DBLε primers Type3epsF and Type3epsF, indicated that type 3 var gene transcripts were present at all asexual cell stages, but were not present in gametocyte RNA (Fig. 5.24). Non-type 3 DBLα domains were amplified with the DBLαAF'/DBLαBR primers from Taylor et al. discussed in chapter 3. All 3D7 cultures showed expression of non-type 3 var genes. Tubulin transcripts were present in all samples demonstrating that the cDNA preparation was successful.
Fig. 5.23 Synchronised 3D7 culture, showing early rings (0-6 hr post-invasion), late rings (12-18 hr), pigmented trophozoites (24-30 hr), schizonts (36-42 hr) and gametocytes (stage II-IV). Parasites were stained with Geimsa. Images taken at 100x magnification.
Fig. 5.24. RT-PCR analysis for synchronised 3D7 strain parasites for type 3 DBLα (left) and tubulin (right) was used for amplification for early rings (ER), late rings (LR), trophozoite (Tr), schizonts (Scz), gametocytes (Gc). CTL (control); no reverse transcriptase was used, providing a control for gDNA contamination. Amplifications for type 3 DBLα domains (primers AF3_type3F and Type3alphaR2) and tubulin (primers tubulin 3’ and tubulin 5’) are shown. A 5 µl sample of all RT-PCR products was run on a 1.5% agarose gel, and products were visualised under UV after staining in 0.5 µg/ml EtBr. Genomic DNA controls were positive for PCR product in all cases indicating PCR was successful. All asexual stages, but not gametocytes, were positive for type 3 DBLα domain RT-PCR (left). All cDNA lanes were positive for tubulin RT-PCR (right). DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).
For a more precise analysis of timing of expression, laboratory strain 3D7 parasites were tightly synchronised by purifying schizonts with percoll, and allowing only 1 hour for invasion of rings, before remaining schizonts were removed by sorbitol lysis. Parasite samples were taken regularly for RNA extraction and cDNA preparation until the first new rings were seen 42-43 hours post-invasion (Fig. 5.25).

RT-PCR analysis showed type 3 var gene transcripts were present across all time points (Fig. 5.26A). This transcription pattern was different from that observed for the non-type 3 DBL\(\alpha\) domains, transcription of which was more easily detectable in rings than pigmented trophozoites. The strongest RT-PCR product for the non-type 3 DBL\(\alpha\) domains was seen at late ring/early trophozoite stage (Fig. 5.26B). RT-PCR for tubulin (Fig. 5.26C) confirmed cDNA had been made successfully for each RNA extraction and tubulin transcription increased with maturity of the parasites. Unfortunately, despite repeated treatment with DNase, and repeated RNA collection and extraction, genomic DNA contamination remained a problem in these cDNA preparations (bands in RT-control lanes, Fig. 5.26 right panels). To resolve this issue, a fresh time course was undertaken from the same frozen parasite aliquots for Northern blot analysis.
Fig. 5.25. Synchronised 3D7 culture for RT-PCR time course. Images taken 4 hr before percoll treatment (-4 hr), after percoll treatment (0 hr), after 1 hr of invasion, and after a further 1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 40 and 42 hr after sorbitol treatment. Parasites were stained with Giemsa and images were taken at 100x magnification.
A. **Type 3 DBLε**

B. **Non-type 3 DBLα**

C. **Tubulin**
Fig. 5.26. RT-PCR analysis for synchronised 3D7 strain parasite time course. cDNA (left panels) or RT control samples (right panels) were used for amplification. Time points were 1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 40 and 42 hr post invasion. 3D7 gDNA was used as a positive control and distilled water as a negative control. A) Type 3 DBLε var gene transcription: RT-PCR with primers Type3epsF and Type3epsF. B) Non-type 3 DBLα var transcription: RT-PCR with primers DBLαAF'/DBLαBR primers (Taylor et al. 2000a; Bull et al. 2005a). C). Tubulin transcription RT-PCR with primers Tub3’ and Tub5’. DNA ladder was φX174 RF DNA/Hae fragments (Invitrogen).

5.5.9 Northern blot analysis: probe design
Probes were designed to recognise type 3 DBLα domain, type 3 DBLε domain, exon 2 and MSP1. Both type 3 domain probes were designed against pfa0015c. The type 3 DBLα domain probe spans only the type 3-specific section of the type 3 DBLα domain (Fig. 5.27). The type 3 DBLε domain probe covers the whole of the type 3 DBLε domain, and the PCR primers Type3epsF/R (section 5.5.3) were used.

The exon 2 probe spans the first 310 nucleotides of the exon 2 of pfa0005w, and has 60.8-100% homology to the exon 2 of 3D7 var genes (mean homology 70.5%, S.D. 10.4%). The MSP probe spans 553 bp of the *P. falciparum* 3D7 MSP1.
Fig. 5.27. Clustal W nucleotide sequence alignment of the DBLα probe sequence (identical to pfa0015c) with the homologous stretch of the three type 3 DBLα domains from 3D7. Primers type3probeF (forwards arrow) and Type3alphaR2 (backwards arrow) are indicated. Shading indicates nucleotides identical to the probe sequence.

PCR products were purified with a spin column and ligated into a PCR II vector. The vector was used to transform E. coli, and after overnight growth and plasmid extraction, the insert was sequenced to verify the sequence before probe production.

The insert was then amplified using the specific forward primer and M13R/M13F primer sites within the vector to assess the direction of the insert (Fig. 5.28). All inserts were amplified with M13R (Fig. 5.29), and therefore SP6 polymerase was used for the reverse transcription reaction. SP6 polymerase was used to create a single stranded RNA complementary to the coding sequence for each insert.
Digoxigenin (DIG) label was attached to the uracil (dUTP) in the dNTP mix, and was thus incorporated into the RNA probe. This allows the probe to be detected using an antibody with affinity for the DIG label, overcoming the need for radioactivity (Fig. 5.28).

Fig. 5.28. Schematic of amplification for probe reverse transcription. PCR amplification using the specific forward primer and M13R/M13F primer sites within the vector was used to assess the direction of the insert. If the PCR with M13R is successful (upper panel), the insert is forwards, and thus the SP6 promoter is required for reverse transcription of the complementary strand with respect to the coding sequence for the probe. If the PCR with M13F is successful (lower panel), reverse transcription requires the T7 promoter. Digoxigenin (DIG) is incorporated into the probe during polymerisation.
Fig. 5.29. PCR amplification using the specific forward primer and M13R was successful for all ligated vectors. Forward primers used were DBLα: type3probeF, DBLε: Type3epsF, exon2: exon2F, MSP1: N2, water control lane: N2. DBLε PCR was unsuccssessful for the first ligation (left hand panel), and so the ligation was repeated (right hand panel).

DIG-labelled probes for DBLα, DBLε, Exon2 and MSP1 were diluted to 1:40, 1:400, 1:4000 and 1:40,000, along with a 1:10 dilution series DIG-labelled control probe for actin (starting concentration 10 ng/µl). 1 µl of each diluted probe was spotted onto nylon membrane, and fixed with UV. The membrane was washed briefly in Maleic acid buffer and blocked (1hr) before incubation with anti-DIG antibody (CDP-Star, Roche), to visualise the probe spots (Fig. 5.30).
Fig. 5.30. DIG-labelled probes for DBLα, DBLε, Exon2 and MSP1 were diluted to 1:40, 1:400, 1:4000, 1:40,000 and 1:400,000, along with a 1:10 dilution series of a DIG-labelled control probe for actin (start concentration 10 ng/µl). 1 µl of each diluted probe was spotted onto nylon membrane, and fixed with UV. Probes were then incubated with anti-DIG antibody (CDP-Star), and probe spots visualised after exposure to X-ray film.

Comparisons to the actin probe of known concentration allowed estimation of the concentrations of the new probes. Estimated probe concentrations:

- DBLα probe: 1:40 dilution ≈ 1 ng/µl ∴ probe 40 µg/ml
- DBLε probe: 1:40 dilution ≈ 100 pg/µl ∴ probe 4 µg/ml
- Exon 2 probe: 1:400 dilution ≈ 10 ng/µl ∴ probe 4000 µg/ml
- MSP1 probe: 1:40 dilution ≈ 5 ng/µl ∴ probe 200 µg/ml
5.5.10 Northern blot analysis: presence of type 3 var gene transcript in laboratory strain 3D7

A Northern blot was used to verify the presence of type 3 var gene transcripts in laboratory strain 3D7. RNA prepared from a 9% mixed stage culture of 3D7 (4% ring, 4% pigmented trophozoite, 1% schizont). RNA was separated by agarose gel electrophoresis on a 1.2 % agarose/formamide gel, and the RNA was transferred to nylon membrane. The membrane was hybridised overnight at a range of temperatures (60°C–68°C) with the type 3 DBLα domain probe. The membrane was then washed in 0.5x SSC/0.2% SDS (see materials and methods), and was incubated in blocking buffer before incubation with an anti-DIG secondary antibody, which binds to the DIG label in the probe. Unbound antibody was washed away with Maleic acid wash buffer. The membrane was then processed in an X-ray developer.

Hybridisation with the type 3 DBLα domain probe at 60°C resulted in detection of a 3.98 kb transcript corresponding to a type 3 var gene (Fig. 5.31A). The probe also cross-reacted strongly to a band at approx 6.5 kb, and faintly to longer var gene transcripts (> 9 kb). Increasing the stringency of the washing (longer time and a range of SSC/SDS concentrations) did not reduce the strength of the 6.5 kb band compared to the 3.98 kb transcript. Hybridisation at higher temperatures resulted in loss of any recognition of RNA by the probe.

To confirm the 3.98 kb transcript detected with the DBLα probe was a var gene, a probe generated against var gene exon 2 probe was also used. This generic var gene exon 2 probe was used to probe fresh nylon membranes from the same RNA
preparation from the mixed stage 3D7 culture. Hybridisation at 50°C resulted in recognition of a type 3 var gene length (3.98 kb) transcript (Fig. 5.31B). Non-type 3 var genes (> 9kb) are also clearly recognised by the generic exon 2 probe. The 6.5 kb transcript (Band X in Fig. 5.31B) is again recognised, although only very faintly. Hybridisation at temperatures above 50°C resulted in loss of transcript recognition.

Hybridisation was attempted with the type 3 epsilon probe with various temperature and washing conditions, but no DIG labelled probe was ever detected upon development of the membrane. This may be due to the low concentration of the probe, or an unsuccessful labelling reaction (faint spots, Fig. 5.30).

A homology search of the 3D7 genome database (http://www.plasmodb.org) suggests the only gene which was any homology to the probe is var1csa (PFE1640w); the DBLa probe has 60% homology with nucleotides 8165–8470 of the 9.45 kb var1csa transcript (Fig. 5.32). This may also be the faint upper band in Fig. 5.31, but the identity of the 6.5 kb transcript (band X) is unclear.
A DBLα probe

B Exon 2 probe

Fig. 5.31. Northern blot showing RNA from 9% mixed stage 3D7 culture (4% ring, 4% pigmented trophozoite, 1% schizont). RNA was separated by agarose gel electrophoresis on a 1.2% agarose gel, and the RNA was transferred to nylon membrane. A) The membrane was hybridised overnight (60°C) with a type 3 var gene DBLα domain probe. RNA corresponding to the type 3 DBLα var transcript (3.98 kb) is marked with an arrow (←). The probe cross reacts strongly to a band at approx 6.5 kb, and faintly to longer var gene transcripts (> 9 kb). B) The membrane was hybridised overnight (50°C) with the var gene exon 2 probe. RNA corresponding to the type 3 DBLα var transcript (3.98 kb) is marked with an arrow (←). Longer var gene transcripts (> 9 kb) are also present and recognised by the probe.
Fig. 5.32. Alignment showing the type 3 DBLα probe and the sections of transcripts recognised by it in 3D7; pfa0015c (nucleotides (nt) 886-1132, 100% homology), pfi1182w (nt 864-1120, 99.2% homology), mal6p1.314 (685-1141, 97.6% homology), and var1csa (nt 8165–8470 59.8% homology).

The RNA from the RT-PCR time courses were processed through the Northern blot analysis method describe above. Unfortunately, this was not successful for DBLα domain, DBLε domain or exon 2 probes. The MSP1 probe recognised the schizont extract at 42 hr post invasion only, suggesting the Northern blot method used was successful (Fig. 5.33). This suggests the gel, transfer and detection procedures were successful, and the buffers were correctly made. Failure to detect RNA with the other probes (type 3 DBLα/ε and exon 2) may have been due to the hybridisation or washing being sub-optimal for the other probes.
Fig. 5.33 MSP1 Northern blot of 3D7 time course. RNA samples were as Fig. 5.24 early rings (ER), late rings (LR), trophozoite (Tr), schizonts (Sz), gametocytes (Gc). RNA was separated by agarose gel electrophoresis on a 1.2 % agarose gel, and the RNA was transferred to nylon membrane. The membrane was hybridised overnight (60°C) with the MSP1 probe. RNA corresponding to the MSP1 transcript (5.12 kb) is marked with an arrow (←).
5.5.11 Type 3 var genes in Mali field isolates

The aim of this section was to determine whether type 3 var genes were commonly transcribed in *P. falciparum* field isolates, using gDNA and cDNA from the 26 field isolates from the Mali study sites examined for non-type 3 DBLα transcription discussed in chapter 3 (Lyke et al. 2004). Data is presented for type 3 var gene presence in Mali field isolate gDNA (section 5.5.11), type 3 var gene DBLα/ε domain transcript detection by RT-PCR (sections 5.5.12 and 13 respectively), and finally a summary of type 3 var genes in the Mali isolates (section 5.5.14). Section 5.5.15 will then discuss the sequences phylogenetically.

5.5.12 PCR of type 3 gDNA var genes from Mali field isolate gDNA

Firstly, type 3 DBLα domain amplification on gDNA from each isolate was performed to assess whether type 3 var genes were present in the Mali isolates. PCR was performed using the alpha domain primer pair AF3_type3F and Type3alphaR2. Type 3 var gene DBLα domain PCR product was detected in all isolates (Fig. 5.34).
Fig. 5.34 Type 3 DBLα domains were amplified from gDNA from the 26 Malian field isolates (9 cerebral malaria, 8 hyperparasitaemia and 9 uncomplicated malaria) using primer pair AF3_type3F and Type3alphaR2. A 5 µl sample of all RT-PCR products was run on a 1.5% agarose gel. G: gDNA controls were positive for PCR product in all cases indicating PCR was successful. H: water control lanes. DNA ladder was 100 bp ladder.

For 10 isolates with a good PCR product (HYP1, HYP2, HYP5, HYP6, HYP7, CM1, CM4, CM6, U1 and U4) the PCR products were cloned and used to transform E. coli. The ligated plasmids from 24 colonies were extracted and sequenced from each of these isolates. The number of distinct (>95% identity) DBLα transcripts per isolate ranged from 1-4 from 5-22 miniprep plasmid sequences per isolate (Table 5.5). On average there were 0.86 type 3 DBLα domains per genotype, but more isolates would need to be studied to confirm these proportions.
Table 5.5. Table showing the number of distinct DBLα domains detected by PCR of gDNA using the type 3 alpha domain primer pair AF3_type3F and Type3alphaR2, with the number of miniprep plasmids sequenced. Also shown is the number of genotypes detected in these isolates (details in chapter 3), the relative number of type 3 var genes detected per genotype for each isolate, and the average number of detected DBLα domains per genotype.

<table>
<thead>
<tr>
<th>Clinical disease category</th>
<th>Isolate</th>
<th>Minipreps sequenced</th>
<th>Distinct type 3 DBLα domains in gDNA PCR</th>
<th>Distinct genotypes detected</th>
<th>gDNA type3 per genotype</th>
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</thead>
<tbody>
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<td>Hyperpara-sitaemia</td>
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</tr>
<tr>
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<td>HYP2</td>
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<td>3</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>HYP5</td>
<td>15</td>
<td>3</td>
<td>4</td>
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</tr>
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<td>HYP6</td>
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</tr>
<tr>
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<td>HYP7</td>
<td>19</td>
<td>2</td>
<td>3</td>
<td>0.67</td>
</tr>
<tr>
<td>Cerebral Malaria</td>
<td>CM1</td>
<td>11</td>
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<td>1</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>CM4</td>
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<td>5</td>
<td>0.20</td>
</tr>
<tr>
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<td>CM6</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>Uncomplicated malaria</td>
<td>U1</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>0.50</td>
</tr>
<tr>
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<td>U4</td>
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<td>4</td>
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<td>Average</td>
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<td></td>
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<td>0.86</td>
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</table>

5.5.13 Type 3 var gene transcription in Mali field isolates: Type 3 DBLα domain RT-PCR

Having established that the isolates all appeared to contain type 3 var genes at the gDNA level, RT-PCR was used to assess how frequently these were transcribed. Type 3 DBLα domain RT-PCR using AF3_type3F and Type3alphaR2 was performed on cDNA from each of the 26 Mali field isolates (prepared in chapter 3). PCR products were obtained for 15/26 isolates; 8/8 hyperparasitaemia isolates, 3/9 cerebral malaria isolates and 4/9 uncomplicated malaria isolates. The PCR products
were purified, cloned and sequenced, to assess the number of distinct type 3 DBLα domain transcripts per isolate. There was a significantly higher number of type 3 DBLα domain transcripts seen in the hyperparasitaemia isolates compared to cerebral malaria isolates (t-test, P=0.003). Table 5.6 shows the number of distinct type 3 DBLα transcripts detected per isolate.

<table>
<thead>
<tr>
<th>Clinical disease category</th>
<th>Isolate name</th>
<th>Type 3 RT-PCR</th>
<th>Minipreps</th>
<th>Distinct type 3 DBLα domains</th>
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</thead>
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</tr>
<tr>
<td></td>
<td>HYP2</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP3</td>
<td>14</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP4</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP5</td>
<td>15</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP6</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP7</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP8</td>
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<td>1</td>
<td></td>
</tr>
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<td>1</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td>CM4</td>
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<td>0</td>
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<td>CM6</td>
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</tr>
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<td>11</td>
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</tr>
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<tr>
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<td>4</td>
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<tr>
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</table>

Table 5.6: Presence and number of transcribed type 3 DBLα domains detected by RT-PCR using primers AF3_type3F and Type3alphaR2 in 8 hyperparasitaemia, 9 cerebral malaria and 9 uncomplicated malaria field isolates, with the number of minipreps sequenced.
5.5.14 Type 3 var gene transcription in Mali field isolates: Type 3 DBLε domain RT-PCR

Type 3 DBLε domain transcripts were also amplified by RT-PCR from the same cDNA preparations, using primers Type3epsF and Type3epsR. PCR products were obtained for 16/26 isolates; 6/8 hyperparasitaemia isolates, 6/9 cerebral malaria isolates and 4/9 uncomplicated malaria isolates. As before, the PCR products were purified, cloned and sequenced, to assess the number of distinct DBLε domain transcripts detected per isolate, shown in Table 5.7.

<table>
<thead>
<tr>
<th>Clinical disease category</th>
<th>Isolate name</th>
<th>Type 3 RT-PCR</th>
<th>Minipreps</th>
<th>Distinct type 3 DBLε domains</th>
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</thead>
<tbody>
<tr>
<td>Hyperparasitaemia</td>
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<td></td>
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<tr>
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<td>HYP2</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>HYP3</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP4</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYP5</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
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<td>HYP6</td>
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<td>1</td>
<td></td>
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<tr>
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<td>HYP7</td>
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<td>CM2</td>
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<td>1</td>
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</tr>
<tr>
<td></td>
<td>CM3</td>
<td>3</td>
<td>1</td>
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<td>CM5</td>
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<tr>
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<td>CM6</td>
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<td>1</td>
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</tr>
<tr>
<td></td>
<td>CM7</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CM8</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CM9</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Uncomplicated malaria</td>
<td>U1</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U2</td>
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</tr>
<tr>
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<td>U3</td>
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<td>0</td>
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<tr>
<td></td>
<td>U4</td>
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</tr>
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<td></td>
<td>U7</td>
<td>6</td>
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<tr>
<td></td>
<td>U8</td>
<td>-</td>
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<tr>
<td></td>
<td>U9</td>
<td>-</td>
<td>0</td>
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</table>
Table 5.7: Presence and number of transcribed epsilon domains detected by RT-PCR using primers type 3 DBLα in 8 hyperparasitaemia, 9 cerebral malaria and 9 uncomplicated malaria field isolates, with the number of minipreps sequenced.

Epsilon domains were very similar (>92% amino acid identity, see section 5.5.15) and no isolates showed multiple DBLe domain transcripts.

5.5.15 Type 3 var gene transcription in Mali field isolates: Summary

In total, evidence of type 3 var gene transcription was detected in 23/26 isolates; all 8 hyperparasitaemia isolates, 8/9 cerebral isolates and 7/9 uncomplicated isolates (Table 5.8). The observation of epsilon but not alpha product in 7 isolates, including 5/9 cerebral isolates, was puzzling. RT-PCR was repeated on all of the cerebral isolates (except CM7 due to cDNA running out) with the first pair of type 3 DBLα domain primers (αAF3_type3F and Type3alphaR1, section 5.5.1). Only CM1 showed a PCR product with these primers. RT-PCR was also repeated on all of the cerebral isolates (except CM7 due to cDNA running out) with the forward type 3 DBLα domain primer and reverse epsilon domains primer (αAF3_type3F and Type3epsR). Again, only CM1 showed a PCR product with these primers. The lack of product indicates the type 3 DBLα domains are either not present, or at very low concentration, in the cerebral malaria isolates. The type 3 DBLe domains are more highly conserved and there is no degeneracy in the forward primer, unlike the forward primer for the type 3 DBLα domains. Thus it is possible that a very low level of type 3 var gene transcription would be picked up by the epsilon but not the alpha domain primers, as the epsilon primers may be a more powerful primer pair.
It is also possible the alpha domain primers could not amplify the alpha domain, though product was seen in the gDNA PCR for all cerebral isolates.

<table>
<thead>
<tr>
<th>Clinical disease category</th>
<th>Isolate name</th>
<th>Type 3 RT-PCR</th>
<th>Estimated transcribed type 3 repertoire</th>
<th>Genotypes*</th>
<th>DBLα1%*</th>
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</thead>
<tbody>
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<td>Hyperparasitaemia</td>
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<td>1 4 0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>HYP2</td>
<td>2 1 2</td>
<td>3 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>HYP3</td>
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<td>HYP4</td>
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<tr>
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<td>HYP7</td>
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<td>3 0.06</td>
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<tr>
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<td>HYP8</td>
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<td>2 0.00</td>
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<td>1 0.83</td>
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<td>U3</td>
<td>0 1 1</td>
<td>3 1.00</td>
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<td>U4</td>
<td>1 1 1</td>
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<td>U7</td>
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<td></td>
<td>U9</td>
<td>0 0 0</td>
<td>1 0.06</td>
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</tbody>
</table>

Table 5.8: Presence and number of transcribed type 3 alpha and epsilon domains detected by RT-PCR in 8 hyperparasitaemia, 9 cerebral malaria and 9 uncomplicated malaria field isolates, and the estimated (minimum) repertoire of transcribed type 3 var gene in each isolate. *Genotypes and DBLα1% data was taken from chapter 3.
There was no significant difference in the proportion of isolates showing type 3 var gene transcription in the three isolate groups (Chi-squared, \( p = 0.37 \)). The relative levels of transcription between isolates cannot be directly compared, as the RT-PCR was not quantitative. However, there was a significantly higher number of type 3 transcripts seen in the hyperparasitaemia isolates compared to cerebral malaria isolates (t-test, \( p = 0.02 \); Fig. 5.35A). This difference is even greater if only the number of distinct DBL\( \alpha_{\text{type3}} \) transcripts detected is compared (t-test, \( p = 0.003 \)). This is in contrast to the findings of chapter 3 which showed an increase in non-type 3 DBL\( \alpha_{1} \) transcripts in the cerebral versus hyperparasitaemia isolates, and shows an association between type 3 transcription and non-cerebral malaria.

There was no correlation between number of estimated transcribed type 3 var genes and DBL\( \alpha_{1} \% \) as calculated in chapter 3 (Spearman Rank correlation: Rho = -0.03, \( p = 0.89 \); Fig. 5.35B). There was also no correlation between number of estimated transcribed type 3 var genes and number of genotypes estimated in chapter 3 (Spearman Rank correlation: Rho = 0.18, \( p = 0.38 \); Fig. 5.35C).
Fig. 5.35. A) Estimated transcribed type 3 var repertoire for the three isolate clinical categories. Hyperparasitaemia isolates showed significantly higher diversity of type 3 var genes than cerebral malaria isolates (t-test p = 0.02). B) Spearman Rank correlation DBLα1% and estimated transcribed type 3 repertoire (Rho = -0.03, p = 0.89). C) Spearman Rank correlation number of genotypes and estimated transcribed type 3 var gene repertoire (Rho = 0.18, p = 0.38).
Comparing the estimated number of transcribed and encoded type 3 var genes, approximately 50-70% percent of the type 3 var genes are transcribed (Table 5.9).

<table>
<thead>
<tr>
<th>Clinical disease category</th>
<th>Isolate</th>
<th>Distinct type 3 DBLα domains in gDNA PCR</th>
<th>Distinct type 3 DBLα domains transcribed</th>
<th>Estimated type 3 var genes transcribed</th>
<th>Transcribed: type 3 DBLα gDNA ratio</th>
<th>Transcribed: type 3 var gene gDNA ratio</th>
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</thead>
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<td>1</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
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<td>HYP2</td>
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<td>2</td>
<td>2</td>
<td>0.67</td>
<td>0.67</td>
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<td></td>
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<td>1.00</td>
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<td>1.00</td>
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<td>0.33</td>
</tr>
<tr>
<td>Uncomplicated malaria</td>
<td>U1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>U4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0.25</td>
<td>0.25</td>
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<tr>
<td></td>
<td>Average</td>
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<td></td>
<td></td>
<td>0.56</td>
<td>0.69</td>
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</table>

Table 5.9. Table showing the number of distinct DBLα domains detected by PCR of gDNA using the type 3 alpha domain primer pair AF3_type3F and Type3alphaR2, with the number distinct DBLα transcripts detected, and estimated number of type 3 var genes transcribed per isolate. The ratio of transcribed:encoded type 3 var genes, and the average of these for all 10 isolates is given.

The lack of product in CM9, U2 and U9 for alpha and epsilon domain RT-PCR indicates no, or little, type 3 var gene transcription. It is unlikely the RT-PCR reaction “failed” due to a poor cDNA preparation, as amplification of non-type 3 DBLα domains had been successful (chapter 3).

The low number of minireps sequenced per isolate for alpha and epsilon RT-PCR mean that it is possible that other type 3 domain transcripts were also present. The
estimated number of type 3 transcripts in each isolate is included in the table of Mali isolate data (Appendix 1).

5.5.16 Type 3 var gene diversity in Mali field isolates

The type 3 domains showed a similarly high level of conservation as previously published type 3 var genes, with amino acid identities of 58.3-100% for the alpha domains (mean 76.3%, SE 0.4%, SD 0.2%) and 94.2-100% for the epsilon domains (mean 97.8%, SE 0.3%, SD 0.002%) as shown in Fig. 5.36 (histograms and box plot) and illustrated pictorially in Fig. 5.37.
Fig. 5.36. Box plot (displaying 10th, 25th, 50th, 75th and 90th percentiles; values <10th percentile and >90th plotted separately) and histograms of percentage amino acid identity between cerebral, hyperparasitaemia and uncomplicated malaria type 3 DBLα and DBLε domains. Percentage identity was calculated using MegAlign. Histograms and box plots of identity were then created in Statview. Note the scale for amino acid identity on the histograms are different.
Fig. 5.37. Amino acid sequence distance charts showing percentage amino acid identity between Mali field isolate DBL$_{\alpha type3}$ and DBL$_{\varepsilon type3}$ domains. Darkness of shading indicates level of identity between domains. Amino acid sequences were created from nucleotide sequences in EditSeq, and percentage identity was calculated using MegAlign.
5.5.17 Phylogenetic analysis of DBLα type 3 domains from Mali field isolates

The DBLα\textsubscript{type3} domains were aligned using clustal W and phenograms were created (Fig. 5.38). As expected from a subgroup of domains with such high sequence similarities, the DBLα\textsubscript{type3} domains cluster separately from the non-type 3 DBLα domains of 3D7 (Fig. 5.38). The DBLα\textsubscript{type3} domains clustered into 6 distinct clades, representing 6 highly conserved DBLα\textsubscript{type3} domains groups DBLα\textsubscript{type3} A-F of >95% nucleotide sequence identity (Fig. 5.38). The consensus sequence for these groups was defined as Type 3 DBLα\textsubscript{type3} A-F so homology comparisons to different sequences could be made. Mal6p1.314 did not fall into one of these groups (nearest homology was 94% to type 3 group C).
Fig. 5.38. Phenograms of field isolate Type 3 DBLα domains created from a clustal W alignment of sequences. Type 3 DBLα domains from the Mali isolates, transcribed or gDNA only are included, with 3D7 DBLα1 domains for comparison. Sequences are grouped into consensus group (>95% identity) A-F. Type 3 DBLα domains are above dotted line. Non-type 3 DBLα domains are below the line. D1
and D2 are defined as a subset of group D, as the sequences of group D2 fall just outside the 95% homology cut-off, but are very similar (94% identity) to those of group D1.

DBLα\textsubscript{type3} were also amplified from gDNA from 6 Kenyan field isolates (gift from Pete Bull, Kilifi, Kenya), resulting in a further 8 DBLα\textsubscript{type3} sequences. It was not possible to count the number of genotypes within each sample, as only a few microlitres cDNA was available, however a range of 1-5 is expected from previous work in this area (Bull \textit{et al}. 1999). Five of these sequences fell into the strictly defined 6 subgroups A-F of DBLα\textsubscript{type3} (>95% nucleotide sequence identity). In addition, 9 other sequences from various global locations (Trimnell \textit{et al}. 2006) also showed high homology to the defined DBLα\textsubscript{type3} subgroups (Fig. 5.39). DBLα\textsubscript{type} group A is particularly highly conserved, with 19 sequences belonging to this group with >99% homology including sequences from vastly different geographic areas (East Africa, West Africa and South America), and a further 2 sequences with >95% homology. One sequence Kilifi gDNA5D was identical to the alpha domain of Mal6p1.314 (Fig. 5.39). A summary of the type 3 DBLα groups A-F is given in Table 5.10.
Fig. 5.39. Phenograms of field isolate Type 3 DBLα domains created from a clustal W alignment of sequences. A) Type 3 DBLα domains from the Mali isolates,
transcribed or gDNA only are included, with Kenya isolates sequences and other published type 3 DBLα domain sequences. Sequences are grouped into consensus group (>95% identity) A-F, including D1 and D2 separately as before.

<table>
<thead>
<tr>
<th>Type 3 DBLα domain group (&gt;95% identity)</th>
<th>Mali type 3 DBLα domains (% identity to group)</th>
<th>Kilifi type 3 DBLα domains (% identity to group)</th>
<th>Published type 3 DBLα domains (% identity to group)</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>U1B (100%)</td>
<td>K1C (99%)</td>
<td>713. W Africa (100%)</td>
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<tr>
<td></td>
<td>U5A (100%)</td>
<td></td>
<td>M5a, W Africa (99%)</td>
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<tr>
<td></td>
<td>U6A (100%)</td>
<td></td>
<td>Ecu, S America (99%)</td>
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<td></td>
<td>U8A (100%)</td>
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<td></td>
<td>U12A (99%)</td>
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<td></td>
<td>HYP1A (100%)</td>
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<td></td>
<td>HYP3D (100%)</td>
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<td>HYP4A (96%)</td>
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<td>HYP5A (99%)</td>
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<td>HYP6B (99%)</td>
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<td>HYP7B (99%)</td>
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<td></td>
<td>HYP2C gDNA (99%)</td>
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<td>CM1B gDNA (100%)</td>
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<td>CM4A gDNA (99%)</td>
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<td>CM6C gDNA (99%)</td>
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<td>pfi1820w, 3D7 (100%)</td>
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<td>Fab6a, S Africa (100%)</td>
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<td>HYP5D gDNA (100%)</td>
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<td>HYP7A (100%)</td>
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<td>CM6A gDNA (98%)</td>
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<td>HYP2A (97%)</td>
<td>K1A (99%)</td>
<td>M2, W Africa (98%)</td>
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<td>HYP3B (95%)</td>
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<td>D10a, PNG (97%)</td>
</tr>
<tr>
<td></td>
<td>HYP5E gDNA (96%)</td>
<td></td>
<td>V1Sa, SE Asia (96%)</td>
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<tr>
<td></td>
<td>CM1A (98%)</td>
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<td></td>
<td>CM7A (99%)</td>
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<tr>
<td>D2 (94% homology to D1)</td>
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<tr>
<td>E</td>
<td>U5C (99%)</td>
<td>None</td>
<td>None</td>
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<td>U8B (99%)</td>
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<td>U11B (99%)</td>
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<td>U4C gDNA (100%)</td>
<td>K1B (95%)</td>
<td>Pfa0015c, 3D7 (100%)</td>
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<td>D10b, PNG (100%)</td>
</tr>
<tr>
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<td>U10A (99%)</td>
<td></td>
<td>V1Sb, SE Asia (100%)</td>
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<td></td>
<td>HYP2B (100%)</td>
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<td></td>
<td>HYP3C (99%)</td>
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<td>Hype5F gDNA (98%)</td>
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</table>
Table 5.10. Six distinct groups of conserved (>95% identity) DBL\(\alpha\)\text{type3} domains A-F, showing the percentage identity of each sequence to the group consensus. Mali field isolate RT-PCR and gDNA sequences, Kilifi gDNA sequences and domains obtained from the NCBI database. Percentage nucleotide homology the defined DBL\(\alpha\)\text{type}

5.5.18 Phylogenetic analysis of DBL\(\varepsilon\) type 3 domains from Mali field isolates

The DBL\(\varepsilon\)\text{type3} domains also cluster separately from non-type 3 domains, forming just one tight group in the phenogram clearly distinct from the non-type 3 epsilon domains from 3D7 (Fig. 5.38). As they all show such high homology to each other they cannot be separated into more distinct subgroups. All type 3 epsilon domains found on NCBI database showed >97% nucleotide homology to this group. This is also reflected in the high sequence homology across the whole repertoire of amplified DBL\(\varepsilon\)\text{type3} domains (Fig. 5.40).
Fig. 5.40 Phenogram of field isolate DBLε<sub>type3</sub> domains (bracketed) with 3D7 epsilon domains for comparison created from a clustal W amino acid alignment. *3D7 type 3 epsilon domains are marked with an asterisk.
5.6 Results 2: Type 3 PfEMP1 protein analysis

5.6.1 Type 3-specific anti-peptide polyclonal antibody production:

**DBL\(\alpha\) domain structure**

As one hypothesis of this chapter was that there might be an association of type 3 DBL\(\alpha\)1 domain expression with cerebral malaria, it was decided to raise an antibody to the type 3 DBL\(\alpha\) domain. This was intended to be a useful tool for confirming whether the type 3 PfEMP1 is translated, and would be used to determine if the type 3 PfEMP1 were surface exposed, using immunofluorescence, and Western blotting with protease treatment of the surface exposed proteins using trypsin. In the future, a type 3 DBL\(\alpha\) domain could potentially be used to determine type 3 DBL\(\alpha\)1 domain function; for example if binding assays suggested the type 3 DBL\(\alpha\)1 domain had affinity for certain ligands, the antibody could be used to block binding, and show specificity of the interaction. It could also be used in culture, for example to determine whether the type 3 DBL\(\alpha\)1 domain has any role in processes such as invasion, or for selection of type 3 PfEMP1-expressing parasites. These future experiments are outside the scope of this chapter (see discussion for further details).

The type 3 DBL\(\varepsilon\) domains could also have been chosen as the target of a type 3 specific antibody as they are also very highly conserved. However, as they are more membrane proximal they might not be as accessible to antibody for example in live cell immunofluorescence assays.

As described in the introduction, DBL\(\alpha_{\text{type 3}}\)domains contain a specific and conserved subdomain, comprising the second half of the domain (Fig. 5.41). Therefore it was
useful to model the structure of the type 3 specific subdomain to predict any structural feature which may influence antibody design, and to predict surface exposed regions.

![Diagram](image)

**Fig. 5.41.** Alignment of 3D7 group A var gene DBLα domains. Type 3-specific and conserved region is boxed in red.

The structure of the DBL domains of EBA175 had previously been published (Tolia et al. 2005). In comparison to EBA175 RII monomer F2 domains (Tolia et al. 2005), the type three specific portion of a DBLα_{type3} domain is equivalent to the F2 subdomain 2. The switch from generic DBLα1 domain to type 3 specific conserved
part of the DBL\textsubscript{\textalpha_{type3}} domain occurs between the two subdomains, specifically between helices T and U as described in Tolia \textit{et al.} (2005) (Fig. 5.42). This is equivalent to between subdomains 2 and 3 of the \textit{P. knowlesi} DBL as defined by Singh \textit{et al.} (2006); subdomains 1 and 2 of the \textit{P. knowlesi} DBL as defined by Singh are equivalent to the first subdomain of the RII EBA175 monomer F2 DBL domain, defined by Tolia.

![Diagram](image)

Fig. 5.42. The EBA175 RII monomer F1 (green) and F2 (purple) subdomains showing alpha helices (rectangles) and disulfide bridges (red lines), adapted from Tolia \textit{et al.} (2005). The conserved type 3-specific region corresponds to the second half of the DBL\textsubscript{\textalpha} domain sequence, corresponding to the second subdomain (circled in red) within domain F2 (purple) of the eba174 RII monomer (Tolia \textit{et al.} 2005). The first subdomain of the F2 DBL domain as defined by Tolia is equivalent to subdomains 1 and 2 of \textit{PkDBL} as defined by Singh \textit{et al.} 2006 and the second Tolia subdomain is equivalent to Singh subdomain 3.
To analyse the structure of the type 3-specific DBLα domain sequence more closely, the structures of EBA175 and the *P. knowlesi* DBL were downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank ([http://www.pdb.org](http://www.pdb.org)). The 3D7 type 3 *var* gene mal6p1.314 sequence was used in structural modeling as a representative type 3 DBLα domain. The mal6p1.314 DBLα domain amino acid sequence was aligned to the amino acid sequence of EBA175 and *P. knowlesi* DBLα by clustal W. The aligned amino acid sequence was then mapped onto the published structures using Cn3d structural modelling software ([http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml](http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml)). This programme superimposes aligned amino acids sequences but does not attempt to model novel structural features, or resolve unaligned sequences. Thus resulting structures are not definitive, but gross features, such as long alpha helices can be confidently positioned. The two DBL structures map very closely onto each other using this technique, showing a high degree of structural identity between these two domains, suggesting there might be a high structural similarity between other DBL domains.

Structural modelling of the type 3-specific subdomain using published structures of EBA175 and *P. knowlesi* DBL ([Tolia et al. 2005; Singh et al. 2006](http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml)) as a scaffold, suggests the type 3-specific region is a disordered stretch, followed by two long helices separated by another unstructured region (Fig. 5.43).
Fig. 5.43. Cn3D generated image of Mal6P.314 amino acid sequences mapped onto known DBL structures. A and B (upper panel) show Mal6P1.314 mapped onto EBA175 F2 domain (PDB ID 1ZRL; Tolia et al. 2005). C and D (lower panel) show Mal6P1.314 mapped onto *P. knowlesi* DBL structure (PDB ID 2c6j; Singh et al. 2006). A and C (left) show the whole DBLα domain. B and D (right) show only the type 3 conserved region. Type 3 conserved regions are highlighted in yellow, showing disordered residues followed by two predicted long alpha helices.
5.6.2 Type 3-specific anti-peptide polyclonal antibody production: peptide choice

Peptides for anti-peptide antibodies should be 12-16 amino acids long (advice from peptide.design@eurogentec.com) with no internal cysteines as these are used for conjugation to the carrier. For production of an anti-peptide antibody for global recognition of all type 3 domains, peptides were chosen that are well conserved in all known type 3 domains but do not occur in other DBLα1 domains or any other parasite or host proteins. The peptides must also be highly antigenic to increase the chance of successful and specific antigen production in the host animal.

Protean software analysis was used to aid peptide design (Fig. 5.44). Alpha helical regions predicted by structural modelling (Fig. 5.43) are also supported by Garnier Robson analysis (Fig. 5.44). Alpha helical regions suggest a conserved structure, and thus potentially a conserved structural epitope for antigen recognition. Helical peptides are good antigenic targets for recognition of proteins in native states, such as by immunoflorescence, as it is likely that the peptide may also form a helix, and thus have a similar conformation to the native protein. However, denatured proteins have lost secondary structure, so antibodies to helices are not necessarily helpful for recognition of denatured proteins by Western blot analysis.
After analysis with Protean/Lasergene, and consultation with the company Eurogentec (peptide.design@eurogentec.com), three peptides were identified as the most promising epitopes:

1) DTDPPVVDYIPQ
2) CNVLNKEIDEMNNQ
3) LVSKWKN(E/Q)FDK

Kyte-Doolittle plots of these amino acids show the hydrophillicity of the regions is high, suggesting that these amino acids are probably surface exposed, in agreement with the Emini surface probability plot (Fig. 5.44). The Jameson-Wolf analysis also shows high antigenic index for most of this region (Fig. 5.44). These peptides are shown on an amino acid alignment of type 3 DBLα domains (Fig. 5.45) and highlighted in a DBLαtype 3 domain structure model (Fig. 5.46). Blast search of the NCBI database suggested these epitopes do not occur in other proteins.
Fig. 5.44. Analysis of the final 133 amino acids of Mal6p1.314 DBLα domain using Protean/Lasergene software (DNAsStar, Inc), showing predictions of alpha helical and beta sheet regions (Garnier-Robson, Chou-Fasman and Eisenberg plots), hydrophobicity (Kyte-Doolittle plot), flexible regions (Karplus-Schulz plot), antigenicity (Jameson-Wolf index) and surface exposure probability (Emini plot). Potential peptides for immunisation (DTDPVVVDYIPQ) (CNVLNKEIDEMNNQ) and (LVSKWKN(E/Q)FDK) are boxed.
Fig. 5.45. Position of three potential immunogenic peptides (DTDPPVVDYIPQ) (CNVLNKEIDEMNNQ) and (LVSKNWKEN(E/Q)FDK) (boxed) on clustal W alignment of type3 amino acid sequences; mal6p1.314, pfi1820w and pfa0015c from 3D7, pfclin379b05 from Ghanaian clinical isolate (www.sanger.ac.uk), ittype3.pot from IT/FCR3 strain and mc var5 from Malayan Camp.
Fig. 5.46. Cn3D generated worm (A) and spacefill (B) images of Mal6P.314 amino acid sequences mapped onto the known structure of EBA175 F2 domain (PDB number 1ZRL) (Tolia et al. 2005). Peptides DTDPPVVDYIPQ (1), CNVLNKEIDEMNNQ (2) and LVSKWKN(E/Q)FDK (3) are indicated in yellow.

Two peptides are better than one for raising polyclonal antigen as this increases the immunization success rate of obtaining antibodies that recognize the protein from 75% to 95% (reference http://uk.eurogentec.com). Due to the E/Q discrepancy in the third peptide LVSKWKN(E/Q)FDK, the two peptides chosen were:

Peptide 1. DTDPPVVDYIPQ
Peptide 2. CNVLNKEIDEMNNQ
As described, both peptides are totally conserved in all available published type 3 DBLα domains, including the three from 3D7, and showed no homology to any other proteins (P. falciparum, human, or otherwise) by NCBI blast homology search. Both are hydrophilic with a high antigenic index (Jameson-Wolf and Kyte-Doolittle plots; Fig. 5.44), and are thus likely to be surface exposed with good antigenic potential. Peptide 2 is likely to resemble native protein secondary structure, as is it predicted helical by structural modelling and Garnier-Robinson prediction. As such, antibodies recognising this peptide are likely to recognise native protein such as in immunoflorescence. Peptide 1 is less likely to resemble native protein conformation as it is predicted to be disordered by Protean analysis. Antibodies recognising this epitope will potentially recognise denatured proteins such as in a Western blot. A terminal cysteine would be added to peptide 1 to facilitate coupling to the carrier (final sequence DTDPPVVDYIPQ +C).

5.6.3 Preimmune sera analysis

Two host animals were required for immunisation. The more commonly used and appropriate host species were hen and rabbit. To decide which species would be used, 5 preimmune hen egg yolks and 5 preimmune rabbit sera were obtained from Eurogentec (Belgium), and the preimmune antibody repertoire from the animals was tested for recognition of infected and uninfected red blood cells.

Preimmune hen egg samples from 5 candidate hens were obtained as fresh yolk. Preimmune hen yolk IgY purification was carried out using the Promega Eggstract
Extracted IgY concentration (2-4 mg/ml) was within the expected concentration of IgY from yolk (Eggstract kit manual).

To determine how pure the sample was, the 1 µl (2-4 µg) of the IgY extractions was run on a reducing SDS-PAGE gel and either stained with SimplyBlue (Fig. 5.47A) or transferred onto a PVDF membrane and probed with a goat anti-chicken HRP-conjugated secondary antibody (Fig. 5.47B). The heavy (67 kDa) and light (27 kDa) IgY chains were detected by SimplyBlue and by goat anti-chicken HRP-conjugated secondary antibody probing. Fig. 5.47 shows that the purified IgY extract was successful and was relatively pure.

Fig. 5.47. IgY extractions (PO38-42). A) 1 µl of each IgY extract (2-4 mg/ml) was run out on a gradient 3-8% reducing tris-acetate SDS-PAGE gel and all proteins were visualized by staining with SimplyBlue. B) Proteins transferred to PVDF membrane and detected with an anti-chicken HRP- conjugated secondary antibody
(1:1000, 1 hr incubation). IgY was visualised using enhanced chemiluminescence and exposure to X-ray film. The protein ladder was a pre-stained precision protein molecular weight marker (Bio-Rad).

5.6.4 Preimmune recognition of IE proteins by Western blot

Western blots were used to determine whether the antibodies in the preimmune sera or yolk extract recognised proteins from infected erythrocytes (IE). Proteins extracted from an asynchronous culture of 3D7 were run on a 3-8% reducing acrylamide gel. The proteins were transferred to PVDF membrane. The membrane was cut into strips for incubation in the sera at 1:100 dilution. The appropriate secondary antibody (anti-chicken or anti-rabbit) conjugated to HRP was used to detect primary antibody binding.

Each IgY extract showed some recognition of IE proteins (Fig. 5.48). PO40 IgY bound the IE proteins the most strongly and PO41 and PO42 showed the least binding in the 155-270 kDa range. The Western blot was repeated to try to improve the quality of protein detection but this was not successful. It was decided to rely on affinity purification to reduce background antibody affinity for IE proteins.
Fig. 5.48. Western blot of an asynchronous culture of 3D7 (gift from JP Semblat) using preimmune IgY extracts. Extracted IE proteins were separated by reducing SDS-PAGE on a 3-8% tris-acetate gel (1 hr, 150 V). Proteins were transferred to PVDF membrane (1 hr, 30V) and the membrane was cut into strips. Each strip of membrane was blocked overnight in milk and then incubated in the IgY extract (PO38-42) at 1:100 dilution for 1 hr. HRP-conjugated anti-chicken secondary antibody (1:1000, 1 hr incubation) was used to bind IgY. Bands were visualised using enhanced chemiluminescence and exposure to X-ray film. The protein ladder was a pre-stained precision protein molecular weight marker (Bio-Rad).

Each preimmune rabbit sera also showed some recognition of IE proteins, with strong recognition of proteins in the PfEMP1 size (155-270 kDa) range (Fig. 5.49). Rabbit S170 showed the lowest levels of host protein recognition. All rabbits serum recognized proteins around size of known type 3 PfEMP1 (153 kDa), and strong recognition of proteins around 100 kDa and 250 kDa.
Fig. 5.49. Western blot of an asynchronous culture of 3D7 (gift from JP Semblat) using preimmune rabbit sera. Extracted IE proteins were separated by reducing SDS-PAGE on a 3-8% tris-acetate gel (1 hr, 150 V). Proteins were transferred to PVDF membrane (1 hr, 30V) and the membrane was cut into strips. Each strip of membrane was blocked overnight in milk and then incubated in the preimmune rabbit sera (S166-S170) at 1:100 dilution for 1 hr. HRP-conjugated anti-rabbit secondary antibody (1:1000, 1 hr incubation) was used to detect bond IgG. Bands were visualised using enhanced chemiluminescence and exposure to X-ray film. The protein ladder was a pre-stained precision protein molecular weight marker (Bio-Rad).
5.6.5 Preimmune recognition of IE proteins on live cells in immunofluorescence assay (IFA)

Samples of *P. falciparum* culture were used to assess whether the preimmune sera or yolk IgY would recognise native proteins in an immunofluorescence assay (IFA). As many parasite proteins that are exported to the surface of the erythrocyte are seen on the surface at pigmented trophozoite stage (e.g. PfEMP1), the IFAs were performed when the culture contained a majority of parasites at the pigmented trophozoite stage (4%), although some ring stage parasites were also present (1%). As such any recognition of host erythrocyte proteins, or *plasmodium* protein on the IE surface at ring or pigmented trophozoite stage, should be detected.

A TM284 *P. falciparum* culture (gift from A Raza) was incubated for 1 hour in preimmune sera (1:10) or preimmune IgY (1:10) in PBS/1% BSA. The IgY was then washed off and the cells were incubated in alexa 488 conjugated secondary antibody 1:1000 and PBS/DAPI/BSA1% for 1 hour in the dark. Secondary incubation was washed off and the live cells were viewed under fluorescence at 100x magnification.

Representative images from each of the IFA are shown in Fig. 5.50 and a summary is given in Table 5.11. Where recognition occurred, DAPI staining revealed fluorescing cells were not specifically infected or uninfected.
Fig. 5.50. Representative images of immunofluorescence assays (IFA) on TM284 infected IE. Cells at 2% haematocrit and cultures at 4% trophozoite, 1% ring stage. A) Cells were incubated with chicken pre-immune IgY extract (1:10) and then with alexa488-conjugated goat anti-chicken secondary antibody (1:1000). B) Cells were incubated with rabbit preimmune serum (1:10) and then with alexa488-conjugated goat anti-rabbit secondary antibody (1:1000).
Table 5.11 Preimmune IFA summary.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Preimmune sera</th>
<th>Immunofluorescence observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hen egg IgY extracts</td>
<td>PO38</td>
<td>Occasional cells fluoresced strongly plus occasional speckles in other cells.</td>
</tr>
<tr>
<td></td>
<td>PO39</td>
<td>Occasional cells fluoresced strongly plus moderate speckles in most cells.</td>
</tr>
<tr>
<td></td>
<td>PO40</td>
<td>All cells fluoresced strongly.</td>
</tr>
<tr>
<td></td>
<td>P041</td>
<td>Rare fluorescence of a few speckles per cell.</td>
</tr>
<tr>
<td></td>
<td>PO42</td>
<td>Moderate speckling in every cell.</td>
</tr>
<tr>
<td>Rabbit Sera</td>
<td>S166</td>
<td>Occasional fluorescence of few speckles per cell.</td>
</tr>
<tr>
<td></td>
<td>S167</td>
<td>No fluorescence.</td>
</tr>
<tr>
<td></td>
<td>S168</td>
<td>Occasional fluorescence of few speckles per cell.</td>
</tr>
<tr>
<td></td>
<td>S169</td>
<td>No fluorescence.</td>
</tr>
<tr>
<td></td>
<td>S170</td>
<td>No fluorescence.</td>
</tr>
<tr>
<td>Secondary controls</td>
<td>Chicken control</td>
<td>No fluorescence.</td>
</tr>
<tr>
<td></td>
<td>Rabbit control</td>
<td>No fluorescence.</td>
</tr>
</tbody>
</table>

5.6.6 Animal choice and justification

Chickens were chosen for immunization. Yolk have more IgY than rabbit sera has IgG and more yolk volume is received from chicken than sera from rabbits. Thus chicken immunization provides more antibody per animal. Avian antibody is expected to be less cross-reactive to mammalian proteins than rabbit antibody. Although the immunofluorescence data suggested rabbit preimmune sera showed lower recognition of IE proteins, all rabbit preimmune sera showed a high level of recognition of proteins around the size of type 3 PfEMP1 (153 kDa).

Chickens SH19 and SH20 were chosen because they showed the lowest levels of preimmune recognition of infected erythrocyte proteins in Western blot and
immunofluorescence. This suggested they might yield specific antibody with low background of non-specific signal. Any remaining non-specific recognition in the post-immunisation polyclonal antibody mix will be reduced by affinity purification against the two immunizing peptides.

5.6.7 Generation of affinity-purified polyclonal antibody

Chickens PO41 and PO42 were immunised with peptides DTDPPVVDYIPQC (peptide 1) and CNVLNKEIDEMNNQ (peptide 2) by Eurogentec (Belgium).

Eggs were collected in weekly batches, and received as fresh pooled yolk. IgY was extracted from days 51-98 post immunization, and extracted IgY reconstituted in PBS to the original yolk volume for each weekly pool (Table 5.2). Affinity purification against a mixture of the two peptides was performed using the Sulfolink technology (Promega) for 4/7 pools. This exploits the free sulfhydryl groups on the terminal cysteines of the peptides to immobilize the peptides on a SulfoLink Resin (see Fig. 5.51 and materials and methods). IgY was eluted in 6 ml elution buffer, due to its low pH, then buffer exchanged into 0.5 ml PBS.

Fig. 5.51. SulfoLink Resin affinity purification method. Diagram taken from Sulfolink kit 20325 manual, Pierce.
Affinity-purified IgY from days 79-85 (dates 25/04-02/05) were used in all following experiments, unless stated otherwise.

5.6.8 Affinity-purified antibody recognises target peptides by ELISA

To determine whether the pre-immune, post-immune and affinity-purified antibodies had affinity for the peptides, an ELISA was performed. Peptides were immobilised onto ELISA plate wells overnight at 4°C. Antibody was incubated in the plates and non-bound antibody was removed by washing before incubation with 150 µl of peroxidase conjugated goat anti-chicken IgG diluted 1:1000, for 1 hour. TMB Substrate was added to each well and the plate was left to develop for 30 min in the dark, before the reaction was stopped with sulfuric acid. The concentration of bound antibody was assessed by absorbance at 450 nm. These are shown as mean ± standard error (SE), Fig. 5.52.

The ELISA data (Fig. 5.52) showed preimmune sera had low affinity for the peptides, and immunisation resulted in IgY with affinity for both peptides. The pool of antibodies from the immune yolks also has increased affinity for BSA and milk block compared to IgY extracted from the yolks of the hens pre-immunisation. However, after affinity purification, the resulting antibody pools from both hens had increased specificity for both peptides, and greatly reduced affinity for BSA and milk block. Both pools of affinity-purified antibodies had equally high affinity for the 2 peptides, suggesting that both peptides had elicited a successful immune response in each hen.
Fig. 5.52. ELISA showing affinity of preimmune, post immune IgY and affinity-purified IgY, purified against both peptides simultaneously. Antibody was incubated in ELISA plate wells coated (overnight at 4°C) with 10 µg/ml peptide 1, 10 µg/ml peptide 2, 1% BSA in PBS or PBS alone. Non-bound antibody was removed by washing before incubation with 150 µl of peroxidase conjugated goat anti-chicken IgG diluted 1:1000. Detection was measured as absorbance at 450 nm after incubation with TMB Substrate then sulfuric acid. These are shown as mean ± standard error (SE).
5.6.9 Affinity purified antibody recognises band of approximately 150 kDa

To assess whether the antibody recognised type 3 PfEMP1 in a protein extract of 3D7 culture. 6.5 µl of protein extract was separated by denaturing SDS-PAGE on a 3-8% tris-acetate gel, and the proteins were transferred to PVDF membrane. The membrane was blocked overnight in milk. At the same time, 1:100 dilution of the immune IgY or affinity purified IgY from chickens PO41 and PO42 was pre-incubated overnight in blocking buffer with a small strip of PVDF membrane to remove antibodies that bound non-specifically to the membrane. The blocked membrane was incubated for 1 hour with the immune or affinity purified IgY. Non-bound antibody was then removed by washing, and bound antibody detected by incubation with secondary HRP-conjugated anti-chicken antibody followed by enhanced chemiluminescence and exposure to X-ray film.

The pre-purification immune IgY from both chickens showed a high level of non-specific affinity to many proteins IE proteins (Fig. 5.53) as was predicted by ELISA (Fig. 5.52). The affinity-purified antibody from hen PO41 did not show any strong protein recognition; a faint band at approximately 150 kDa may be type 3 PfEMP1. The affinity-purified antibody from hen PO42 recognised a protein of approximately 150 kDa on SDS-PAGE by Western blot that is specific to infected erythrocytes and may be type 3 PfEMP1. The three type 3 PfEMP1 in 3D7, Mal6p1.314 (153.3 kDa), Pfa0015c (153.3 kDa) and pf1820w (152.0 kDa), are all too close in size to differentiate these by migration. There is also a strong band at approximately (180 kDa).
Fig. 5.53. Western blot of an asynchronous culture of 3D7 using immune IgY extract and affinity-purified IgY from PO41 (left) or PO42 (right). Triton soluble protein extraction from uninfected erythrocytes (u) and erythrocytes infected with a 10% mixed stage culture of 3D7 (i) were run on a reducing SDS-PAGE on a 3-8% tris-acetate gel (1 hr, 150 V). Proteins were transferred to PVDF membrane (1 hr, 30V) and the membrane was cut into 4 strips. Each strip of membrane was blocked overnight in milk and then incubated with immune IgY extract or affinity-purified IgY from PO41 (left) or PO42 (right) at 1:100 dilution for 1 hr and 1 hr secondary incubation with HRP-conjugated anti-chicken secondary antibody (1:1000). Bands were visualised using enhanced chemiluminescence and exposure to X-ray film. An approximately 150 kDa protein, the size of a type 3 PfEMP1, is indicated (arrow). The ladder was pre-stained precision protein molecular weight marker (BioRad).
5.6.10 Affinity purification against peptides separately

Affinity purification was also carried out on the two peptides separately for immune IgY from chicken PO42. Only chicken PO42 IgY was used as the PO42 IgY affinity purified against both peptides showed higher recognition of the band of 150 kDa, which was thought to be the type 3 PfEMP1, than the PO41 IgY affinity purified against both peptides. Affinity purification was performed as before using Sulfolink resin affinity purification method. The purified antibody showed recognition of the appropriate peptide, and low affinity for PBS/BSA (Fig. 5.54). Purification with peptide 2 and both peptides together were successful, with enriched IgY with affinity for the appropriate wells. Purification against peptide 1 separately was less successful, with low specific affinity for peptide 1.
Fig. 5.54. ELISA showing affinity of post immune non-purified PO42 IgY and PO42 IgY purified with both peptide, or peptides 1 or 2 separately. Antibody was incubated in ELISA plate wells coated (overnight at 4ºC) with 10 µg/ml peptide 1, 10 µg/ml peptide 2, 1% BSA in PBS or PBS alone. Non-bound antibody was removed by washing before incubation with 150 µl of peroxidase conjugated goat anti-chicken IgG diluted 1:1000. Detection was measured as absorbance at 450 nm after incubation with TMB Substrate then sulfuric acid. These are shown as mean ± standard error (SE).
To assess the recognition specificity for IE proteins of the peptide1- and peptide 2-purified antibodies a Western blot was performed. The 3D7 culture protein extract used in Fig. 5.53 was separated again by denaturing SDS-PAGE on a 3-8% tris-acetate gel, and the proteins were transferred to fresh PVDF membrane. The membrane was blocked overnight in milk. At the same time, 1:100 dilution of chicken PO42 immune IgY affinity purified against peptide 1 or peptide 2 was pre-incubated overnight in blocking buffer with a small strip of PVDF membrane to remove antibodies that bound non-specifically to the membrane. The blocked membrane was incubated for 1 hour with the affinity purified IgYs. Non-bound antibody was then removed by washing, and bound antibody detected by incubation with secondary HRP-conjugated anti-chicken antibody followed by enhanced chemiluminescence and exposure to X-ray film (Fig. 5.55).

Antibody purified against both peptides independently recognised a 150 kDa protein in *P. falciparum* protein extract but not in the uninfected erythrocyte controls (Fig. 5.55). Antibody affinity purified against peptide 1 showed weak recognition of type 3 protein, and also cross reacted with a host cell-derived protein at 240 and 260 kDa (possibly spectrin). Antibody affinity purified against peptide 2 gave a much stronger recognition of the type 3 protein at 150 kDa, but also cross-reacted with a parasite-derived protein at approx 270 - 300 kDa, possibly non-type3 PfEMP1. Recognition of the triton-soluble fraction by the antibody purified against both peptides (Fig. 5.53, above) more closely resembles that purified against peptide 2 only. Thus, it is likely that the majority of IgY in the purified antibody pool recognises peptide 2.
Fig. 5.55. Triton soluble (TS) and Triton insoluble (TI) protein extractions from erythrocytes infected with a 6% trophozoite stage culture of 3D7 and uninfected erythrocytes control were run on a reducing 3-8% tris-acetate gel SDS-PAGE and proteins were transferred to PVDF membrane. The membrane was blocked overnight in milk and probed with 1:1000 dilution of PO42 IgY affinity-purified against peptide 1 (left) or peptide 2 (right). Secondary incubation was with HRP-conjugated anti-chicken secondary antibody (1:1000). Bands were visualised using enhanced chemiluminescence and exposure to X-ray film. A protein of the correct size for a type 3 PfEMP1 (153 kDa) is indicated (arrow). The protein ladder was a pre-stained precision protein molecular weight marker (Bio-Rad).
5.6.11 The 150 kDa band detected with the antibody appears to be trypsin insensitive

The pigmented trophozoite culture was treated with 0.1-1mg/ml trypsin for 5 min, and then treated with trypsin inhibitor for 5 min, before protein extraction. The 150 kDa band detected with the antibody appears to remain trypsin insensitive, indicating it may be internal (Fig. 5.56).

In an attempt to verify the trypsin worked, the blot was repeated and probed for non-type 3 PfEMP1 with an antibody with affinity for the ATS region and complement receptor 1 which are expected to be trypsin sensitive at >10 µg/ml (Miller et al. 1977; Sharling et al. 2007). Unfortunately the Western blots were unsuccessful due to high background (PfEMP1 ATS) or no signal (CR1). However, the type 3 antibody cross-reacts with bands in the 270-300 kDa range in the 3D7 triton insoluble protein extracts which may be non-type 3 PfEMP1. These bands are reduced on addition of the trypsin at 1 mg/ml (Fig. 5.56), suggesting that the trypsinisation was effective.
Fig. 5.56. Triton soluble (TS) and Triton insoluble (TI) protein extractions from uninfected erythrocytes (uRBC) and erythrocytes infected with a 6% trophozoite stage 3D7 culture (3D7) were run on a denaturing SDS-PAGE and probed with affinity-purified anti-type 3 antibody. Samples marked (+) were incubated with 1mg/ml trypsin for 5 min before protein extraction. The 153 kDa protein is marked with an arrow. Bands thought to correspond to non-type 3 PfEMP1, which reduce in intensity after trypsinisation, are indicated (*). The protein ladder was a pre-stained precision protein molecular weight marker (Bio-Rad).
5.6.12 Antibody recognises a protein of 150 kDa in HB3; a strain without a type 3 PfEMP1

The recent whole genome sequencing of parasite strain HB3 revealed that this strain was a natural knock-out for the type 3 PfEMP1. This explains “failure” of the type 3 alpha and epsilon PCR in HB3 gDNA in section 5.5.4.

HB3 parasites were cultured and protein samples collected from a culture of 7% trophozoites. Proteins were loaded on to four reducing 3-8% tris-acetate acrylamide gels, separated by SDS-PAGE, and transferred to PVDF membrane for Western blot (3 gels), or stained in SimplyBlue to record total protein loaded (1 gel). The three PVDF membranes were probed with preimmune IgY, immune IgY affinity purified against peptide 1, or immune IgY affinity purified against peptide 2. Probing with immune IgY affinity purified against peptide 2 showed strong recognition of a 150 kDa protein in HB3, as well as in 3D7 rings and pigmented trophozoites (Fig. 5.57). This suggests the 150 kDa band is specific to infected erythrocytes, but may not be specific to a type 3 PfEMP1.

In order to see if this cross reactivity was a problem due to using reducing SDS-PAGE, the Western blot with the same antibody preparation was repeated using native (non-reducing) gels. No proteins were recognized by the antibody after membrane were probed with preimmune IgY or immune IgY affinity purified against peptide 1 or 2.
Fig. 5.57. Triton soluble (TS) and Triton insoluble (TI) protein extractions from uninfected erythrocytes (U), HB3 culture 7% trophozoites (ht), 3D7 culture 6% rings (3r) and 3D7 culture 6% pigmented trophozoites (3t) were loaded onto reducing 3-8% tris-acetate acrylamide gels, separated by SDS-PAGE, and transferred to PVDF membrane for Western blot or stained in SimplyBlue (top right) to record total
protein loaded. Membranes were blocked in 5% milk o/n and probed with preimmune IgY (top left), immune IgY affinity purified against peptide 1 (bottom left), or immune IgY affinity purified against peptide 2 (bottom right) for 1 hr. After washing, secondary incubation was with HRP-conjugated anti-chicken secondary antibody (1:1000). Bands were visualised using enhanced chemiluminescence and exposure to X-ray film. A protein of 153 kDa is recognised in all infected erythrocyte lanes, including HB3, by peptide 2-affinity purified antibody (indicated with an arrow). The protein ladder was a pre-stained precision protein molecular weight marker (Bio-Rad).

5.6.13 Antibody recognises a protein on HB3 by fixed IFA

A fixed IFA was used to assess whether the antibody would recognise uninfected erythrocytes, and erythrocytes infected with 3D7 or HB3. 3D7 and HB3 *P. falciparum* cultures were smeared on to a glass slide and fixed with acetone. The slides were incubated for 1 hour with preimmune IgY or immune IgY (affinity purified against both peptides) both 1:10 in PBS/1% BSA, or with PBS/1% BSA alone. Unbound antibody was then washed off and the slides were incubated in alexa 488 conjugated secondary antibody 1:1000 and PBS/DAPI/1%BSA for 1 hour in the dark. Secondary incubation was washed off and the fixed cells were viewed under fluorescence at 100x magnification. The IFA on 3D7 revealed the affinity-purified antibody recognized infected erythrocytes only (Fig. 5.58). However, repeating the IFA on HB3-infected erythrocytes also resulted in recognition of IE by the affinity-purified antibody (Fig. 5.58). For both 3D7 and HB3 cultures, uninfected erythrocytes did not fluoresce with preimmune IgY or PBS/1%BSA alone (Fig.
As only infected erythrocytes were recognised by the affinity purified antibody, the cross reactivity must be to a parasite-derived protein, as seen with the Western blot (Fig. 5.58). It is likely there is cross reactivity to high molecular weight PfEMP1, as suggested by faint bands (>250 kDa (*), Fig. 5.56), as well as with the band of 150 kDa in HB3, which is also likely to exist in 3D7. All erythrocytes, infected and uninfected, fluoresced with immune IgY in live IFA (data not shown). Whether there is any specific recognition of the type 3 PEMP1 in 3D7 is not clear.
Fig. 5.58. *P. falciparum* cultures of 3D7 (6% pigmented trophozoites; upper 3 panels) and HB3 (7% pigmented trophozoites; bottom) were smeared onto slides and fixed with acetone. Slides were incubated for 1 hour in preimmune IgY, immune IgY extract affinity purified against peptides 1 and 2 together (all 1:10 in PBS/1% BSA), or PBS/1%BSA alone. The culture was then washed and the slides were incubated in alexa 488 conjugated secondary antibody 1:1000 and PBS/DAPI/1/%BSA for 1 hour in the dark. Secondary incubation was washed off and the fixed cells were viewed under fluorescence at 100x magnification. Representative images of 3D7 with preimmune IgY, affinity purified immune IgY extract and PBS/1%BSA alone, and HB3 with affinity purified immune IgY extract are shown. HB3 did not fluoresce with preimmune IgY or PBS/1%BSA alone.

5.7 Discussion

This chapter examined the type 3 var genes and PfEMP1 in a range of laboratory and field isolates. The hypotheses to be examined in this chapter were threefold. Firstly, that there would be a high level of conservation in all type 3 var genes in laboratory strains and field isolates, but that the timing of expression may be unusual due to the lack of a regulatory intronic promoter which controls the silencing of other var genes (Deitsch 2001; Calderwood *et al.* 2003; Frank *et al.* 2006). Secondly that there might be a link between type 3 var gene transcription and cerebral malaria, due to the association of group A var transcription with cerebral malaria isolates discussed in chapter 3. Thirdly, due to the high conservation of type 3 PfEMP1 it was hypothesised that type 3 PfEMP1 may not be surface exposed, and design of a type
3-specific antibody was attempted to address this question. The data obtained in answer to the above hypotheses will be discussed.

PCR of gDNA revealed 4 of the 9 laboratory isolates examined encode a type 3 var gene. Transcripts were only detected in TM267, Palo Alto and 3D7, suggesting expression was not constitutive, as had been hypothesised due to the lack of regulatory intron (Deitsch 2001; Calderwood et al. 2003; Frank et al. 2006). Another conserved var gene family, var1csa, also lacks the regulatory intron, and appears to be regulated uniquely, possibly outside of the mutually exclusive var gene promoter system (Kyes et al. 2003; Winter et al. 2003; Kyes et al. 2007). Transcription of type 3 var genes was not associated with rosetting in TM267 or Palo Alto, or with IgM binding in 3D7.

RT-PCR data for laboratory strain 3D7 suggests that type 3 var genes are expressed in both ring and trophozoite stages of erythrocytic development. Northern blot confirmed the presence of the type 3 var gene transcript in a mixed stage culture, but due to technical difficulties a precise Northern blot time course could not be completed. There was no evidence for a unique mechanism of transcriptional control. No type 3 transcripts were detected in gametocytes, in agreement with a previous study (Sharp et al. 2006).

The type 3 DBLα domains were omitted in the field isolate transcription profiling study (chapter 3) as in many similar var gene studies (Fowler et al. 2002; Bull et al. 2005a; Albrecht et al. 2006; Barry et al. 2007; Kraemer et al. 2007), due to disparity
between type 3 and non-type 3 reverse primer site sequences (Taylor et al. 2000a; Bull et al. 2005a). Most (23/26) of the field isolates examined in this study show evidence of type 3 var gene transcription, supporting recent observations that the vast majority of isolates contain a type 3 var gene (Trimnell et al. 2006). Type 3 var sequences show an extremely high level of conservation within and between isolates. The almost total conservation of the second half of DBLα<sub>type3</sub> domains suggests a very particular and conserved function. The first half of the DBLα<sub>type3</sub> domain sequence, though not entirely conserved, appears to be constrained in sequence, and six conserved subgroups of DBLα<sub>type3</sub> domain (A-F) were defined. This indicates a functional constraint on the type 3 PfEMP1 alpha domain, and suggests that there may be a limited repertoire of DBLα<sub>type3</sub> sequences. DBLε<sub>type3</sub> domains are also highly conserved, with nucleotide sequence homology >97% across the whole domain. DBLε<sub>type3</sub> domains are so similar that different domains from different type 3 var genes may not easily be distinguished, and no isolate showed multiple distinct epsilon transcripts in the RT-PCR analysis.

The high similarity of DBLε<sub>type3</sub> domain sequences partially explains the discrepancy between the number of alpha and epsilon domains detected by RT-PCR. However, why some isolates showed DBLα<sub>type3</sub> domain but not DBLε<sub>type3</sub> domain transcription, or vice versa, cannot easily be explained. It is formally possible DBLα and DBLε domains are regulated alternately, though this would be very unusual and unexpected. Alternatively, it could be a PCR artefact. The most likely explanation is that there is a very low level of type 3 var gene transcript in these isolates, and the alpha domain PCR is less powerful than the epsilon domain PCR due to the
degeneracy of the forward primer, and so cannot efficiently amplify the type 3 DBLα domain transcript by RT-PCR. Type 3 transcription in field isolates could be studied in more detail, especially where alpha and epsilon data were inconsistent, to explore this observation more thoroughly.

The second hypothesis, that there would be a strong correlation between type 3 DBLα1 domain transcription and cerebral malaria, did not appear to be true for type 3 DBLα1 domains. Type 3 var gene transcripts were not more commonly detected in cerebral malaria isolates. Though type 3 var gene DBLε domain transcripts were detected in 8/9 cerebral isolates, type 3 var gene DBLα domain transcripts could not be detected, suggesting a low level of transcription. Furthermore, all 8 hyperparasitaemia isolates and 7/9 uncomplicated isolates showed evidence of type 3 var gene DBLε domain transcription, with type 3 var gene DBLα domain transcripts also detectable in all hyperparasitaemia and 5/7 of these uncomplicated malaria isolates. The data suggests a correlation between increased diversity of type 3 var gene transcription with hyperparasitaemia. As the total RT-PCR product was not quantifiable, it was not possible to directly compare abundance of type 3 transcripts in different isolates, and so it is not possible to conclude an overall higher level of type 3 var gene transcription in hyperparasitaemia isolates compared to cerebral malaria isolates. It is intriguing that the uncomplicated malaria isolates fall between the hyperparasitaemia and cerebral malaria isolates in the diversity of type 3 var gene transcripts observed. This is reminiscent of the level of DBLα1 domain transcription in uncomplicated malaria isolates falling between that of cerebral malaria isolates and hyperparasitaemia malaria isolates reported in chapter 3. This
strengthens the observation of a discrepancy in diversity of type 3 \textit{var} gene transcripts in the two other groups, as uncomplicated malaria can be considered an earlier stage in disease progression, before expansion to hyperparasitaemia, or development of severe symptoms, as discussed in chapter 3.

It is interesting that genes with the same upstream region (group A type 3 \textit{var} genes versus group A non-type 3 \textit{var} genes; both UpsA) appear to be regulated differently in terms of level of expression in field isolates. It may be possible that parasite populations in an infection tend to have a certain proportion of parasites activating group A \textit{var} gene expression; when this is a non-type 3 group A \textit{var} gene, severe symptoms are common, but when this is a type 3 group A \textit{var} gene, severe symptoms are less likely, and hyperparasitaemia can develop. There was not a significant negative correlation between type 3 group A \textit{var} gene transcript diversity and non-type 3 DBL\(\alpha\)1 domain transcription proportion, though data on absolute type 3 \textit{var} gene versus non-type 3 group A transcription level is not available.

This association between the DBL\(\alpha\)1-type 3 domain with hyperparasitaemia may indicate that only DBL\(\alpha\)1(non-type3) are involved in severe disease pathology, for example through processes such as rosetting (Rowe \textit{et al.} 1995; Russell \textit{et al.} 2005), which tend to be mediated through non-type 3 DBL\(\alpha\)1 domains (Rowe, JA, unpublished data). Alternatively, it could be the large variety of domains in the long and complex group A PfEMP1 that are crucial to disease pathology, rather than the DBL\(\alpha\)1 domain directly. The reduced number of extracellular domains in the type 3 PfEMP1 may result in reduced adhesive potential. This is supported by \textit{in vitro} data
that no type 3 var genes were upregulated when parasites were selected for adhesion to a human bone marrow endothelial cell line (Jensen et al. 2004). Type 3 var gene transcription was not found to be associated with rosetting or IgM binding in this study, and the type 3 epsilon domain is not capable of IgM binding (Semblat, JP et al. in prep). In addition, and unlike many other group A var genes, the type 3 var genes were not increased in transcription after selection for “severe malaria phenotype” by affinity for pools of semi immune plasma (Jensen et al. 2004). These observations suggest that type 3 var genes are not likely to be specifically involved in severe disease pathogenesis, though very little data on type 3 var transcription in vivo is available. Parasites from experimentally infected volunteers expressed type 3 var, though transcription levels were low, and tended to decrease with time of infection, unlike many non-type 3 group A var genes (Lavstsen et al. 2005). In a subsequent study, type 3 var gene transcription was found to be significantly higher in severe malaria cases than in asymptomatic malaria, though this increase may simply reflect the 7 fold higher parasitaemia in the severe malaria group (Rottmann et al. 2006). It would have been interesting to compare this severe malaria type 3 var gene transcription to an appropriate hyperparasitaemia patient group with similar parasitaemia.

The third hypothesis examined type 3 PfEMP1 and possible surface exposure. The high level of conservation of the type 3 var genes (for example all epsilon domains share > 97%, and the 6 groups of highly conserved type 3 DBLα domains) appears to be at odds with its membership of a family of variant surface antigens. Of the two other well-conserved PfEMP1 families, VAR2CSA is conserved due to a crucial role
of CSA-binding in placental malaria (discussed in chapter 4), but the role of VAR1CSA is still uncertain. It is generally believed that conserved proteins are selected for a particular purpose, thus the conservation and frequency of the type 3 var genes between isolates indicates an important function, although this study identified 5 laboratory strains in which a type 3 var could not be amplified from gDNA (Muz12, Dd2, HB3, TM180 or 7G8) suggesting they cannot be essential. The role of the type 3 var genes cannot be determined without first studying the encoded proteins. At present there is no published data determining whether the type 3 PfEMP1 are expressed at a protein level, or if they are exported to the infected erythrocyte surface. It is conceivable such a conserved var gene family might be a liability to a strategy of immune evasion; if a copy was commonly expressed on the surface of every P. falciparum isolate antibodies might be raised which could recognise all other isolates on subsequent infections. It is possible such a conserved gene would remain internal.

To attempt to address this possibility, a polyclonal antibody to the type 3 var was designed and raised in chickens. The DBLα_{type3} domain was chosen for antibody production rather than the DBLε_{type3} domain. The type 3-specific portion of the DBLα_{type3} domain is highly conserved and so it was possible to identify type 3-specific peptide epitopes for immunisation. It would also have been possible to use peptide epitopes from the epsilon domain to create a type 3 PfEMP1-specific antibody. However, DBLα_{type3} domains also contain a variable region, whereas DBLε_{type3} domains are almost identical across the whole domain. Thus different DBLα_{type3} domains are more likely to have different binding properties than different
DBL\(_{\text{type3}}\) domains. In addition, DBL\(\alpha\) domains are more N-terminal and thus lie further away from the erythrocyte surface. Being more exposed they might have an increased binding potential. DBL\(\alpha\) domains which bind host molecules have already been defined in the case of rosetting (Rowe et al. 1997; Russell et al. 2005), and so the interaction of conserved DBL\(\alpha_{\text{type3}}\) domains with other proteins is not unlikely. Thus the binding properties of DBL\(\alpha_{\text{type3}}\) domains appeared to be more interesting to study than that of DBL\(_{\text{type3}}\) domains, especially due to the association between non-type 3 DBL\(\alpha1\) domains and cerebral malaria. Therefore an antibody raised against the DBL\(\alpha_{\text{type3}}\) domains was considered potentially more useful.

Hens rather than rabbits were chosen for immunisation due to the better pre-immune sera data for hens, specifically for hens PO41 and PO42. Hen egg yolks also give an increased yield of antibody compared to rabbit sera. Avian antibodies are also less likely to cross react with mammalian erythrocyte proteins than rabbit antibodies.

After consultation with Eurogentec, 2 highly conserved type 3-specific peptides were chosen for immunisation. Structural modelling on EBA175 (Tolia et al. 2005) and \(P.\) knowelsi DBL\(\alpha\) (Singh et al. 2006) allowed one helical and one disordered peptide to be chosen. This was to give a high chance of eliciting specific antibodies to recognise type 3 PfEMP1 in immunofluorescence (native state) and in Western blot (denatured state). ELISA confirmed a specific recognition of both peptides with affinity-purified antisera, though the level of recognition was not very high compared to non-specific binding to BSA/PBS in the immune serum prior to affinity purification. Affinity-purification against both peptides together gave a similar
affinity profile to antisera purified against peptide 2 only. This suggests peptide 2 elicited a more efficient immune response in the hens. The majority of antibodies in the resulting affinity-purified antisera thus probably recognise peptide 2.

The affinity-purified anti-peptide antisera recognised a protein of approximately 150 kDa on a Western blot in protein extracts from ring and pigmented trophozoite stages, indicating that type 3 PfEMP1 (153 kDa) may be recognised by the antibody at both ring and pigmented trophozoite stage. In general, PfEMP1 are expressed at ring stage and are packaged in single small vesicles and exported to Maurer’s clefts under the erythrocyte plasma membrane (Haeggstrom et al. 2004; Knuepfer et al. 2005; Frankland et al. 2006). If type 3 PfEMP1 acted as “normal” they would be expected to appear at the erythrocyte surface, usually in knobs approximately 16 hours post-invasion (Horrocks et al. 2005). The mechanism of export of PfEMP1 is controversial, but appears to involve an N-terminal hydrophobic stretch and a PEXEL, motif or vacuolar transport signal (VTS), located within the first DBL domain (Hiller et al. 2004; Marti et al. 2004; Knuepfer et al. 2005). The PEXEL/VTS are present in the type 3 PfEMP1. This suggests trafficking of type 3 PfEMP1 is, at least in part, through a similar mechanism to other PfEMP1.

There is evidence that at least some type 3 PfEMP1 reaches the surface, as it can be labelled with sulfo-N-hydroxysuccinimide (NHS)-LC-biotin (Sharling et al. 2007). Sulfo-NHS-LC-biotin is an ester vitamin that reacts with and labels lysines and the N-termini of proteins via primary amino groups. Uptake of biotin through the novel permeation pathway (Baumeister et al. 2003) was prevented using furosemide (Kirk
et al. 1994; Sharling et al. 2007), thus the biotin labelling was specific to surface-exposed proteins. Surface proteins were separated by SDS-PAGE and a band of approximately 150 kDa was detected with HRP-conjugated streptavidin. Detection using a generic antibody to PfEMP1 ATS region suggested this protein was probably type 3 PfEMP1, suggesting the type 3 PfEMP1 was on the erythrocyte surface.

Surface-exposed PfEMP1 is sensitive to cleavage by trypsin at concentration 0.1 mg/ml (Leech et al. 1984; Sharling et al. 2004), and trypsinisation results in loss of PfEMP1-mediated adhesion in trypsin-sensitive PfEMP1 (Nielsen et al. 2007). This study suggested that 150 kDa band recognised by the type 3 antibody is resistant to trypsin at 0.1-1 mg/ml. Higher molecular weight proteins (> 270 kDa) in the triton insoluble fraction which cross reacted with the antibody and are probably non-type 3 PfEMP1, decreased in intensity in the trypsin-treated lanes. This suggests the trypsinisation was successful. In addition, type 3 PfEMP1 trypsin-insensitivity was also observed in a study of PfEMP1 biotinylation (Sharling et al. 2007). This indicates the type 3 PfEMP1 is resistant to trypsin cleavage, or is not exported to the erythrocyte surface.

If the type 3 PfEMP1 is surface-exposed, an alternative reason for trypsin insensitivity could be simply its length. The epsilon domain and second half of the alpha domain of type 3 PfEMP1 may be hidden. EBA175, a dimer of two DBL domains, has a predicted length of 8.6 nm, extrapolating from a Stoke’s radius of 4.3 nm (Tolia et al. 2005). Thus, the extracellular portion of a type 3 PfEMP1, also having just two DBL domains, may be expected to have a similar length. The
glycocalyx, a network of polysaccharides on cellular components of the blood, has a mean thickness on erythroctyes of 5.9 nm (Linss et al. 1991). Speculatively, this fits well with the approximately 3/4 of the type 3 PFEMP1 being conserved, possibly leaving the N-terminal portion of the DBLα domain open to immune pressure and the resulting diversifying selection.

Unfortunately, these results were all questionable due to the antibody recognising a band of the same size in HB3, a strain without a type 3 var gene. Whether the antibody cross-react with something in HB3, but also recognises the type 3 PfEMP1 in 3D7 is not clear. If the type 3 PfEMP1 is recognised, as suggested by recognition of the peptides through ELISA, then the intensity of the Western blot band should decrease if the type 3 PfEMP1 is sensitive to trypsin, even if the antibody also cross-reacts with another protein of a similar size. Affinity purifying the antibody against the type 3 PfEMP1 protein directly might be helpful.

The function of the type 3 PfEMP1 remains unclear. Their conserved sequence suggests an important function, although their absence in certain strains such as HB3 suggests they are not essential. As they appear to remain internal, a role in sequestration is unlikely. A role in invasion is possible, as invasion proteins would be expected only to be surface exposed at merozoite stage, such MSP-1 or AMA-1 (O'Donnell et al. 2000; Triglia et al. 2000). If affinity purification of the antibody against the type 3 PfEMP1 directly was successful, then future experiments could help elucidate the function of the type 3 PfEMP1. Using the antibody in a type 3 PfEMP1-expressing culture may reveal a function in processes such as invasion,
merozoite release or schizont rupture, or in binding phenotypes such as rosetting or platelet-mediated clumping. Binding-affinity in type 3 PfEMP1-expressing strains could also be tested through expression of DBLα$_{\text{type3}}$ domains, or entire type 3 PfEMP1 in in vitro expression systems. Expression could be verified using the antibody, and type 3 PfEMP1-specific adhesion could be confirmed using additional antibody to reverse binding. The antibody may also help to address type 3 binding specificity by inhibiting adhesion of type 3-expressing parasites to ligand-coated plates.

In conclusion, the experiments outlined in this chapter revealed that type 3 var genes are commonly transcribed in laboratory strains and field isolates, and are highly conserved, largely falling into 6 highly conserved groups on the basis of sequence polymorphism in the first half of the alpha domain. Surprisingly, transcription was associated with hyperparasitaemia rather than cerebral malaria, as was the case for other non-type 3 DBLα1 domains in chapter 3, suggesting an altered regulation of this subpopulation of group A var genes. Unfortunately, attempts to raise an antibody to investigate type 3 PfEMP1 expression timing and protein location were unsuccessful due to cross-reactivity of the antibody produced. The type 3 var genes/PfEMP1 remain an intriguing and highly conserved family, and further investigation of these var genes/PfEMP1, for example with regard to function, would be of great interest in the future.
Chapter 6

Curdlan sulfate: potential for treatment of severe malaria symptoms caused by rosetting \textit{P. falciparum} strains

Some of the results presented in this chapter were published as

6.1 Abstract

Gene sequence associations with severe malaria disease manifestation, such as the link between group A var gene transcription cerebral childhood malaria (discussed in chapter 3) and var2csa transcription placental malaria in pregnant women (discussed in chapter 4), provide information on the pathogenesis of the disease and offer data for informed drug and vaccine design to improve medical treatment available to infected individuals. Vaccines usually target conserved parasite antigens to elicit a protective immune response and drugs can be designed to disrupt processes associated with severe disease. The spontaneous binding of infected erythrocytes to uninfected erythrocytes to form rosettes is a property of some strains of P. falciparum that is linked to severe complications of malaria. Rosette disrupting drugs such as heparin and fucoidan have high anticoagulant activity, preventing their use as treatment. Curdlan sulfate (CRDS) is a sulfated glycoconjugate compound that is chemically similar to heparin but with much lower anticoagulant activity. CRDS has previously been shown to have antimalarial activity in vitro and is safe for clinical use. Previous work in the Rowe laboratory revealed that CRDS significantly inhibited rosetting in seven P. falciparum laboratory strains. In this chapter the ability of CRDS to inhibit rosetting in a group of 18 African clinical isolates was examined. CRDS disrupted rosettes in vitro in all 18 P. falciparum African field isolates examined. The kinetics of CRDS anti-rosetting action in laboratory strain Palo Alto was also examined, revealing fast action on rosettes (< 2 min) and reversibility of rosette disruption. The strong ability to inhibit rosetting suggests that CRDS has the potential to reduce the severe complications and mortality rates from
P. falciparum malaria among African children, supporting further clinical trials of CRDS.

**6.2 Introduction**

Despite decades of research, malaria still claims an estimated million lives per year in sub-Saharan Africa, and severe complications such as cerebral malaria remain relatively common (WHO 2000). Eradication of Plasmodium remains a long-term goal. Meanwhile, it is crucial to target factors that contribute to severe disease and reduce clinical complications of malaria. Rosetting, which is estimated to be a factor in 25-50% of severe malaria cases in sub-Saharan Africa (Rowe 2005), is one obvious target for therapy to reduce incidents of severe disease.

**6.2.1 Molecular basis of rosetting**

Rosetting is the spontaneous binding of non-infected erythrocytes by erythrocytes infected with mature asexual blood stage Plasmodium parasites (reviewed in Rowe 2005). The molecular basis of rosetting centres on PfEMP1, the high-molecular weight transmembrane protein, encoded by the plasmodium var multigene family, discussed in chapters 3-5. Rosetting is a strain-specific property (Wahlgren et al. 1990) and it is the N-terminal DBLα domain of a subset of PfEMP1 that mediates the rosetting interaction (Rowe et al. 1997; Chen et al. 2004). There is little sequence similarity between rosetting DBLα domains, though sequence analysis suggests the majority are DBLα1 domains, and that rosetting PfEMP1 are frequently Group A var genes (J.A.Rowe unpublished data; Bull et al. 2005a; Kyriacou et al. 2006; Normark et al. 2007 and as discussed in chapter 3).
Rosetting is a heterogeneous phenomenon, and a variety of molecules on the erythrocyte surface may participate in rosette formation, including heparan sulfate (HS)- like glycosaminoglycans (GAG) (Chen et al. 1998a; Barragan et al. 1999; Vogt et al. 2003), complement receptor 1 (Rowe et al. 1997), and the trisaccharide glycan of blood group A antigen (Barragan et al. 2000). CD36, a scavenger receptor on many host cells including erythrocytes (van Schravendijk et al. 1992), has also been shown to occasionally support rosetting, acting as the receptor for Malayan Camp strain rosettes (Handunnetti et al. 1992). Low molecular weight “rosettins” may also be involved, though there is little data on these (Helmby et al. 1993).

6.2.2 Rosette frequency (RF) is linked to severe disease in African field isolates

In African *P. falciparum* malaria field isolates, there is a compelling link between rosette frequency (RF) and severe disease (Carlson et al. 1990; Rowe et al. 1995), and higher rosetting was observed in the cerebral malaria compared to hyperparasitaemia or uncomplicated Malian field isolates discussed in chapter 3. Rosette-like aggregates have been observed in congested cerebral vessels (Riganti et al. 1990), but are infrequently reported in cerebral malaria postmortem. Rosettes are not observed in arterioles (high wall sheer stress), but rapidly reform in the venules (low wall sheer stress), demonstrating rapid reformation after disruption in an artificially perfused rat microvasculature model of *P. falciparum*–infected erythrocyte sequestration (Kaul et al. 1991).
However, an association between rosetting and severe malaria disease is not the case in Asia, where complement receptor 1 (CR1) deficiency is common. CR1 deficient erythrocytes show reduced RF with 5/5 laboratory isolates (Rowe et al. 1997), including Malayan Camp which rosettes through CD36- perhaps pointing to a synergistic interaction between CD36 and CR1.

All other members of human-infective *plasmodium* family also rosette, for example almost all *P. vivax* isolates show some degree of rosetting (Udomsanpetch et al. 1995; Angus et al. 1996; Chotivanich et al. 1998; Lowe et al. 1998). However, it is only in *P. falciparum* that rosetting is linked to severe disease, probably because of the additional cytoadhesion property which may act in concert with rosetting to cause vessel obstruction (Rowe 2005) as demonstrated in a rat model (Kaul et al. 1991). Human erythrocyte polymorphisms such as CR1 deficiency in Asia (Cockburn et al. 2004; Thomas et al. 2005) and blood group O (Rowe et al. 2007) in malaria-endemic areas have been postulated to be selected for because of their protective effect in malaria due to reduced rosetting, and a resulting inverse association with severe malaria. It has not so far been possible to prove a causative link between rosetting and severe malaria, but the evidence is compelling.

### 6.2.3 Rosette disruption by heparin and heparan sulfate (HS)

Heparan sulfate (HS) and heparin are members of the sulfated glycosaminoglycan (GAG) family. HS and heparin are both composed of alternating glycosamine and uronic acids, linked 1→3 or 1→4 (Fig. 6.1). The glucosamine can be N-sulfated or N-acetylated and can contain O-sulfate ester on the second carbon. The uronic acids
can be glucuronic acid or iduronic acid and may also be O-sulfated. Binding properties of distinct GAGs are associated with the sulfate and carboxyl groups. As sulfation states of heparin and HS vary, the two can be very similar, but in general heparin is a highly sulfated form of HS, with a more uniform sulfation across the molecule and more extensive modification. HS is found on almost all cells including endothelial cells, whereas heparin is only present in granules of connective tissue, mast cells and basophils. HS was reported to be present on erythrocytes in 2004 (Vogt et al. 2004), confirming HS as a potential rosetting receptor.

Fig. 6.1 Glycosaminoglycan (GAG) structures: Rosette disrupting GAGs; heparin/heparan sulfate (HS), and the main component of fucoidan, sulfated fucose.
Non-rosette disrupting GAGs; hyaluronic acid (HA) and chondroitin sulfate A (CSA).

Previous work has shown that rosettes can be disrupted by glycosaminoglycans or sulfated glycoconjugate compounds such as heparin and fucoidan in a strain dependent manner (Carlson et al. 1992; Rowe et al. 1994; Barragan et al. 1999). Heparin and HS consistently disrupt rosettes in approximately 50% of rosetting field isolates (Carlson et al. 1992; Rowe et al. 1994; Chen et al. 1998a; Barragan et al. 1999). Fucoidan has a more wide ranging action than heparin (9/10 isolates sensitive to fucoidan, compared to 5/10 sensitive to heparin in Rowe et al. 1994). Some isolates are more resistant to rosette disruption, e.g. PAR+ is more sensitive to heparin and fucoidan than TM284+ (Carlson et al. 1992; Rowe et al. 1994).

Heparin was a promising candidate for adjuvant therapy for severe disease, and clinical trials with heparin have shown some success (Munir et al. 1980; Rampengan 1991). However, a high level of heparin-induced intracranial bleeding has led to this treatment being discouraged and the anti-coagulant effects of these drugs prevent their use as treatment for severe malaria (WHO 2000).

6.2.4 Reducing anticoagulant activity of rosette disrupting compounds

The anticoagulant properties of GAGs are an intrinsic chemical property of each individual GAG, based on the chemical structure, size and modifications. The different members of the family show a range of anticoagulant activity, so it is possible to design molecules with reduced anticoagulant activity, whilst retaining
rosette disruption ability. A number of ways have been attempted to reduce the anticoagulant activity of heparin-based derivatives (Fig. 6.2).

Fig. 6.2. Strategies for reducing anticoagulant activity of rosette disrupting heparin derivatives.

Firstly, fractionation can be used to separate heparin according to molecular weight or affinity for antithrombin III, resulting in heparin-derived fractions with varying rosette disruption ability and anticoagulant properties (Carlson and Wahlgren 1992). The rosette inhibition activity was not associated with anti-coagulant properties, suggesting it should be possible to find a fraction with the optimum combination of high anti-rosette activity but low anticoagulant nature.

Secondly, depolymerisation of heparin results in reduction to composite ‘building blocks’ of novel depolymerised glycosaminoglycans (dGAGs). These lack anti-coagulant activity but still disrupt rosettes and inhibit merozoite invasion (Vogt et al. 2006). Rosette disruption with small disaccharides as well as with monomer and long polymers had previously been reported (Barragan et al. 1999). There was evidence of
reduced sequestration in macaques and rats, but so far without convincing extrapolation to humans (Vogt et al. 2006).

Thirdly, modifications of heparin/HS based compounds can drastically alter their chemical properties. The N-sulfate groups on heparin/HS are important for rosette disruption activity and the extent of N-sulfation further increases rosette disruption activity (Barragan et al. 1999). This is supported by chemical data showing the sulfation state of polysaccharides is responsible for anti-coagulant activity (Chaidedgumjorn et al. 2002). All sulfated carbohydrate-based heparin derivatives have some degree of anti coagulant and antithrombin activity, but this differs between compounds, and is due to specific interactions rather than to the general anionic character (Alban and Franz 2000). 1 → 3 linked polysaccharides exhibit 75% reduction in plasma mediated antithrombin activity compared to heparin activity, and the 6-sulfate group largely determines the level of anticoagulant activity (Chaidedgumjorn et al. 2002).

6.2.5 Curdlan sulfate has low anticoagulant properties

Curdlan sulfate (CRDS, Fig. 6.3) is a sulfated 1-→3 β-D glucan; MW: 402.329. CRDS was predicted to have rosette disruption properties (Havlik et al. 2005) due to its polyanionic nature and chemical similaritites to other sulfated polysaccharides (Xiao et al. 1996), see structure in Fig. 6.1. CRDS has a much more favourable toxicity profile than heparin as it has 10-fold lower anticoagulant activity, measured as activated partial thromboplastin time (APTT) compared to heparin (Kaneko et al. 1990).
Fig. 6.3. Structure of curdlan sulfate (CRDS), a sulfated glycosaminoglycan with rosette disrupting potential.

The exact mechanism of CRDS anticoagulant activity is unclear. Unlike heparin, CRDS does not accelerate thrombin inactivation by anti-thrombin but there is evidence it may bind to fibrinogen, causing steric hindrance preventing interaction between thrombin and fibrinogen and preventing fibrin deposition (Alban and Franz 2000).

6.2.6 Curdlan sulfate is safe to use

CRDS is safe to use, and completed phase 1 clinical trials in HIV therapy over 10 years ago (Gordon et al. 1994; Gordon et al. 1997). In HIV, CRDS appears to block HIV adsorption on target CD4+ T cells (Kaneko et al. 1990; Aoki et al. 1992), via binding to the V3 loop of gp120 (Jagodzinski et al. 1994), therefore preventing virus invasion. A similar adsorption mechanism exists in merozoite invasion of *P. falciparum*, and CRDS almost completely blocks merozoite invasion (Havlik et al. 2000).
1994). CRDS may act as an analogue of the glycophorin sialic acid which is required for merozoite invasion (Orlandi et al. 1992; Evans et al. 1998). A similar mechanism may cause heparin-induced invasion disruption (Kulane et al. 1992). CRDS may also inhibit sporozoite invasion of hepatocytes, as reported for other sulfated glycans (Pancake et al. 1992). CRDS does not affect trophozoite maturation but does delay schizont rupture (Evans et al. 1998).

6.2.7 Curdlan sulfate and malaria

A clinical trial of the effect of CRDS in severe/cerebral P. falciparum malaria by Havlik et al. (2005) suggested that CRDS may be able to reduce disease severity, as fever clearance time was reduced as was coma resolution time, though the trial was not conclusive, probably due to the small number of patients in the trial (Havlik et al. 2005).

As CRDS inhibits merozoite invasion but does not affect trophozoite development (Havlik et al. 1994; Evans et al. 1998), it is likely that the CRDS modifies erythrocyte surface proteins. A role in rosette disruption has also been postulated, due to its polyanionic character (Havlik et al. 2005). Because of the chemical similarities between CRDS and heparin, and the 10 fold lower anticoagulant effects of CRDS (Kaneko et al. 1990), it was hypothesised that CRDS might be effective against P. falciparum rosetting and could form the basis of an adjunctive treatment for severe malaria (Havlik et al. 2005).
CRDS is also active against *Babesia*, a tick-borne parasite which is an economically important infection of cattle in the tropics and sub tropics. The mechanism is unclear but *Babesia* has a similar mechanism of infection as *P. falciparum* (Igarashi *et al.* 1998).

CRDS thus has the chemical properties that suggest it may disrupt rosettes and is safe for clinical use (Kaneko *et al.* 1990; Jagodzinski *et al.* 1994; Havlik *et al.* 2005). This study tested the ability of CRDS to inhibit rosetting in *P. falciparum* field isolates, and shows universal rosette inhibition at therapeutic doses of CRDS. CRDS disrupted rosettes in 18/18 *P. falciparum* field isolates tested in addition to 7/7 laboratory isolates previously examined in the laboratory. The combined data present CRDS as a strong candidate for adjunct therapy in severe malaria from rosetting *P. falciparum*.

### 6.3 Aim of chapter

The aim of the chapter was to assess the ability of curdlan sulfate (CRDS) to disrupt rosettes in field isolates of *P. falciparum*, to complement *in vitro* work on laboratory isolates carried out in the Rowe laboratory. The ultimate aim of this work was to examine CRDS as a potential therapeutic agent against severe malaria among African children caused by rosetting strains of *P. falciparum*. 
6.4 Materials and methods

Materials and methods that are specific to chapter 6 are listed below. General materials and methods and a list of suppliers are given in chapter 2.

6.4.1 Parasites

Wild isolates were collected from Kilifi District Hospital, Kenya. Fresh isolates were cultured by standard methods (chapter 2) for at least 18 hours in vitro to the mature pigmented trophozoite stage. Frozen rings and trophozoites had been cryopreserved in glycerolyte and stored in liquid nitrogen, before thawing for either immediate use (frozen troths), or culturing to the mature pigmented trophozoite stage (frozen rings). Cryopreservation of mature trophozoites does not affect the surface antigens of the trophozoite-infected erythrocytes (Kinyanjui et al. 2004). Thus the rosetting frequency of a single isolate does not change significantly pre-freezing and post-thawing (Kyriacou et al. 2007). Two sets of field isolates were used; those collected Jan-March 2006, and those which had been previously collected and cryopreserved (J.A. Rowe) in 1993. Summary of field isolates used: Collected Jan-March 2006: 6566Fztr, RF48; 6394Fztr, RF15; 6399Fztr, RF12; 6547Fztr, RF5; 6542Fztr, RF52; 6794Fzrng, RF33; 6921Fsh, RF27.5; 6962Fsh, RF24.5; 6908Fsh, RF27 and collected in 1993: KR1Fzr, RF53.5; KR7Fzr, RF68; KR9Fzr, RF14; KR10Fzr, RF25; KR11Fzr, 27; KR14Fzr, RF37.5, KR17Fzt, RF61; KR18Fzt, 28; KR20Fzr, RF6 (Fsh, fresh trophs; Fztr, frozen trophs, Fzrng, frozen rings. RF, pretreatment rosette frequency).
Rosetting laboratory strains used were TM284+, TM180, Muz12R+, HB3, R29, A4R and PAR+. Laboratory strains were cultured by standard methods and rosetting was regularly selected by percoll treatment (see chapter 2).

6.4.2 Assessment of Rosette Frequency

All rosette assays were performed on mature pigmented trophozoites, determined using a Giemsa smear (details in chapter 2). Rosette frequency (RF) varies from cycle to cycle, but is stable within a given cycle (Rowe et al. 1994). Parasites were resuspended at 1-2% haematocrit in complete binding medium (Bicarbonate-free RPMI, supplemented as RPMI), as this has a more stable pH when ungased. The culture suspension was pre-stained with ethidium bromide (EtBR), or acridine orange (AO), to identify infected erythrocytes. These staining methods did not alter rosette frequency (PAR+: EtBr staining: mean RF 69.0%, SE 1.1%; AO staining: mean RF 68.8%, SE 0.8%).

6.4.3 Curdlan sulfate drug rosetting inhibition assay

Curdlan suphate (CRDS) was dissolved in sterile phosphate buffered saline (PBS) and added to 18 µl pigmented trophozoites (RF > 50%) culture suspension to give final concentrations 0, 10, 50 and 100 µg/ml. Final concentrations of 100 µg/ml fucoidan and 100 µg/ml hyaluronic acid (both from Sigma, UK) were used as positive and negative controls for rosette inhibition (Rowe et al. 1994).

All aliquots of P. falciparum were incubated for 30 min. Field isolates were rotated for 30 min at room temperature. Laboratory isolates were incubated at 37 °C, and cells
were resuspended every 15 min by flicking the tubes. These methods do not alter the rosette frequency (PAR+: Incubation with EtBr 30 min rotation, RT, RF 69.0% SE 1.1; incubation with EtBr 30 min not rotating, but “flicking” at 0, 15 and 30 min, RF 68.5%, SE 0.65). Wet preparation slides of the parasites were prepared and viewed under fluorescence. Two hundred infected erythrocytes were counted, and scored for number of rosettes; at least two uninfected red blood cells bound constitutes a rosette. Rosette frequency was then expressed as a percentage of total infected erythrocytes. All slides were counted blind. For laboratory strains, data were collected at least three times for every culture and condition used. For field isolates, isolates were only counted once for each condition.

6.4.4 Kinetics of curdlan sulfate rosette disruption
CRDS was dissolved in sterile phosphate buffered saline (PBS) and added to 180 µl culture suspension of PAR+ or TM284+ pigmented trophozoites to give final concentration of 50 µg/ml or 0 µg/ml (PBS control). Cultures were incubated at 37°C, and RF recorded after 0, 2, 5, 30, 60 and 120 min. The assay was repeated 5 times.

6.4.5 Rosette reformation after curdlan sulfate rosette disruption
Two 36 µl aliquots of culture suspension of PAR+ pigmented trophozoites were incubated at 37°C with a final concentration of 50 µg/ml CRDS, 100 µg/ml fucoidan, 100 µg/ml hyaluronic acid or with 2 µl PBS. After 30 min one culture for each condition was washed 4 times with 750 µl incomplete binding media, to wash away the CRDS, fucoidan, hyaularonic acid or PBS, and the culture was resuspended in
complete binding media and incubated at 37 °C. RF was recorded after 0, 2, and 120 min (after washing) and 120 min (without washing). The assay was repeated 4 times.

6.4.6 Statistics

Relative rosette frequency was calculated as a proportion of post-treatment rosette frequency, and given as mean ± SD or SE as appropriate. Paired t-tests were used to compare RF in different treatment groups of time points as the data were normally distributed. Combined data for field isolate RF after treatment was also analysed using ANOVA.

6.5 Previous work: CRDS-mediated inhibition of rosetting in laboratory strains

The following laboratory work (section 6.5 only) was carried out by Ahmed Raza and Katie E Steen (Edinburgh), but data analysis, statistics and production of the figures was carried out personally.

The effect of CRDS was tested in eight different laboratory parasite strains. Cultures were pigmented trophozoites (RF > 50%). Three doses of CRDS were used (10, 50, 100 µg/ml), alongside a PBS control (i.e. 0 µg/ml CRDS). Positive and negative controls for rosette inhibition, fucoidan and hyaluronic acid, were also included. Rosette frequency was significantly reduced (t-test, P<0.05) by CRDS at the highest dose (100 µg/ml) in all parasites (7/7). At 50 µg/ml CRDS, rosetting was significantly reduced in all parasites apart from TM284+ (6/7). At 10 µg/ml CRDS,
rosette frequency was reduced (t-test, P<0.05) in 3/8 parasites. Data for each laboratory isolate is given in Fig. 6.4, and a summary table is shown in Fig. 6.5.

The mean RF for all parasites combined at each treatment were 62.3% for PBS (Standard Error (SE) 2.8), 21.9% for 10 µg/ml CRDS (SE:6.6), 14.6% for 50 µg/ml CRDS (SE:4.6) and 8.7% for 100 µg/ml CRDS (SE:2.7). As expected hyaluronic acid provided a reliable negative control for rosette inhibition (mean RF 65.2%, SE 2.8) and fucoidan a reliable positive control (mean RF 5.4%, SE 1.0).
Fig. 6.4. Individual data for inhibition of rosette frequency in 7 laboratory strains of *P. falciparum* by 0, 10, 50 and 100 µg/ml CRDS in PBS. Each assay was counted blind and performed in triplicate. Post-treatment RF, calculated as proportion of pre-treatment RF, were logged to normalise the data then compared to pre-treatment RF using paired t-tests. p values are indicated with asterisks as *P<0.05 and **P<0.005.
<table>
<thead>
<tr>
<th>(P. \text{falciparum} ) strain</th>
<th>Origin</th>
<th>RF as proportion of pre-treatment RF after addition of CRDS:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µg/ml</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>TM284+</td>
<td>Thailand</td>
<td>*</td>
</tr>
<tr>
<td>TM180</td>
<td>Thailand</td>
<td>*</td>
</tr>
<tr>
<td>Muz12R+</td>
<td>PNG</td>
<td>**</td>
</tr>
<tr>
<td>HB3</td>
<td>Honduras</td>
<td>*</td>
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<tr>
<td>R29</td>
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<tr>
<td>A4R</td>
<td>SE Asia</td>
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</tr>
<tr>
<td>PAR+</td>
<td>SE Asia</td>
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| > 96 % | 51-95 % | 11-50 % | 0-10 % |

Fig. 6.5. Summary table for rosette disruption in laboratory strains of \(P. \text{falciparum}\) by 0, 10, 50 and 100 µg/ml CRDS in PBS. Each assay was counted blind and performed in triplicate. Post-treatment RF, calculated as proportion of pre-treatment RF, is indicated by shading as >96%, 51-95%, 11-50% and 0-10%. Post-treatment RFs were logged to normalise the data then compared to pre-treatment RF using paired t-tests. p values are indicated with asterisks as *P<0.05 and **P<0.005.
Parasites responded to CRDS in a dose dependent manner, though there was variation in the response of the parasite strains to CRDS. Some isolates, such as PAR+, are very sensitive to CRDS, with significant inhibition at only 10 µg/ml (Fig. 6.4, 6.5 and 6.6). This may be due to differences in the strength of the rosettes, depending on the precise molecular interactions involved, as seen for fucoidan and heparin fucoidan (Rowe et al. 1994).

Fig. 6.6. 10µg/ml CRDS significantly reduced rosetting in *P. falciparum* laboratory strain PAR+ compared to a control culture. Live wet-preparation images taken with 40x objective lens on a bright field microscope. Infected erythrocytes can be identified by the presence of malaria pigment, seen as black dots within the erythrocytes. Images taken by Alex Rowe.
6.6 Results 1: CRDS inhibition of rosetting in laboratory strains

After the initial rosette disruption experiments described above (section 6.5), I wanted to further define the rosette-disrupting ability of CRDS.

6.6.1 CRDS inhibition of rosetting in laboratory strains: kinetics of CRDS

The kinetics of CRDS rosette disruption was assessed in PAR+ (very CRDS sensitive; Fig. 6.4) and TM284+ (less CRDS sensitive; Fig. 6.4) over 2 hr with 50 µg/ml CRDS.

PAR+ (mean RF 67.6%, SE:2.6%) was treated with 50 µg/ml CRDS and rosette frequency recorded over 2 hours. Rosettes were disrupted within 2 min (relative RF 11.9%, SE:3.3%). After 2 hr no rosettes were present in the CRDS treated samples (relative RF 0%, SE 0%). The PBS control samples showed high RF (relative RF 95.9%, SE 1.9%). Fig. 6.7A shows mean relative rosette frequencies for 5 repeats of the 6 time points.

TM284+ (mean RF 67.6%, SE:2.6%) was also treated with 50 µg/ml CRDS and rosette frequency recorded over 2 hours. Rosettes were also disrupted within 2 min, but to a lesser extent that for PAR+, reflecting the reduced sensitivity of TM284+ rosettes to CRDS treatment (at 2 min: relative RF 55.4%, SE:2.9%). Rosettes were still present in the CRDS treated samples after 2 hr (at 2 hr: mean RF 32.9%, SE 0.6%). Rosetting in the PBS control samples remained high (PBS at 2 hr: mean RF
87.3%, SE 6.7%). Fig. 6.7B shows mean relative rosette frequencies for 5 repeats of the 6 time points.

A

![Graph showing PAR+ parasites treated with 0 or 50 µg/ml CRDS.](image)

**Fig. 6.7. Speed of action of CRDS. PAR+ parasites were treated with 0 or 50 µg/ml CRDS. The RF was assessed at 0, 2, 5, 30, 60 and 120 min post treatment. RF given as proportion of pre-treatment RF, as mean ± SE for 5 repeats of the experiment.**
Post-treatment RFs were compared to pre-treatment RF using paired t-tests. p values are indicated with asterisks as **P<0.005.

6.6.2 CRDS inhibition of rosetting in laboratory strains: Rosette reformation after CRDS treatment

The ability of rosettes to reform after CRDS disruption was also investigated. Rosette reformation assays were also carried out on PAR+ parasites. After 30 min CRDS treatment (maximum disruption), the CRDS was thoroughly washed out with incomplete binding medium (as binding medium, but with no added serum), and the erythrocytes re-suspended in fresh complete binding medium. Rosette frequency returned to 102% (SE:6.5%) original RF (Fig. 6.8). This shows similar kinetics to fucoidan (Rowe et al. 1994), which I found to return to 100% (SE:8.2%) original RF after washing.
Fig. 6.8. Rosette reformation assay. PAR+ were incubated at 37°C with final concentration of 50 µg/ml CRDS, 100 µg/ml fucoidan, 100 µg/ml hyaluronic acid or with 2 µl PBS. After 30 min the drug treatment was washed out. RF was recorded after 0, 2, and 120 min (after washing) and 120 min (without washing). The assay was repeated 4 times. RF is given as proportion of pre-treatment RF as mean ± SE for 4 repeats of the experiment.

6.7 Results 2: CRDS-mediated rosette disruption in rosetting field isolates

To validate the use of CRDS in a clinical trial, the same four concentrations of CRDS (0, 10, 50 and100 µg/ml), alongside fucoidan and hyaluronic acid, were used on rosetting field isolates from Kilifi District Hospital, Kenya.
6.7.1 CRDS-mediated rosette disruption in rosetting field isolate samples from Kilifi, Kenya collected Jan-March 2006

Nine rosetting isolates were cultured to mature trophozoite stage. CRDS consistently disrupted rosetting in a concentration dependent manner in all parasites (Fig. 6.9). Fucoidan and hyaluronic acid were used for positive and negative controls for rosette inhibition in all isolates.

CRDS seems to be at least as effective at rosette disruption as fucoidan, reported to be active against 9/10 isolates in a previous study (Rowe et al. 1994), and to have a wider range of action than heparin, which was effective in only 50% isolates in that same study and in others (Carlson et al. 1992; Rowe et al. 1994; Chen et al. 1998a; Barragan et al. 1999).

As with laboratory isolates there is evidence for high and low responders to CRDS, with some isolates reacting strongly to a low dose, whereas others required the highest dose for effective rosette inhibition. This may have implications for dose needed for therapy.
Fig. 6.9. CRDS-mediated rosette disruption in 9 rosetting field isolates from Kilifi, Kenya, collected Jan-March 2006. RF shown as proportion of pre-treatment RF for comparison: Relative rosette frequency, Y-axis. Summary of field isolates used: 6566Fztr, RF48; 6394Fztr, RF15; 6399Fztr, RF12; 6547Fztr, RF5; 6542Fztr, RF52; 6794Fzrng, RF33; 6921Fsh, RF27.5; 6962Fsh, RF24.5; 6908Fsh, RF27. (Number, Clinical Brady Number from Kilifi District Hospital; Fsh, fresh trophs; Fztr, frozen trophs, Fzrng, frozen rings. RF, pretreatment rosette frequency).
Rosette frequency was expressed relative to initial (pre-treatment) rosette frequency, so that all nine field isolates could be compared (Fig. 6.10). There was a significant inhibition (P < 0.05) across all field isolates combined at 10 µg/ml CRDS, and this rose in significance (P < 0.005) for 50 and 100 µg/ml CRDS conditions. Difference in RF compared to PBS control for all concentrations was significant by ANOVA (P<0.0001).

Fig. 6.10. Combined data for 9 field isolates of *P. falciparum* from Kilifi, Kenya, collected Jan-March 2006 treated with 0, 10, 50, 100 µg/ml CRDS in PBS, 100 µg/ml fucoidan or with 100 µg/ml hyaluronic acid. Mean ± SD is shown. * p < 0.05. ** p < 0.005 for paired t-tests compared to 0 µg/ml CRDS (PBS control). Relative rosette frequency was calculated as a proportion of each isolate’s own pre-treatment rosette frequency.
6.7.2 CRDS-mediated rosette disruption in rosetting field isolate samples from Kilifi, Kenya 2- samples collected and cryopreserved in 1993

It was not possible to examine any more field isolate in Kenya due to the lack of malaria during Jan-March 2006 because of a severe drought. However, in order to increase the sample size, further field isolates from Kenya that had been frozen in 1993 (J.A. Rowe) were thawed and cultured to mature trophozoite stage as above.

Consistent with the previous Kenya field isolate samples (section 6.7.1), CRDS consistently disrupted rosetting in a concentration dependent manner in all parasites (Fig. 6.11), again showing evidence for high and low responders to CRDS.

As for the previous field isolate samples, rosette frequency was expressed relative to initial (pre-treatment) rosette frequency, so that all nine field isolates could be compared (Fig. 6.12). There was a significant inhibition ($P = 0.006$) across all field isolates combined at 10 µg/ml CRDS, and this rose to ($P < 0.005$) significance for 50 and 100 µg/ml CRDS conditions. Difference in RF compared to PBS control for all concentrations was significant by ANOVA ($P<0.0001$).
Fig. 6.11. CRDS-mediated rosette disruption in 9 rosetting field isolates from Kilifi Kenya, collected 1993. RF shown as proportion of pre-treatment RF for comparison:

Fig. 6.12. Combined data for 9 field isolates of *P. falciparum* from Kilifi, Kenya collected in 1993 treated with 0, 10, 50, 100 µg/ml CRDS in PBS, 100 µg/ml fucoidan or with 100 µg/ml hyaluronic acid. Mean ± SD is shown. * p < 0.05. ** p < 0.005 for paired t-tests compared to 0 µg/ml CRDS (PBS control). Relative rosette frequency was calculated as a proportion of each isolate’s own pre-treatment rosette frequency.
6.7.3 CRDS-mediated rosette disruption in rosetting field isolates: combined data

Rosette frequency was expressed relative to initial (pre-treatment) rosette frequency, so that all 18 field isolates could be compared (Fig. 6.13). There was a significant inhibition (P<0.005) across all field isolates combined at each CRDS condition compared to PBS. The effect of CRDS was also significant by ANOVA (P<0.0001).

Fig. 6.13. Combined data for all 18 field isolates of *P. falciparum* from Kilifi, Kenya treated with 0, 10, 50, 100 µg/ml CRDS in PBS, 100 µg/ml fucoidan or with 100 µg/ml hyaluronic acid. Mean ± SD is shown. * p < 0.05. ** p < 0.005 for paired t-tests compared to 0 µg/ml CRDS (PBS control). Relative rosette frequency was calculated as a proportion of each isolate’s own pre-treatment rosette frequency.
6.7.4 PfEMP1 expression in rosetting field isolates

Attempts were made to analyse the var gene transcription in the Kenyan rosetting field isolates, by separating the rosetting and non-rosetting infected erythrocytes using percoll (Fig. 6.14, details in chapter 2).

Fig. 6.14. Pigmented trophozoite infected erythrocytes can be separated from uninfected and ring stage parasite infected erythrocytes using a percoll gradient, as trophozoite infected erythrocytes have a lower density, and form a discrete layer above the 60% percoll after spinning (4000 rpm, 15 min). However, rosettes sink beneath the percoll due to the weight of uninfected erythrocytes, causing rosetting pigmented trophozoite infected erythrocytes to remain with the uninfected or ring stage infected cells. Rosetting and non-rosetting pigmented trophozoite infected erythrocytes can thus be separated.
Unfortunately, the small packed cell volume of the field isolates which was available meant that only a few microlitres of non-rosetting parasites were retrieved, and so there was not enough for RNA extraction, cDNA preparation and RT-PCR for a control (non-rosetting) set of var gene transcripts for comparison. Without this control group, analysis of the var gene transcripts from the remainder of the culture would be hard to interpret, as the contributions of the different PfEMP1 to rosetting would not be clear. An interesting follow-up study to this would be to examine the link between rosetting and expressed PfEMP1 in field isolates, and between expressed PfEMP1 and efficiency of CRDS action.

6.8 Discussion

CRDS was found to disrupt rosettes in 18/18 field isolates at doses suitable for clinical use, showing a significant effect on rosette frequency in two groups of Kenyan field isolates. The analysis of CRDS on field isolates discussed in this chapter complements data on laboratory clones (outlined in section 6.5), together showing that CRDS effectively and consistently disrupts rosettes in a wide range of strains.

At present we cannot prevent infection, or predict severe disease manifestation, so a key area of therapy is to reduce severe disease complications. High levels of rosetting (>30% RF) are estimated to occur in approximately 25% of severe malaria cases in Kenya, and around 50% of severe malaria cases in Mali (Alex Rowe, personal communication). If these figures are representative across the whole of sub-Saharan Africa, rosetting may play a role in an estimated 500,000 P. falciparum
malaria deaths per year in sub-Saharan Africa (Rowe 2005). Rosette inhibiting drugs could thus have a substantial impact on malaria mortality and morbidity. The strong ability to reduce rosetting suggests CRDS may be able to reduce the severe complications and lower mortality of *P. falciparum* malaria.

Who would CRDS therapy be for? A clinical trial of CRDS in severe and cerebral malaria reported a moderate decrease in fever and coma resolution time (Havlik *et al.* 2005). However, the patients studied were from Thailand, where rosetting is not associated with disease severity, possibly due to the CR1 deficiency across Asia (al-Yaman *et al.* 1995; Cockburn *et al.* 2004). Rosette disruption via CRDS treatment might not be appropriate in Asia. Rosette-disruption via CRDS therapy is also not relevant where *P. vivax* is the dominant infective *Plasmodium*. Although rosetting is a common property of *P. vivax* field isolates, and heparin (and therefore potentially also CRDS) reverses *P. vivax* rosettes, rosetting of *P. vivax* is not associated with severe disease (Chotivanich *et al.* 1998). As such there may not be any therapeutic benefit from disrupting the *P. vivax* rosettes.

However, in Africa, and especially in African children, there is compelling evidence that rosetting is involved in the pathogenesis of severe disease (Carlson *et al.* 1990; Rowe *et al.* 1995; Rowe *et al.* 2002c and also reported in chapter 3). In the case of cerebral malaria, parasitized erythrocytes adhering to the cerebral endothelium are associated with coma which is fatal in up to 30% of cases (MacPherson *et al.* 1985; White 1987; Brewster *et al.* 1990; Berendt *et al.* 1994a; Newton *et al.* 1998; Silamut *et al.* 1999). However, if the infected cells are cleared, a full recovery is made with
neurological impairment in only 12% of survivors (Brewster et al. 1990). As rosetting parasites are associated with cerebral malaria (Carlson et al. 1990; Rowe et al. 1995; Rowe et al. 2002c), it seems logical to conclude that the rosettes participate in the obstruction of the cerebral capillaries. Thus, disruption of rosettes may quicken the clearance of infected erythrocytes from the cerebral vasculature. This may reverse the coma more rapidly and reduce chance of focal lesions, or ring haemorrhages, within the cerebral hemisphere which can lead to neurological sequelae (Brewster et al. 1990; Newton et al. 1998). Ideally CRDS treatment could be targeted towards isolates with a high rosette frequency, though the 24 hr culturing required to mature peripheral ring parasites to pigmented trophozoites would be inappropriate for field situations. Furthermore, many patients do no have 24 hr to wait before treatment as most malaria deaths occur within 24 hr of admission (Jaffar et al. 1997), before standard anti-malarial drugs are effective. Thus there is an urgent need for drugs to tackle phenomena causing severe malaria disease symptoms within this short time window. As rosetting is consistently associated with all forms of severe disease in Africa (though not in Asia) (Carlson et al. 1990; al-Yaman et al. 1995; Rowe et al. 1995; Rowe et al. 2002b) CRDS could be given to all patients showing severe complications in Africa.

In addition to rosetting, it is possible CRDS may have a direct effect on sequestration. HS can also mediate sequestration, as HS is present on endothelial cells although the possibility of HS as a ligand for sequestration for infected erythrocytes has been controversial. Xiao et al. (1993) reported HS does not influence cytoadherence to HMEC or C2 melanoma cells, as heparinase treatment
did not abolish adhesion. However, the heparinase treatment used may not have completely digested the heparin, leaving enough HS chains on the endothelium to support IE adhesion. Vogt et al. (2003) reported adhesion of FCR3 and wild type clinical isolates to HUVEC and HLEC cells lines via heparin/HS, through a DBLα:HS interaction, and field isolates with affinity for HS have been reported (Hedini et al. 2001). Sporozoite invasion of hepatocytes is via CSP adhesion to heparan sulfate proteoglycans on the hepatocyte surface (Frevert et al. 1993). This may also be disrupted by CRDS.

Even if CRDS does not directly inhibit sequestration, removal of rosettes is likely to reduce vascular blockage. For example disruption of Malayan Camp rosettes dramatically decreased microvascular obstruction and cytoadherence in a rat mesocaecum vasculature model with *P. falciparum* infected sequestered erythrocytes (Kaul et al. 1991), though the rosetting ligand in this case was CD36, a common endothelial sequestration receptor, and not affected by CRDS (Evans et al. 1998). However, CD36 binding is associated with sequestration in non-vital organs, and with PfEMP1 expressed in non-severe malaria, as discussed in chapter 3.

Other ligands present on cell types such as endothelium or platelets, which may aid sequestration by bridging IE and activated endothelium, may also be affected by CRDS. Chondroitin sulfate A and hyaluronic acid, implicated in placental malarial sequestration, as discussed in chapter 4, are both sulfated glycoconjugates and thus chemically similar to heparin. Though these molecules do not inhibit rosetting (Rowe et al. 1994), it remains possible that CRDS may disrupt their use as adhesion
receptors for infected erythrocytes. Thus it is possible CRDS may also play a role in reversal of adhesion in placental malaria.

Further experiments characterising the effect of CRDS on laboratory isolates revealed that rosettes were able to reform after CRDS was removed. The short half-life (2-3 hr; Gordon et al. 1994) and rosette reformation after washing has implications for administration of drug, suggesting that the drug should be administered regularly throughout treatment. The effect on merozoite invasion was not examined in this study, but it has previously been reported that CRDS can inhibit merozoite invasion (Havlik et al. 1994; Evans et al. 1998), as can many sulfated glycoconjugates (Kulane et al. 1992; Xiao et al. 1996).

At present there is no data on whether GAGs influence commitment to gametocytogenesis, which is an important consideration for any drug administered to infected individuals, as reducing the asexual parasitaemia can sometimes be associated with an increase in sexual stage parasites, and thus an overall increase in transmission, such as has been observed with chloroquine (Buckling et al. 1999).

It is important to emphasise that CRDS has already been shown to be well tolerated in clinical trials and was able to reduce fever clearance time in a recent clinical trial of *P. falciparum* malaria (Havlik et al. 2005). CRDS is able to inhibit *P. falciparum* growth *in vitro*, through inhibiting merozoite invasion (Havlik et al. 1994; Evans et al. 1998). In addition CRDS has also been shown to down-modulate cytokine effects in HIV infection (Naito et al. 2001). This is promising for auxiliary therapy for
severe malaria treatment, as some of the clinical symptoms may be due to the immune response to infection (Grau et al. 1989; Kwiatkowski et al. 1990), making CRDS an even more potent agent against severe disease symptoms of clinical malaria. This data, together with previous reports, suggests that CRDS is a strong candidate for adjunctive therapy in severe malaria, and support further clinical trials of CRDS, especially in African children where rosetting is linked to severe disease.
Chapter 7

Discussion
Discussion

The general aim of the thesis was to investigate the hypothesis that PfEMP1 expression influences clinical malaria disease due to *P. falciparum* infection. The work presented in the thesis focussed on conserved PfEMP1 subgroups and clinical disease presentation in African field isolates, and development of a drug that could use this information to prevent onset of severe symptoms due to *P. falciparum* infection. In the case of childhood malaria, I have shown that there was a significantly higher frequency of group A *var* gene transcripts in cerebral malaria field isolates compared to isolates from children with equally high parasite burdens but no symptoms or signs of severe malaria (hyperparasitaemia), suggesting that group A *var* genes are important determinants of parasite virulence (chapter 3). In work on placental malaria, I detected *var2csa* transcripts in all 6 placental malaria field isolates examined, but not in a control group of 10 childhood malaria isolates, suggesting that *var2csa* expression is a critical factor in the onset of clinical malaria disease in pregnant women (chapter 4), consistent with other recent studies (Tuikue Ndam *et al.* 2005; Duffy *et al.* 2006a). I also found that type 3 *var* genes, an atypically small and conserved subset of group A *var* genes, were commonly transcribed in blood-stage parasites, showing a possible association with hyperparasitaemia rather than cerebral malaria field isolates, and sequence analysis confirmed a very high level of conservation across this *var* gene subfamily (chapter 5). In addition, I found a non-toxic compound, curdlan sulfate, to be effective at disrupting rosettes in all rosetting field isolates examined (chapter 6), suggesting potential for use in treatment of severe malaria due to rosetting *P. falciparum*
isolates. This final chapter aims to link together these findings and discuss the role of conserved PfEMP1 and clinical malaria disease.

The link between group A var gene expression and severe malaria (chapter 3) is consistent with data from previous field isolate studies (Kirchgatter and Portillo Hdel 2002; Rottmann et al. 2006) and with upregulation of group A var gene transcription in 3D7 after selection for severe malaria-type VSA (Jensen et al. 2004). Severe malaria has also been linked to “common” var genes through sera agglutination studies (Bull et al. 1999), and the resistance to severe malaria which develops throughout childhood suggests common epitopes are present in severe malaria-causing parasites, allowing protective cross-reactive antibodies to develop. Group A var genes tend to have more cross-reacting epitopes than group B or C (Joergensen et al. 2006), and transcripts that were present in multiple isolates in the chapter 3 analysis were more frequently DBLα1 than DBLα0. Thus the association between group A PfEMP1 and severe malaria is consistent with a link between “common” PfEMP1 and severe malaria.

Multiple adhesive phenotypes can be associated with severe malaria (Heddini et al. 2001). Group A PfEMP1 tend to have more domains than group B and C PfEMP1 (Gardner et al. 2002; Kraemer et al. 2007), and thus have an increased binding potential, for example for ligands such as ICAM-1 (Smith et al. 2000a; Springer et al. 2004) or IgM (Semblat et al. 2006). Furthermore, binding to molecules on uninfected erythrocytes, such as CR1 (Rowe et al. 1997; Rowe et al. 2000), ABO blood group antigens (Carlson and Wahlgren 1992; Chen et al. 1998a; Barragan et
Rosetting interactions may cause rosetting PfEMP1 to have conserved structural or sequence features which may provide common epitopes for recognition by cross-reactive antibodies. Natural rosetting-disrupting antibodies can form upon infection, and have been associated with protection from cerebral malaria (Carlson et al. 1990). Antibodies raised against the rosetting DBLα domains of FCR3S1.2 (the rosetting PfEMP1 of FCR3) disrupt FCR3 rosettes (Chen et al. 2004) and also show cross-reactive rosette disruption against R29 rosettes (Moll et al. 2007). These antibodies also showed recognition of a panel of Ugandan field isolates, with increased recognition of those causing severe disease (Normark et al. 2007) suggesting common antibody epitopes are present across diverse rosetting PfEMP1. The interaction of many diverse rosetting PfEMP1 with host rosetting ligands can also be inhibited by drugs such as heparin (Carlson et al. 1992; Rowe et al. 1994) and curdlan sulfate (chapter 6), also suggesting common features are present between many rosetting interactions.
Conversely, group B and C PfEMP1 are commonly expressed in non-severe malaria (this study chapter 3, Kaestli et al. 2006; Rottmann et al. 2006). Group B and C PfEMP1 commonly bind to CD36, which is associated with non-severe malaria, and with immunosuppression (Urban et al. 1999) and sequestration in non-vital organs (Serghides et al. 2003). Group B and C PfEMP1 are commonly expressed in individuals who have reached a high level of immunity to clinical disease (asymptomatic adults e.g. Kaestli et al. 2006, or hyperparasitaemia; this study) suggesting cross-reactive protective antibodies are rare. Parasites from clinically immune individuals, or patients with mild or asymptomatic malaria infection, are less likely to be agglutinated by heterologous sera (Bull et al. 2000), also indicating a lower level of epitope cross-reactivity. Cross-reactive epitopes between group B and C PfEMP1 of 3D7 are uncommon (Joergensen et al. 2006), and few DBLα0 were found in multiple isolates in the Mali childhood malaria isolates discussed in this thesis (chapter 3). Thus DBLα0 domains or group B and C PfEMP1, though present in the majority of PfEMP1, are not promising vaccine candidates, as they are associated with non-severe malaria and do not share a high number of cross-reactive epitopes.

If a vaccine were to be raised to PfEMP1, then this data suggests group A PfEMP1 should be targeted. Immunisation of Aotus monkeys with the CIDR domain of Malayan Camp caused protection against subsequent infection with the same strain, showing PfEMP1 domains can be immunogenic and elicit a protective antibody response (Baruch et al. 2002). As all known group A PfEMP1 have a DBLα1
domain, cross-reactive epitopes within DBLα1 domains might be a promising focus for vaccine design. DBLα0 domain immunisation in Sprague Dawley rats also elicited a protective immune response against rosetting and sequestration, with some degree of cross-reactivity to other strains, providing evidence that DBLα domain immunisation can be effective (Chen et al. 2004; Moll et al. 2007; Normark et al. 2007). Normark et al. (2007) also suggested certain motifs are common between PfEMP1 associated with rosetting and severe malaria isolates, and using structural modelling predicted that some of these motifs lie within a pocket of the DBLα1 domain that is homologous to the fold used by Pk-DBLα for DARC-recognition. This fold is also conserved in EBA-175 and may be a conserved area for receptor binding that could provide a cross-reactive structural antibody target. However, there was little overall cross-reactivity of antibodies raised against various domains of 3D7 (Joergensen et al. 2006) and the question remains whether PfEMP1 is too polymorphic to be an effective vaccine candidate.

The type 3 PfEMP1 are a special case of group A PfEMP1, having only 2 extracellular domains and an atypically short intron which appears to be without a regulatory promoter. Theoretically, the type 3 PfEMP1 subfamily makes an excellent vaccine candidate as type 3 PfEMP1 are highly conserved. However, though most or many P. falciparum strains encode a type 3 var gene, and most field isolates show type 3 var gene expression (chapter 5), there is no evidence that they are expressed on the infected erythrocyte surface, or that their expression is essential, and as such a vaccine might not be effective. Furthermore, the surprising observation of greater diversity of type 3 var gene expression in hyperparasitaemia isolates compared to
cerebral malaria isolates suggests that these atypical group A PfEMP1 may not be commonly involved in severe disease pathogenesis (chapter 5).

At present the only PfEMP1 which shows potential as a vaccine target is VAR2CSA specifically in the context of placental malaria, as discussed in chapter 4. There is a strong association between var2csa transcription and placental malaria field isolates (chapter 4, and e.g. Tuikue Ndam et al. 2005; Duffy et al. 2006a). In addition, VAR2CSA has affinity for CSA, which is associated with placental sequestration (Fried and Duffy 1996; Reeder et al. 2000; Muthusamy et al. 2007). The rise in anti-VAR2CSA antibodies, which coincides with protection from placental malaria upon subsequent pregnancies (Salanti et al. 2004; Tuikue Ndam et al. 2006), suggests a VAR2CSA vaccine could effectively mimic this natural immunity. Not all peptide stretches of VAR2CSA are targets of natural antibodies (Dahlback et al. 2006), and so high sequence homology appears to be insufficient to ensure an protective antibody response. A motif EIEKD within a variable region (V2) was noted by Dahlback et al. (2006) to be common in VAR2CSA from primigravidae and was predicted to lie within a surface exposed loop, and thus provide a promising vaccine target. The motif EIEKD is uncommon in multigravidae where GIEGE is found at this position (Dahlback et al. 2006). Although this suggests EIEKD-VAR2CSA variants may be more virulent or more efficient at sequestration and thus a good vaccine target, loss of these variants may simply result in selection of GIEGE-VAR2CSA variants and would not necessarily result in an overall decrease of placental infection. As discussed in chapter 4, there still remains some sequence diversity between var2csa homologues, and there is evidence of surprisingly low
cross-reactivity between antibodies raised against different VAR2CSA (e.g. (Bockhorst et al. 2007; Oleinikov et al. 2007). In addition, the contribution of other PfEMP1 is unclear, and there is evidence of a high number of non-\textit{var2csa} \textit{var} genes being transcribed at similar or higher frequency to \textit{var2csa} (chapter 4). Furthermore, other (non-PfEMP1) proteins have also been associated with placental malaria (Francis et al. 2007). If VAR2CSA is not the only protein responsible for placental sequestration, then the relative contributions of other proteins must also be taken into account in order to determine the efficacy of an anti-VAR2CSA vaccine.

In addition to vaccines, drugs can also be used to target sequestration in malaria. At present there are no drugs designed to directly target sequestration of \textit{P. falciparum}-infected erythrocytes. However, advances in the development of a drug to target rosetting (curdlan sulfate, as described in chapter 6) are promising. The benefits of a rosette-disrupting drug are that it is quick acting and may have an immediate effect in reducing microvascular obstruction due to rosetting and help to relieve symptoms of anaemia by releasing bound uninfected erythrocytes back into the circulation. Curdlan sulfate could be administered to all patients with severe malaria disease in Africa, where rosetting is associated with all forms of severe disease (Rowe et al. 1995; Rowe 2005), and so patients would not require expensive or time-consuming tests to assess rosette frequency before drug administration. As many parasite isolates do not rosette, there may be little pressure for drug resistance to develop against this anti-rosetting drug, as is seen with more conventional antimalarials, which aim to eradicate the parasite. Thus drug resistance to curdlan sulfate may be slow to build up in a population. The effect of curdlan sulfate on other
glycosaminoglycan interactions, such as sequestration to CSA in placental malaria, is at present unknown, and would be an interesting follow-up study. There is also recent evidence that CSA is involved in ookinete invasion in the *anopheles* mosquito (Dinglasan et al. 2007). In addition to disrupting placental sequestration via CSA, this may provide another opportunity for any drugs which can disrupt CSA-binding, whether developed from the VAR2CSA/placental malaria perspective, or from research on CSA/proteoglycans and rosetting.

The links between conserved PfEMP1 and clinical disease are not independent. Interactions between the major associations discussed above can be represented in a network of relationships between PfEMP1 group and clinical disease (Fig. 7.1). This highlights the connections between various factors associated with severe/cerebral clinical disease and group A PfEMP1, such as “commonness”, rosetting and multiple-binding phenotypes, as distinct from factors associated with non-severe clinical disease/hyperparasitaemia, such as CD36-binding and group B or C PfEMP1. VAR2CSA, CSA-binding and placental malaria form a separate triangle, with a possible link to group A PfEMP1 due to DBLγ domain interactions. The possible therapeutic interventions with curdlan sulfate are also indicated in Fig. 7.1. These include rosette inhibition, a general role in severe malaria treatment for example through other roles such as merozoite inhibition (Havlik et al. 1994; Evans et al. 1998), and the possibility of inhibition of other interactions such as CSA-binding, as discussed in chapter 6. The putative link between type 3 PfEMP1 and non-severe malaria/hyperparasitaemia discussed in chapter 5 is also included.
Fig. 7.1. Relationships between PfEMP1 group and clinical disease. Positive associations between factors associated with severe or clinical disease and group A PfEMP1 (red), between non-severe clinical disease/hyperparasitaemia and group B or C PfEMP1 (blue), between VAR2CSA, CSA binding and placental malaria (green) and possible therapeutic interventions with curdlan sulfate (purple) are indicated. Dotted lines show negative associations. Bold arrows between group A PfEMP1 and severe/cerebral malaria, between group B/C PfEMP1 and non-severe malaria, between VAR2CSA and placental malaria and between type 3 PfEMP1 (orange) and non-severe malaria, and the bold line between curdlan sulfate and rosette inhibition, indicate relationships based on data from this thesis.
This network highlights the associations between multiple factors and raises questions about intervention strategies. For example, does knocking out a bridging point between group A PfEMP1 and severe malaria (such as rosetting or other binding phenotypes) reduce the strength of the link between group A PfEMP1 expression and severe disease?

In conclusion, the data presented in this thesis demonstrate significant relationships between var gene/PfEMP1 expression and clinical outcome in *P. falciparum* malaria disease pathogenesis. Research on malaria is highly multidisciplinary, and benefits from all areas of research working collectively towards the common goal of malaria disease control and eradication. This work highlights the need for continued research on var gene/PfEMP1 expression involvement in clinical disease in the effort to reduce the severity of complications due to *P. falciparum* infection, in the fight against this devastating disease.
References
References


Appendix
Appendix 1. Table of Mali childhood malaria field isolate details
Data on the 26 Mali isolates for clinical disease manifestation, age, parasitaemia, rosette frequency, platelet-mediated clumping and multiplicity of infection is given, along with percentage of DBLα transcripts detected that were of the DBLα1-type (discussed in chapter 3), and the estimated number of type 3 transcripts present (discussed in chapter 5).

Appendix 2. Phylogenetic network of DBLα transcripts in Mali childhood field isolates (discussed in chapter 3)
A phylogenetic network was created by Dr Graham Stone (University of Edinburgh) to replace the phenogram of childhood isolate DBLα domain transcripts (chapter 3, Fig. 3.13). The network was produced from an initial Clustal W alignment of the amino acid sequences, converted to a nucleotide alignment. The network and phenogram both show separation of sequences into two clades, signifying DBLα1 versus DBLα0-like sequences. Sequences from the cerebral malaria patients (red in network; appendix 2) are significantly concentrated into a ‘cerebral malaria clade’ (P<0.001, chi-squared test). Sequences from the hyperparasitaemia patients (blue) are significantly concentrated in a ‘hyperparasitaemia clade’ (P<0.025, Chi-squared test). Sequences from the uncomplicated malaria patients (green) showed no bias in distribution between the two main clades of the network.
Appendix 3. Publications arising from this thesis

1: Arising from the work presented in chapter 3

**Title**: Differential *var* gene transcription in *Plasmodium falciparum* isolates from patients with cerebral malaria compared to hyperparasitaemia.


**Journal**: Molecular and Biochemical Parasitology (2006) 150(2): 211-8

**Publisher**: Elsevier

2: Arising from the work presented in chapter 4

**Title**: Transcribed *var* genes associated with placental malaria in Malawian women.

**Authors**: Michael F. Duffy, Aphrodite Caragounis, Rintis Noviyanti, Helen M. Kyriacou, Ee Ken Choong, Katja Boysen, Julie Healer, J. Alexandra Rowe, Malcolm E. Molyneux, Graham V. Brown, and Stephen J. Rogerson.


**Publisher**: American Society for Microbiology
3: Arising from the work presented in chapter 6

**Title:** *In vitro* inhibition of *Plasmodium falciparum* rosetting formation by curdlan sulphate.

**Authors:** Helen M. Kyriacou*, Katie E. Steen*, Ahmed Raza, Monica Arman, Pete C Bull, Ivan Havlik and J. Alexandra Rowe. *Joint first authors.


**Publisher:** American Society for Microbiology

4: Arising from work with Lisa Sharling during the course of my PhD but not discussed in this thesis.

**Title:** Rapid and specific biotin labelling of the erythrocyte surface antigens of both cultured and *ex-vivo Plasmodium* parasites.

**Authors:** Lisa Sharling, Kordai M.P. Sowa, Joanne Thompson, Helen M. Kyriacou and David E. Arnot.


**Publisher:** BioMed Central
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**Appendix 1:** Table showing isolate name, clinical disease manifestation, age (mo: months), parasitaemia (Pt, %), rosette frequency (RF, %) platelet-mediated clumping (PMC, %), multiplicity of infection (genotypes), percentage of amplified DBLα transcripts that were of the DBLα1-type (DBLα1%), and the estimated number of type 3 transcripts present (type 3 transcripts) for the 26 Mali childhood malaria isolates discussed throughout the thesis.
**Appendix 2.** Phylogenetic network produced by Dr Graham Stone from a Clustal W alignment of Mali isolate DBLα domain transcripts described in chapter 3. Relationships between *var* gene DBLα sequence tags transcribed by *P. falciparum* isolates, generated using Neighbour-Net (Bryant and Moulton 2004). Sequences transcribed by isolates from African children with cerebral malaria (CM, red), hyperparasitaemia (HYP, blue), and uncomplicated malaria (U, green) are compared to a selection of *var* genes from the laboratory clone 3D7. For the 3D7 genes, the gene name is preceded by A, B, C, BA or BC to indicate the group to which the gene belongs. The sequences fall into 2 major clades, with the DBLα0-like sequences to the left of the dotted line and the DBLα1-like sequences to the right. For the predominant gene from 9 of the Malian isolates, the *var* gene upstream region was determined by PCR and is indicated as a boxed letter.