Plasmodium falciparum transfection technology for the analysis of *var* gene regulation and knockout investigation

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Table of Contents

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Summary

Malaria is an infectious disease caused by protozoans of the genus *Plasmodium,* which are injected by the bite of an infected female *Anopheles* mosquito during a blood meal. Out of the four species that infect humans, *P. falciparum* is the most important. About 40% of the world's population is at risk and 500 million cases of malaria occur every year, mainly in sub-Saharan Africa. Due arising resistance of mosquitoes against insecticides, the lack of a malaria vaccine, and emerging resistance of parasites against established drugs, research into new drugs and vaccine targets is most important.

Morbidity is associated with adherence of infected red blood cells (iRBC) to endothelial tissue thereby obstructing the blood flow. The major protein conferring this cytoadherence is the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) anchored in the erythrocyte membrane of infected red blood cells (iRBCs). *Pf*EMP1 is encoded by the *var* gene family that consists of approximately 60 members in the haploid genome of the 3D7 strain. *var* genes are expressed mutually exclusive, i.e. only one *var* gene is expressed in a parasite at a time and the rest is silenced. In this thesis we were interested in the regulation of expression and silencing of *var* genes. For this purpose we generated transgenic parasite lines that harbored plasmids expressing luciferase under the control of various fragments of the *var* gene upstream region. By comparing luciferase activities in the different lines we identified the core promoter, two activator-binding sites and a repressor-binding site. Additionally, we identified a regulatory sequence on the *var* upstream region that interacts with the *var* intron during silencing. Using quantitative RT-PCR with specific primers for every *var* gene we were unable to confirm that the *var* upstream regions on the transfected plasmids were recognized by the machinery that ensures mutually exclusive transcription.

In the second part of this thesis, we evaluated phosphodiesterase 1 (PDE1) as a possible drug target in *P. falciparum* by creating a knockout parasite

line. PDEs are known drug targets in humans where selective PDE inhibitors are being used to treat a wide range of diseases. In trypanosomiasis research PDE inhibitors are promising drug candidates against sleeping sickness, Nagana or Chagas' disease. Out of the four PDEs described for *P. falciparum* we focused on *Pf*PDE1, which is expressed in blood stage parasites and in gametocytes and sporozoites. We observed a slightly faster growth of the knockout parasite line compared to the wildtype indicating that the knockout parasite had a shorter erythrocytic lifecycle. We found that *Pf*PDE1 is responsible for 20% of the total cGMP activity observed in late blood stage parasites and that there is no rescue mechanism of the remaining PDEs to compensate for the loss of activity. We were not able to localize *Pf*PDE1 in the parasite. The fact that we could delete *Pf*PDE1 clearly shows that it is not an essential gene in blood stage forms of *P. falciparum* and hence not a good drug target. Nevertheless we created a useful tool to investigate the role of *Pf*PDE1 in the development of sexual parasite forms.

Zusammenfassung

Malaria ist eine Infektionskrankheit, die von Protozoen der Gattung *Plasmodium* verursacht wird, welche beim Stich einer weiblichen *Anopheles* Mücke übertragen werden. Von den vier Spezies, die den Menschen befallen können, ist *P. falciparum* die Wichtigste. Etwa 40% der Weltbevölkerung leben in Risikogebieten und jährlich treten 500 Millionen Krankheitsfälle auf, meist südlich der Sahara. Insektizidresistenzen der Mücken, ein fehlender Impfstoff, sowie Resistenzbildung des Parasiten gegen vorhandene Malariamittel machen die Suche nach Zielstrukturen für neue Malariamittel und Impfstoffe dringend.

Das Anheften der infizierten roten Blutkörperchen (iRBC) an Endothelgewebe löst die Krankheit aus unter anderem durch das daraus folgendem Verstopfen der Blutkapillaren. *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), das vermeintliche Hauptprotein bei Entstehung der sogenannten Cytoadherence, ist in der Erythrocytenmembran verankert. *Pf*EMP1 wird von der *var* Genfamilie kodiert, die im haploiden Genom des 3D7 Stamm etwa 60 Mitglieder hat. *var* Gene werden in einer sich gegenseitig ausschließender (mutually exclusive) Art exprimiert, d.h. nur ein *var* Gen ist jeweils in einem Parasiten angestellt und der Rest ist abgestellt (silencing). In dieser Doktorarbeit wurden die Regulation der Expression und das Silencing von *var* Genen untersucht. Hierfür haben wir transgene Parasiten Linien geschaffen, die mit einem Plasmid transformiert wurden, in welchen das Luciferase-Gen unter der Kontrolle verschiedener Fragmente der *var* Gen upstream Region exprimiert wurde. Beim Vergleich der Luciferase-Aktivität der verschiedenen Linien haben wir den Kern-Promotor, zwei Aktivator Bindungs-Stellen, sowie eine Repressor Bindungs-Stelle identifiziert. Zusätzlich konnten wir eine Regulierungs-Sequenz auf der *var* Gen upstream Region identifizieren, welche zusammen mit dem *var* Intron das Ausschalten der *var* Gene verursacht. Mittels quantitativer RT-PCR mit spezifischen Primern für jedes einzelne *var* Gen konnten wir nicht nachweisen, ob die upstream Regionen

auf den transfizierten Plasmiden von der Maschinerie zur auscchliesslichen Transkription (mutually exclusive Transcription) gewährleistet, erkannt werden können.

Im zweiten Teil dieser Dissertation wurde Phosphodiesterase 1 (PDE1) als mögliches Zielmolekül für ein Malariamedikament gegen *P. falciparum* evaluiert. Hierzu wurden eine Knockout Parasiten Linie generiert. PDEs sind bekannte Zielmoleküle für Medikamente im Humangebrauch, die durch selektive Inhibition der PDEs bei einer Vielzahl von Krankheiten wirken. PDE Inhibitoren sind vielversprechende Zielstrukturen in Trypanosomen und könnten als Medikamente gegen Schlafkrankheit, Nagana oder Chagas Krankheit eingesetzt werden. Von den vier bekannten PDEs in *P. falciparum* haben wir uns auf *Pf*PDE1 konzentriert, welches in Blutstadien aber auch in Gametozyten und Sporozoiten exprimiert ist. Die Parasiten Knockout Linie wuchs im Vergleich zum Wildtyp schneller, was auf einen verkürzten Erythrozytenzyklus des Knockouts Parasitens hinweist. Wir fanden, daß *Pf*PDE1 für 20% der gesamten cGMP Aktivität in späten Blutstadien des Parasiten verantwortlich ist, und daß die übrigen PDEs den Verlust an Aktivität nicht kompensieren können. Wir konnten *Pf*PDE1 im Parasiten nicht lokalisieren. Die Tatsache, daß wir *Pf*PDE1 deletieren konnten weist darauf hin, daß es kein essentielles Gen in Blutstadien ist und daher als Zielmolekül für ein Medikament ungeeignet ist. Dennoch haben wir ein nützliches Hilfsmittel geschaffen, das zur Untersuchung der Funktion von *Pf* PDE1 in der Entwicklung von sexuellen Parasiten Formen dienen kann.

Chapter 1: Introduction

The introduction of this PhD thesis is composed of three parts. The first part focuses on the role of *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) and the regulation of expression of *var* genes using transfection technology. The second part focuses on the generation of a *Pf*PDE1 knockout parasite line also using transfection technology, whilst the third part deals with the technology itself.

Part 1 – *Pf*EMP1 and *var* gene regulation

1.1 *Plasmodium* and Malaria

Malaria is caused by infection with a protozoan parasite of the genus *Plasmodium*, which is transmitted by an infectious bite of a female Anopheles mosquito. *Plasmodia* are members of the phylum *Apicomplexa,* characterized by the presence of an apical complex, which contains an apicoplast (a structure in juxtaposition to the nucleus and related to the chloroplast), a polar ring organizing the microtubules, and vesicles called micronemes, rhoptries and dense granules. The genus *Plasmodium* contains more than 100 species of which four infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Out of these four species, *P. falciparum* is the only one where severe pathogenesis such as cerebral malaria, severe anaemia, renal failure and pulmonary affection are frequently seen. The reason for *P. falciparum*'s virulence originates in the ability to invade red blood cells (RBCs) of all ages causing very high parasitaemia, reaching high multiplication rates (up to 24 merozoites as compared to 8-10 merozoites in *P. vivax*) and enhanced growth, and the capacity to adhere to vascular endothelium through the process of sequestration. Every year around 500 million clinical cases occur and out of these one million die (Snow et al., 2005). Almost 80% of these cases occur in sub-Saharan Africa, where mainly children younger than five years and pregnant women are affected. Mortality is not the only problem; morbidity leads to major social and economic losses in endemic areas. There are multiple channels by which malaria impedes development, including effects on fertility, population growth, saving and investment, worker productivity, absence, premature mortality and medical costs.

1.2 Lifecycle of Plasmodium falciparum

The life cycle of *Plasmodium* is complex (Figure 1). It involves two hosts, a female mosquito of the genus *Anopheles* and a vertebrate (e.g. a human). The cycle can be divided into three consecutive phases of multiplication: Two phases of schizogony (asexual multiplication) in the vertebrate host first in hepatocytes then in RBCs and one phase of sporogony (sexual multiplication) in the mosquito. The vertebrate host gets naturally infected by the bite of a mosquito injecting the parasite in the sporozoite form. Sporozoites rapidly migrate to the liver via the blood circulation. They invade hepatocytes where they develop into hepatic schizonts (reviewed in Baldacci and Menard, 2004; Kappe et al., 2004). Every schizont produces up to 10,000 merozoites in a few days, which are released into the blood stream where they infect RBCs. In the RBC they multiply giving raise to up to 24 merozoites, which are released and again invade RBCs thereby maintaining the erythrocytic cycle. In parallel a few parasites differentiate into male or female sexual forms called gametocytes. Once ingested by a blood sucking mosquito gametocytes give rise to gametes, which fuse in the midgut lumen. The zygotes formed by this fertilization develop into motile ookinetes, which invade and traverse the midgut epithelium. Diploid ookinetes undergo meiosis and, on reaching the basal side of the midgut, transform into oocysts, thereby undergoing several rounds of mitosis as they mature. Each oocyst releases thousands of haploid sporozoites into the mosquito hemocoel, from where they are transported through the hemolymph and invade the salivary glands. Sporozoites are finally transmitted to a new vertebrate host during an infective bite (reviewed in Whitten et al., 2006).

The life cycle of *Plasmodium* thus consists of three invasive stages: the ookinetes traversing the intestinal cells in the mosquito, the sporozoites infecting the mosquito salivary gland, and the vertebrate hepatocytes, and the merozoites infecting the vertebrates' erythrocytes. The sporozoites and the hepatic stages are called the pre-erythrocytic stages. The hepatic stage is asymptomatic in humans and takes approximately one week in the case of *P.*

falciparum. Clinical symptoms that can be very severe are solely due to the erythrocyte stages. Almost all antimalarial drugs currently in use are directed against this stage (Fidock et al., 2004).

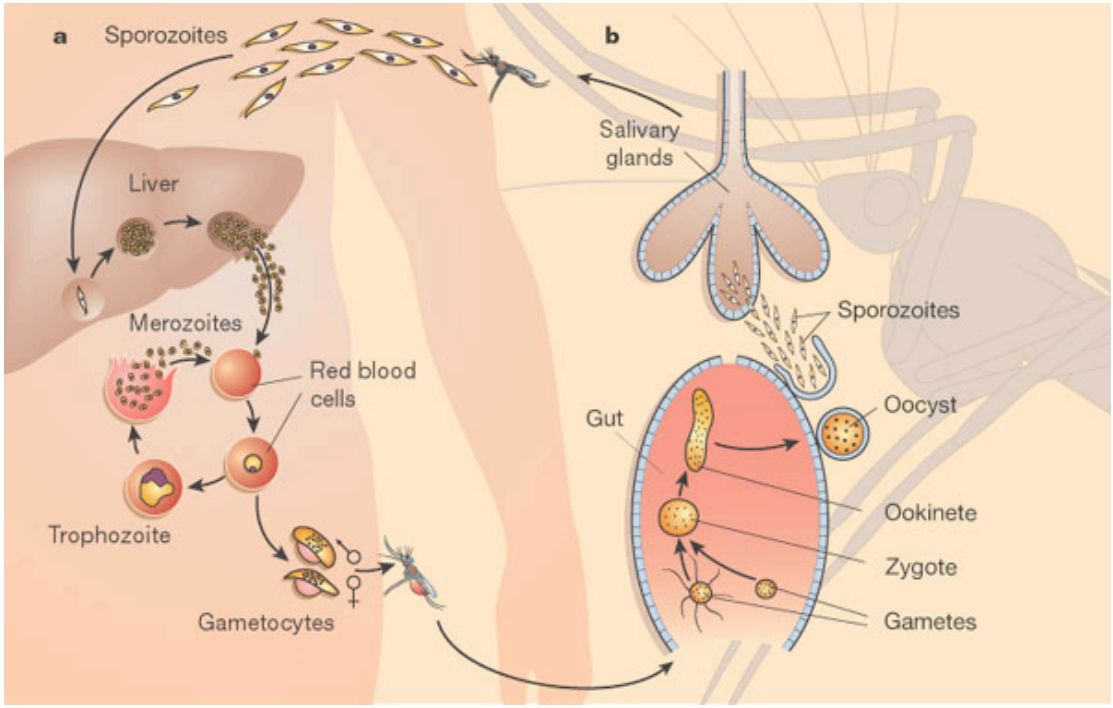


Figure 1. Life cycle of *P. falciparum* (Source: Wirth, 2002).
The life cycle of the *Plasmodium* parasite is divided between the human host where asexual replication takes place (a) and the mosquito where the sexual reproduction occurs (b). Details are given in the text.

1.3 Cytoadherence and *Pf*EMP1

During the blood stage of infection, the infected RBCs (iRBC) sequester from the blood circulation by binding to host endothelium, a process known as cytoadherence. One of the main mediators of cytoadherence is the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Leech et al., 1984), encoded by the *var* gene family (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). *var* genes are large (6-13kb) and have a two-exon structure interrupted by a conserved *var* intron. The first exon encodes an extremely diverse extracellular region with different domains that are responsible for binding, and a predicted transmembrane domain. The second exon encodes for a more conserved cytoplasmic tail (acidic terminal sequence, ATS), anchoring the protein to the knob structure on the red RBC surface. The exposure on the RBC allows *Pf*EMP1 to interact with host cell receptors thereby avoiding clearance in the spleen but at the same time renders the parasite vulnerable to the host immune system. To evade an antibody response, the *Pf*EMP1 family undergoes clonal antigenic variation (Kyes et al., 2001). There are approximately 60 *var* genes per parasite genome but only one *var* gene is transcribed in a single parasite at a time, known as mutually exclusive transcription (Chen et al., 1998b; Scherf et al., 1998). The extracellular binding domain of *Pf*EMP1 is highly variable, but predominantly assembled from four types of building blocks: the semi-conserved N-terminal segment (NTS) located at the amino terminus, the Cysteine-rich Interdomain Region (CIDR), the Duffy Binding-like (DBL) domain and the C2 domain (Figure 2). The original Duffy Binding Protein (DBP) is an important invasion ligand for RBC invasion in *P. vivax* and *P. knowlesi* (Gaur et al., 2004). Another protein family in *P. falciparum*, the Erythrocyte Binding Antigen (EBA) family, is also implied in RBC invasion and contains a DBL domain as well. DBL and CIDR domains can be classified into different types (α, β, γ, δ, ε, and X) according to their sequence similarity. Different DBL types are associated with different binding properties: nearly all CIDR-α type domains bind to CD36, whereas CIDR-β domains do not bind to this receptor, many DBLβC2 bind

intercellular adhesion molecule-1 (ICAM-1) (reviewed in Kraemer and Smith, 2006).

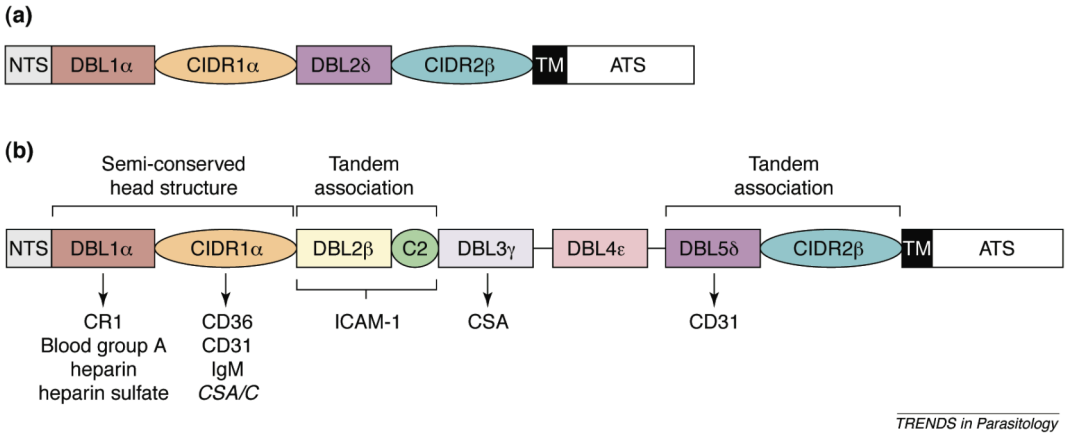


Figure 2. *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) protein architecture and binding domains.

(a) The prototypical *Pf*EMP1 extracellular region consists of an NTS and DBL1α–CIDR1 'semiconserved head structure' followed by a DBL2δ–CIDR2 tandem.

(b) Larger *Pf*EMP1 proteins also include the DBLβ, γ and ε types arrayed differently. Mapped binding traits for receptors are indicated with the domain that is responsible for binding.

Abbreviations: ATS, acidic terminal segment; CIDR, cysteine-rich interdomain region; CR1, complement receptor 1; DBL, Duffy-binding-like domain; ICAM-1, intracellular adhesion molecule-1; NTS, N-terminal segment; *Pf*EMP1, *P. falciparum* erythrocyte membrane protein 1; TM, transmembrane domain

(Source: Smith et al., 2001).

Several other receptors serve as binding partner for iRBCs but often it is unknown which *Pf*EMP1 domain or even which protein is responsible for the binding (Table 1) (Kyes et al., 2001). Some of the domains that have been associated with binding to host receptors are indicated in Figure 2. The binding ability of a *Pf*EMP1 variant is due to the composition of its domains, which in turn determine its virulence. Generally CD36 binding is common in mild infections, whereas ICAM-1 (Smith et al., 2000) and CSA binding (Fried

and Duffy, 1996) is more often associated with either cerebral or placental malaria, as these receptors are mainly expressed in cerebral blood vessels or the placenta, respectively.

var genes can be further classified into five distinctive types (upsA, upsB, upsC, upsD and upsE) according to their promoter sequence, as well as their chromosomal localization and orientation, (Gardner et al., 2002). In 3D7, the *P. falciparum* strain that has been sequenced completely, upsC genes are localized exclusively in chromosome-central clusters, whereas all but one upsB genes are located at the telomere ends, being transcribed towards the centromere. The remaining groups are also subtelomeric, but transcribed towards the telomere. The *var* genes are not the only gene family localized at the teleomers. The repetitive interspersed family (*rifin*) and the subtelomeric variable open reading frame family (*stevor*) are localized adjacent to the *var* genes (Figure 3) (Gardner et al., 2002). Both families show antigenic variation and are associated with the RBC membrane (Lavazec et al., 2006) but their function is not yet clear. The genomic organization of the distinctive *var* gene types might allow the different groups to recombine more often within rather than between different groups. upsA *var* genes are more closely related to each other than to other *var* genes, all encoding non-CD36-binding type CIDR domains.

Table 1. Host molecules to which parasitized red blood cells bind.

Abbreviations: ICAM-1 (intercellular adhesion molecule-1); VCAM-1 (vascular cell adhesion molecule-1); PECAM-1 (platelet-endothelial cell adhesion molecule-1); TSP (thrombospondin); CSA (chondroitin sulfate A); CR1 (complement receptor 1); LFA-1 (leukocyte function antigen-1); VLA-4 (very late antigen 4); PSGL-1 (P-selectin glycoprotein ligand -1; CD62P); LDL (low density lipoprotein); GPIIb (glycoprotein IIb).

[b]Not an exhaustive or exclusive list.

[c]GAGs = some glycosaminoglycans, such as heparin, sulfated glycolipids, heparan sulfate proteoglycans.

(Source: Kyes et al., 2001).

Host receptor	Normal host ligand[b]	Parasite ligand	Phenotype association	Proportion of field isolates
Ig superfamily				
ICAM-1 (Berendt et al., 1989)	LFA-1, MAC-1	*Pf*EMP1(Baruch et al., 1996; Smith et al., 2000)	Endothelial binding	Most
VCAM-1 (Ockenhouse et al., 1992)	VLA-4	Unknown	?	Rare
PECAM-1 (Treutiger et al., 1997)	CD31	*Pf*EMP1(Chen et al., 2000)	Rosetting?	?
IgM		Unknown	Rosetting	Medium
Other				
CD36 (Barnwell et al., 1989)	TSP, LDL	*Pf*EMP1(Baruch et al., 1996; Baruch et al., 1997)	Endothelial binding	Nearly all
E-selectin (Ockenhouse et al., 1992)	Sialyl Lewis x & a	Unknown	?	Rare
P-selectin (Udomsangpetch et al., 1997)	Sialyl Lewis x, PSGL-1	Unknown	?	?
TSP (Roberts et al., 1985)	CD36, $\alpha_v \beta_3$ GPIIb/IIIa, GAGs[c]	Modified band 3 (Lucas and Sherman, 1998)	?	Nearly all
$\alpha_v \beta_3$ (integrin) (Siano et al., 1998)	Vitronectin receptoe, TSP	Unknown	?	?
CR1 (Rowe et al., 1997)	C3b, C4b	*Pf*EMP1 (Rowe et al., 1997)	Rosetting	Medium
Glycosaminoglycans				
CSA (Robert et al., 1995; Rogerson et al., 1999)	Thromomodulin	*Pf*EMP1 (Buffet et al., 1999; Reeder et al., 1999; Degen et al., 2000)	Placental binding	Rare
Heperan sulfate (Chen et al., 1998a)		*Pf*EMP1 (Chen et al., 1998a)	Rosetting	Medium
Sulfated glycoconjugates (Xiao et al., 1996)		Unknown	Rosetting	Medium
Blood group A and B (Carlson and Wahlgren, 1992)		*Pf*EMP1 (Chen et al., 2000)	Rosetting	Medium
Hyaluronic acid (Beeson et al., 2000)	CD44	Unknown	Placental	Rare binding

Table 1.

In contrast, nearly all *Pf*EMP1 proteins in the upsB and upsC groups have CD36-binding CIDR domains (Kraemer and Smith, 2006). Even though *var* genes of one single genome are highly diverse, two conserved *var* genes (*var*1CSA and *var2*CSA) have been found that also exist in field isolates where they are associated with placental malaria (Rowe et al., 2002; Salanti et al., 2003). Their conserved sequence structures make a possible vaccine target for preventing placental malaria (Rowe and Kyes, 2004).

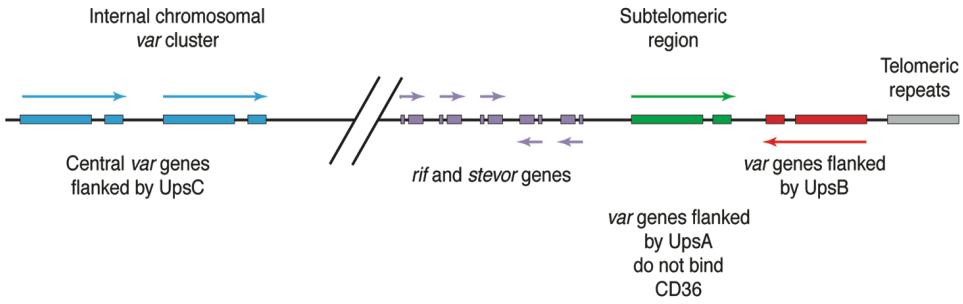


Figure 3. Typical arrangement of *var*, *rifin* and *stevor* genes.
Within the sequenced 3D7 genome, internal chromosomal clusters of *var* genes vary between one and seven copies found on five different chromosomes. Subtelomeric regions contain zero to three *var* genes, found in either orientation, the *rif* and *stevor* genes can be in either orientation in close proximity to var genes. Five types of flanking sequences, referred to as upsA, upsB, upsC, upsD and upsE are found upstream of *var* genes depending on their location and orientation. For simplicity only the first three types of flanking regions are indicated.
(Source: Deitsch and Hviid, 2004).

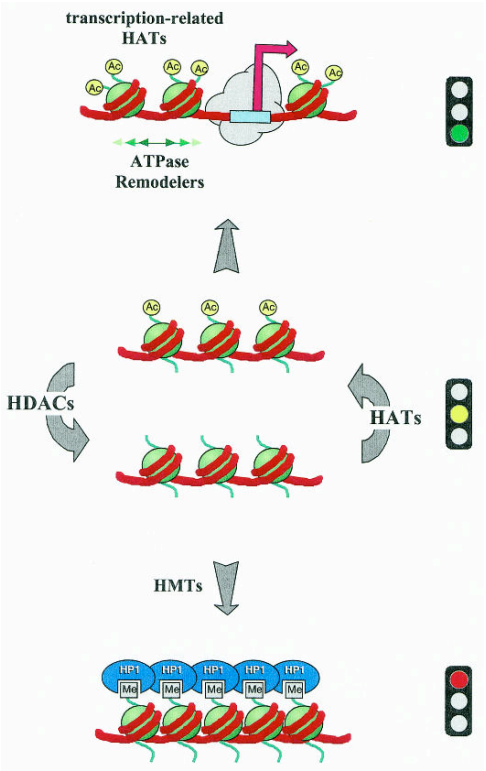
1.4 Regulation of *var* gene expression

Although there are 60 *var* genes present in the 3D7 *Plasmodium* strain, only one is expressed at a time, resulting in one functional *Pf*EMP1 variant on the RBC surface. Mutually exclusive transcription is a common feature in several organisms, i.e. VSG expression in African trypanosmes (Borst and Ulbert, 2001) and the immuno globulin heavy-chain gene expression in humans (Corcoran, 2005). In the case of *P. falciparum*, the switch in *var* gene expression is regulated *in situ* without any sequence alterations or repositioning of the gene in the genome context (Scherf et al., 1998). *var* gene expression in the asexual blood-stage is only found 3 to 18 hours post infection (Kyes et al., 2000). Although only a single mRNA will be translated to a functional *Pf*EMP1, early transcripts from the 5' end of most *var* genes can be detected (Chen et al., 1998b; Scherf et al., 1998). The nature of the early 5' transcripts is controversial. Comparison of RT-PCR data, using validated 5' universal primers with Northern blots, suggests that even in young ring stages, only a single full-length transcript exists and that the remaining are terminated early (Taylor et al., 2000). It seems possible that shortly after invasion, transcriptional initiation is promiscuous, but proper elongation fails to occur (Kyes et al., 2001). Another set of non full-length *var* gene transcripts has been described consisting of exon 2 and some *var* intron sequences (Su et al., 1995). The relative abundance of these 'sterile' transcripts suggests that they are derived from several *var* genes. The function of the sterile transcripts remains to be determined.

Gel mobility shift assays identified protein binding sequences in upsC and upsB upstream regions that are bound by unknown nuclear proteins (Voss et al., 2003). Transcriptional start point mapping disclosed that *var* mRNAs contain rather long 5' upstream regions of about 1kb (Deitsch et al., 1999). Promoters removed from the chromosomal context and cloned into plasmids can drive luciferase or chloramphenicol acetyl transferase expression (Deitsch et al., 1999; Voss et al., 2000; Vazquez-Macias et al., 2002). This is surprising as the most frequent state of a *var* promoter in the genome is silent, indicating

that a silencing element is missing in the promoter. Deitsch et al., (2001) identified the *var* intron as such a silencing element. They showed that a *var* promoter is only able to drive luciferase expression when it is not paired with a *var* intron. The silencing effect is not dependent on the orientation of the *var* intron but transfected parasites need to pass the S-phase to achieve complete silencing. During the S-phase chromatin structures are relaxed to enable replication of the whole genomic DNA. After replication, DNA is packed again into chromatin and due to rearrangement previously silenced genes can be activated. The *var* intron is composed of a three partite structure, whereby the central part contains bidirectional promoter activity and is essential for silencing (Calderwood et al., 2003).

Figure 4. The histone acetylation switch.



Targeted histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities negotiate the acetylation status of chromatin. Acetylation establishes a structure that permits ATP-dependent chromatin remodeling factors to open promoters. Deacetylation, frequently followed by histone methylation, may form a solid base for highly repressive structures, such as heterochromatin. Acetylated histone tails are shown as yellow circles. Methylations are indicated as gray rectangles. HMT, histone methyltransferase; HP1, heterochromatin protein 1 (Source: Eberharter and Becker, 2002).

Alteration in chromatin structure goes along with phosphorylation, methylation and acetylation of histone proteins, thereby changing the accessibility of various DNA sequences to transcription factors (Eissenberg and Wallrath,

2003). For illustration see Figure 4. A homologue of the yeast deacetylase Silent mating type Information Regulation-2 (Sir2), that mediates heterochromatin formation by removing an acetyl group from histone molecules, has been identified in *P. falciparum* and termed *Pf*Sir2 (Freitas-Junior et al., 2005). *Pf*Sir2 binds to the subtelomeric region of the chromosome, extending into the regulatory sequences of the *var* genes. *Pf*Sir2 does not bind to the active *var* gene, but only to silent ones supporting a role in the mutually exclusive transcription of the *var* genes. It has to be noted, that *Pf*Sir2 is only associated with subtelomeric *var* genes but not with central ones, suggesting a different silencing mechanism for centrally located *var* genes. The same group showed that histone H4 acetylation was associated with the active *var* gene, in agreement with the general notion that histone hyperacetylation promotes gene activation while hypoacetylation generates repression (Freitas-Junior et al., 2005). Recently, these results have been complemented by another group which noticed that trimethylated histone H3 at lysine-9 (H3K9me3) is significantly enriched at silent *var* gene loci (Chookajorn et al., 2007). These observations are supported by a study from (Duraisingh et al., 2005), who inserted a human dihydrofolate reductase (*h*dhfr) gene into the Rep20 locus which is a special element of the telomere associated repeat elements (TAREs) that tether chromosome ends into clusters. They observed that chromatin packaging is more compact at the *h*dhfr transgene locus when the gene is silenced than when it is active. In addition, deletion of *Pf*Sir2 resulted in upregulation of a subset of members of the *var* and *rifin* gene families (Duraisingh et al., 2005).

Telomeres and the adjacent *var* genes cluster into clusters of six to eight chromosome ends near the periphery of the nucleus, where the heterochromatin is in a more condensed form (Freitas-Junior et al., 2000). Central *var* genes have also been localized in the nuclear periphery (Ralph et al., 2005) and recently it has been shown that they colocalize with telomeric clusters (Voss et al., 2006). Activation of a specific *var* promoter was accompanied by physical repositioning of the chromosome end to a hypothetical nuclear domain competent for transcription in the nuclear periphery. Such a nuclear body, that allows transcription of a single gene from a gene family, is known for the VSG genes of *Trypanosoma brucei* (Navarro

and Gull, 2001). Electron microscopic studies identified a zone of relaxed euchromatin within the mostly condensed heterochromatin of the nuclear periphery (Ralph et al., 2005), supporting this theory. Generally it has been observed that active *var* promoters colocalize significantly frequent with other active promoters in the nuclear periphery (Duraisingh et al., 2005; Ralph et al., 2005; Voss et al., 2006). A schematic representation of the nuclear architecture and the possible localization of active and silent *var* promoters is shown in Figure 5.

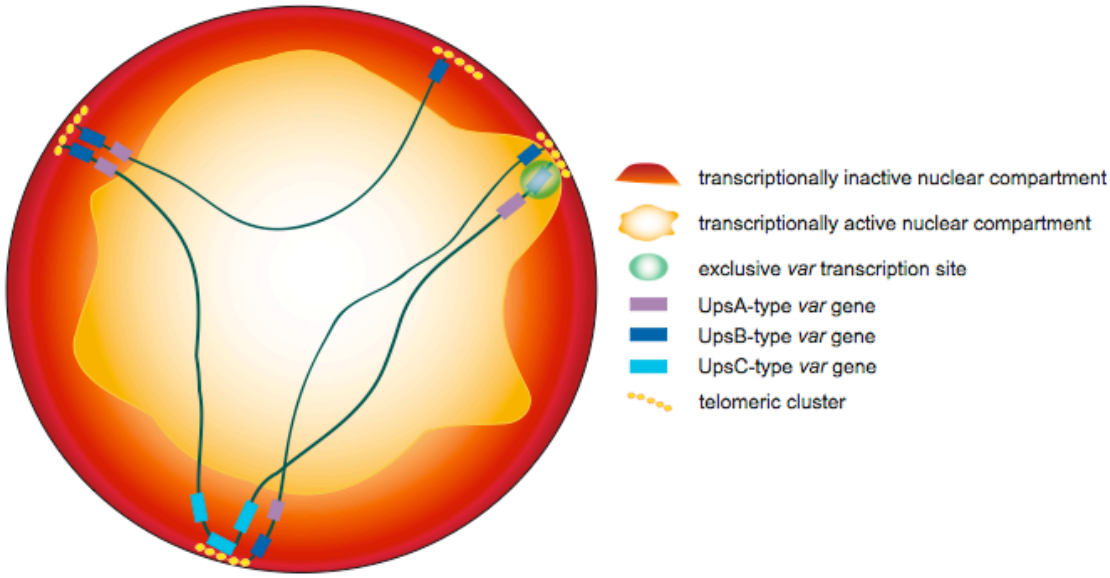


Figure 5. Model for mono-allelic *var* gene transcription.
A simplistic display of a *P.falciparum* nucleus divided into the transcriptionally incompetent nuclear periphery (red) and a transcriptionally active central region (yellow) that extends to the nuclear membrane at one site. Silent internal upsC *var* genes (light blue) cluster with silent telomeric upsB and upsA *var* genes (blue and purple, respectively) in the transcriptionally incompetent nuclear periphery (Ralph et al., 2005). The previously described active perinuclear zone (Duraisingh et al., 2005) includes in this figure a telomeric cluster and the proposed exclusive *var* gene transcription site (green). *var* gene transcription occurs only in the *var* gene transcription site within the active perinuclear zone. Figure adapted from Voss et al., 2006)

1.5 Objectives

Despite a growing body of knowledge, many questions about the *var* gene regulation remain open and more and more are arising: There are still no transcriptional regulation factors for the *var* genes identified so far, the function of the *var* intron has to be specified, and the role of the nuclear body needs further investigation. In my PhD I was mainly interested in the interaction between the *var* intron and the *var* upstream region. The aim was to identify the interaction region of the *var* intron on the *var* upstream region. During my MSc studies I have already transfected *P. falciparum* transiently with various constructs containing different *var* upstream region fragments driving luciferase expression paired or unpaired with a *var* intron. Unfortunately, luciferase activity was rather weak and varied much between different experiments. In the course of my PhD I optimized these experiments by using stable instead of transient transfection to reduce variations between different experiments. The advantage of stable transfections is that every parasite contains the desired plasmid. Thus luciferase expression is stronger making comparisons between different plasmid constructs more relevant.

Another aspect of my PhD thesis was to test whether the mutually exclusive transcription machinery needs the *var* intron to recognize *var* promoters localized episomally on plasmids as suggested by (Frank et al., 2006). For this purpose we did quantitative RT-PCR on cDNA from transfected parasites with primer pairs for every endogenous *var* gene as well as for the luciferase gene located on the plasmids.

Part 2 - PDE knock out

1.6 Signal transduction

Signal transductions are processes where one type of a signal or stimulus is changed into another type. Through this transduction the cell is able to adapt to actual conditions and needs by regulation of cell-conformation and movement as well as metabolism and gene expression. Membrane-bound receptors transfer extracellular signals via second messengers that enhance the stimulus to signal cascades in the cell. Important features are conformational changes of signaling proteins, whereby a protein switches from an activated to an inactivated form or vice versa. Protein kinases are well known mediators of such conformational changes phosphorylating the hydroxyl group in the side chain of Tyrosine, Serine or Threonine of the target protein.

1.7 Cyclic nucleotide transduction pathway

Cyclic nucleotides have been extensively studied as second messengers of intracellular events initiated by activation of many types of hormone and neurotransmitter receptors. A general overview is given in Figure 6 with explanations in the text below. Receptors that stimulate the conversion of adenosine triphosphate (ATP) to cyclic 3', 5'-adenosine monophosphate (cAMP) are associated with G proteins and thus termed G protein coupled receptors (GPCR). Binding of the hormone or neurotransmitter to its membrane-bound GPCR induces a conformational change in the GPCR that leads to activation of the α-subunit of the G protein and subsequent dissociation of the α-subunit from the βγ subunit of the G protein. The activated α-subunit can either stimulate ($G_s\alpha$) or inhibit ($G_i\alpha$) an adenylyl cyclase (AC). Stimulation of AC catalyzes the conversion of cytoplasmic ATP to cAMP. cAMP activates cAMP-dependent protein kinases, including protein kinase A (PKA). By catalyzing the phosphorylation (activation or deactivation) of intracellular enzymes, cAMP-dependent kinases elicit a wide array of metabolic and functional processes. Negative regulation can occur in the pathway when PDEs catalyze the hydrolysis of cAMP to adenosine-5'-monophosphate (5'-AMP).

Cyclic guanosine monophosphate (cGMP) serves as a second messenger in a manner similar to that observed with cAMP. Peptide hormones, such as the natriuretic factors, activate membrane-bound guanylate cyclases (GC) directly (Wedel and Garbers, 2001). Receptor activation of GC leads to the conversion of guanosine triphosphate (GTP) to cGMP. Nitric oxide (NO) also stimulates cGMP production by activating soluble GC, perhaps by binding to the heme moiety of the enzyme. Similar to cAMP, cGMP mediates most of its intracellular effects through the activation of specific protein kinase G (PKG). PDEs act as regulatory switches by catalyzing the degradation of cGMP to guanosine-5'-monophosphate (5'-GMP).

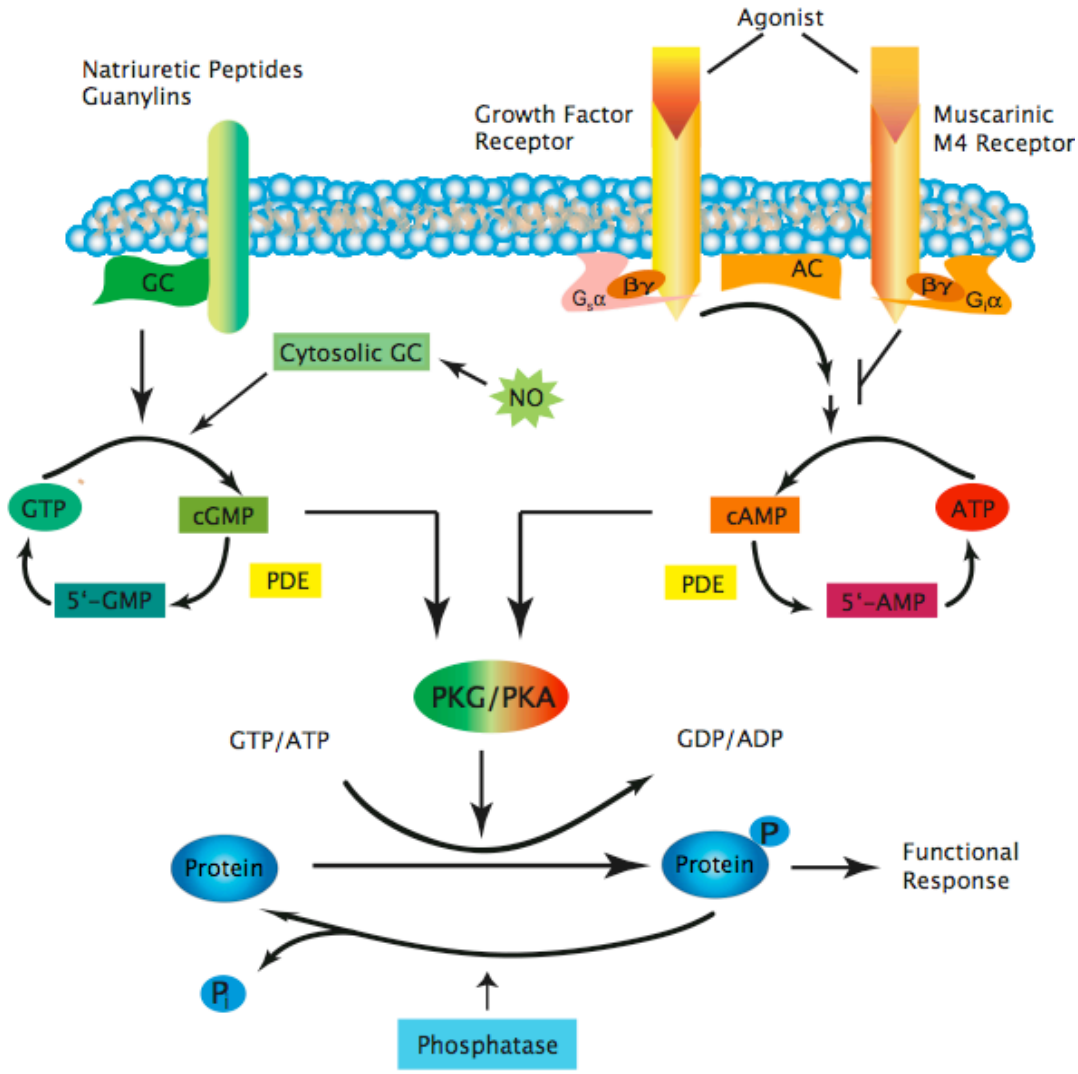


Figure 6. Cyclic Nucleotide Metabolism.

The metabolism pathway is indicated on the left hand site for cGMP and on the right hand site for cAMP. Detailed explanations are given in the text. Abbreviations: 5'-AMP, adenosine-5'-monophosphate; AC, adenylyl cyclase; cAMP, cyclic 3', 5'-adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PKG, cGMP dependent protein kinases; 5'-GMP, guanosine-5'-monophosphate; GC, guanylate cyclase; NO, nitric oxide; PDE, phosphodiesterase; PKA, protein kinase A;

Figure and text adapted from SIGMA-ALDRICH:

http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Signaling/Scientific_Resources/Pathway_Slides___Charts/Cyclic_Nucleotide_Metabolism_cAMP.html

1.8 Roles of cyclic nucleotides in the parasite

As discussed above, cyclic nucleotides play an important role in gene expression and metabolism. In the malaria parasite, evidence suggests that cAMP might be involved in promoting differentiation of bloodstream asexual parasites into male and female gametocytes, a step that is essential for transmission. It has been observed that development of gametocytes in *P. falciparum* blood-stage cultures with high parasitaemia can be triggered either by addition of cAMP (Kaushal et al., 1980) or addition of PDE inhibitors (Brockelman, 1982; Trager and Gill, 1989). In other instances, cAMP has been observed to have an inhibitory effect on gametocyte development (Inselburg, 1983).

Not all laboratory strains have the same ability to produce gametocytes. Some parasite clones are good gametocyte producers while other clones hardly ever produce gametocytes *in vitro*. Comparison of two parasite clones showed that both have similar basal levels of AC activity but the cAMP-dependent PKA activity was significantly lower in non-gametocyte producers than in gametocyte producer clones (Read and Mikkelsen, 1991).

A G-protein dependent pathway that takes part in switching to sexual development has been suggested for *P. falciparum* (Dyer and Day, 2000). However, in malaria genome project (Gardner et al., 2002) no recognizable heterotrimeric G protein homologues was identified nor did a homology search based on Hidden Markov Models derived from phylogenetic classified human G protein coupled receptors (GPCRs) (Fredriksson and Schioth, 2005). It has been proposed that the cAMP signaling pathway of the RBC could play a role in malaria infection (Harrison et al., 2003). Host GPCRs and Gα subunits appear to be associated with the parasite vacuole and addition of peptides blocking interaction between these two, decreased parasitaemia.

Studies with PDE inhibitors have suggested a role for the cGMP signalling pathway in exflagellation (Martin et al., 1978; Kawamoto et al., 1990; Kawamoto et al., 1993). This process occurs in the mosquito midgut when eight flagellated male gametes emerge from a single infected cell. *In vitro*,

exflagellation can be induced by a decrease in temperature together with a rise in pH (Nijhout and Carter, 1978; Sinden, 1983) or by the gametocyte-activating factor xanthurenic acid (XA) (Billker et al., 1998; Garcia et al., 1998), a product of tryptophan catabolism.

1.9 Guanylyl cyclases and Adenylyl cyclases in *Plasmodium falciparum*

Two GCs have been identified in *P. falciparum* (*Pf*GCα and *Pf*GCβ) (Carucci et al., 2000). Peptides and mRNA of both GCs were detected in asexual blood stages, gametocytes and sporozoites (Kappe et al., 2001; Bahl et al., 2003).
Activators of plasmodial cyclases have yet to be identified, as there are no obvious G protein homologues identified so far (Bahl et al., 2003; Fredriksson and Schioth, 2005).
Two ACs have been identified in *P. falciparum* (*Pf*ACα and *Pf*ACβ) that are not very closely related to each other (Muhia et al., 2003). Low levels of *Pf*ACα mRNA are expressed in gametocytes, sporozoites and asexual blood stages (Le Roch et al., 2003). The protein has a single catalytic domain and the six membrane helices correspond to transmembrane segments of voltage-dependent potassium channels (Weber et al., 2004). 21 introns interrupt the *Pf*ACα gene and there are several splice variants that might correspond to different environmental signals each resulting in a defined change in intracellular levels of cAMP (Muhia et al., 2003). *Pf*ACβ is expressed at high levels in schizonts (Le Roch et al., 2003). The protein is related to a small class of soluble ACs. The mammalian soluble AC is involved in sperm activation; therefore *Pf*ACβ could play a role in activation of *P. falciparum* microgametocytes.
Not only the synthesizer of plasmodial cyclic nucleotides have been identified, but also some of their targets. The second messengers cAMP, cGMP, and diacyl glycerol (DAG) regulate a PK family consisting of the protein kinase A, protein kinase G, and protein kinase C, termed AGC family (Hanks et al., 1988). Four to five malarial kinases cluster within this group, according to genome based clustering of the kinome by two independent research groups (Ward et al., 2004; Anamika, 2005). Two AGC kinases have been earlier characterized: the cAMP-dependent PfPKA (Syin et al., 2001) and the cGMP-dependent PfPKG (Deng and Baker, 2002) Another PKA has been previously shown to be required for the development of the parasite (Li and Cox, 2000).

Protein kinase C (PKC) homologues have not been identified in the parasite (Ward et al., 2004; Anamika, 2005).

1.10 PDEs in mammals

The importance of phosphodiesterases (PDEs) was for the first time observed in 1886 by Henry Hyde Salter. An asthmatic, he noted that when he drank a strong cup of coffee on an empty stomach, his breathing eased, an effect attributed to the bronchodilator properties of caffeine. Although the mechanism of action at the time was unknown, it has since been shown that caffeine was acting as a non-selective, though weak, PDE inhibitor.

The discovery of cyclic nucleotides occurred not till over half a century later. In 1958 cAMP was discovered in liver extracts (Sutherland and Rall, 1958) and five years later cGMP in rat urine and simultaneously PDE was identified as the enzyme capable of inactivating cAMP (Ashman et al., 1963). It was shown that this enzyme could be activated by magnesium ions and importantly, could be inhibited by caffeine providing a plausible mechanism of action for the diverse activities of this drug (Sutherland and Rall, 1958).

From a very early period, it was hypothesized that there were a number of different isoforms of PDE distinguished primarily by their substrate specificity and sensitivity to calcium-calmodulin. Diverse isoforms of the PDEs have been differentiated in rat and bovine tissue (Beavo et al., 1970) and have been further characterized by selective inhibitors (Hidaka and Endo, 1984; Nicholson et al., 1991). Even more isoforms of the PDEs have been identified and characterized by selective inhibitors over the years (reviewed in Boswell-Smith et al., 2006). Today 11 isoenzyme groups, encompassing over 50 isoforms, have been identified in mammals. PDE activity is found in every cell in the body, although there is distinct cellular and subcellular distribution. Their selectivity for substrate, localization and different inhibition profiles makes them good targets for therapeutics (reviewed by Lugnier, 2006). Today selective PDE inhibitors are being investigated in a wide range of diseases like sepsis, sexual dysfunction in females, cardiovascular disease, pulmonary hypertension, asthma, allergic rhinitis, psoriasis, multiple sclerosis, depression, Alzheimer's disease and schizophrenia.

1.11 PDEs in Plasmodium

Four putative *Pf*PDE genes have been postulated by *in silico* analysis of the *P. falciparum* 3D7 genome (Bahl et al., 2003). All these gene products contain a sequence consistent with the class I PDE signature sequence HDX_2HX_4N (Beavo and Reifsnyder, 1990). The four putative *Pf*PDEs do not belong to any PDE family previously described thus constituting a new family of PDEs. Out of these four PDEs the *Pf*PDE1 (PFL_0475w) has been described in more detail, whereby several authors found non-identical cDNA sequences. PlasmoDB (Bahl et al., 2003) predicts a four exons, three introns structure, the same organization, as well as a three exons, two introns structure has been found by Wentzinger (personal communication), yet another group identified a two exons, one intron structure (Yuasa et al., 2005). Different numbers of predicted transmembrane domains, ranging from three to six have been identified by the same three groups. A schema for the different RNA sequences is shown in Figure 7. Transcription levels of mRNA in blood stage parasites were highest in ring stages (Yuasa et al., 2005) or schizonts (Le Roch et al., 2003).

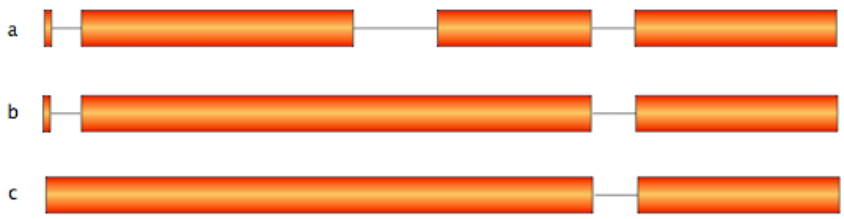


Figure 7. Different splice variants of *Pf*PDE1.
*Pf*PDE1 has been found in three different splice variants consisting of different numbers of exons (red bars) and introns (black lines). Initially, PlasmoDB postulated variant a) containing three transmembrane (TM) domains. Wentzinger found a similar structure to a) but with four predicted TM domains and observed a structure with six predicted TM domains b) (unpublished data). Variant c) with six TM domains has been found by the group of Yuasa et al., (2005).

Recombinant *Pf*PDE1 produced in bacteria exhibited enzymatic activity *in vitro* for cGMP but not for cAMP (Yuasa et al., 2005). *In vivo*, cGMP hydrolytic activity in *P. falciparum* iRBcs was highest in the membrane fraction, while uninfected RBCs showed no significant cGMP hydrolytic activity at all (Yuasa et al., 2005). Wentzinger has confirmed these results. The three remaining *Pf*PDEs do also have predicted transmembrane domains and could additionally account for the hydrolytic activity found in the membrane fraction. Yuasa et al., (2005) also showed that PDE 1 activity is lost by mutagenesis of the conserved Asp^{762}, which is predicted to be involved in the formation of a metal-binding pocket essential for class I PDEs. Most likely cGMP-dependent protein kinase (*Pf*PKG) is the only effector of cGMP in *Plasmodium* as there are no known parasitic cyclic nucleotide-gated ion channels or cGMP-regulated PDEs as found in mammals.

1.12 Objectives

The potential of PDE inhibitors has long been discovered in human medicine. Today a variety of drugs are on the market that target specifically PDE families or even PDE isoforms, despite considerable sequence conservation between catalytic domains of different isoforms. Among these are PDE inhibitors that are used against cardiovascular disease, pulmonary hypertension, asthma, allergic rhinitis, multiple sclerosis, depression, Alzheimer's disease and schizophrenia (Lugnier, 2006). Despite the potential of PDE inhibitors as chemotherapeutics, little is known about PDEs of parasites.

Research in the protozoan parasite *Trymanosoma brucei*, the causative agent of sleeping sickness in humans and Nagana in animals, identified the presence of two families of PDEs. The *Tb*PDE2 family is essential for proliferation of bloodstream form (Zoraghi et al., 2001; Rascon et al., 2002; Zoraghi and Seebeck, 2002), whereas the *Tb*PDE1 family does not appear to be essential for bloodstream forms (Kunz et al., 2004). Four *Tb*PDE2 inhibitors were identified that inhibit proliferation of bloodstream form trypanosomes in culture (Zoraghi et al., 2001). Two different PDE families have also been identified in *T. cruzi*, the causative agent of Chagas' disease that display also sensitivity to PDE inhibitors (D'Angelo et al., 2004; Kunz et al., 2005; Alonso et al., 2006; Diaz-Benjumea et al., 2006).

The search for inhibitors that could block the activity of trypanosome PDEs is on its way. Meanwhile, little is known about the four identified PDEs in *P. falciparum* although they might present an important target for chemotherapeutics. Drug resistance to the commonly used drugs such as chloroquine, mefloquine and pyrimethamine makes the development of new antimalarial drugs crucial (Cunha-Rodrigues, 2006).

In this PhD thesis we wanted to created a *Pf*PDE1 knockout parasite line and analyse the resulting phenotype to determine its potential as drug target as well as gain more information about cyclic nucleotide signalling in *P. falciparum* parasites.

Part 3 - Transfection

1.13 Horizontal gene transfer

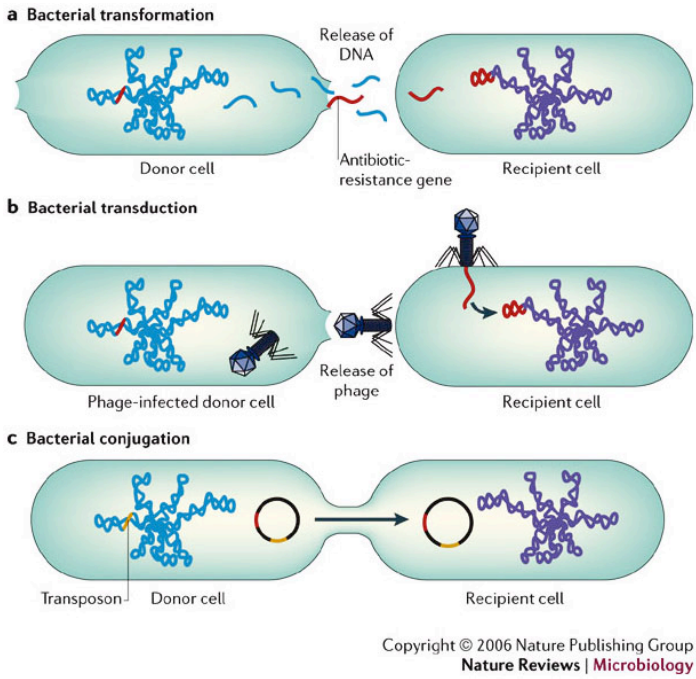
The first evidence for horizontal gene transfer (HGT) was observed in *Streprococci pneumoniae* by Fred Griffith in the late 1920s. He showed that virulence determinants can be transferred between pneumococci in infected mice (Griffith, 1928). The causing agent was identified as DNA. These studies, together with the model of the structure of DNA (Watson and Crick, 1953) hardened the concept of DNA as genetic material. While in prokaryotes, HGT has contributed to 10-20% of the genome (Koonin et al., 2001; Lawrence and Ochman, 2002), HGT occurred much less frequently in eukaryotes and donors were mainly bacteria.

HGT naturally occurs in bacteria via three different processes: (1) transformation, whereby naked extracellular DNA is taken up by competent bacteria and integrated into their genome, (2) transduction, in which the donor DNA transfer is mediated by a virus, i.e. bacteriophage, and (3) conjugation, in which the transfer involves cell-to-cell contact and a conjugative plasmid in the donor cell (Figure 8).

1.14 Physical Methods for gene transfer

High efficiency natural transformation is found only in few bacteria. In order to make *Escherichia coli*, the most used bacterial strain in the laboratory, competent for genetic engineering, empirical studies with variations in culture medium, temperature and other factors have been applied. It has been found that when *E. coli* is treated with high concentrations of calcium ions and then stored in the cold, it becomes transformable at low efficiency upon short exposure to 42°C (heat-shock).

Figure 8. Transformation, transduction and conjugation in bacteria.



a) Transformation occurs when naked DNA is released on lysis of an organism and is taken up by another organism. The antibiotic-resistance gene can be integrated into the chromosome or plasmid of the recipient cell. b) In transduction, antibiotic-resistance genes are transferred from one bacterium to another by bacteriophages and can be integrated into the chromosome of the recipient cell. c) Conjugation occurs by direct contact between two bacteria: plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of antibiotic-resistance genes by the recipient cell. Transposons are sequences of DNA that carry their own recombination enzymes that allow for transposition from one location to another; transposons can also carry antibiotic-resistance genes. (Source: Furuya and Lowy, 2006)

A newer method to artificially transform cells is electroporation. This technique was first applied by Neumann, who artificially transferred DNA into murine cells (Neumann et al., 1982). Many others followed his lead and used electoporation as a delivery system for many settings *in vitro*, as well as *in vivo* (reviewed in Gehl, 2003). During electroporation, the cell membrane is exposed to high-intensity electrical pulses that can cause transient and localized destabilization of the membrane barrier. Due to this destabilization, the cell membrane becomes highly permeable to exogenous molecules present in the surrounding media. The extent of permeabilization (area of membrane which is permeabilized) can be controlled by pulse amplitude, and the degree of permeabliization can be controlled by the pulse duration and number (Gabriel and Teissie, 1997). Due to the permeabilization of the membrane, DNA is taken up as a result of electrostatic forces that drag the negatively charged DNA through pores in the membrane (Sukharev et al., 1992). The formation of these pores is a very quick event, happening in microseconds but the resealing happens over a range of minutes whereby the cytoskeleton has an important function (Teissie and Rols, 1994). Direct interaction of DNA with the membrane facilitates pore formation (Spassova et al., 1994). When DNA is artificially introduced into mammalian cells it is called transfection.

Electroporation is not the only method for artificial transfer of DNA into cells. A variety of techniques has been established and applied in various settings. Mechanical methods include microinjection of single cells and the gene gun, where naked DNA precipitated onto microparticles is propelled at a sufficient velocity into the target cell. Naked DNA can be administered successfully to muscle and liver cells. To introduce genes into other tissues, the plasmid DNA is coated with positively charged lipids. The charge helps the construct, called a lipoplex, stick to cell membranes and place genes inside the cell (reviewed in Ferber, 2001). Other physical methods include sonoporation whereby cells are permeabilized via ultrasound and laser irradiation, and magnetofection, a fairly new technique to enhance the introduction of gene vectors into cells. Many of these techniques are used in the medical field for immunization and cancer treatment. For further information see Mehier-Humbert and Guy (2005)

1.15 Transfection in *Plasmodium*

Goodewardene was the first to transfect malaria parasites in 1993. He succeeded in transiently expressing luciferase in *P. gallinaecium* (Goonewardene et al., 1993). The first transfection in *P. falciparum* was achieved two years later (Wu et al., 1995). Unlike *P. berghei, P. falciparum* can only be transfected with circular plasmid DNA and the efficiency of transfection is estimated to be 10^{-6} (O'Donnell et al., 2002). These first transfections were only transient with no marker to select for parasites harboring the plasmid. The first targeted chromosomal integration was performed in the subsequent year using the dihydrofolate reductase-thymidylate synthase gene (*dhfr-ts*) from *Toxoplasma gondii* that confers pyrimethamine and cycloguanil resistance (Wu et al., 1996). Today, selection cassettes like the *dhfr-ts* gene from *T. gondii* are mainly used for transfections in *P. berghei*, while human *dhfr* (*hdhfr*) is widely used in *P. falciparum*. *hdhfr* confers resistance to pyrimethamine or the drug WR99210 (Fidock and Wellems, 1997; Zhang and Rathod, 2002). Other markers have also been successfully developed. Blasticidin S deaminase (Mamoun et al., 1999), neomycin phosphorotransferase (Mamoun et al., 1999) and puromycin-N-acetyltransferase (de Koning-Ward et al., 2001) confer resistance to blasticidin S, geneticin (G418) and puromycin, respectively. With these four positive selectable markers it is possible to design plasmids for numerous experiments.

Generic vectors with only one selectable marker can be used for stable transgene expression (Crabb et al., 1997b; Frank et al., 2006) in promoter studies where a promoter of interest drives a reporter gene like chloramphenicol acetyl transferase (CAT) or the Firefly luciferase. Single marker vectors have also been used in gene knock-outs (Crabb et al., 1997a), whereby the plasmid is integrated via single cross-over into the chromosome disrupting the target gene. The implication of a protein in drug susceptibility can be studied by over expression of the protein in question and subsequent sensitivity tests to the drug compared to the wild type. Transgenic parasites

should be less sensitive to the drug than wild type parasites (Gardiner and Good, 2006). The fusion of a protein of interest with a green fluorescent protein (GFP) reporter gene allows for localization, orientation, and trafficking studies (Khattab and Klinkert; Waller et al., 2000; Wickham et al., 2001). Franke-Fayard et al., (2004) integrated a GFP gene controlled by a strong promoter into the genome of *P. berghei.* GFP is constitutively expressed in a growth responsive manner in the parasites cytoplasm. These parasites can be used for drug sensitivity assays (Vennerstrom et al., 2004), the study of host-parasite interaction by *in vivo* imaging (Heussler and Doerig, 2006) and generally in applications that involve FACS analysis.

In order to facilitate segregation of vector plasmids between daughter cells the Rep20 sequence has been introduced into plasmid constructs (O'Donnell et al., 2002). Rep20 belongs to the telomere associated repeat elements (TAREs) at the end of each chromosome. Plasmids that contain Rep20 are physically tethered to terminal chromosome clusters and thus more efficiently segregated.

The introduction of the herpes simplex virus thymidine kinase (tk) gene makes it possible to select for double crossover events (Duraisingh et al., 2002). Tk is the key enzyme in the pyrimidine salvage pathway catalyzing the transfer of a phosphate from ATP to thymidine to produce thymidine monophosphate. The guanosine analog ganciclovir serves as a substrate for tk (Balzarini et al., 1993). Once phosphorylated, these nucleoside analogs are further phosphorylated into nucleoside triphosphates that inhibit DNA synthesis after incorporation into nascent DNA (Reardon, 1989). Thus, parasites that express the tk gene are sensitive to ganciclovir and the tk gene can be used as a negative selection marker (Kokoris and Black, 2002). After positive selection for parasites that contain a plasmid, negative selection pressure is put on to select for parasites that have integrated the positive selection cassette specifically into the chromosome via double crossover and consequently lost the negative selectable marker. All parasites that contain the plasmid episomally are lost. In order to prove that an observed phenotype of a knock out parasite is really due to the loss of the gene, the deleted gene can be complemented and the wild type phenotype restored. With the availability of numerous positive selectable markers, the deleted gene can be expressed

episomally from a plasmid with a different positive selectable marker than the one used for the knock out (Maier et al., 2006). Recently an elegant system has been developed in *P. berghei,* where the positive and negative selectable marker are combined in a bi-functional protein (Braks et al., 2006). In this system the positive-negative selection cassette disrupts the gene under investigation and a phenotype is established. In the second step positive selection pressure is removed and negative selection pressure is turned on to select for the rare event where parasites have undergone a second recombination event resulting in the loss of the selection cassette and the restoration of the original phenotype.

The use of RNA interference has been attempted in *P. falciparum* (Gardiner et al., 2000; McRobert and McConkey, 2002) but it is not clear if the observed effects are really due to specific RNA interference. So far no plasmodial homologues to known proteins from the classical RNAi pathway like Dicer, Piwi, PAZ or RdRp have been identified (reviewed in Ullu et al., 2004).

Chapter 2: Objectives

1 To optimize transfection efficiency and reduce variability between luciferase assays
2 To characterize the *var* gene upstream region
3 To identify the interaction site of the *var* intron on the *var* upstream region
4 To establish knockout-technology by deleting *Pf*PDE1
5 To establish the phenotype of the *Pf*PDE1 knockout parasite line

Chapter 3: Silencing in *Plasmodium falciparum var* genes -searching for an interaction site of the *var* intron on the *var* upstream region

INTRODUCTION

Malaria together with HIV and tuberculosis is one of the most important infectious diseases worldwide. *Plasmodium falciparum* is the most virulent form of the four human plasmodia species with an estimated 500 million clinical cases every year (Snow et al., 2005). *P falciparum* expresses variant surface antigens on the erythrocyte membrane enabling the parasite to adhere to different receptors on host endothelial cells. This so called sequestration helps avoiding clearance of the infected red blood cells (iRBC) in the spleen (Miller et al., 1994; Kyes et al., 2001). Cytoadhesion of iRBCs is responsible for a majority of severe disease symptoms such as cerebral and placental malaria. The main protein involved in this cytoadherence is *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Leech et al., 1984), which is encoded by the *var* gene family that consists of over 60 members in 3D7 (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995; Gardner et al., 2002). In order to evade the host immune system, the parasite is able to undergo antigenic variation by switching expression from one *var* gene to another (Biggs et al., 1991; Roberts et al., 1992). In this process, the parasite switches the expression of one *Pf*EMP1 to another variant, thus avoiding recognition by antibodies induced by previously expressed *Pf*EMP1 variants (Kyes et al., 2001). Expression is regulated at the level of *var* gene transcription where only a single *var* gene out of 60 copies per genome is expressed in a single parasite at a time, known as mutually exclusive transcription (Chen et al., 1998b; Scherf et al., 1998). The switching rate for each *var* gene seems to be variable (Horrocks et al., 2004) and divers virulence and binding properties can be attributed to different *Pf*EMP1 variants (Kraemer and Smith, 2006).

Transcriptional regulation of the *var* genes is still not fully understood. Changes in *var* gene expression are not due to alterations in the sequence or repositioning of the gene in the genome context but occur *in situ* (Scherf et al., 1998). Changes in the expression of specific *var* genes have been linked to alterations in chromatin structure implicating an epigenetic mechanism for *var* gene regulation (Deitsch et al., 1999). Studies in other organisms showed that in epigenetic gene regulation modification of chromatin structure, in particular

histone tail acetylation and methylation, is a common feature. Recently, a histone deacetylase homologue to yeast silent information regulator 2 (SIR2) has been identified in *P. falciparum* and its localization has been associated with the silencing of telomeric *var* genes (Duraisingh et al., 2005; Freitas-Junior et al., 2005). The position of the *var* genes in the nucleus is linked to expression, as experiments with *var2csa* revealed (Ralph et al., 2005). Silent *var* genes cluster at the periphery of the nucleus where condensed heterochromatin is located, whereas active *var* genes dissociate from the cluster to a discrete region of euchromatin found within the nuclear periphery. The condensed heterochromatin structure plays an important role in silencing, as silent genes are packed tightly in heterochromatin rendering them inaccessible for transcription factors. Chromatin structure rearrangement happens during the S-phase of the cell cycle. The importance of the *var* intron in silencing has been established by demonstrating that an episomal *var* promoter is constitutively active unless paired with a *var* intron in a S-phase dependant manner (Deitsch et al., 2001; Calderwood et al., 2003; Gannoun-Zaki et al., 2005). Recent work revealed that one *var* intron can only silence a single *var* promoter at a time and that *var* promoters unpaired with an intron are not recognized by the machinery that maintains mutually exclusive transcription (Frank et al., 2006).

In this study, we wanted to characterize the upstream region of an upsC *var* gene and identify an interaction site of the *var* intron on the *var* upstream region. For this purpose we stably transfected *P. falciparum* parasites with various constructs containing different *var* upstream region fragments driving luciferase expression in the presence or absence of the *var* intron. Luciferase activity can be used as a direct indication for the transcription efficiency of a promoter. Additionally, we were interested if the mutually exclusive expression machinery that guarantees the expression of a single *var* gene in one parasite recognizes the active *var* promoter on our transfection constructs and hence silence all endogenous *var* genes. For this purpose we performed quantitative RT-PCR on cDNA from transfected parasite lines with active or silent *var* promoters on plasmids to monitor the transcription levels of every *var* gene by using specific primer pairs for the endogenous *var* genes (Salanti et al., 2003)

METHODS

Transfection plasmid constructs for optimization of transfection efficiency and reduction of variability between luciferase experiments

To reduce the variability between different luciferase experiments the dual reporter assay system from Promega was tested. In this system, two plasmids are transfected in parallel: one plasmid expresses Firefly luciferase under the promoter of interest while a control plasmid that expresses *Renilla* luciferase under control of a standart promoter (*cam*) serves to adjust for different transfection efficiencies between samples. pGL3-Basic vector and pRL-null vector from Promega were used as backbone for cloning. These vectors contain either a Firefly luciferase reporter gene (pGL3-Basic) or a *Renilla* luciferase reporter gene (pRL-null) without any regulatory elements. The *hrp*2 3'region combined with the *var* intron was PCR amplified from the pVLH/INT vector previously described (Deitsch et al., 2001) using the primers hrp2F-XbaI (5'-GCTATCTAGAAGATCGCCGTGTAAGCTTATTTA-3') and hrp2R-HpaI (5'-GGGCGTTAACAGAATACTCAAGCTATGCATCCAAC-3'). This fragment was *Xba*I and *Hpa*I digested and cloned into *Xba*I,*Hpa*I-digested pGL3-Basic or pRL-null vectors resulting in pGLhrp/int or pRLhrp/int, respectively. The calmodulin (*cam*) promoter sequences were PCR amplified from the pHTK vector (Duraisingh et al., 2002) using the primers camF-BglII (5'-GGGAAGATCTGAGCTTCTTCTTTGTTAACCATTT-3') and camR-NcoI (5'-GCATGCCATGGTCCTGATATATTTCTATTAGGTATT-3') for the Firefly luciferase construct and primers camF-EcoRI (5'-GCCGGAATTCTGAGCTTCTTCTTTGTTAACCATTT-3') and camR-NheI (5'-GCTAGCTAGCTCCTGATATATTTCTATTAGGTATT-3') for the *Renilla* luciferase construct. The PCR fragments were either *Bgl*II/*Nco*I or *Nhe*I/*Eco*RI digested and cloned into *Bgl*II/*Nco*I digested pGLhrp/int or *Nhe*I/*Eco*RI digested pRLhrp/int resulting in pGLcam/int and pRLcam/int, respectively. The *var* intron was removed by *Pst*I-digestion and subsequent ligation resulting in pGLcam and pRLcam, respectively. Vector maps are shown in Figure 1.

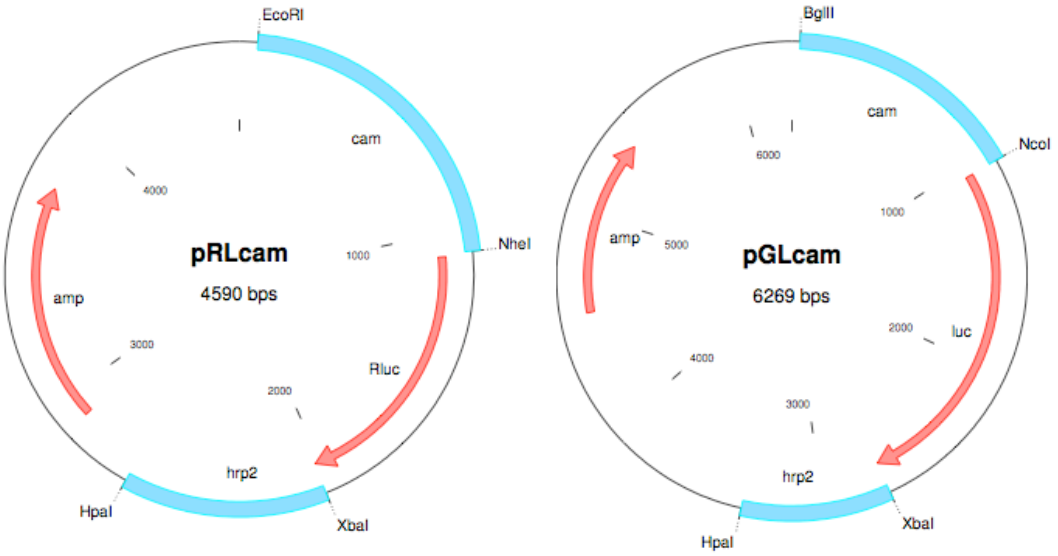


Figure 1. Maps of transfection constructs to optimize transfection efficiency.

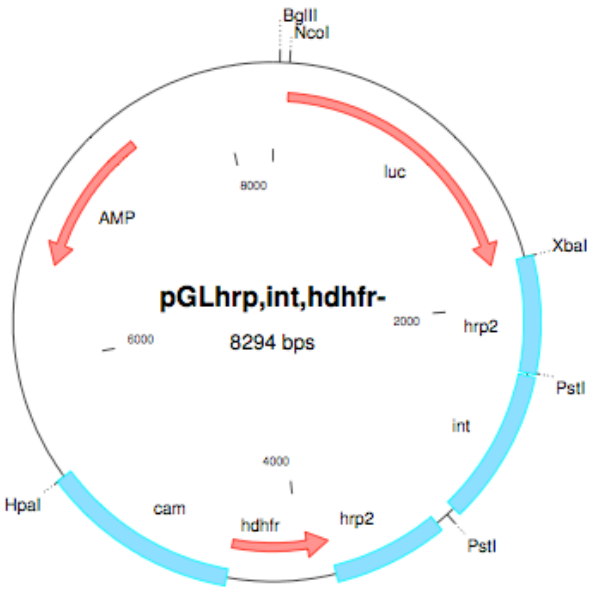
The Firefly luciferase vector is on the left, the *Renilla* luciferase vector on the right. Shown are the calmodulin promoter (cam), the luciferase gene, the hrp2 3' region, and the ampiciline resistance gene (amp). The restriction sites used for cloning are indicated. Details of cloning are explained in the text.

Transfection plasmid construction for *var* promoter analysis

To identify regulatory sequences and investigate the role of the *var* intron different *var* promoter constructs were designed. pGL3-Basic vector from Promega was used as a template for cloning. This vector contains a Firefly luciferase reporter gene without any regulatory elements. The *hrp*2 3'region combined with the *var* intron was PCR amplified from the pVLH/INT vector previously described (Deitsch et al., 2001) using the primers hrp2F-XbaI (5'-GCTATCTAGAAGATCGCCGTGTAAGCTTATTTA-3') and hrp2R-HpaI (5'-GGGCGTTAACAGAATACTCAAGCTATGCATCCAAC-3'). This fragment was *Xba*I and *Hpa*I digested and cloned into *Xba*I,*Hpa*I-digested pGL3-Basic vector resulting in pGLhrp/int. The *h*dhfr cassette containing a *cam* promoter, the *h*dhfr gene and the *hrp*2 3'region was cut out of the pHTK (Duraisingh et

al., 2002) vector with *Hpa*I and *Cla*I, overhangs were filled using Klenow Polymerase and ligated into *Hpa*I-digested pGLhrp/int. To reduce background of pHTK, the ligation was digested with *Aat*II before electroporation. Due to the non-directional cloning, constructs with tail-to-head orientation (with the *cam* promoter next to the *var* intron, phGLhrp/int-) and with tail-to-tail orientation (with the *hrp*2 3' region next to the *var* intron phGLhrp/int+) were obtained. phGLhrp/int+ was used as a negative control. phGLhrp/int– was used as template for the different *var* promoter fragments (vector map is shown in Figure 2). *var* upstream sequences were obtained by PCR amplification using primers 5B1.F.BamHI (5'-GCGCGGATCCGACTCACTATAGGG-3') and 5B1.R.Nco1 (5'-CATGCCATGGCCTTTTGTTTTTTGTTTATCG-3') on pCAT5B1, pCAT5B1Δ, pCAT5B1 1879 and pCAT5B1 1508 (Voss et al., 2003). The fragments were digested with *Bam*HI and *Nco*I and cloned into *Nco*I, *Bgl*II-digested phGLhrp/int- (to reduce the background ligations were digested with *Bgl*II prior to electroporation) resulting in phGL-WTint, phGL-Δint, phGL-18int and phGL-15int respectively. The *var* intron was removed by *Pst*I-digestion and subsequent ligation resulting in phGL-WT, phGL-Δ, phGL-18 and phGL-15.

Figure 2. Vector map of phGLhrp/int-



This vector was used as template for all transfection constructs by inserting various *var* promoter fragment upstream of the luciferase gene. Shown is the Firefly luciferase, the *h*dhfr and the ampiciline gene in red, the *cam* promoter, both *hrp*2 3' regions and the *var* intron are indicated in blue. Restriction sites used for the cloning are given. Details of cloning are given in the text.

In vitro cultivation of *Plasmodium falciparum*

All experiments utilized the *P. falciparum* 3D7 line cultivated at 5% haematocrit as described previously (Trager and Jensen, 1976) in RPMI 1640 medium supplemented with 25mM HEPES, 0.5% Albumax, 50mg/l hypoxanthine, 0.25% sodium bicarbonate, and 50 mg/ml neomycin sulphate. Parasites were incubated at 37 °C in an atmosphere of 3% oxygen, 5% carbon dioxide, and 92% nitrogen.
Growth synchronization was achieved by sorbitol lysis (Lambros and Vanderberg, 1979).
Thin red blood smears were used to examine the parasitaemia and the status of the culture throughout culturing. Thin red blood smears were fixed with methanol and stained for 15min in Giemsa solution.

Transfection of *P. falciparum* cultures

For transfection 10ml of cultured 3D7 *P. falciparum* ring-stage parasites (approximately 10% parasitaemia) were washed once in incomplete cytomix (120mM KCl, 0.15mM $CaCl_2$, 2mM ethylene glycol tetra-acetic acid, 5mM $MgCl_2$, 10mM K_2HPO_4/KH_2PO_4 25mM Hepes, PH 7.6) (van den Hoff et al., 1992) and the pellet was subsequently resuspended in 800µl incomplete cytomix containing 40pmol of plasmid DNA and transferred to an electroporation cuvette (2mm or 4mm cuvettes, BioRad). Parasites were transfected by electroporation using the EasyjecT equipment (Equibio). Electroporation with the control plasmid pVLH (Deitsch et al., 2001) to improve transfection efficiency was conducted by applying one of the following three conditions: low voltage: 310V/960µF (Fidock and Wellems, 1997), high voltage: 2500V/25µF (Wu et al., 1995) or by a double impulse: 1500V/25µF followed by 310V/1050µF with 0.5s inter-pulse delay (Voss et al., 2000). The last electroporation protocol with the 4mm cuvette gave the best results and subsequently all other plasmids were transfected according to this protocol. For stable transfection, selection with 10nM WR99210 was started eight hours post transfection (Fidock and Wellems, 1997).

Real-Time PCR for determination of plasmid copy numbers on gDNA

To detect the average number of plasmids in a parasite, we performed quantitative RT-PCR on gDNA from transfected parasite lines.

10ml of late stage parasites were Saponin lysed and washed twice with 1xPBS. The pellet was resuspended in 500µl TE with 0.8% SDS, 0.01M EDTA and 0.2mg/ml Proteinase A (SIGMA) and incubated over night at 60°C. Phenol/chloroform extraction was performed twice followed by an additional extraction with chloroform only. The aqueous phase was precipitated with Sodium acetate and Ethanol and finally resuspended in TE. The quality and concentration of the gDNA obtained was checked on an agarose gel.

Quantitative real-time PCR using Brilliant SYBR Green QRT-PCR master mix two step (STRATAGENE) was performed on a TaqMan 7500 (Applied Biosystems, ABI) according to provider's manual, using the seryl-tRNA synthetase gene (PF07_0073: primers p90_for 5'-TCAATTTGATAAAGTGGAACAATTC-3' and p90_rev 5'-GCGTTGTTTAAAGCTCCTGA-3') as endogenous control and the luciferase gene (primers Luciferase forward 3 5'-GCTGGGCGTTAATCAGAGAG-3' and Luciferase reverse 3 5'-GTGTTCGTCTTCGTCCCAGT-3') as estimate for plasmid copy number per parasite. PCR conditions were as follows: 10min 94°C followed by 41 cycles of 30sec at 94°C, 30sec at 58°C, and 35sec at 70°C. Subsequently the dissociation curve was established. Primers were tested on 10-fold dilutions of 3D7 gDNA and plasmid DNA. Primers amplified fragments of the expected size and T_m, amplification efficiencies (E) was 4.9 for the luciferase primers and 5.3 for the p90 primers [$E = 10^{(-1/\text{slope of 10-fold-dilution gDNA standard curve})}$]. Efficiencies of target and reference were approximately equal; absolute value of the slope of log input amount verses ΔCT equaled 1.72×10^{-6}.

To estimate the plasmid copy number, the ΔCT was determined by subtracting the CT value for the luciferase gene from the CT value for the control seryl-tRNA synthetase gene (ABI, User Bulletin 2). ΔCTs were then converted to relative copy numbers with the formula $2^{\Delta Ct}$.

Luciferase activity detection

Promoter activity was measured by the amount of luciferase produced by each transfected parasite population. For luciferase assays, saponin-released parasites were lysed in reporter lysis buffer provided with the luciferase assay system (E1500, Promega) 20h post erythrocyte infection. The lysate was freeze–thawed once. Luciferase activity in parasite lysates was determined using the luciferase assay system (E1500, Promega) and the MicroLumat *Plus* LB 96V luminometer from Berthold. Briefly, to 20µl parasite lysate 50µl Luciferase assay substrate was added automatically and light emission was measured over a period of 10sec.
For combined detection of Firefly and *Renilla* luciferase the Dual Luciferase Reporter assay system (E1920, Promega) was used. Parasites were prepared identical to the luciferase assay alone. To 20µl parasite lysate 50µl LARII substrate (Promega) was added and light output was measured over a period of 10sec. 50µl Stop & Glo/EReagent (Promega) was added to the tube and light emission was measured identical to Firefly luminescence.

Statistics

To ascertain the exact amount of parasites for each luciferase experiment, the number of cells (RBCs and iRBCs) to be lysed was estimated in a Neubauer chamber and parasitaemia was determined on Giemsa stained thin blood smears. Luciferase counts were divided by the number of parasites lysed and by the predicted plasmid copy-number for each parasite line resulting in luciferase counts per plasmid.

Immuno Fluorescence Assay (IFA)

To determine what proportion of the parasite populations express luciferase, we performed IFAs with a rabbit polyclonal α-luciferase antibody (Sigma), a monoclonal mouse α-luciferase antibody (Sigma) or monoclonal α-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Daubenberger et al.,

2003) antibodies as a positive control. 800µl of parasite culture with 5% mixed stages was washed twice with 1ml culture medium. The pellet was afterwards resuspended in 375µl culture media, 22.5µl paraformaldehyde, 25µl 10xPBS and 2.5µl Triton x-100 in PBS (10%). 40µl parasite mixture were pipetted onto each well of a 10 well microscope slide (Erie scientific company) and incubated for 30min at room temperature. Parasites were washed 5 times with 50µl culture media and then blocked with 50µl 1xPBS with 1% BSA (blocking buffer) for 15min in a humid chamber. The blocking buffer was replaced by 30µl blocking buffer containing the primary antibody (α-GAPDH; 1:1,000, or mouse or rabbit α-luciferase in dilution series; 1:100, 1:1,000 and 1:10,000) and incubated for 1h at room temperature in a humid chamber. Parasites were washed 5 times with 50µl blocking buffer and then incubated with 30µl secondary antibody (FITC conjugated goat α-mouse IgG, 1:300 or Cy3 conjugated goat α-rabbit IgG, 1:500 from Sigma) and Hoechst dye (1:100) in blocking buffer for 1h at room temperature in a humid chamber. Parasites were washed 5 times with 50µl 1xPBS, covered with a glass cover slip and sealed.

Fluorescence microscopy was performed using a Leica DM5000 fluorescent microscope and documented with a Leica DC200 digital camera system using x100 oil immersion objectives.

Western blot analysis

Western blot analysis was performed to detect the cause of the cross-reactivity of rabbit α-luciferase antibodies observed in IFAs and test whether the monoclonal mouse α-luciferase antibody recognizes luciferase produced in transfected *P. falciparum* lines. 10ml synchronized ring stage *P. falciparum*-infected RBCs were lysed in 1.5ml 0.1% saponin in 1xPBS and washed twice with 1ml 1xPBS. The parasite pellet was resolved in sample buffer (0.1M TrisHCl PH 6.8, 20% glycerol, 2% Sodium dodecyl sulfate (SDS), 0.1M β-mercapto-methanol, bromphenol-blue). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide), transferred to a nitrocellulose membrane (Hybond-C extra; Amersham

Biosciences) for 3h using a Trans-Blot semidry electroblotter (Bio-Rad), and probed with either antiserum from mice immunized with GAPDH (1:1,000, Daubenberger et al., 2003) rabbit α-luciferase antibodies (Sigma) (1:1,000) or mouse α-luciferase antibodies (Sigma) (1:1,000) followed by horseradish peroxidase-conjugated secondary antibodies (goat α-mouse immunoglobulin G (IgG), 1:10,000 or goat α-rabbit IgG, 1:10,000 from Sigma). The membrane was developed according to the ECL western blot detection kit manual (Amersham).

Southern Blot and detection

Southern blot analyses were performed to check whether the plasmids had integrated into the *var* PFL1960w locus that has the same *var* promoter sequence as the plasmid constructs or into any locus on the chromosome. gDNA was either digested with *Eco*RV*, Nco*I and *Pvu*II and probed with the *h*dhfr gene or with *Not*I and *Eco*RV and probed with the *var* PFL1960w gene, digestion status was monitored on agarose gels. Agarose gel, blot and detection followed the protocol of the ECL Direct Nucleic Acid Labelling And Detection Systems (Amersham Biosciences). Briefly, 2-10µg of restriction enzyme digested gDNA was loaded on a 1% Agarose gel and electrophoreses was performed over night in 1x TAE buffer at 1V/cm. The gel was depurinated in 200ml 250mM HCl, denatured in 200ml 1.5MNaCl/0.5MNaOH and neutralized in 200ml 1.5M NaCl/0.5M Tris-HCl (pH 7.5) before blotting on a Hybond N+ membrane over night. The DNA was UV cross-linked to the membrane. Horseradish peroxidase was cross-linked with glutaraldehyde to 100ng probe. Either an amplicon of the *Pf*EMP1 gene or the *h*dhfr gene was used as probes. The *h*dhfr probe was PCR amplified from the pHTK vector by primers hdhfr_forw (5'-GGATCCATGCATGGTTC-3') and hdhfr_rew (5'-GGCTGTACAGTGTATAAACC-3') with following conditions: 20sec denaturation at 94°C, 20sec annealing at 54°C, and elongation 1.5min at 66°C, the cycle was thirty timed repeated. A 735-basepair fragment derived from the 5' end of PFL1960w was PCR amplified from gDNA according to (Voss et al., 2006).

Hybridization and stringency washes were performed in hybridization tubes in an incubator with an integral rotisserie device. After a minimum of 15min prehybrization with hybridization buffer the labeled probe was added and hybridized over night at 42°C. The blot was washed first with wash buffer containing 6M Urea, 0.4%SDS and 0.5x SSC and second with 2x SSC. The signal was generated by incubating the blot for 1min with an equal amount of detection reagent 1 and 2. A BioMax light film (Kodak) was placed on the blot in a film cassette for an appropriate length of time. The film was afterwards developed.

Cloning and sequencing of the truncated intron sequence

We detected an unexpected double band in the phGL-18int transfected parasite line in Southern blot analysis indicating a deletion. To analyze this deletion PCR amplification of the *hrp*2 3' region adjacent to the luciferase gene and the *var* intron was performed with primers hrp2F-XbaI (5'-GCTATCTAGAAGATCGCCGTGTAAGCTTATTTA-3') and hrp2R-HpaI (5'-GGGCGTTAACAGAATACTCAAGCTATGCATCCAAC-3') on gDNA. The amplicon was ligated into TOPO cloning vector (Invitrogen) according to manufacturer's protocol. Two clones were sent to Macrogen for sequencing with primers M13F and M13R.

RNA isolation and cDNA

cDNA was produced from RNA of transfected parasite cultures to detect the levels of *var* gene RNA transcribed in the population.
40ml of culture of 5% late ring stage parasites were Saponin lysed and washed twice with 1xPBS. The pellet was then resuspended in 2ml Trizol (Invitrogen) and RNA was extracted with 0.2ml chloroform and precipitated with isopropanol. The extraction was repeated in half of the original volume Trizol to reduce contamination with gDNA. Residual gDNA was twice digested with RQ 1 DNase (Promega) according to the manufacturer's protocol in a total volume of 50μl. RNA was subsequently extracted with 180μl Trizol and

40µl chloroform. Before cDNA synthesis, eventual gDNA contamination was tested by PCR. For the PCR 1 µl of RNA was used as template with the primer pair DBLα5' and DBLα3' that amplified most of the *var* genes (Kaestli et al., 2004). As a positive control an additional PCR with the same primers but addition of 1µl gDNA from 3D7 to the RNA was performed. PCR conditions were as follows: 30sec denaturation at 94°C, 30sec annealing at 54°C, and 1min elongation at 60°C. The cycle was repeated thirty times.
cDNA synthesis was performed with M-MuLV Reverse Transcriptase (New England BioLabs) with random primers (Invitrogen) as described by the manufacturer. cDNA was synthesized from 800ng total RNA in a reaction volume of 50µl. For each cDNA synthesis reaction, a control reaction without reverse transcriptase was performed with identical amounts of template.

RT PCR on cDNA to detect transcripts of all *var* genes

Transcript detection for *var* genes was done in collaboration with R. Dzikowski and K. Deitsch from the Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, USA according to their previously published method (Dzikowski et al., 2006):
For realtime quantitative RT-PCR reactions to detect transcription from all *var* genes present in the 3D7 genome, we employed the primer set of (Salanti et al., 2003) with the following modifications. We added an additional primer pair for PF08_0107: 5′-CCTAAAAAGGACGCAGAAGG-3′ and 5′-CCAGCAACACTACCACCAGT-3′ and designed separate primer sets for PFD1005c: 5′-ACGATTGGTGGGAAACAAAT-3′ and 5′-CCCCATTCTTTTATCCATCG-3′ and for PFD1015c: 5′-AAAGGAATTGAGGGGGAAAT-3′ and 5′-TAAACCACGAAACGGACTGA-3′.
All reactions included the three control genes published by these authors: seryl-tRNA synthetase (PF07_0073), fructose biphsphate aldolase (PF14_0425), and actin (PFL2215w); however, we added another two control sets: arginyl-tRNA synthetase (PFL0900c) using 5′-AAGAGATGCATGTTGGTC-3′ and 5′-GTACCCCAATCACCTACA-3′ and

glutaminyl-tRNA synthetase (PF13_0170) using 5′-GGCACTTCAAGGGTACCT-3′ and 5′-TAATATAGCCTCACAAGC-3′.

Amplification efficiency was verified by performing amplifications using different concentrations of genomic DNA as templates. Reactions were performed at a final primer concentration of 0.5 µM using Biorad ITAQ SYBR green Supermix in 20µl reactions on an ABI Prism 7900HT. The Δ Ct for each individual primer pair was determined by substracting the measured Ct value from the Ct value of the control seryl-tRNA synthetase (User bulletin 2, Applied Biosystems, http://www.appliedbiosystems.com). Δ Cts were then converted to relative copy numbers with the formula $2^{\Delta Ct}$.

RESULTS

Optimization of the luciferase transfection system

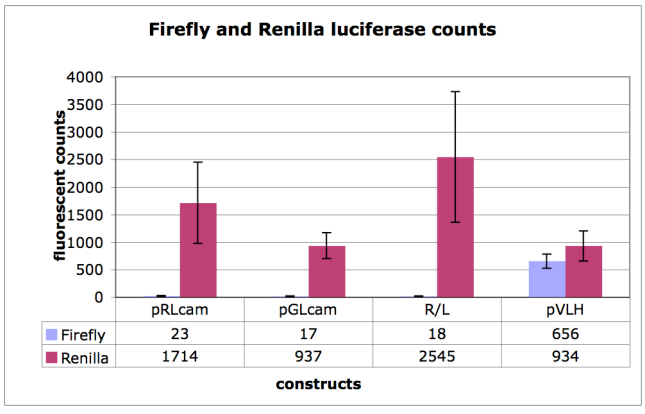
Luciferase expression and subsequent enzyme activity can be used as a direct measurement for the activity of a promoter of interest. In previous experiments we transiently transfected *P. falciparum* with plasmids containing *var* promoters driving luciferase expression. We found high variability between different experiments making interpretation of the results difficult. To improve transfection efficiency and reduce variability between different luciferase assays, several options have been tested.

Electroporation efficiency was estimated to be very low in *P. falciparum* cultures, i.e. only 10^{-6} (O'Donnell et al., 2002). To improve this various electroporation protocols and different cuvettes were tested. A previously described plasmid with a *var* promoter transcribing luciferase (pVLH) (Deitsch et al., 2001) was transfected and transiently expressed for 72 hours. Electroporation with an exponential impulse was varied in the strength of pulse voltage (V) and intensity of the capacity (in microfarad, µF). Parasites were electroporated using the following conditions: low voltage; 310V/960µF (Fidock and Wellems, 1997), high voltage; 2500V/25µF (Wu et al., 1995) or by a double impulse combining both conditions; 1500V/25µF followed by 310V/1050µF (Voss et al., 2000). The double impulse was found to be the most efficient. 2mm and 4mm cuvettes from BioRad were tested, out of which the latter have been found to be more suitable as luciferase counts of the transiently transfected pVLH were highest in this combination.

To minimize variability between different luciferase assays the dual-reporter assay system from Promega was tested. In this system, two plasmids are transfected in parallel: one plasmid expresses Firefly luciferase under the promoter of interest while a control plasmid that expresses *Renilla* luciferase serves to adjust for different transfection efficiencies between samples. Firefly luciferase is a protein isolated from beetles (*Photinus pyralis*), while *Renilla* luciferase is a protein from sea pansy (*Renilla reniformis*). These enzymes differ in their substrate and cofactor requirements. Firefly luciferase produces

a greenish yellow light in the 550–570nm range, whereas *Renilla* luciferase produces a blue light of 480nm. These enzymes can thus be used in dual-reporter assays due to their differences in substrate requirements and emission. To test this dual-reporter assay system two constructs were designed that expressed either Firefly luciferase (pGLcam) or *Renilla* luciferase (pRLcam) under the control of a calmodulin (*cam*) promoter terminated by the histidine-rich protein 2 (*hrp*2) 3' region. Equal amounts (20pMol) of each plasmid were co-transfected via double impulse and analyzed 72 hours later. Both, Firefly and *Renilla* luciferase activity was measured for 10 seconds (Figure 3). Unexpectedly, pGLcam transfected parasites did not exhibit any Firefly luciferase activity upon addition of Firefly specific substrate, nor did the pRLcam-transfected parasites, as expected. Parasites transfected with a control plasmid (pVLH) showed normal Firefly luciferase activity. Parasites transfected with *Renilla* luciferase plasmids showed high levels of *Renilla* luciferase activity when incubated with *Renilla* substrate. Surprisingly, high *Renilla* luciferase activity was detected also in parasites transfected with Firefly luciferase constructs (pGLcam, pVLH). Due to the high background as well as large differences between the various experiments with *Renilla* luciferase this approach was abandoned. Instead of this co-transfection approach we chose stable transfection of plasmids using a positive selectable marker.

Figure 3. Firefly and Renilla luciferase activity in dual-reporter assay system.



Plasmids expressing either Firefly luciferase (pGLcam) or *Renilla* luciferase (pRLcam) were transfected individually or combined (R/L). Plasmid pVLH was used as a control for transfection expressing exclusively Firefly luciferase by a *var* promoter. Means ±SD of four experiments are shown.

Stable transformation of *Plasmodium falciparum* with different *var* upstream region constructs and determination of plasmid copy numbers

In order to investigate *var* promoter activity and interaction with the *var* intron different *var* 5' region fragments were cloned upstream of the luciferase gene. A 2522bp upstream region of a varC *var* gene (PFL_1960w) (Voss et al., 2003) served as full-length reference for transcription activity (phGL-WT). Three transfection constructs with truncated forms of the same *var* upstream region were used to identify possible regulatory units: in one construct a protein-binding site (chromosome-central *var* gene promoter element, CPE) previously identified (Voss et al., 2003) was deleted (phGL-Δ), whereas in the other two constructs different stretches of the 5' region were removed (phGL-18 and phGL-15). Details and maps of the constructs are shown in Figure 4. To examine the interaction of the *var* intron with the different *var* upstream region fragments, two transfection constructs variants were designed for each fragment, one without *var* intron (phGL-x) and another one with the *var* intron downstream of the luciferase gene (phGL-xint) (Figure 4).

For stable transfection of the parasites a selection cassette allowing to screen for parasites containing the plasmid was used (Fidock and Wellems, 1997). The advantage, compared to transient transfection is that every single parasite contains at least one copy of the plasmid consequently increasing the sensitivity and reproducibility of luciferase experiments. Parasites were transfected with the different plasmids containing a human dihydrofolate reductase (*h*dhfr) selection cassette driven by a *cam* promoter and put under WR99210 selection.

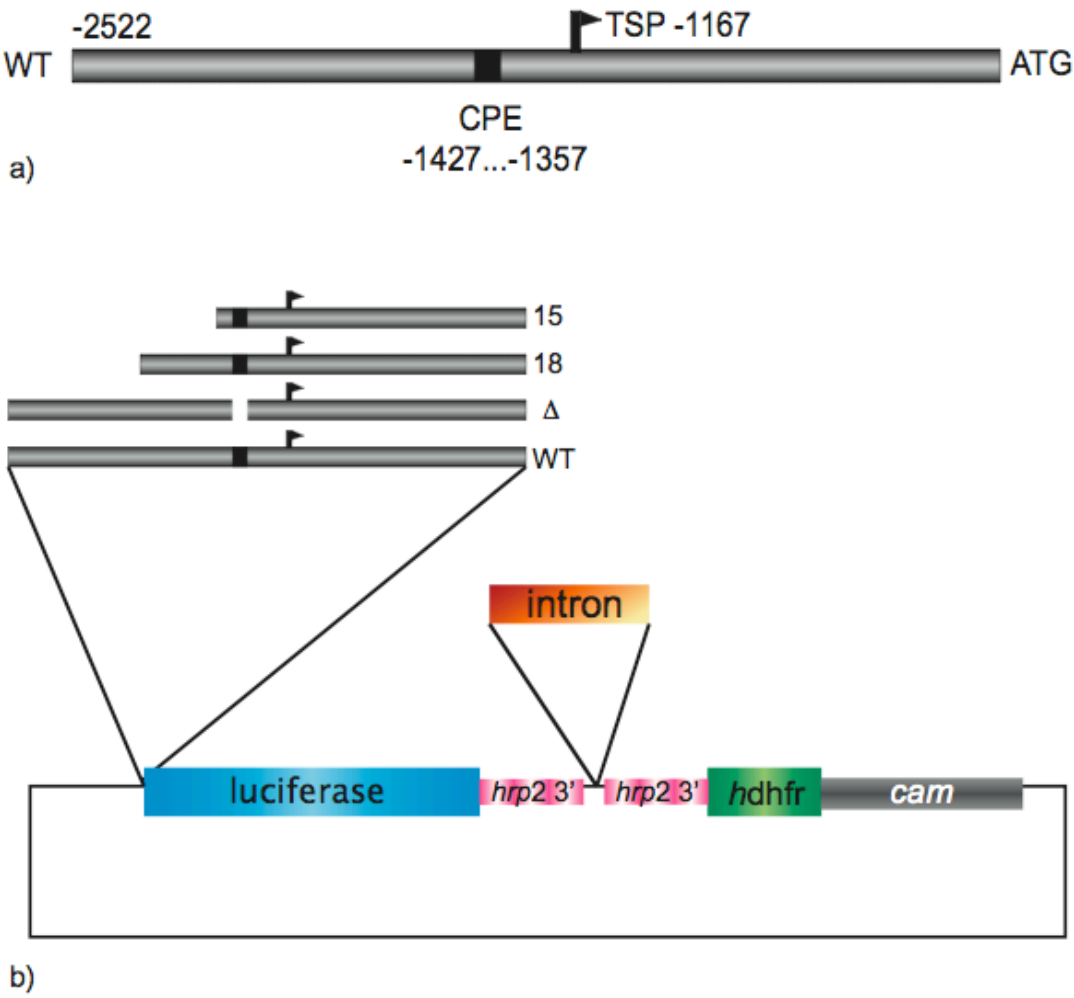


Figure 4. Transfection plasmids.

a) Wildtype (WT) full-length *var* upstream region indicating the position of the transcriptional start point (TSP) and of the central promoter-binding element (CPE).

b) Map of the different transfection constructs. The human dihydrofolate reductase (*h*dhfr) gene is expressed under a calmodulin promoter (*cam*) and terminated by the histidine-rich protein 2 (*hrp*2) 3' region (brindled boxes), similar to the luciferase gene but in opposite direction. The *var* intron, if present is localized between the two 3' terminator sequences. The different *var* upstream region fragments are shown seperately. The small flag indicates the TSP and the box the CPE.

After an average of 6 weeks selection stably transformed parasites were detected. To assess plasmid copy number for each parasite line we performed quantitative RT-PCR on gDNA for the luciferase gene and the seryl-tRNA synthetase control gene. Plasmid copy numbers were determined by the formula $2^{\Delta Ct}$. Copy numbers varied between 3.7 and 39,3 plasmids per parasite (Table1). Generally, the plasmid copy number was higher in parasite lines transfected with constructs containing the *var* intron.

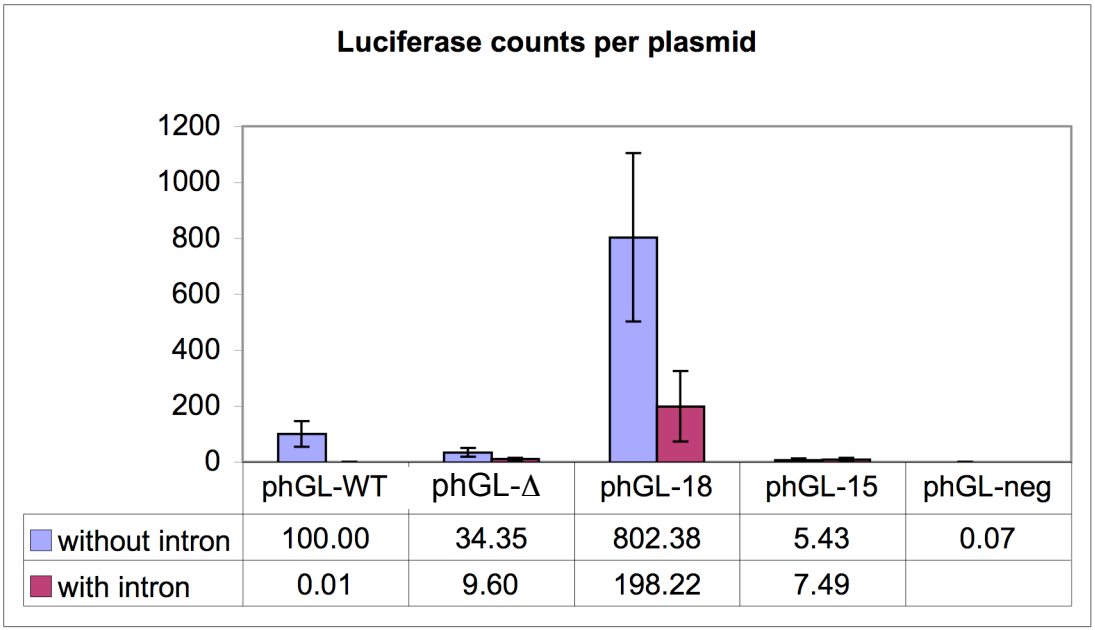
	WT	Δ	18	15	neg
without intron	13.7	7.7	3.7	5.3	n.a.
intron	39.3	14.5	9.0	6.5	5.1

Table 1. Average plasmid copy number per parasite.

var gene promoter analysis

To gain some information about the function of different sequence sections, we mapped the upstream region of a *var* gene promoter. In addition, we were interested in the interaction site of the *var* intron in the upstream region of the *var* gene. Promoter activity of the phGL-WT construct with the full-length upstream region was set as 100% (Figure 2). Plasmid phGL-Δ, where the CPE is deleted, showed a reduction of luciferase activity to 34%. Removal of 700bp (phGL-18) led to an eightfold increase of promoter activity to 802%. Deletion of a further 300bp (phGL-15) reduced the luciferase production to 5%. This weak luciferase expression let us hypothesize that region between the transcriptional start point (1167bp) and 1508bp upstream of the ATG contains the core promoter of the *var* genes. The negative control with no *var* upstream region shows a background of 0.07% luciferase activity.

The role of the *var* intron in *var* gene silencing was investigated by comparing the luciferase activity from the same *var* upstream region fragment in the presence or absence of the *var* intron. The presence of the *var* intron reduced luciferase activity of the full-length upstream region 10,000 times (Figure 5). This result confirms previous work where *var* promoters were silenced in the presence of the *var* intron in either transient (Deitsch et al., 2001) or stable transfected parasites (Calderwood et al., 2003; Frank et al., 2006), as well as when integrated (Dzikowski et al., 2006). There was no effect of the *var* intron on the shortest *var* upstream region construct (phGL-15 and phGL-15int) where expression levels were already reduced to basic transcription background without intron. Luciferase activity of the constructs phGL-Δ and phGL-18 was reduced in the presence of the *var* intron (3.58 times and 4.05 times, respectively). Interestingly, luciferase activity of phGL-18int containing the *var* intron was higher than activity of phGL-WT without *var* intron.



	phGL-WT	phGL-Δ	phGL-18	phGL-15	phGL-neg
without intron	100.00	34.35	802.38	5.43	0.07
with intron	0.01	9.60	198.22	7.49	

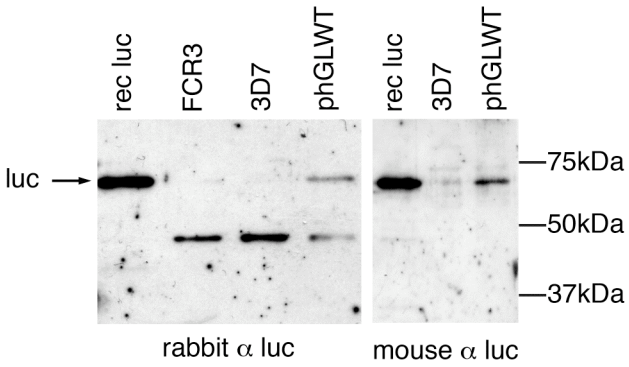
Figure 5. Luciferase counts per plasmid.

Luciferase activity of lysate from late ring stage parasites transfected with plasmids phGL-WT, phGL-WTint, phGL-Δ, phGL-Δint, phGL-18, phGL-18int, phGL-15, phGL-15int or phGL-neg was measured. Luciferase activity was adjusted for plasmid copy number. Experiments were done in triplicates and the mean ±SD of three experiments is shown in percentage of the wildtype promoter without the intron. Constructs without *var* intron are shown in blue and constructs with *var* intron are shown in red.

Estimation of the percentage of luciferase producing parasites in a parasite line

After transfection and selection with WR99210, each parasite contains at least one plasmid copy. This does not necessarily mean that every parasite in the population does also express the luciferase gene. Luciferase activity measured could be produced by all parasites in the population or by a subset of parasites only. To address this question we performed IFAs with polyclonal rabbit α-Firefly luciferase antibodies. Every parasite in a transfected culture (phGLWT) gave a signal but so did also every non-transfected 3D7 wildtype parasite (data not shown). In Western blot analysis it became apparent that the rabbit α-Firefly luciferase antibodies recognized an uncharacterized parasite protein in addition to luciferase (Figure 6). Experiments were repeated with a monoclonal mouse α-Firefly luciferase antibody. With this antibody no signal was detected in IFAs although a band specific for luciferase was detected in Western blots (Figure 6). No cross-reactivity with parasite proteins was detected for this antibody.

Figure 6. Western blot analysis for luciferase expression.



Recombinant Firefly luciferase protein and total protein extracts from *P. falciparum* parasite lines FCR3, 3D7 and 3D7 transfected with phGL-WT were subjected to Western blot analysis and probed with rabbit α-Firefly luciferase antibodies (left) or mouse α-Firefly luciferase antibodies (right). The predicted size (62kDa) of the recombinant Firefly luciferase (rec luc) protein is indicated by an arrow; an unknown parasite protein below 50kDa is additionally recognized by the rabbit α Firefly luciferase antibodies.

Integration of plasmids into the chromosome

Integration of the plasmids into the endogenous *var* locus could lead to complementation of the truncated *var* upstream region constructs, falsifying the obtained results. There are a number of plasmodial sequences on the plasmid where integration into the chromosome is possible: The *var* promoter, the *cam* promoter, and the *hrp*2 3' region. In order to test whether one of the constructs had integrated into the chromosomes we performed Southern blot analysis. The most likely integration locus is the *var* promoter as it contains the longest plasmodial sequence. We performed Southern blot analysis with a specific probe for the PFL1960w *var* gene locus, the origin of our plasmid promoter sequence. An additional Southern blot was performed with an *h*dhfr probe to detect free and integrated plasmids into another locus. With the *h*dhfr probe we detected only the plasmids, but no additional band indicating integration (Figure 7a). With the probe for the *var* gene locus, we found that the phGL-WTint plasmid had integrated into the chromosome via single crossover of the *var* promoter (Figure 7b).

Figure 7. Southern blot analysis for detection of plasmid integration
gDNA of parasite lines transfected with plasmid phGL-WTint, phGL-WT, phGL-18int or phGL-18 was digested with *Pvu*II, *Eco*RV and *Nco*I and probed with the *h*dhfr gene (a) or the gDNA was digested with *Eco*RV and *Not*I and probed with part of the 5' end of *var* gene PFL1960w (b). A schematic drawing Southern blot b) is shown in c): plasmid phGL-WTint, the endogenous *var* PFL1960w locus in 3D7 and phGL-WTint integrated into the *var* locus via single cross over at the 5' region is given. The *var* upstream region is indicated in dashed boxes, the *h*dhfr cassette in a green box, the *var* intron in blue and the luciferase and the endogenous *var* gene in white boxes. Restriction sites for this Southern blot, predicted fragment sizes and probe targets for Southern blot are indicated (restriction enzyme sites: *Not*I (N) and *Eco*RV (E) dotted line, DBL1α probe). Expected plasmid fragment sizes for Southern blot shown in a): 9920bp for phGL-WT, 10769bp for phGL-WTint, 4285bp for phGL-18 and 5132bp for phGL-18int.

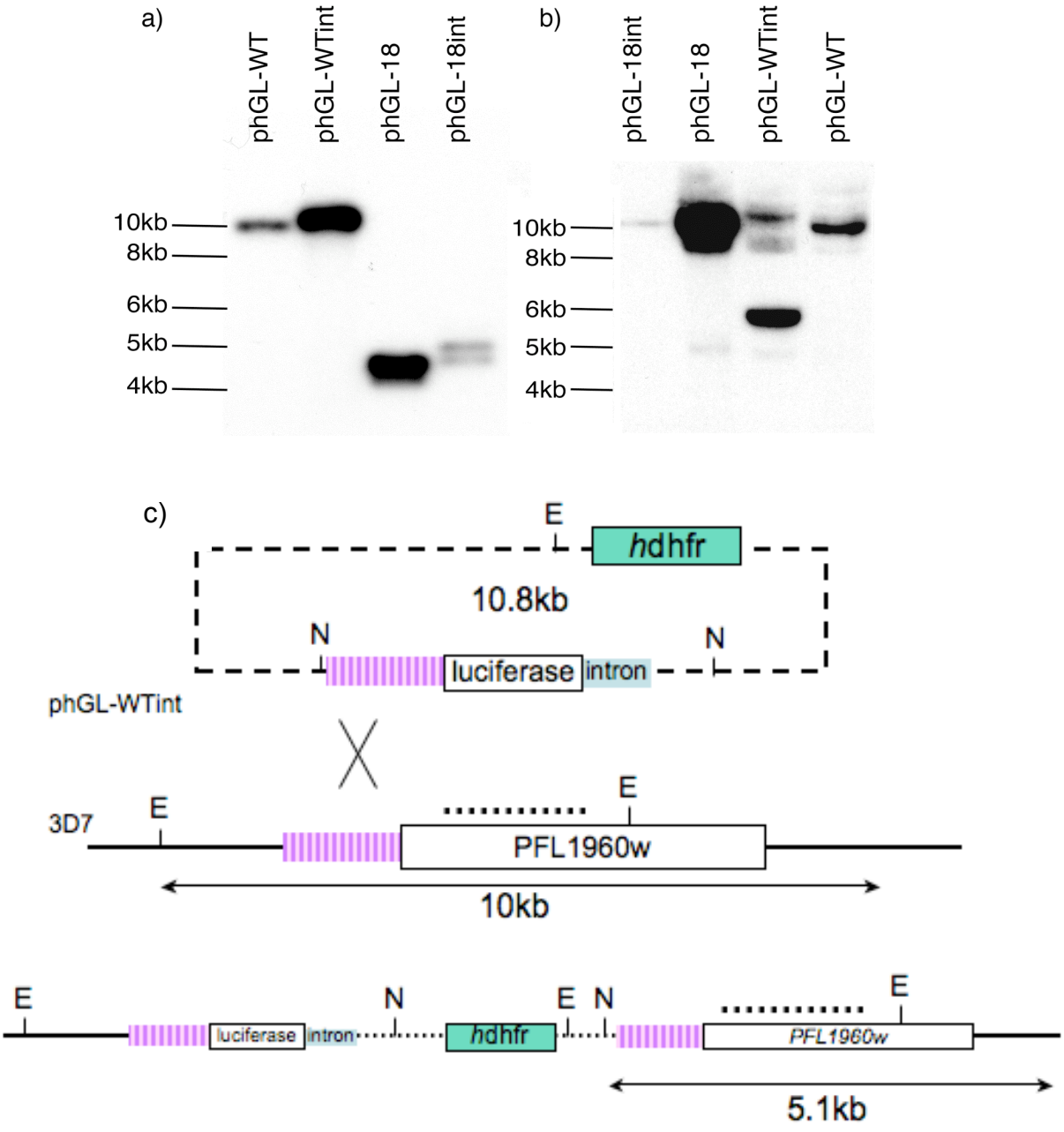

a)
phGL-WT
phGL-WTint
phGL-18
phGL-18int
10kb
8kb
6kb
5kb
4kb
b)
phGL-18int
phGL-18
phGL-WTint
phGL-WT
10kb
8kb
6kb
5kb
4kb
c)
E
hdhfr
10.8kb
N
N
luciferase
intron
phGL-WTint
3D7
E
E
PFL1960w
10kb
E
N
E
N
E
luciferase
intron
hdhfr
PFL1960w
5.1kb

Figure 7.

Unexpectedly, we detected a double band with the *h*dhfr probe for the phGL-18int. To identity this additional band, the *var* intron and the upstream *hrp*2 3' region of this plasmid (see Figure 4) was PCR amplified on gDNA. Two bands were amplified by PCR: one of the expected sizes for the intact intron and a smaller one. This suggested the presence of two different plasmids in the parasite population. To determine what part of the sequence was missing in the smaller PCR product it was cloned into a TOPO vector and sequenced. Alignment (ClustalW, (Thompson et al., 1994)) of the obtained sequences with the original *var* intron sequence revealed a 462bp deletion in the phGL-18int parasite line. Comparison with a previously reported spontaneous deletion in the *var* intron sequence of a transfection plasmid (Gannoun-Zaki et al., 2005) revealed exactly the same sequence deletion in our study with the exception of two nucleotides. An alignment of three intron sequences is shown in Figure 8.

Alignments of *var* introns showed a highly conserved structure that consists of three discrete regions (1 to 3) with distinct base pair compositions (Calderwood et al., 2003). The middle region (region 2) is highly AT-rich, displays promoter activity on its own and is sufficient to silence an associated *var* promoter. In our study the complete middle region of the intron was deleted except for 40bp. Therefore, it is very likely that the silencing capacity of the *var* intron was lost in parts of the population.

Figure 8. Comparative alignments of the *var* intron sequences.

The original *var* intron, a previously identified truncated intron (INTdel2' (Gannoun-Zaki et al., 2005)) and the sequenced intron from phGL-18int transfected parasites (INT18del) were aligned using CLUSTAL W software. Regions 1 (yellow) and 3 (green) of the intron are indicated.

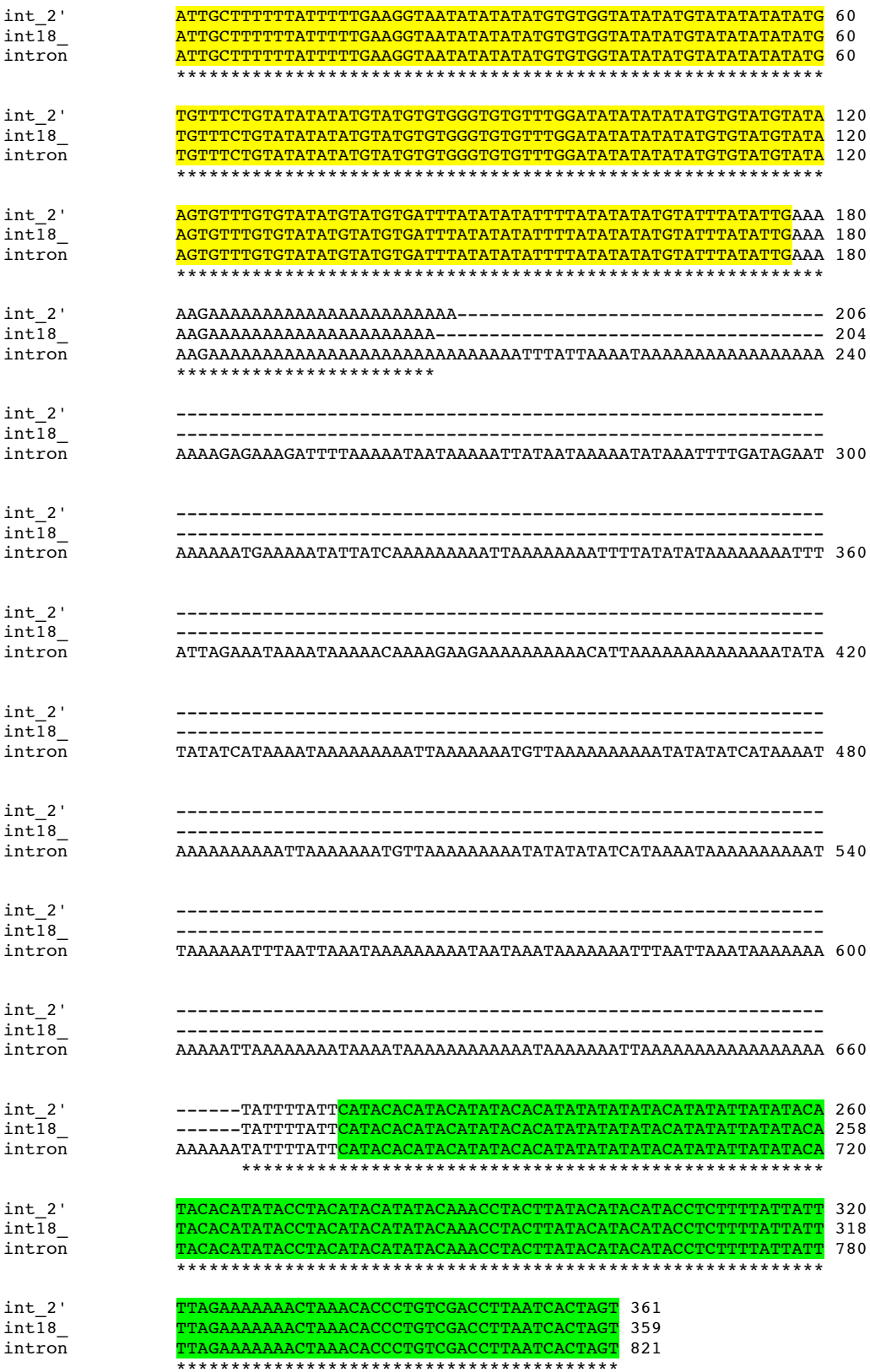

int_2' ATTGCTTTTTTATTTTTGAAGGTAATATATATATGTGTGGTATATATGTATATATATATG 60
int18_ ATTGCTTTTTTATTTTTGAAGGTAATATATATATGTGTGGTATATATGTATATATATATG 60
intron ATTGCTTTTTTATTTTTGAAGGTAATATATATATGTGTGGTATATATGTATATATATATG 60
int_2' TGTTTCTGTATATATATGTATGTGTGGGTGTGTTTGGATATATATATATGTGTATGTATA 120
int18_ TGTTTCTGTATATATATGTATGTGTGGGTGTGTTTGGATATATATATATGTGTATGTATA 120
intron TGTTTCTGTATATATATGTATGTGTGGGTGTGTTTGGATATATATATATGTGTATGTATA 120
int_2' AGTGTTTGTGTATATGTATGTGATTTATATATATTTTATATATATGTATTTATATTGAAA 180
int18_ AGTGTTTGTGTATATGTATGTGATTTATATATATTTTATATATATGTATTTATATTGAAA 180
intron AGTGTTTGTGTATATGTATGTGATTTATATATATTTTATATATATGTATTTATATTGAAA 180
int_2' AAGAAAAAAAAAAAAAAAAAAAAAA---------- 206
int18_ AAGAAAAAAAAAAAAAAAAAAAAA---------- 204
intron AAGAAAAAAAAAAAAAAAAAAAAAAAAAAAATTTATTAAAATAAAAAAAAAAAAAAAAA 240
int_2' ----------
int18_ ----------
intron AAAAGAGAAAGATTTTAAAAATAATAAAAATTATAATAAAAATATAAATTTTGATAGAAT 300
int_2' ----------
int18_ ----------
intron AAAAAATGAAAAATATTATCAAAAAAAAATTAAAAAAAATTTTATATATAAAAAAAATTT 360
int_2' ----------
int18_ ----------
intron ATTAGAAATAAAATAAAAACAAAAGAAGAAAAAAAAAACATTAAAAAAAAAAAAAATATA 420
int_2' ----------
int18_ ----------
intron TATATCATAAAATAAAAAAAAATTAAAAAAATGTTAAAAAAAAAATATATATCATAAAAT 480
int_2' ----------
int18_ ----------
intron AAAAAAAAAATTAAAAAAATGTTAAAAAAAAATATATATATCATAAAATAAAAAAAAAT 540
int_2' ----------
int18_ ----------
intron TAAAAAATTTAATTAAATAAAAAAAAATAATAAATAAAAAAATTTAATTAAATAAAAAA 600
int_2' ----------
int18_ ----------
intron AAAAATTAAAAAAAATAAAATAAAAAAAAAAATAAAAAAATTAAAAAAAAAAAAAAAA 660
int_2' ------TATTTTATTCATACACATACATATACACATATATATATACATATATTATATACA 260
int18_ ------TATTTTATTCATACACATACATATACACATATATATATACATATATTATATACA 258
intron AAAAAATATTTTATTCATACACATACATATACACATATATATATACATATATTATATACA 720
int_2' TACACATATACCTACATACATATACAAACCTACTTATACATACATACCTCTTTTATTATT 320
int18_ TACACATATACCTACATACATATACAAACCTACTTATACATACATACCTCTTTTATTATT 318
intron TACACATATACCTACATACATATACAAACCTACTTATACATACATACCTCTTTTATTATT 780
int_2' TTAGAAAAAAACTAAACACCCTGTCGACCTTAATCACTAGT 361
int18_ TTAGAAAAAAACTAAACACCCTGTCGACCTTAATCACTAGT 359
intron TTAGAAAAAAACTAAACACCCTGTCGACCTTAATCACTAGT 821

Figure 8.

Recognition by the *var* gene machinery

Frank et al., (2006) postulated that the *var* intron needs to be in close proximity of the *var* promoter in order to be recognized by the *var* gene machinery that ensures mutually exclusive transcription. Their hypothesis is based on the finding that an integrated *var* promoter without a *var* intron continuously expressed luciferase while *var* gene transcripts from the endogenous *var* genes were still detected by quantitative RT-PCR on cDNA. Results by Viebig et al., (2005) support these findings where disruption of the *var2CSA* gene by a selectable marker renders the *var* promoter constitutively active and unrecognized by the mutually exclusive machinery. Construct phGL-18int containing a truncated upstream region paired with the *var* intron, expressed luciferase comparable to the wildtype promoter without intron (Figure 4). In order to test whether the *var* gene machinery recognizes construct phGL-18int, we did quantitative RT-PCR on cDNA to check for the status of every *var* gene. To compare the mRNA levels directly with the luciferase activity, we isolated parasites for both assays from the same culture dish at the same time. To assess the plasmid copy number we performed quantitative RT-PCR on gDNA for the luciferase gene and on the glutaminyl tRNA synthetase gene for each parasite line. Relative plasmid copy numbers were determined by the formula $2^{\Delta Ct}$ and are indicated in Table 2. RNA levels detected by RT-PCR were normalized to the glutaminyl tRNA synthetase transcript level and adjusted for the plasmid copy number estimated on gDNA to generate the relative transcription level (RTL) for each promoter. The luciferase counts were calculated for the number of promoters present in the assay.

	WT	18
without intron	13.1	10.1
with intron	39.	19.8

Table 2. Relative copy numbers of transfected plasmids

Luciferase transcription was highest for the wildtype promoter (almost 6 RTL) while the construct with the shortened promoter (phGL-18) showed a RTL of 2.6 (Figure 9). In the presence of the *var* intron the transcription level of the wildtype promoter (phGL-WT) was 12 times reduced to 0.5 RTL and transcription level for the shortened promoter (phGL-18) was almost two times reduced to 1.4 RTL. Transcription levels of the endogenous *var* genes were very low (RTL below 0.4) in all transfected parasite lines regardless of the state of the episomal *var* promoter driving luciferase activity. Six *var* genes were always among the 10 most transcribed *var* genes out of which one (PFL1830c) was always dominant (Figure 9).

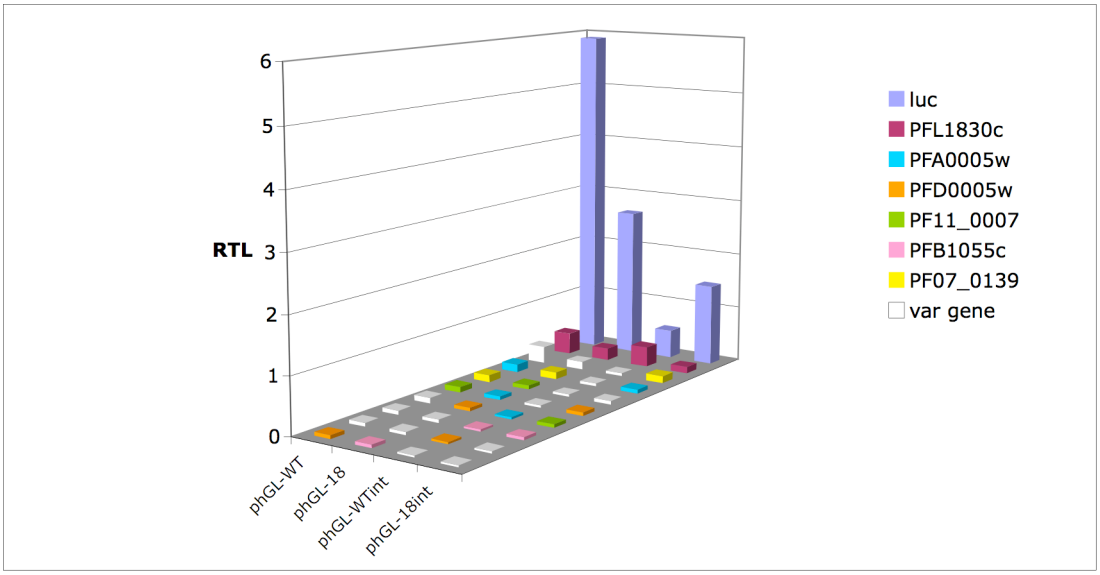


Figure 9. Transcription levels for *var* gene promoters.

Transcription levels of all *var* genes were monitored by quantitative RT-PCR with primers specific for each *var* gene on cDNA from late ring stages of transfected parasite cultures (x-axis). The transcription level is relative to the housekeeping gene glutaminyl t-RNA synthetase (y-axis, RTL). Shown is the mean of two experiments for the ten highest transcribed *var* promoters. Genes were sorted according to their level of expression (z-axis) and the seven most frequently transcribed genes are labeled.

Luciferase activity was highest in the parasite lines transfected with the plasmid containing the wildtype promoter (phGL-WT) and the presence of the *var* intron reduced the luciferase activity to 0.04%. Luciferase activity was halved in the parasite lines transfected with the truncated promoter construct (phGL-18). The presence of the *var* intron in this construct (phGL-18int) reduced luciferase activity in one experiment but not in the other. Results are summarized in Figure 10.

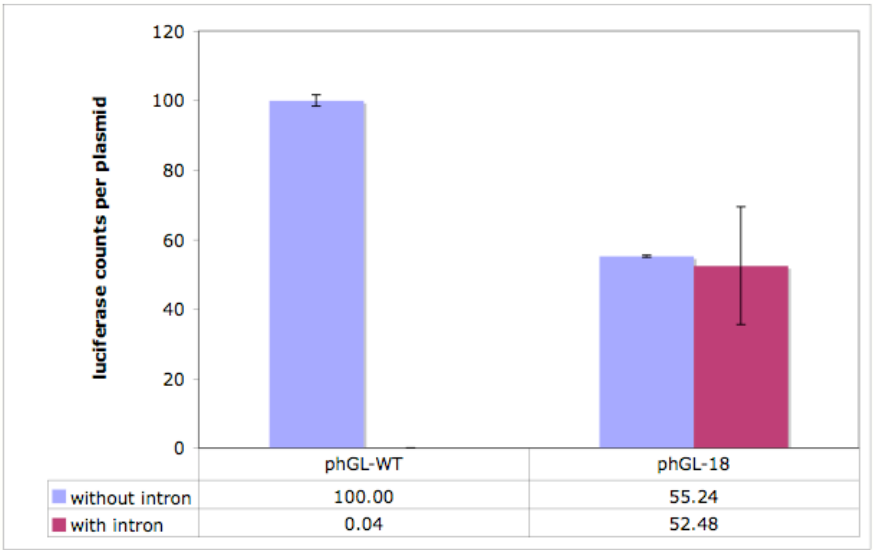


Figure 10. Luciferase activities from of transfected parasite cultures. Luciferase activity of lysate from late ring stage parasites transfected with plasmids phGL-WT, phGl-WTint, phGL-18 or phGL-18int was measured. The luciferase activity per plasmid copy was calculated. The mean ±SD of two experiments is shown in percentage of the wildtype promoter (phGL-WT) without the intron. Constructs without *var* intron are shown in blue and constructs with *var* intron are shown in red.

DISCUSSION

The aim of this part of the thesis was to identify regulatory sequences of central *var* genes and identify an interaction site of the *var* intron on the *var* upstream region. We mapped the region of the core promoter and found some possible repressor and activator sites on the *var* promoter. In addition, we identified a potential interaction site for the *var* intron on the upstream region of the *var* promoter. We were not able to determine whether the allelic exclusion machinery recognizes our transfection constructs.

Improvement of transfection efficiency

Transfection in *P. falciparum* is still a time-consuming and not very efficient method to study plasmodial biology. In previous experiments with transient transfection of various luciferase reporter plasmids, we encountered large variations in luciferase activity between different experiments. To reduce variability we tried to improve transfection efficiency and increase luciferase activity. Luciferase expression obtained with the previous expression vectors was always low, making examination of differences between transfection constructs difficult. To ensure integrity of the used luciferase reporter gene, we started cloning with a new vector that contained only the luciferase gene and evaluated different transfection setups with this already established plasmid (pVLH). In electroporation, the pulse amplitude (voltage) controls what area of the membrane is permeabilized and pulse duration and number control the degree of permeabilization (Gabriel and Teissie, 1997). In our hands a double impulse that combines a pulse with high voltage (1500V/25µF) followed by a longer pulse with low voltage (310V/1050µF) worked best (Voss et al., 2000).
For future experiments transfection efficiency might be further improved by varying the proportion of plasmid and parasites transfected and by optimizing parasite *in vitro* culturing conditions after transfection by using erythrocyte concentrate instead of full blood (unpublished observation).

Minimizing variations between experiments

Even if transfection efficiency is improved, variations between different transfections are still possible. To compare transfection efficiency between different transfections a control plasmid can be co-transfected with the plasmid of interest. We tested such a dual reporter assay system from Promega. In our approach, a calmodulin (*cam*) promoter drove expression of the *Renilla* luciferase gene. The *cam* promoter is constitutively active and thus a good control for co-transfection. The *Renilla* control plasmid was simultaneously transfected with the test plasmid where a promoter of interest drives Firefly luciferase expression. Firefly luciferase activity can then be put in relation to *Renilla* luciferase activity to adjust for different transfection efficiencies. We encountered high background activity of *Renilla* luciferase in our experiments, making evaluation of the results impossible. This approach was hence discarded and we used stable transfection instead. In this setting a positive selection cassette (the human dihydrofolate reductase gene, *h*dhfr) allows for selection of parasites that harbor a plasmid of interest. The advantage of this system is that every parasite contains at least one plasmid copy and thus luciferase activity is much higher. The disadvantage is that establishment of a stably transfected parasite line was long, taking between three to eight weeks in our hands. Another problem was cloning of the AT-rich constructs in *E. coli*. The introduction of an *h*dhfr cassette into the plasmid adds 2100bp to the already large constructs, which make the plasmids even more difficult to transfect. It is also known that AT-rich sequences are difficult to clone in *E. coli*.

Transfection efficiency is not the only problem encountered with transgenic parasites. Due to differences in parasite cultures and in the processing of parasite material (e.g protein lysate), variations between experiments are still likely to happen. It is therefore important that parasite-derived material is always prepared identically for every experiment. Variations in luciferase expression between experiments with the same parasite line might also be due to the composition of parasite populations expressing luciferase. Parasites are not forced to produce luciferase and therefore not necessarily every parasite expresses the gene and variations in expression over time in the

same population are likely (discussed in more detail later). Measurement of luciferase activity is a convenient and fast method but it lacks an internal control for the condition of the parasite population and for downstream processing of parasite proteins. Hence, Luciferase activity measured is absolute and not in relation to a control protein. This is the advantage of quantitative RT-PCR where a number of control genes can be used to adjust for the amount of parasites lysed and the composition of the population. Replacement of the luciferase gene by a positive selectable marker forcing activation of all *var* promoters in a parasite population could be an approach to base experiments on RT-PCR results instead of luciferase activity.

Identification of regulatory sequences in a central *var* gene upstream region

To identify regulatory sequences of central *var* genes, we compared luciferase activity driven by different *var* upstream region fragments in stably transfected parasite populations. Promoter activity of the phGL-WT construct with the full-length upstream region of *var* gene PFL_1960w was used as a standard (100%). Its transcriptional start point (TSP) has been mapped to 1167bp upstream of the ATG (Deitsch et al., 1999). The shortest *var* upstream region construct contained the sequence from the ATG to the TSP plus an additional 340bp (phGL-15). These 340bp were sufficient to drive basic luciferase activity (20 times less than the reference) indicating that the core promoter lies within this sequence (Figure 11). A protein binding element (central promoter binding element, CPE) was previously identified in this sequence (Voss et al., 2003). Additional 371bp (phGL-18) increased luciferase activity 147-times compared to the core promoter activity. This suggests the presence of an activator binding sequence in these additional 371bps. The luciferase activity for the 1879bp of the phGL-18 upstream region was 8-times higher than for the wildtype (phGL-WT) indicating the presence of a repressor binding site upstream of 1879bp from the ATG. Deletion of the CPE resulted in a 3-fold reduction of luciferase activity compared to the wildtype promoter suggesting another activator binding site at the CPE. The activation potential of this CPE is smaller than that of the activator between 1879 and 1508. It has to be noted

that the repressor-binding site is only present in the CPE deletion construct but not in the truncated upstream region constructs, hence the activation potential of the CPE might be higher but is abolished by the repressor. It would be interesting to test this hypothesis using a construct where the repression site as well as the CPE is missing.

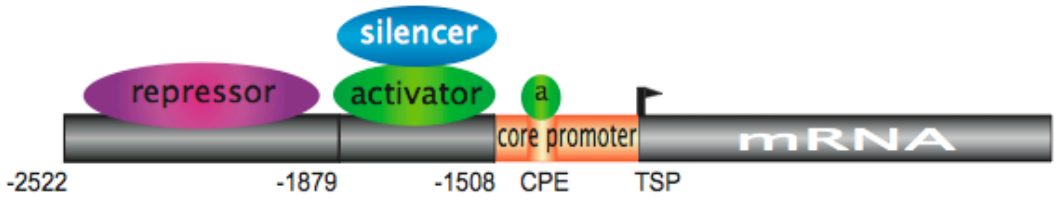


Figure 11. Model for possible *var* gene regulation mechanism.
The full-length *var* gene upstream region investigated in this thesis is shown. Possible proteins that might bind to the DNA are indicated as follows: activators in green, a possible repressor in red and the silencer in blue. The core promoter is colored in orange. Distances to the ATG are indicated in base pairs.

The *var* intron plays an important role in *var* gene silencing (Deitsch et al., 2001). Therefore we wanted to determine what part of the *var* promoter is responsible for the interaction with the *var* intron. We measured luciferase activity for all promoter fragments in the presence and absence of the *var* intron on the plasmid. As expected, the *var* intron silenced the wildtype promoter completely. In the shortest construct no impact of the *var* intron on luciferase expression could be detected. Thus, it is very likely that the intron does not interact with the core promoter. Addition of 371bp led to a 4-fold reduction of luciferase activity when the *var* intron was present (phGL-18 versus phGLint-18). Luciferase activity in the presence of the intron for the phGLint-18 construct was still 2-fold higher than the wildtype promoter without the intron, which was statistically not significant (two-tailed unpaired t test P=0.1). The presence of the *var* intron did not influence luciferase activity of the promoter sequence where the CPE was deleted (3.5-times). Consequently, the interaction site for the *var* intron can be mapped to a sequence between 1508bp to 1879bp relative to the ATG (Figure 11).

Complete silencing of the luciferase gene by the *var* intron could only be achieved with the wildtype promoter indicating that the complete upstream region is involved in the silencing mechanism.
One of the activator binding sites and the silencer site are locating to the same 371bp sequence (Figure 11). It is possible that the two proteins bind to different stretches on this sequence or that they compete for the same binding site.

Distribution of luciferase expression in the population

As mentioned above the luciferase system raises questions about the homogeneity of the transfected parasite lines: Does every parasite in a transfected population express an equal amount of luciferase or is it only a subset of parasites that produces the luciferase measured in an assay? To address this question we performed IFAs and Western blot analysis with the transfected parasite lines using anti–luciferase antibodies. We tested two different anti-luciferase antibodies: A polyclonal rabbit antibody from Sigma (L0159) and a mouse monoclonal antibody from Sigma (L2164). The rabbit antibody was cross-reactive with an unidentified parasite protein in IFAs and Western blot and the mouse antibody detected only a signal in Western blot. The reason why no parasites were detected in IFAs with the mouse antibody could be that parasites accumulate luciferase in the food vacuole or vesicles, which are not accessible for antibodies with the conditions we used for the IFAs. It is also possible that only a minority of the parasites expressed luciferase and was therefore not found in IFAs.
To test whether luciferase activity can be detected by FACS analysis, parasites were incubated with luciferin salt and analyzed by FACS for luciferase activity. No activity could be detected with this method (data not shown).
Due to the problems outlined above, we were not able to determine what part of the population did express luciferase. For all future experiments we assumed that the populations were homogenous. Luciferase activity was therefore normalized to the plasmid copy number for every construct. With the plasmid copy number, the average number of promoters in a single parasite is

established. However it is not known whether every single promoter is active and shows the same expression level.

Integration of the constructs into the parasite chromosome

To rule out the possibility that one of the transfected plasmids had integrated into the complementary *var* locus on the chromosome and thereby complemented the truncated *var* promoter driving luciferase expression we performed Southern blot analysis using the DBL1α domain of the endogenous PFL1960w *var* gene as a probe. None of the truncated promoter fragments had integrated and the luciferase counts obtained for the truncated promoters (phGL-18, phGL-18int) were thus genuine. However, the phGL-WTint construct had integrated into the *var* locus. As silencing was only complete for this construct proper embedding of the *var* locus in the chromosome might be necessary to achieve complete silencing. Although we observed complete silencing for a transient plasmid with the same *var* upstream region and the *var* intron before (data not shown). Southern blot analysis probed with the *h*dhfr gene detected only a single band for episomal plasmids but no additional band for an integration event. Due to the multiple plasmid copy numbers the intensity of the plasmid bands would be 10 to 40 times stronger than an additional band indicating integration. This is probably also the reason why no additional band was detected for the phGL-WTint transfected parasite line in this Southern blot although a previous Southern blot with the *var* gene probe showed an integration event (Figure 7). To verify that none of the other plasmids had integrated into the chromosome, additional Southern blots with probes specific for different possible integration loci need to be performed. For studies in this thesis integration is most likely not a problem, as long as it does not affect the *var* promoter or the *var* intron.

Deletion of *var* intron region 2 in phGL-18int

Southern blot analysis for integration of plasmids into the chromosome revealed an unexpected double band for the parasite line transfected with phGL-18int. After cloning and sequencing of the *var* intron region of this

plasmid we identified a 462bp deletion in the *var* intron. The deletion corresponds to the middle region (region 2) of the *var* intron, which is highly AT-rich, displays promoter activity on its own and is sufficient to silence an associated *var* promoter (Calderwood et al., 2003). The same deletion has been observed in a parasite line transfected with a plasmid where a *var* promoter was driving *h*dhfr gene expression episomally with a *var* intron located down-stream of the *h*dhfr gene (Gannoun-Zaki et al., 2005). In contrast to this construct in which the *var* promoter needed to be active to survive selection by WR99210, in our study there was no need for an active *var* promoter, as it was only driving luciferase expression. The *h*dhfr gene was under the control of an additional *cam* promoter. Promoters in close proximity can influence the activity of each other (Crabb et al., 1997b). It has been demonstrated that an active *var* promoter enhanced the activity of an adjacent heat shock protein 86 (*hsp*86) promoter driving blasticidin deaminase (*bsd*) expression (Voss et al., 2006). We observed the same effect between the *var* gene and the *cam* promoter: mRNA levels of the *h*dhfr for each *cam* promoter were highest where luciferase expression was highest and vice versa (data not shown).

Areas of electron-dense material consistent with heterochromatin and gene silencing as well as non-condensed areas allowing transcription are present at the nuclear periphery (Ralph et al., 2005). Silenced *var* genes, regardless if subtelomeric or central, are localized to the electron-dense nuclear periphery while active *var* genes seem to locate to a privileged site that is competent for transcription (Duraisingh et al., 2005). In order to transcribe the *h*dhfr gene, the transfected plasmids must be in a transcriptional active zone. An active *var* promoter might localize the plasmid to the nuclear periphery where transcription is enhanced and thereby increase the expression of the *cam* promoter. Therefore, even if there is no direct selection pressure to express luciferase, the activation of the *var* promoter may possibly benefit the parasite. The plasmid copy number estimated for each parasite line gives additional support to this theory as parasites with a non-active *var* promoter (phGL-WTint) have more plasmid copies than parasites with an active *var* promoter (e.g. phGL-WT). It is also possible that the *var* promoter on a plasmid benefits from the active *cam* promoter. The plasmids must be in a trancriptionally

active compartment to express the *h*dhfr gene and therefore the chromatin structure is relaxed and permits expression of the luciferase gene by the *var* promoter. The presence of the *var* intron might reduce this effect by working as a boundary element that prevents the spreading of euchromatin from the active *cam* promoter.

The partial loss of the intron discussed above has also an impact on the interpretation of the results obtained for the parasite line transfected with phGL-18int. Region 2 of the *var* intron was probably only lost in half of the *var* introns present on the plasmid in the parasite population, as two bands with equal intensities have been detected by Southern blot (Figure 7). The influence of the intact *var* intron in construct phGL-18int might hence be stronger than measured and luciferase activity might have been reduced even more in parasites containing intact *var* introns. This observation strengthens the hypothesis of a possible *var* intron interaction site located between 1879 and 1509bp upstream of the ATG.

The fact that exactly the same sequence deletion in the *var* intron was found in two different studies independently, suggests that it is directed and confers a benefit to the transformed parasite. Region 1 and 3 of the *var* intron contain repeat sequences (TGTATGTG, ACATACAC) that are complementary to each other (Calderwood et al., 2003). It is therefore possible that region 1 and 3 form a hairpin facilitating the deletion of region 2.

Mutually exclusive transcription

var genes are expressed in a mutually exclusive manner. Only one *var* promoter is active at one time in a single parasite. We assumed that the *var* promoter on the plasmids is active in every parasite, rising the question what the state of the endogenous *var* genes is. Recent work suggested that the machinery that mediates mutually exclusive transcription does not recognize active *var* promoters on a plasmid without the *var* intron allowing for transcription of an endogenous *var* gene (Gannoun-Zaki et al., 2005; Frank et al., 2006). Frank et al., (2006) postulated that the mutually exclusive transcription machinery recognizes an active *var* promoter driving luciferase activity only when the *var* intron is present in the construct. To test if the

mutually exclusive transcription machinery recognizes our constructs, we performed quantitative RT-PCR on cDNA isolated from ring stage parasites with primers specific for each *var* gene (Salanti et al., 2003). The relative transcription level (RTL) of every *var* gene was calculated in relation to the transcription level of the glutaminyl tRNA synthetase control gene. No *var* gene was transcribed higher than the control gene (RTL below 0.4), while luciferase transcripts of constructs with active *var* promoters were higher than the control gene (RTL above 1). RTLs of the silent *var* promoter (phGL-WTint) were below 0.5 and consequently in the same range as the endogenous *var* genes. This suggests that the mutually exclusive transcription machinery did not recognize the active *var* promoters on the constructs and transcription of endogenous *var* genes was normal. These results are contradictory to previous data where a *var* promoter driving a selectable marker was able to silence all endogenous *var* genes (Dzikowski et al., 2006; Voss et al., 2006).

In our study we did not select for a homogeneous population and therefore it is possible that we looked at a heterogeneous population as discussed above. Active *var* promoters on the plasmids might thus be recognized and endogenous *var* genes silenced but the transcription of endogenous *var* genes in parasites where the *var* promoter on the plasmid was silenced falsified the analysis. Without the possibility to select for a homogenous population either by introduction of an additional positive selectable marker under the control of the *var* gene promoter, or by cloning the parasite lines using limiting dilution or by single cell analysis, it is not possible to draw conclusions regarding this question.

RT-PCR analyis of *var* gene expression showed that *var* gene PFI1830c was predominantly expressed in all parasite lines examined. This finding supports data from our group where *var* gene switching of 3D7 was examined over one year in *in vitro* parasite cultures. This same *var* gene was dominantly expressed throughout the experiment (master thesis K. Wittmer). The question arises whether our 3D7 strain displays a normal switching behavior or if there is some defect in the *var* gene switching machinery. In our experiments *var* gene expression detected by quantitative RT-PCR was generally low compared to *var* gene ΔCT values obtained by another group (Dzikowski et al., 2006).

The detected transcripts of the endogenous *var* genes might also be none full-length or a RT-PCR artifact. Early transcripts from the 5' end of most *var* genes can be detected by quantitative RT-PCR (Chen et al., 1998b; Scherf et al., 1998) but comparison of RT-PCR data with Northern blots suggested that even in young ring stages, only one single full-length transcript exists and that the remaining ones detected by RT-PCR are terminated early (Taylor et al., 2000).

Differences between results from luciferase assays and quantitative RT-PCR on cDNA

To compare quantitative RT-PCR and luciferase assays we isolated and processed RNA and protein from the same parasite population at the same time. We found some quantitative differences between transcription levels and protein activity. Still, phGL-WT was most active and phGL-WTint was least active in both approaches. While we detected almost no luciferase activity for the phGL-WTint transfected parasite lines (2500-fold reduction compared to phGL-WT), we still found mRNA transcripts (12-fold reduction compared to phGL-WT). mRNA and protein levels need not always be concordant as protein expression can also be regulated at a translational level and not only at the transcriptional level. We expect that post translational regulation of the luciferase reporter gene is the same for all constructs as they all have the same sequence downstream of the TSP.
It is possible that quantitative RT-PCR is much more sensitive than the luciferase assay detecting transcripts that are not going to be translated. Some bidirectional transcription activity was found in the *var* intron in an earlier study (Calderwood et al., 2003) and part of the luciferase mRNA detected in our experiments might be transcribed from the other direction by the *var* intron.

Determination and influence of the relative copy number on luciferase activity

To determine how many plasmids and consequently *var* promoters are present in a parasite, we estimated the relative copy number of each

transfected parasite line by quantitative RT-PCR on gDNA. This estimation was repeated several times over a two-year period whereby the parasite cultures have been frozen and re-established in between. Plasmid copy numbers varied between different time points (Tables 1 and 2) but also between experiments conducted within two weeks (data not shown). Variation is therefore not only a result of changes in the population but also due to problems with reproducibility of the RT-PCR results. Estimation of the plasmid copy number is very important as luciferase activity as well as RTLs is adjusted to this. Luciferase activity was adjusted to the plasmid copy number determined by RT-PCR at the same time. Due to discrepancies between the estimated plasmid copy numbers (3.7 and 10.1) between different experiments, luciferase activity for phGL-18 transfected parasite lines was once higher than in the control (phGL-WT) and in the next experiment lower. The first data set is more robust as experiments were repeated twice in triplicates and not only once like in the second data set. For that reason, we think that the proposed regulatory sequences based on the first data set hold true.

Transcription levels detected by quantitative RT-PCR have also been adjusted to the copy number estimated for every single *var* and control gene on gDNA. One ΔCT value difference corresponds only to a doubling of the amount of template if the amplification efficiencies [$E = 10^{(-1/\text{slope standard curve})}$] of target and reference are approximately equal (ABI, User Bulletin 2). Not all primer pairs used for the amplification of the *var* transcripts, the luciferase gene and the control genes have been characterized in the original publication (Salanti et al., 2003). Additionally, amplification efficiencies of the same primer pair might vary on different substrates (plasmid, cDNA or gDNA). Copy numbers estimated for each transfected parasite line must therefore be handled with care.

Another method to establish plasmid copy numbers is Southern blot analysis with a probe for the plasmid and a second probe for a control gene on the parasite chromosome. The intensities of the resulting bands can then be compared. While this method is not as quantitative as RT-PCR it can serve as a control. Relative transcription levels could also be assessed by Northern blot

analysis similar to Southern blot detection though it is difficult to determine the intensity of a band when saturation is reached.

Conclusions

We improved transfection efficiency of *P. falciparum* cultures and generated nine distinctive stable parasite lines expressing luciferase under the control of different *var* gene upstream regions. Thanks to stable transfection technique we could reduce the variability between different luciferase assays. By comparing the luciferase activities from different parasite lines, we were able to identify two possible activator-binding sites and a repressor-binding site on a central *var* upstream region. In addition, we propose an interaction site for the *var* intron between 1879bp and 1508bp upstream of the ATG. We could not determine what proportion of the transfected parasite lines did express luciferase nor if the mutually exclusive transcription machinery recognized the expressed *var* promoter on the plasmid and consequently silenced all endogenous *var* genes. We learned that it is important to control the intactness and location of transfected plasmids in parasites, as deletion and integration events are likely to happen.

Chapter 4: Characterization of *Plasmodium falciparum* Phosphodiesterase 1

INTRODUCTION

Malaria is still a major health problem with about 500 million new clinical cases and at least one million people dying every year (Snow et al., 2005). Human malaria is caused by four different *Plasmodium* species (*Plasmodium falciparum, P. vivax, P. ovale,* and *P. malariae*) that are transmitted by a female anopheles mosquito. Out of these four species, *P. falciparum* causes the most severe form of pathogenesis. A wide range of chemotherapeutics is used against all species that display a big spectrum of targets. The most frequently used antimalarials are quinolines, antifolate drugs, artemisinins, atovaquone, and antibiotics such as tetracyclines (Cunha-Rodrigues et al., 2006). Quinolines, with its most famous member chloroquine, have been used excessively and with great success throughout the 20th century. Quinolines are thought to inhibit the dimerization of heme and/or prevent the disposal of dimers from the food vacuole to the cytoplasm, where hemozoin is formed (Fitch, 2004). The accumulation of free heme in the intra-erythrocytic parasite becomes highly toxic. Due to the long and extensive use of chloroquine and others, quinolines have lost their efficacy to a large degree and therefore need to be replaced. Antifolates inhibit the synthesis of folate cofactors that are required for nucleotide synthesis and amino acid metabolism during schizogony in RBCs and hepatocytes by acting on dihydrofolate reductase (dhfr (Ferone et al., 1969)) or dhihydropteroate synthase (dhps (Zhang and Meshnick, 1991)). Antifolates act more slowly against *Plasmodium* than quinolines and are eliminated gradually from the blood circulation allowing resistance to develop rapidly by mutating the target enzymes (Dieckmann and Jung, 1986). Even though antifolates are mainly used in drug combinations (FANSIDAR, MALARONE, LAPDAP), resistance is dramatically emerging (Looareesuwan et al., 1992; Wongsrichanalai et al., 2002). Atavaquone inhibits electron transport in plasmodial mitochondria (Fry and Beesley, 1991) and depolarizes the membranes of plasmodial mitochondria (Srivastava et al., 1997). Drug resistance arises due to mutations in the cytochrom c reductase gene (Looareesuwan et al., 1996; Kessl et al., 2004). Artemisins display activity against young rings (Skinner et al., 1996) and gametocytes in blood

stage forms (Chen et al., 1994). Parasites are thought to be killed by the generation of free radicals through activation of artemisins by ferrous heme or exogenous free iron (Meshnick, 2002). Artemisins are the fastest working antimalarials with a very short half life (White, 1997) and the only antimalarial drugs where no resistance has emerged so far. Although *Plasmodium* is a eukaryote, they carry a plastid, known as apicoplast, which contains elements deriving from prokaryotic transcription and translation systems. Thus antibiotics can also be used as antimalarials by inhibiting RNA synthesis. Because there are no new malaria vaccines to be expected in the near future development of new drugs against malaria is crucial (Greenwood et al., 2005).

Cyclic nucleotide signaling is a common regulatory mechanism and involved in a multitude of biological functions. In *Plasmodium* it has been implicated that cyclic nucleotides signaling could be involved in the differentiation of the parasite within both the human host and the insect vector. Different stages of the signaling cascade could be used as possible drug targets, namely cyclases that catalyze the conversion of ATP and GTP to cyclic nucleotides, phosphodiesterases (PDEs) that catalyze the hydrolysis of cyclic nucleotides to monophosphates, and kinases, the actual targets of the cyclic nucleotides. We have focused on the PDEs as possible new drug target.

Today 11 PDE groups, encompassing 50 isoforms, have been identified in humans. They show considerable sequence conversation between catalytic domains of different families but vary in their substrate specificity and sensitivity to calcium-calmodulin. PDE activity is found in every cell in the human body, but there is a distinct cellular and subcellular distribution of the 11 groups, which provides many possibilities for selective therapeutic targets (Lugnier, 2006). PDE inhibitors are widely used in human medicine as they can specifically interact with a PDE family or even PDE isoform. Another advantage of PDE inhibitors is that they do not have to compete with very high levels of endogenous substrate, as cyclic nucleotides are usually low concentrated in the cell (Bender and Beavo, 2006). Despite the potential of PDE inhibitors as chemotherapeutics, little is known about PDEs of parasites as possible drug targets.

With trypanosomes, the potential of PDE inhibitors as drugs against sleeping sickness, Nagana or Chagas' disease has been tested. Four PDE inhibitors

were identified that inhibit proliferation of bloodstream form of *Trypanosoma brucei* in culture (Zoraghi et al., 2001) and two different PDE families have been identified in *T. cruzi*, that also display sensitivity to PDE inhibitors (D'Angelo et al., 2004; Kunz et al., 2005; Alonso et al., 2006; Diaz-Benjumea et al., 2006).

In contrast little is known about the four PDEs identified in *P. falciparum* although they might present an important target for chemotherapy. The fact that the four *Pf*PDEs show little homology to human PDEs and that they build a unique group of *Pf*PDEs suggests that they might become targets for inhibitors of PDEs with little side effects on human PDEs.

As part of this PhD thesis a transgenic *P. falciparum* parasite was generated which has a deletion of *Pf*PDE1. The resulting clone was analyzed for its phenotype and PDE activity. This clearly showed that *Pf*PDE1 is not an essential gene in the asexual bloodstage form of *P. falciparum* and no rescue or compensation was observed with the remaining three PDEs which implicates that the main function of *Pf*PDE1 might not lie in the asexual bloodstages.

METHODS

Transfection construct for the *Pf*PDE1 knockout

Cloning of the transfection constructs for the *Pf*PDE1 knockout and for GFP localization were done by L. Wentzinger in Bern as follows: Based on the sequence obtained from plasmoDB (www.plasmodb.org) for the *Pf*PDE1 gene PFL0475w, primers were designed to amplify the predicted open reading frame: LWPF01F (5'-CATATGAATGAGTATAATAATGATAATATGGAACAGGAGAA-3') and LWPF01R (5'-GGATCCTTATAATTTTCCATGTTTTATTTCTTCTTTAAAATT-3');

The cDNA synthesis is performed as follow: 1 μg of total RNA is denatured at 65°C for 5 min together with gene specific primers and quickly cooled on ice. A mix containing 4μl 5x superscript buffer, 200 U superscript III, 1μl dNTP 10mM, 1μL DTT 0.1M and 1μL RNase inhibitors (Roche) is added to the denatured RNA and incubated 1h at 46°C. PCR amplification was carried out in a total volume of 25μl with 0.5μl of the obtained cDNA, 1x PfuUltra PCR reaction buffer (Stratagene), 2.5U PfuUltra (Stratagene), 200μM gene specific primers (same than those used for reverse transcription step), 300μM dNTPs and 3mM Mg^{2+}. PCR conditions for the PDE were the following: 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 68°C for 3min. PCR products were cloned into the TA-TOPO cloning vector (Invitrogen) and sequenced.

The verified 5' UTR and 3' UTR from PfPDE1 were PCR amplified from the TA-TOPO cloning vectors (PfPDE1_5UTR-f: 5'-ACTGTAAAAAATATAAAGATTTATACAAAAAAAAAGAACGTAC-3' and PfPDE1_5UTR-r AGATCTTTTATTTTGTTTTTTTACAAATGTTTATGTGTG; PfPDE1_3UTR-r: 5'-GAATTCAAATATACAAGGAGAAAAATATATTTGATGATTG-3' and PfPDE1_3UTR-r 5'-CCATGGATATATTATTTGTCCACTTGTTTTATTTTTATATAAGAAG-3'). The PCR products for the 5'UTR were digested with *Spe*I and *Bgl*II and for the 3'UTR with *Eco*RI and *Nco*I and cloned into the adequately digested pHTK vector (Duraisingh et al., 2002).

For the GFP fusion constructs the pARLmGFPmT vector served as backbone. This vector contains the GFP sequence flanked by two multiple cloning sites and an *h*dhfr selection cassette. The PDE sequences were amplified from cDNA prepared as described above with following primers: PDE1_GFP.F (5'-AGATCTTCATGATGGATACAAAAGTAGATCAAAC-3') and PDE1_460_GFP.R (5'- CCTAGG TACATATTCATAATATAAAGACATGATAATCTT -3') for the shorter construct (460) and primers: PDE1_GFP.F (5'-AGATCTTCATGATGGATACAAAAGTAGATCAAAC -3') and PDE1_610_GFP.R (5'- CCTAGGATGCATATTAGAAAAAGAATTAAAAGAT -3') for the larger construct (610). PCR products were digested with *Bgl*II and *Avr*II and cloned into the adequately digested pARLmGFPmT vector.

In vitro cultivation of *Plasmodium falciparum*, stable transfection, selection for integrants and limiting dilution

All experiments were done with the *P. falciparum* 3D7 strain cultivated at 5% haematocrit as described previously (Trager and Jensen, 1976) in RPMI 1640 medium supplemented with 25mM HEPES, 0.5% Albumax, 50mg/l hypoxanthine, 0.25% sodium bicarbonate, and 50 mg/ml neomycin sulphate. Parasites were incubated at 37°C in an atmosphere of 3% oxygen, 5% carbon dioxide, and 92% nitrogen.

Growth synchronization was achieved by sorbitol lysis (Lambros and Vanderberg, 1979). Stable transfection of cultured 3D7 *P. falciparum* ring-stage parasites with the pHTK vector for *Pf*PDE1 knockout was obtained by electroporation as described previously (Voss et al., 2000) and drug selection using 10nM WR99210 (Fidock and Wellems, 1997). A population of stable transfected parasites was established after 6 weeks. To enrich the population for integrants, three cycles of on/off WR99210 selection was applied. After the last round, ganciclovir was added to the population in a final concentration of 400nM eliminating parasites that contained the plasmid episomally and only parasites with an integrated plasmid survived (Duraisingh et al., 2002). Parasites were analyzed by Southern blot to test for integrated plasmids. The population consisted of parasites that had either integrated the plasmid by

single cross over or by double cross over. To get a homologous population of parasites that had integrated the plasmid by double cross over only, parasites were diluted to single clones by limiting dilution. Parasites were diluted to 10, 1 or 0.1 parasites per well in separate 96 well plates for each dilution and cultured with a haematocrit of 2.5% in a volume of 200µl culture media. When the color of the least diluted plate turned from red to dark red, the status of each well for the other dilutions was checked with thick blood smears. The content of examined wells that harbored parasites was transferred to 24 well plates and cultures with 5% haematorcit in a volume of 2ml culture media. 17 clones were expanded to 10ml plates and tested for plasmid integration status by Southern blot.

Genomic DNA extraction

To gain gDNA from *Plasmodium* cultures 10ml of late stage parasites were saponin lysed and washed twice with 1xPBS. The pellet was resuspended in 500µl TE with 0.8% SDS, 0.01M EDTA and 0.2mg/ml proteinase A (SIGMA) and incubated over night at 60°C. Phenol/chloroform extraction was performed twice followed by an additional extraction with chloroform only. The aqueous phase was precipitated with sodium acetate and ethanol and finally resuspended in TE. The quality and concentration of the obtained gDNA was checked on an agarose gel.

Southern Blot and detection

Southern blot analyses were performed to check whether the plasmid had integrated correctly into the *Pf*PDE1 locus on the chromosome. gDNA was digested with *Sau*96I and *Drd*I for 2 to 3 days, digestion status was monitored on agarose gels. Agarose gel, blot and detection followed the protocol of the ECL Direct Nucleic Acid Labelling And Detection Systems (Amersham Biosciences). Briefly, 2-10µg of restriction enzyme digested gDNA was loaded on a 1% Agarose gel and electrophoreses was performed over night in 1x TAE buffer at 1V/cm. The gel was in depurinated in 200ml 250mM HCl, denatured in 200ml 1.5MNaCl/0.5MNaOH and neutralized in 200ml 1.5M NaCl/0.5M Tris-

HCl (pH 7.5) before blotting on a Hybond N+ membrane over night. The DNA was UV cross-linked to the membrane. Horseradish peroxidase was cross-linked with glutaraldehyde to 100ng probe. Either a 500bp amplicon of the *Pf*PDE1 gene or the *h*dhfr gene was used as probes. The *h*dhfr probe was PCR amplified from the pHTK vector by primers hdhfr_forw (5'-GGATCCATGCATGGTTC-3') and hdhfr_rew (5'-GGCTGTACAGTGTATAAACC-3') with following conditions: 20sec denaturation at 94°C, 20sec annealing at 54°C, and elongation 1.5min at 66°C, the cycle was thirty timed repeated. The *Pf*PDE1 probe was amplified with primer LWPf30f (5'-GGATCCATGAATAGTCTTAACAGAATATCTTTTAATTC-3') and LWPf30r (5'-GCGGCCGCTTATTCAAATTTGATGAGCTCA-3') with following PCR conditions: 30sec at 94°C, 30sec at 50°C, and 1min at 68°C, the cycle was 32 times repeated. Hybridization and stringency washes were performed in hybridization tubes in an incubator with an integral rotisserie device. After a minimum of 15min prehybrization with hybridization buffer the labeled probe was added and hybridized over night at 42°C. The blot was washed first with wash buffer containing 6M Urea, 0.4%SDS and 0.5x SSC and second with 2x SSC. The signal was generated by incubating the blot for 1min with an equal amount of detection reagent 1 and 2. A BioMax light film (Kodak) was placed on the blot in a film cassette for an appropriate length of time, usually 2min. The film was afterwards developed.

FACS analysis

FACS analysis was used to monitor the parasite growth over time, as it is a convenient method to estimate parasitaemia. Stock solutions (10mg/ml) of hydroethidine (HE) in di-methyl sulfoxide (DMSO) was prepared and stored at -20°C. 150µl culture was spun and the pellet was resuspended in 150µl HE (1/1000 in 1x PBS) and then incubated for 30min at 37°C in the dark. HE is taken up by the parasite and metabolized to ethidium, a nucleic acid-binding fluorochrome (van der Heyde et al., 1995). 1ml of FACS Flow was mixed with the RBCs and out of this 200µl were pipetted to 1ml of FACS Flow in tubes for FACS analysis. Flow cytometry data aquisition and analysis were performed

on a FACS instrument (FACSScan, Becton-Dickinson). The detectors for forward- and side-scatter of the FACS were set to E-01 and 227, respectively; and both detectors were set to the logarithmic scale. The FL2 detectors were also adjusted to bring events within the detection range of the instrument (generally 433 for FL2). First uninfected RBCs were gated to exclude burst and dead RBCs, subsequently early and late stages were gated separately. A negative control of uninfected RBCs only was used to estimate the number of auto-fluorescent RBCs. These cells appeared in the gate for the early stages and were thus subtracted from the samples. The parasitaemia and the percentage of early and late stages were detected using the CellQuest Software (Becton-Dickinson). A total of 100,000 cells were analyzed per sample.

Microscopy

Thin red blood smears were used to examine the parasitaemia and the status of the culture throughout culturing. Thin red blood smears were fixed with methanol and stained for 15min in Giemsa solution. To determine the proportion of early stages and late stages in the short time course experiment, a minimum of 100 iRBC per slide were examined by microscopy. To ensure unbiased counting, slides were blinded and counted after all samples have been taken. All slides were counted twice.

Fluorescent microscopy

Parasites transfected with GFP constructs were examined for their production and localization of GFP by fluorescent microscopy. Fluorescence microscopy was performed using a Leica DM5000 fluorescent microscope and documented with a Leica DC200 digital camera system using x100 oil immersion objectives. Parasitized RBCs expressing GFP were mounted wet on a glass slide, covered by a glass coverslip, sealed, and imaged.

Western blot analysis

Western blot analyses were performed to detect recombinant GFP in *Plasmodium* culture lysate transfected with a plasmid expressing recombinant GFP. 10ml asynchronous *P. falciparum*-infected RBCs were lysed in 1.5ml 0.1% saponin in 1xPBS and washed twice with 1ml 1xPBS. The parasite pellet was resolved in sample buffer (0.1M TrisHCl PH 6.8, 20% glycerol, 2% Sodium dodecyl sulfate (SDS), 0.1M β-mercapto-methanol, bromphenol-blue). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide), transferred to a nitrocellulose membrane (Hybond-C extra; Amersham Biosciences) for 3h using a Trans-Blot semidry electroblotter (Bio-Rad), and probed with antiserum from mice immunized with recombinant MAHRP1 (1:1000) to show equal loading of parasite lysate or mouse α-GFP (1:1000, Sigma) followed by peroxidase-conjugated goat α-mouse immunoglobulin G, (1:10,000, Sigma). The membrane was developed according to the ECL western blot detection kit manual (Amersham).

RNA isolation and cDNA

RNA was isolated to perform Northern blots and serve as template for the synthesis of cDNA to provide a template for quantitative RT-PCR.
40ml parasite culture containing 5% late ring stage were saponin lysed and washed twice with 1xPBS. The pellet was then resuspended in 2ml Trizol (Invitrogen) and RNA was extracted with 0.2ml chloroform and precipitated with isopropanol. The extraction was repeated in half of the original volume Trizol to reduce contamination with gDNA. gDNA was twice digested with RQ 1 DNase (Promega) according to the manufacturer's protocol in a total volume of 50μl. RNA was subsequently extracted with 180μl Trizol and 40μl chloroform. Before cDNA synthesis, eventual gDNA contamination was tested by PCR. For the PCR 1 μl of RNA was used as template with the primer pair DBLα5' and DBLα3' that amplified most of the *var* genes (Kaestli et al., 2004). As a positive control an additional PCR with the same primers but addition of 1μl gDNA from 3D7 to the RNA was performed. PCR conditions were as

follows: 30sec denaturation at 94°C, 30sec annealing at 54°C, and 1min elongation at 60°C. The cycle was repeated thirty times. cDNA synthesis was performed with M-MuLV Reverse Transcriptase (New England BioLabs) with random primers (Invitrogen) as described by the manufacturer. cDNA was synthesized from 800ng total RNA in a reaction volume of 50µl. For each cDNA synthesis reaction, a control reaction without reverse transcriptase was performed with identical amounts of template.

RT-PCR for PDE RNA level determination

Quantitative real-time PCR was used to check for differences in the expression levels of every PDE in the knockout compared to the wildtype parasite strain. Quantitative real-time PCR using QuanTitect SYBR Green PCR master mix (QIAGEN) was performed on a TaqMan 7500 (Applied Biosystems, ABI) according to provider's manual, using the seryl-tRNA synthetase gene (PF07_0073) as endogenous control with two different primer pairs (primers p90_for 5'-TCAATTTGATAAAGTGGAACAATTC-3' and p90_rev 5'-GCGTTGTTTAAAGCTCCTGA-3' and primers LWPf26f 5'-TGCCGAACTTGATGACTTTGAA-3' and LWPF26r 5'-GTAGGAGATGTAGATACCTGTTGAGATGA-3') and specific primers for each PDE gene (PDE1: LWPf23f 5'-GTCTCCACGCAGCACAGGTA-3' and LWPf23r 5'-TAAGCAAAATTCGTCAATAGCTGAAA-3'; PDE2: LWPf22f 5'-TATTCTTTCCCTCACGGACCAA-3' and LWPf22r 5'-TTGGCGGAACCTACTAATATGATG-3'; PDE3: LWPf24f 5'-CGATCCACGCAGCTATGGT-3' and LWPf24r 5'-GCACCTAGTTCGTTATCCCTAAGAA-3'; PDE4: LWPf25f 5'-ATGGTGCTACAGTATGTCACTTATCAAA-3' and LWPf25r 5'-TGGATGTCCTACATCATGTGCTATAGAT-3'). PCR was performed on cDNA in duplicates for three dilutions (1:2, 1:20, and 1:200) and CT values for the seryl-tRNA synthetase varied between 18 (1:2) and 27 (1:200) and for the different PDEs between 20 (1:2, *Pf*PDE2) and 34 (1:200, *Pf*PDE3). PCR conditions were as follows: 2min at 50°C for activation of the Uracil-DNA glycosidase followed by initial denaturation for 10min at 96°C, then followed 41 cycles of 15sec 95°C and 1min 59°C. The dissociation curve was

subsequently established. Undetected PCR products were set to CT 40. To estimate the relative copy number, the ΔCT was determined by subtracting the CT value for the PDE genes from the CT value for the control seryl-tRNA synthetase gene (ABI, User Bulletin 2). ΔCTs were then converted to relative copy numbers with the formula $2^{\Delta Ct}$.

Northern Blot and detection

Total RNA was prepared as described above. Equal amounts of RNA were loaded on a 0.7% agarose gel and blot and detection were prepared according to the ECL Direct Nucleic Acid Labelling And Detection Systems (Amersham Biosciences). For details see Southern blot detection. Either the merozoite surface protein 2 (*msp2*) gene or the PDE genes were used as probes. Following primer pairs were used to amplify the PDE genes from gDNA: *Pf*PDE1: LWPf30f (5'-GGATCCATGAATAGTCTTAACAGAATATCTTTTAATTC-3') and LWPf30r (5'-GCGGCCGCTTATTCAAATTTGATGAGCTCA-3'), *Pf*PDE2: LWPf31f (5'-GAATTCAAATGAATTGTTTAACATATTTTGATGAATC-3') and LWPf31r (5'-GCGGCCGCTTAATCGGAAACATTTTTTATAAA-3'), *Pf*PDE3: LWPf32f (5'-GAATTCAAATGATAGATGAAAAATCAAAAATGTATTC -3') and LWPf32r (5'-GCGGCCGCTTATTTTTTTTGTGTTTTGTAAATATTC -3'), and PfPDE4: LWPf33r (5'- GCGGCCGCTTATGTCATTTTTTCTGTGTTATAAAAC -3') and LWPf33.a.f (5'- GGATCCAAATGATAGCATACGAAGTTGAAGTATTG -3') with following PCR conditions: 30sec at 94°C, 30sec at 50°C, and 1min at 68°C, the cycle was 32 times repeated. The probe for the *msp*2 gene was amplified with primer pair S1 and S4 described by (Foley et al., 1992). A BioMax light film (Kodak) was placed on the blot in a film cassette for 1min in case of the *msp*2 probe and over night for the PDE probes.

Growth inhibition assay

Increasing concentrations of different PDE inhibitors (zaparinast and BG-1) and cyclic nucleotide analogues (8Br-cAMP, cpt-cAMP, 8BR-cGMP and cpt-

cGMP), dissolved in di-methyl sulfoxide (DMSO), were tested for their inhibitory effect on the *P. falciparum* intraerythrocytic development of wildtype (NF54) and *Pf*PDE1 knockout strains (A6 and G5).

200µl of parasites with an initial parasitaemia of 0.3% and a hematocrit of 1.25% were allowed to grow at 37°C for 48h in a 96 well plate, then 50µl ^{3}H-hypoxanthine (0.5 µCi) was added per well. After 24h incubation period, plates were harvested on glass fibre filters using a Betaplate™ cell harvester (Wallac, Perkin Elmer). 10ml scintillation liquid was added to the dried filters and counted in a Betaplate™ liquid scintillation counter (Wallac, Perkin Elmer).

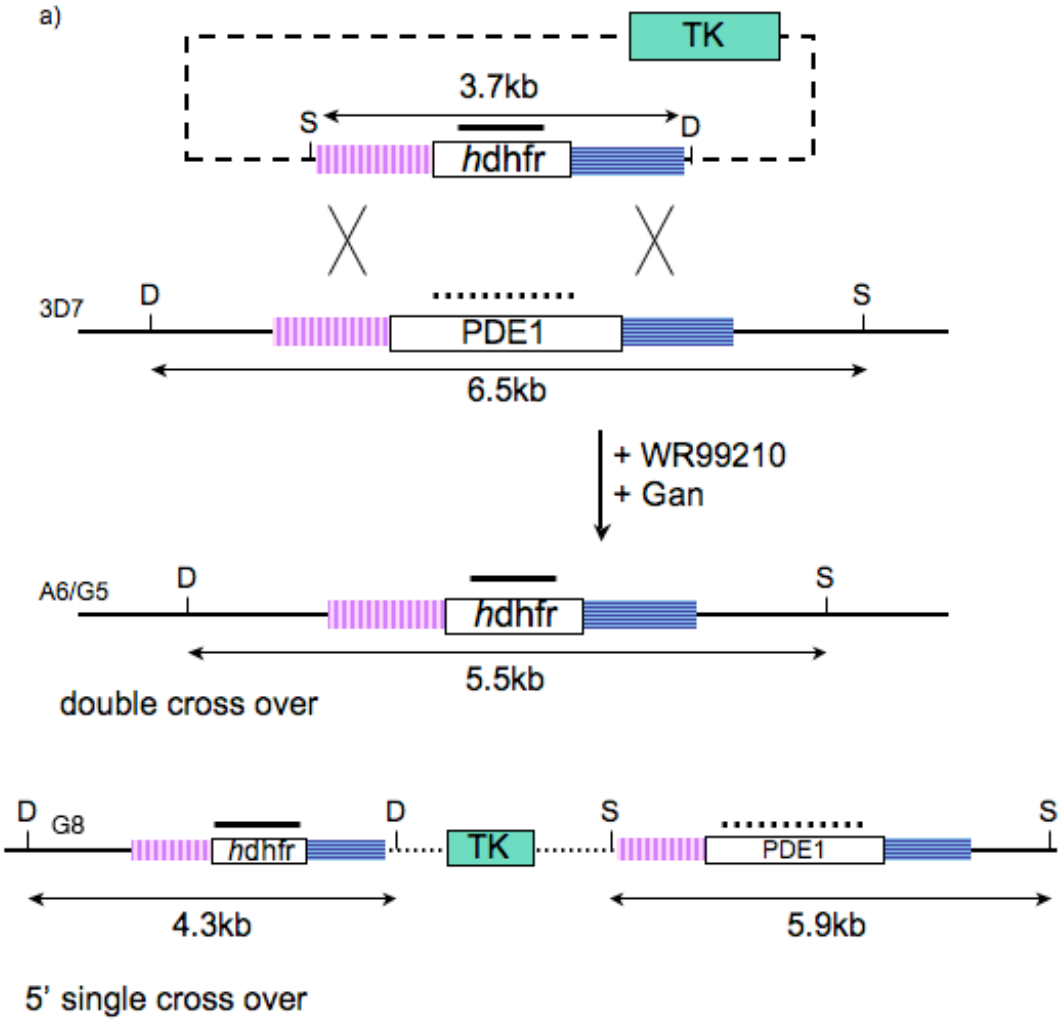
Growth inhibition in percent was calculated from the parasite-associated radioactivity. 100% ^{3}H-hypoxanthine incorporation was determined from a control culture grown in the absence of drugs. Values for the IC_{50} were determined in duplicates as described by (Desjardins et al., 1979).

RESULTS

Knock out of the *Pf*PDE1 gene by double cross-over

Very little is known about the role of phosphodiesterases (PDE) in *P. falciparum*. In order to describe the function of one of the four predicted PDEs, we replaced the endogenous gene coding for *Pf*PDE1 (PFL0475w) with the human dihydrofolate reductase gene (*h*dhfr) conferring resistance to WR99210. In a collaboration with the institute of Cell biology, University of Bern (T. Seebeck and L. Wentzinger) we constructed a plasmid that contained the *h*dhfr expression cassette flanked by approximately 300bp of the 5' and 3' untranslated region of the PDE1 gene. In addition to the positive selectable marker, the presence of a negative selection cassette allowed for elimination of parasites that have not integrated the plasmid into the genome by double crossover. The negative selection cassette consisted of the thymidine kinase (tk) gene from the herpes simplex virus which phosphorylates the guanosine analogue gancyclovir to nucleoside triphosphates that inhibit DNA synthesis after incorporation into nascent DNA (Reardon, 1989). The plasmid map and chromosomal location of PDE1 in *P. falciparum* is shown in Figure 1a.

After transfection of the plasmid into the *P. falciparum* 3D7 strain, drug selection was applied and a population that harbored the plasmid episomally was established. Three rounds of on and off drug cycling with WR99210 was performed and subsequently gancyclovir pressure was introduced, eliminating parasites, which had not integrated the plasmid into the chromosome. Southern blot analysis revealed that a heterogeneous population of parasites existed that had integrated the plasmid either by double cross over or by single cross over at the 5' end (Figure 1). To select parasites in which a double cross over occurred, single-cell cloning was employed and the lines were checked for their integration status by Southern blot hybridization (Figure 1b). Based on this screen, all subsequent experiments were performed with the two PDE1 knockout lines A6 and/or G5 that showed identical patterns in Southern blots (Figure 1b).


a)
TK
3.7kb
S
D
hdhfr
3D7
D
PDE1
S
6.5kb
+ WR99210
+ Gan
A6/G5
D
hdhfr
S
5.5kb
double cross over
D
G8
hdhfr
D
TK
S
PDE1
S
4.3kb
5.9kb
5' single cross over

b)

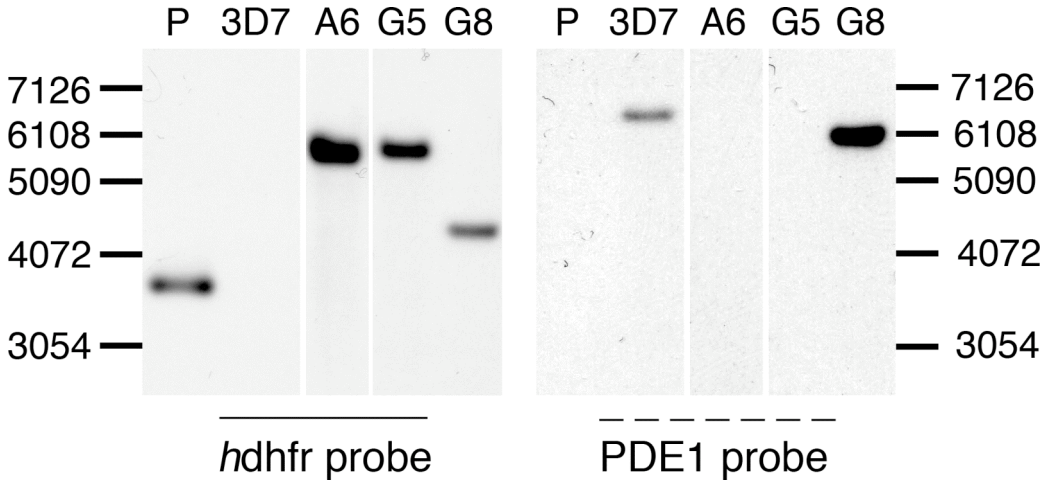

P
3D7
A6
G5
G8
P
3D7
A6
G5
G8
7126
6108
5090
4072
3054
7126
6108
5090
4072
3054
hdhfr probe
PDE1 probe

Figure 1.

Figure 1. Disruption of *Pf*PDE1 gene by double cross over.

a) Schematic representation of the 3D7 parental locus and the disrupted PDE1 loci, for the double cross over (A6/G5) as well as for the 5' single cross over event (G8). The human dihydrofolate reductase gene (*h*dhfr) conferring resistance to WR99210 has been inserted in the PDE1 locus by either double cross over, resulting in the parasite lines A6 and G5, or by 5' single cross over (G8). The herpes simplex virus thymidine kinase (tk) gene serves as a negative selectable marker. Parasites that have not integrated the plasmid (P) into the chromosome are susceptible to the guanosine analogue gancyclovir. Restriction sites, predicted fragment sizes and probe targets for Southern blot are indicated (restriction enzyme sites: D, *Drd*I; S, *Sau*96I; solid line, *h*dhfr probe; dotted line, PDE1 probe).

b) Southern blot analysis of the parental 3D7 line and the knockout lines A6 and G5, as well as the 5' single cross over line G8. DNA has been digested with *Drd*I and *Sau*96I and probed with fragments of the *h*dhfr or the PDE1 gene. Predicted sizes with the PDE1 probe: 6.5kb for 3D7; 5.9 for G8. For the *h*dhfr probe the predicted size for the plasmid (P) is 3.7kb, 5.5kb for the knockout lines A6 and G5 and 4.3 for G8. The size of the marker is given in base pairs.

Characterization of the *Pf*PDE1 knockout line by comparison of its growth rate with the 3D7 wildtype.

*Pf*PDE1 is not an essential gene in *P. falciparum*, as its deletion is not lethal to the parasite. No morphological difference between the parental 3D7 line and the knockout line was observed by microscopy of Giemsa stained thin blood smears (data not shown). Both lines developed an average of 18 merozoites per schizont and variations in merozoite-numbers between experiments within the same line were larger than between different lines (Figure 2).

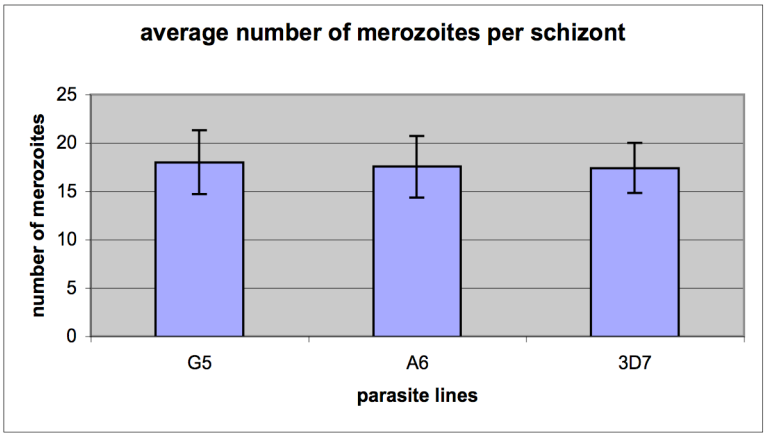


Figure 2. Average number (±SD) of merozoites per schizont.
For each parasite line the number of merozoites from 11 schizonts was counted Shown is the mean of four independent experiments.

We used FACS analysis to monitor growth rates of wildtype and knockout parasite lines over time. Parasites were stained with hydroethidine (HE) that labels the cytosol of the cell and intercalates with DNA. DNA is only present in iRBC, as RBCs do not have a nucleus. The life stages of the parasite population can be distinguished by the intensity of the HE fluorescence of the cells. Early rings are barely distinguishable from RBC, but as the parasite grows and replicates, DNA accumulates and fluorescence increases (van der Heyde et al., 1995; Jouin et al., 2004). An example for the FACS analysis is given in Figure 3.

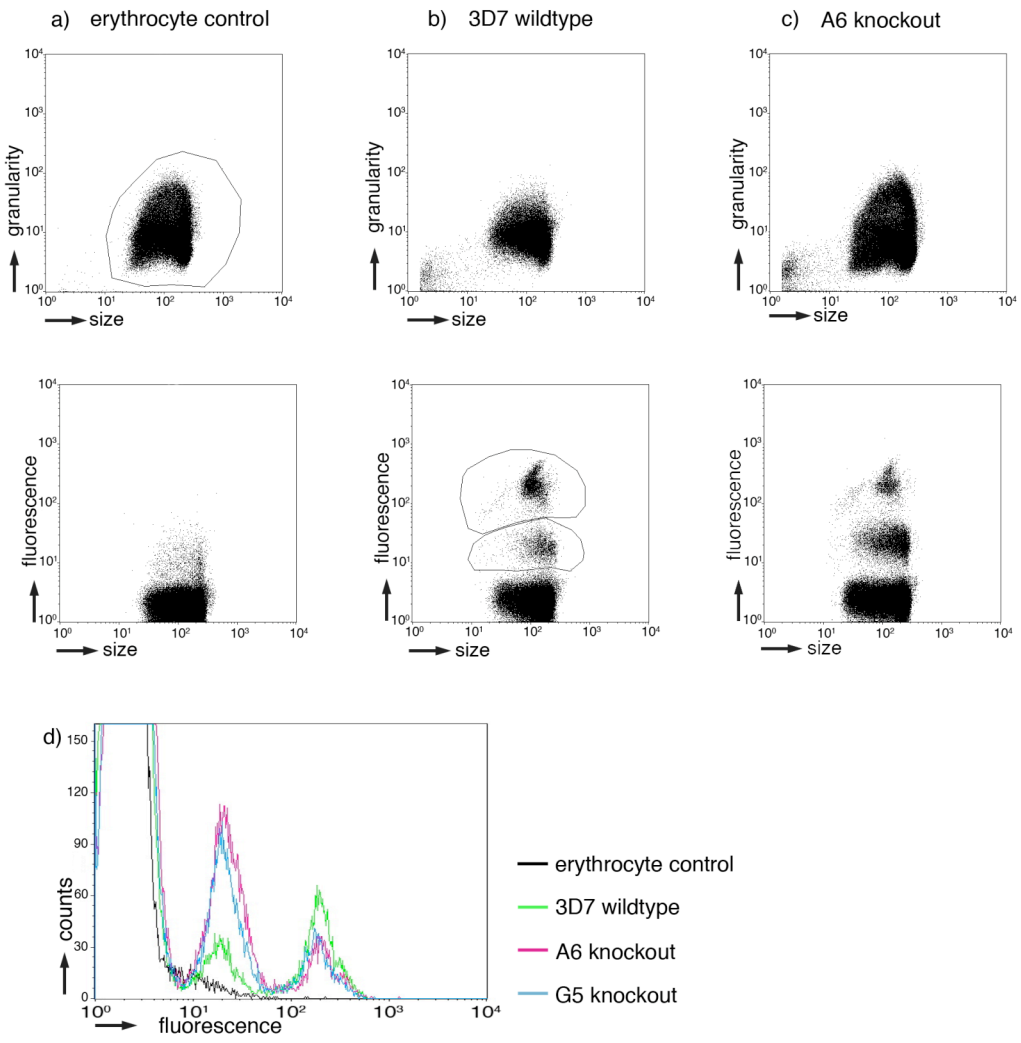
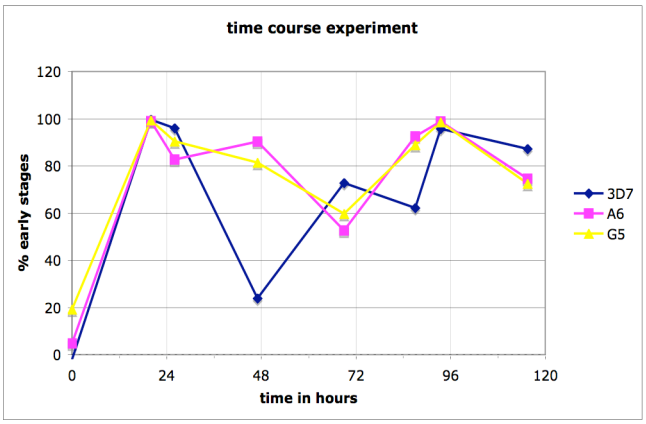


Figure 3. FACS analyses for growth rates of wildtype and knockout.
Parasites were stained with hydroethidine (HE) and analyzed by FACS technology. Shown are dot plots of a) non-infected erythrocytes, b) 3D7 wildtype strain and c) A6 knockout line. In a) to c), the x-axis corresponds to forward scatter (FSC-H) specifying the size of the cells. In the upper row, the y-axis corresponds to side scatter (SSC-H) specifying the granularity of the cells and in the lower row the y-axis corresponds to HE fluorescence (FL2-H). Cells were gated based on the first dot plot in a) excluding burst or dead RBCs. To determine the ratio of early parasite stages to late stages, the corresponding cells were gated in row two and examples for the evaluation of the statistics are shown in Figure 4 and 5. A histogram of the distribution of the fluorescent intensity for all three lines and the RBC control is shown in d).

To compare the growth rate of wildtype and knockout strains, time course experiments were conducted. Parasites were synchronized simultaneously and diluted to equal parasitaemia. Parasites were then examined at least once a day over a time period of several days and the experiment was repeated three times over a period of 4 months. We were mainly interested in the change in proportion of early stages (rings) and late stages (trophozoites and schizonts) and not in the increase of parasitaemia. In two experiments, the parasite lines differed significantly 70h after synchronization, once after 90h and one culture differed initially after 24h but not after. FACS results of parasite stages were confirmed by staining the population on Giemsa stained thin blood smears. Two knockout lines were always monitored in parallel (A6 and G5) and no difference was seen between these two lines. One experiment is shown in Figure 4. The first timepoint was taken 22h after synchronization. At 0h all three lines were late stages, at 20h all lines have become rings. At 26h the knockout lines already started to turn into late stages in contrast to the wildtype. At 47h, the wildtype line had become late stages while the knockout lines completed this life cycle already and had turned into early stages. Thus, the lifecycle of the knockout lines seemed to be shorter than the lifecycle of the wildtype.

Figure. 4. Percentage of early stage parasites in a FACS time course experiment.

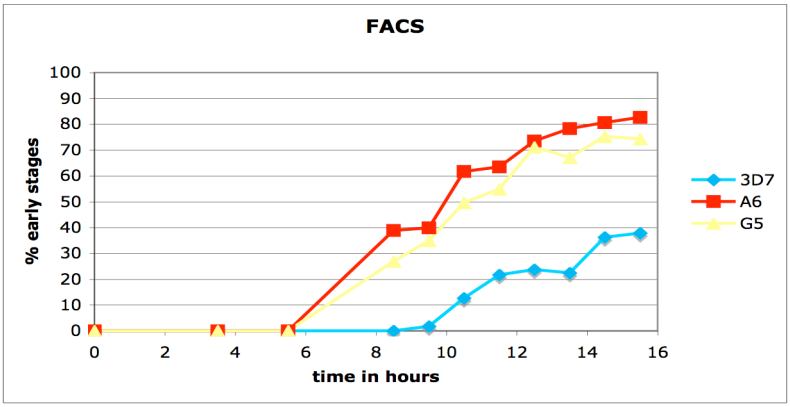


The parental parasite line 3D7 and the PDE1 knockout lines A6 and G5 have been synchronized twice (8h interval) and the first time-point was taken 22h after the last synchronization. The parasites lines were tested over the next 120h by FACS analysis and time-points o sampling are indicated. A background resulting from uninfected erythrocytes has been subtracted from the early stages. The main difference can be seen at time point 47h, i.e. 69h post synchronization.

Although a difference between the cycle durations of the parasite lines was detected, it was not possible to determine the exact length of the parasite cycles by long-time FACS analysis because we could not reproduce the results obtained for each experiment. To estimate the difference in the duration of the lifecycles between the wildtype and the knockout strains, a growth-assay was performed where parasites were monitored every hour. At the same time it was tested whether FACS analysis is less sensitive than microscopy by applying both methods in parallel. Parasites were synchronized twice and 17h from the last synchronization the monitoring started. The results are shown in Figure 5. A clear difference can be seen between the wildtype and the knockout parasites regardless of the technique applied. Both knockout strains finished their lifecycle about four hours earlier and started to invade new RBC before the wildtype parasites.

In microscopy, ring stage parasites could be detected at the first time point in slides of all three parasite lines. The first discrepancy between the wildtype and the knockout lines could be seen at 5.5h using microscopy while in FACS analysis a difference could be seen only at 8.5h (Figure 5). After this initial discrepancy, both techniques showed a similar pattern in detecting ring stage parasites. Both techniques showed that the knockout parasites were about four hours in advance of the wildtype parasites. However, it must be noted that the differences obtained in two other experiments were only two hours and even no difference between the strains (Data not shown).

A



B

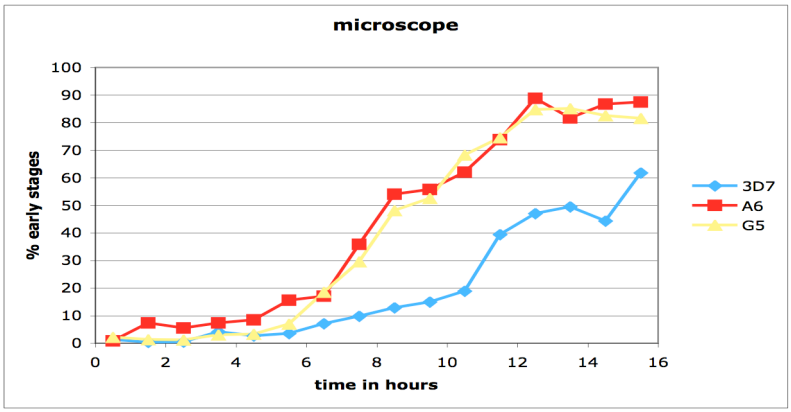
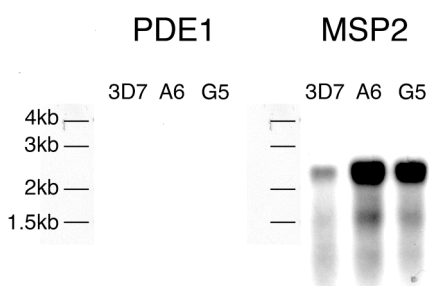


Figure 5. Increase of early stages in a time course experiment.
Parasite populations have been synchronized twice (8h interval) and the first timepoint was taken 17h post synchronization. 3D7 is the parental line and A6 and G5 are PDE1 knockout lines. Shown is the increase of early stages for FACS analysis (A) and for microscopy (B).

Comparison of PDE mRNA levels between wildtype and knockout parasites

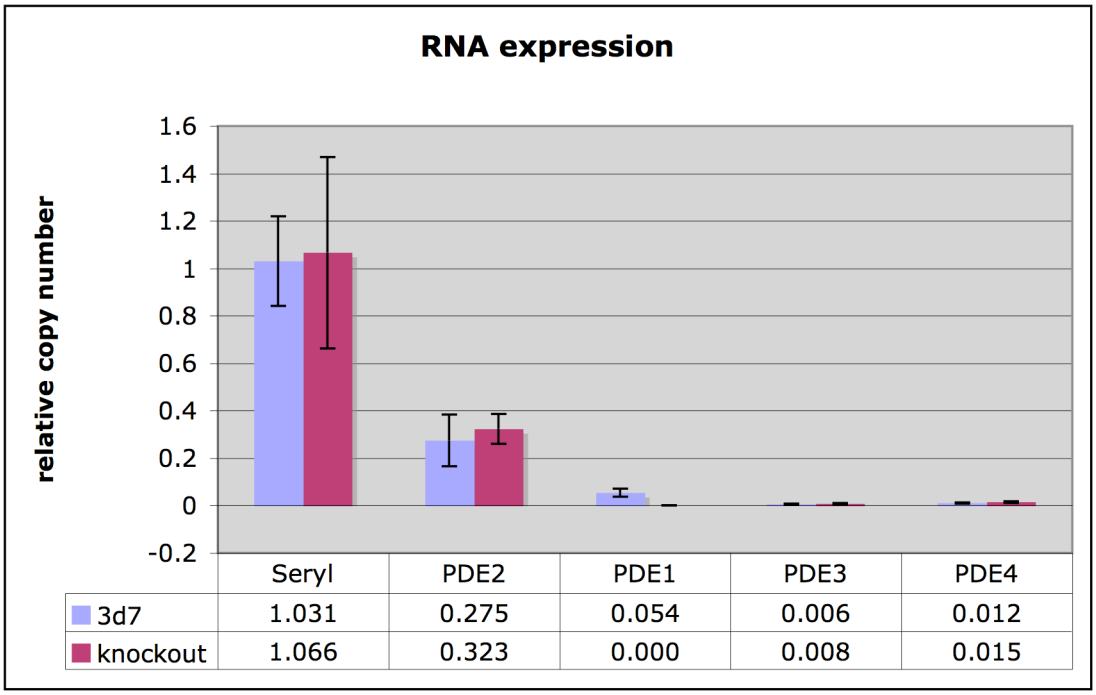
Four PDEs have been predicted for *P. falciparum* (Bahl et al., 2003) with stage specific expression patterns for each PDE. PDE3 and PDE4 are predicted to be almost exclusively expressed in gametocytes, PDE1 is thought to be expressed in late asexual bloodstage forms as well as in gametocytes and sporozoites, and PDE2 shows highest expression levels in asexual bloodstage forms but also some low level expression in gametocytes and sporozoites (Le Roch et al., 2003). Northern blot analysis of late bloodstage parasites with specific probes for each PDE did not give any signal, probably due to very low expression levels also observed by (Le Roch et al., 2003). One example is shown in Figure 6. Because it might be possible that one of the remaining three PDEs would compensate the function of the deleted PDE, we isolated RNA for quantitative RT-PCR assays of all PDEs. We isolated late stage RNA where PDE1 expression is highest during the asexual bloodstage cycle. The isolated RNA was reverse transcribed into cDNA and quantitative RT-PCR was performed with four primer pairs specific for each PDE and two additional primer pairs for the seryl-tRNA synthetase gene as a control. For comparison of the different RNA levels, the relative copy number for each gene was established. Individual CT values were subtracted from the CT value obtained with the P90 primer pair for the seryl-tRNA synthetase gene. ΔCTs were then converted to relative copy numbers with the formula $2^{\Delta Ct}$.

Figure 6. Northern blot analysis of *Pf*PDE1 transcripts.



RNA from late stage *P. falciparum* cultures (3D7 wildtype and A6 and G5 knockout lines) was probed with either phosphodiesterase 1 (PDE1) or merozoite surface protein 2 (MSP2). The observed band for MSP2 RNA corresponds to the expected size (Kyes et al., 2002).

The RT-PCR results for the 3D7 wildtype strain confirmed the data from (Le Roch et al., 2003). PDE2 was expressed three-times less than the seryl-tRNA synthetase control gene, PDE1 was already expressed 20 times less and the other two PDEs were barely detectable (100-200 times less expression) (Figure 7). As expected, PDE1 was not detected in the knockout. There was no marked difference in expression for the three remaining PDEs between the wildtype and the knockout strain.



	Seryl	PDE2	PDE1	PDE3	PDE4
3d7	1.031	0.275	0.054	0.006	0.012
knockout	1.066	0.323	0.000	0.008	0.015

Figure 7. Relative RNA levels of the four PDEs for wildtype and knockout *P. falciparum* parasites.

Wildtype 3D7 parasites are indicated in blue and knockout parasites in red. The relative copy numbers are in relation to the expression of the seryl-tRNA synthetase gene. Shown is the mean of two experiments (±SD) with three dilution series each.

Abbreviations: KO, knockout; PDE, phosphodiesterase; Seryl, seryl-tRNA synthetase

Growth inhibition assay with PDE inhibitors

Inhibitors for mammalian PDEs are often very specific for a subclass of PDEs (Hidaka and Endo, 1984; Nicholson et al., 1991) and are thus commonly used as drugs (reviewed in Lugnier, 2006). Different PDE inhibitors and cyclic nucleotide analogues were tested on the knockout clones and NF54, the parental isolate of 3D7 in parallel to identify possible inhibitors of *Pf*PDE1. The experiment was run in duplicates to establish IC_{50} values and repeated once. *Pf*PDE1 is considered to be cGMP substrate specific (Yuasa et al., 2005). Zaparianst is a cell-permeable, selective inhibitor of cGMP-specific PDE in humans (PDE V; IC_{50} of 450nM, PDE IX: IC_{50} of 35μM). Zaparinast did not inhibit growth of the parasites at a starting concentration of 50μM, (data not shown). This is in contrast to results obtained by Yuasa, who found zaparinast to be an effective antagonist of the recombinant *Pf*PDE1 enzyme *in vitro* (IC_{50} of 3.8 ± 0.23μM) and endogenous cGMP PDE activity in *P. falciparum in vivo* (IC_{50} of 35 ± 4.2μM) (Yuasa et al., 2005). Knockout parasites were less tolerant to cAMP analogues (IC_{50} = 10μM for 8Br cAMP, and IC_{50} = 35μM for 4- chlorophenylthio (cpt) cAMP) than the wildtype parasites (IC_{50} = 19μM for 8Br cAMP, and IC_{50} = 49μM for cpt cAMP) (Fig. 8). Although the differences between knockout and wildtype parasites were significant (two tailed unpaired students t-test: p=0.013 for 8Br cAMP and p=0.0036 for cpt cAMP) the statistical relevance is neglectable due to the small sample size. For the cGMP analogues we observed the opposite. Wildtype parasites were slightly less tolerant to cGMP analogues (IC_{50} = 9.5μM for 8Br cGMP, and IC_{50} = 22μM for cpt cGMP) than the knockout parasites (IC_{50} = 16μM for 8Br cGMP, and IC_{50} = 28μM for cpt cGMP). The IC_{50} for the 8Br cGMP from the wildtype parasites is in agreement with observations from Yuasa, who estimated the IC_{50} to be less than 10μM (Yuasa et al., 2005). BG-1 inhibits parasite growth equally in all three strains with an IC_{50} of 15μM. BG-1 is a potential cAMP specific PDE inhibitor of PDE4 in humans (IC_{50} = 0.4nM) and PDE2 in *T. brucei* (IC_{50} = 4nM) where it also inhibits bloodstream growth (IC_{50} = 30nM) (personal communication L. Wentzinger)

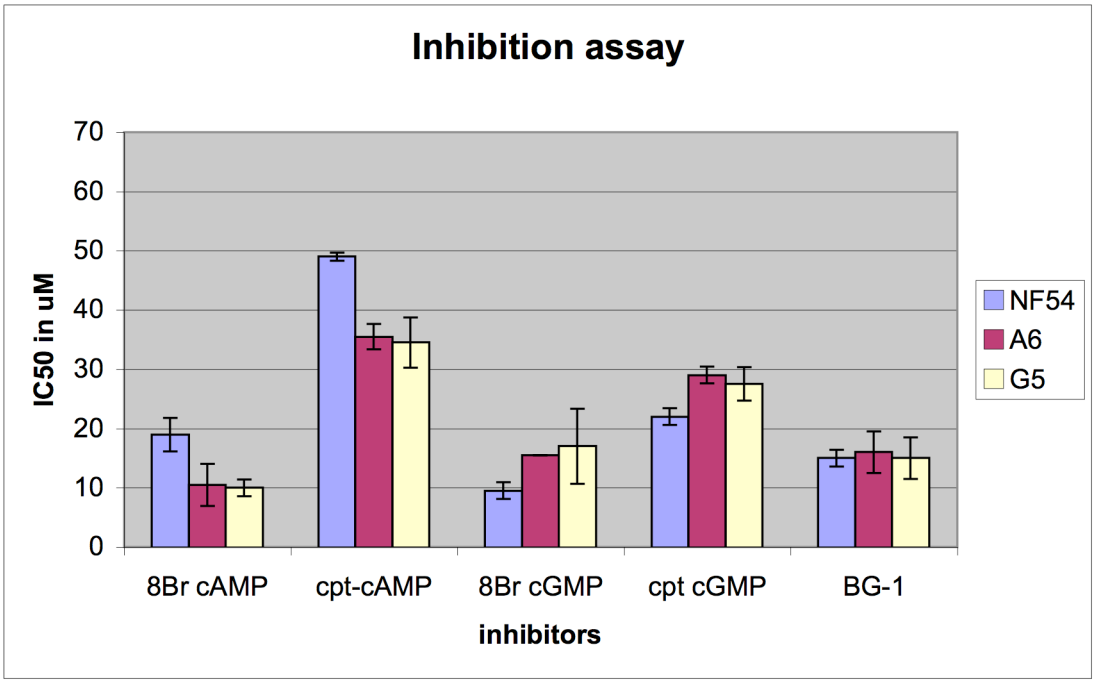


Figure 8. Growth inhibition assay with PDE inhibitors and cyclic nucleotide analogues.

Inhibitors are indicated at the bottom, blue bars indicate the NF54 strain, red and yellow bars indicate the *Pf*PDE1 knockout lines A6 and G5. IC_{50} values have been calculated from two dilution series. Shown is an IC_{50} average of two replicates (±SD) with inhibition concentrations starting at 50μM. IC_{50} values are given in μM on the y-axis. Differences between knockout and wildtype parasites were significant in a two-tailed unpaired students t-test for following inhibitors: 8Br cAMP (p=0.013), 8Br cGMP (p=0.0361) and cpt cAMP (p=0.0036)

Localization of the *Pf*PDE1 in the parasite

In silico analysis of *Pf*PDE1 predicted six transmembrane α-helices (Bahl et al., 2003), suggesting that it is a transmembrane protein. In order to localize *Pf*PDE1 in the parasite, L. Wentzinger designed GFP fusion plasmids to express *Pf*PDE1-GFP hybrids episomally but stable in the parasite. In one construct the catalytic domain where no signal sequences are expected, was replaced by GFP (termed 610). In a second construct the catalytic domain and 150 amino acids in addition were replaced by GFP (termed 460). A control plasmid (pARLmGFPmT) that expresses GFP alone, i.e. with no signal sequence or transmembrane domains derived from *Pf*PDE1, was also transfected (termed GFP). All three transfected parasite lines could be established but only in the GFP control line green fluorescent parasites could be detected in immuno fluorescence assays (IFAs) using anti-GFP antibodies (data not shown). Fluorescence microscopy of non-fixed cells without antibody labeling has been used to examine the location of the GFP protein in the control line at different stages of growth (Figure 9). In early ring to early trophozoite stages GFP is equally distributed throughout the parasite cytosol (Figure 9 a to d). In late trophozoites and schizonts a structure around the newly synthesized nuclei can be seen (Figure 9 e to h). Not all parasites in the population do show fluorescence even though all must have the plasmid. Fluorescent and non-fluorescent parasites can invade the same RBC simultaneously (Figure 9b).

Figure 9. Expression of GFP at different stages of the intraerythrocytic cycle of *P. falciparum*.
Shown are differential interference contrast (DIC) images, DAPI staining of the nucleus, green fluorescent protein (GFP), and an overlay of all signals. The parasite matures from top to bottom, from ring stages in a) to schizonts in h). The intensities of the images were adjusted to optimize the fluorescence signal at each parasite stage.

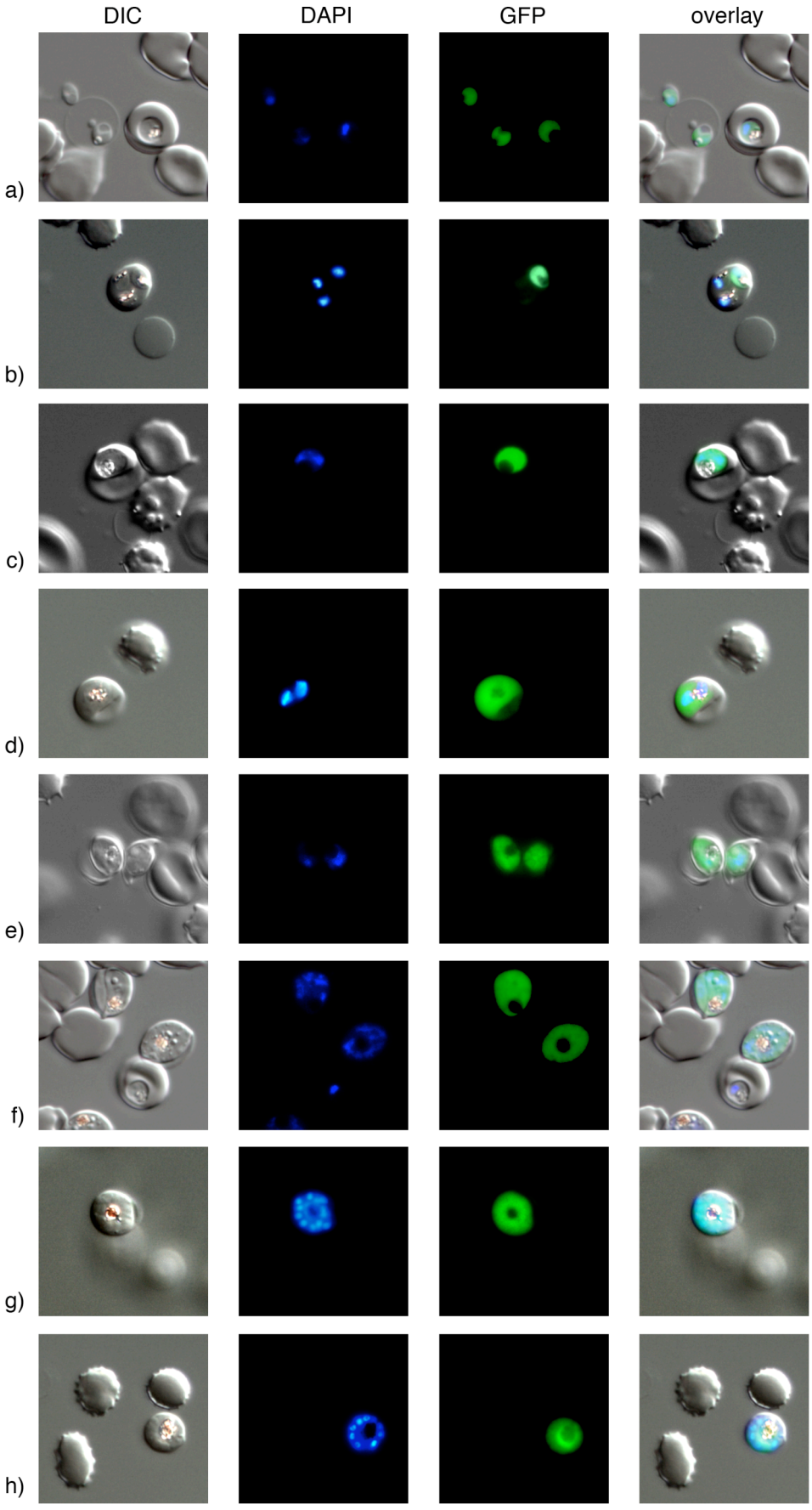


Figure 9.

To determine if the transfected but non-fluorescent parasite lines do express GFP at low levels that could not be detected in IFAs, Western blot analysis was performed. In Western blot with parasite lysate from the three different transfected lines, αGFP antibodies could only detect a protein in the lysate from parasites transfected with the control plasmid (Figure 10). The detected protein is 27kDa in size as expected for the GFP tag alone. A loading control with a mouse αMAHRP1c antiserum (Spycher et al., 2003) detected equal amounts in all three lysates of a protein of approximately 40kDa, corresponding to MAHRP1. Thus, the two fusion proteins (610 and 460) were not expressed in the transfected *P. falciparum* lines. Closer examination of the cloning strategy applied revealed that the promoter was deleted during the cloning procedure.

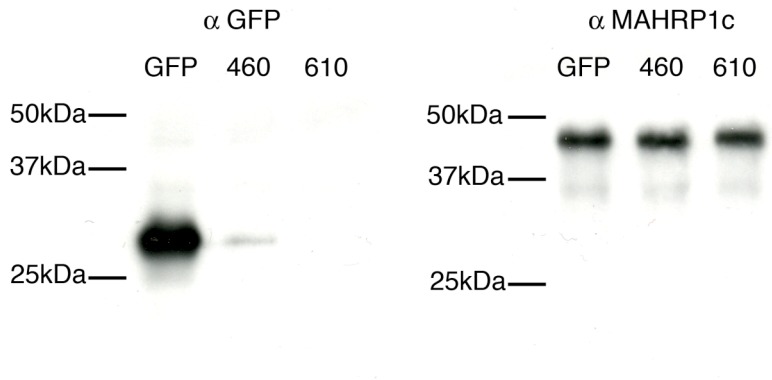


Figure 10. Western blot analysis of parasite proteins showing expression of *Pf*PDE1-GFP chimeras.

Total protein extracts from *P. falciparum* 3D7 transfected parasite lines were subjected to Western blot analysis and probed with α GFP (left) or α MAHRP1c mouse serum (right). The faint band in the 460 line probed with α GFP is probably due to some carryover from the GFP line as the size corresponds to the GFP alone and a bigger band would be expected for the fusion protein.

DISCUSSION

Although many chemotherapeutics against *P. falciparum* and other *Plasmodii* are on the market, resistance to almost all of them has already arisen and the need for new chemotherapeutics is therefore urgent. The aim of this study was to characterize one of the four PDEs postulated for *P. falciparum* to evaluate its potential as an antimalarial chemotherapeutic and gain more insight into the signaling process. For PDE it was already shown that PDE inhibitors could act on PDE2 in *T. brucei*. Therefore, in collaboration with T. Seebeck from the University of Bern we investigated PDEs in *P. falciparum* by generating knockout parasites. Initially we attempted to disrupt *Pf*PDE4 as well as *Pf*PDE1 but abandoned the project because the group of D. Baker already disrupted *Pf*PDE4 and *Pf*PDE3.

Growth rate of asexual blood stage parasites

*Pf*PDE1 is not an essential gene in *P. falciparum* asexual blood stage forms, as its deletion is not lethal to the parasite in culture. *Pf*PDE1 knockout parasites showed no morphological differences to the wildtype 3D7 strain by microscopy of Giemsa stained thin blood smears. The loss of the cGMP specific *Pf*PDE1 does not impede the knockout strains, as its growth rate occurs to be higher than in the wildtype strain. Faster growth of the knockout strains is not due to higher multiplication rate, as single parasites of both strains produced comparable numbers of merozoites per schizont. The different number of merozoites per schizont was probably more influenced by the quality of media and blood, since merozoite numbers increased and decreased in the knockout and the wildtype in parallel over time.

The growth advantage of the knockout parasites compared to the wildtype parasites was noted during preparation of synchronized parasite cultures for stage specific RNA extraction. Determination of the exact cycle-length of the knockout as well as for the wildtype parasites failed. Repetition of experiments was not coherent although they mostly showed a trend of the knockout strain to grow faster than the wildtype strain. One reason for the different outcomes

might be the synchronization of the populations. Synchronization with saponin kills parasites older than 20 to 24 hours post infection. To get a good synchronization the parasites must be treated twice with saponin, the bigger the interval between synchronizations the tighter the synchronization of the population. Parasite populations are not homogenous before synchronization in terms of proportions of different stages (rings, trophozoites and schizonts) and even synchronized populations are never absolutely homogenous. Additionally, not every parasite in the population behaves exactly the same way in terms of its cycle-length. In the time course experiment analyzed by microscopy, the majority of the knockout populations showed a difference in cycle-length of four hours compared to the wildtype population (Figure 5). However, the first rings appeared at the earliest time-point taken for wildtype as well as knockout parasites, indicating that single parasites do have the same cycle-length in both lines. In order to determine the exact cycle-length of a strain, the synchronization method must be optimized. Magnetic cell separation (MACS) allows for selection of trophozoites and schizonts due to accumulation of haemozoin in the developing parasite. When schizonts burst there are no new RBCs to invade and merozoites can be isolated. Merozoites of different strains can thus be added to fresh RBCs at the same time and the development of the parasites can be monitored by microscopy. Another approach might be time-lapse experiments where single cells can be monitored over time.

Longer time course experiments have been performed by FACS analysis, which is a convenient method to estimate parasitaemia because it is possible to count a large number of cells in a short time. In addition, intercalation of HE with DNA allows differentiation and gating of early stages from late stages by their DNA content. Because almost 0.5% of uninfected RBCs is auto-fluorescent and appears in the gate for the early stages, the parasitaemia of the examined parasite population needs to be above 0.5% or must consist of late stages only (Figure 3).

FACS analysis is a suitable method to compare growth rates or monitor the ratio of late stages to early stages between different parasite lines over a long time-period. The lag in detecting young rings is the same for all populations and thus neglectable. FACS analysis is not a suitable method to monitor the

length of a single parasite lifecycle and catch the transition from schizonts to ring stages, as there will be a delay until ring stages can be detected. Although an overall decrease in the older stages was detectable, it did not become visible in the ratio between early and late stages because the early stages were not detectable. FACS analysis might be improved by additional staining of iRBCs with labeled antibodies against *P. falciparum* to reduce the background of uninfected RBCs and become more sensitive.

Biochemical effects of deletion of *Pf*PDE1

The knockout parasite showed evidence of a shorter lifecycle causing faster growth. Why would the loss of a PDE increase growth? The acceleration of growth observed in the knockout line compared to the wildtype line might simply be an artifact. If the growth rate would be truly increased the shortening of the lifecycle must be caused by reduction of any of the four phases of the cell cycle (G1, S, G2 and M).

*Pf*PDE1 is cGMP substrate specific. If *Pf*PDE1 is deleted cGMP might be accumulated in the cell. cGMP levels have not been measured in this thesis, and hence there is no evidence for increased cGMP levels and all of the following assumptions are speculative.

Increase in cGMP levels in the cell is either associated with a delay of the G1/S transition in the cell cycle via inhibition of cyclines in vascular smooth muscle cells (Fukumoto et al., 1999) or delay in mitosis in Novikoff hepatoma cells (Zeilig and Goldberg, 1977) arguing for a delay rather than a shortening of the lifecycle. On the other hand, cGMP is likely to be a secondary messenger molecule for autocrine signal molecules in the regulation of cell survival and promotion of proliferation in *Tetrahymena thermophilia* (Christensen et al., 1996). The role of cyclic nucleotides in the cell cycle of *Plasmodium* has not been investigated so far.

An increase in cGMP might also lead to an increase in protein kinase G (PKG) expression. PKG is involved in motility and invasion in *Toxoplasma* and *Eimeria* (Wiersma et al., 2004) and the PKG inhibitor staurosporine prevents invasion of RBCs by *P. falciparum* (Miller et al., 1994; Ward et al., 2004).

Invasion of RBCs is a very fast event and accelerated invasion by the knockout parasites is not very likely to account for a faster live cycle.
The parasite might prevent an accumulation of cGMP by reducing the number of guanylate cyclases (GCs) or another *Pf*PDE might be involved in the regulation of cGMP levels in the parasite. The most likely candidate to take over *Pf*PDE1 expression would be *Pf*PDE2 which is highest expressed in asexual blood-stage parasites in contrast to *Pf*PDE3 and *Pf*PDE4 that are only expressed in gametocytes. No such rescue mechanism has been observed, as quantitative RT PCR with cDNA revealed no differences in PDE RNA expression between the knockout and the wildtype parasite.
Experiments revealed a 20% reduction in the cGMP hydrolytic activity of membrane fractions from knockout parasites compared to wildtype parasites (L. Wenzinger, personal communication). This suggests that *Pf*PDE1 is responsible for 20% of the cGMP activity in the parasite even though its expression is very low. Another PDE, possibly *Pf*PDE2, hydrolyses the major part of cGMP in blood-stage parasites. It has to be noted that the mRNA levels do not necessarily correlate with the protein levels observed.

Localization of PDEs in *P. falciparum*

The localization of any of the plasmodial PDEs is unknown. There is strong evidence that *Pf*PDE1 is a membrane protein. Three independent groups have predicted transmembrane domains although there are discrepancies in the number of predicted domains. The reason for the discrepancies lies probably in the identification of various splice variants of *Pf*PDE1 and the use of different prediction programs by the various groups (Bahl et al., 2003; Yuasa et al., 2005; Wentzinger, unpublished data).
Signaling cascades are possible at different locations in the parasite as a stimulus from the outside would have to travel all the way to the nucleus in mammalian cells where transcription is regulated. In the case of *P. falciparum*, the signal must thus be transported from the RBC membrane through the RBC cytosol, traverse the parasitophorous vacuole thereby crossing two membranes and pass through the plasmodial cytosol to reach the nucleus by traversing another membrane. Many PDEs show distinct cellular or subcellular

distribution in the human host (Bender and Beavo, 2006) and *Pf*PDE1 could be localized at any of the previously mentioned membranes. Taking the presence of different splice variants into account, *Pf*PDE1 could even be localized in different membranes, depending on the isoform.
The localization of *Pf*PDE1 in the parasite could not be evaluated. IFAs with anti-*Pf*PDE1 antibodies could not be performed, as antibody production against a recombinant *Pf*PDE1 failed. In a second approach, we fused the N terminal *Pf*PDE1 sequence to a GFP tag and expressed the plasmids episomally in the parasite. No recombinant protein was expressed because the promoter driving expression of the fusion protein was deleted during the cloning procedure.

*Pf*PDE1 as drug target

To evaluate the potential for PDEs as drug targets in *P. falciparum*, different known PDE inhibitors were tested for their impact on growth of wildtype and knockout parasites. Although significant differences between the knockout and the wildtype parasites were identified, the relevance of these findings is minor as the experiments were only repeated once. To get statistical relevant results, the experiments would need to be repeated more often. Generally, the activity of these inhibitors was rather weak compared to known antimalarials such as chloroquine (IC_{50} = 9nM) or artemisin (IC_{50} = 2nM). Surprisingly, zaparinast did not inhibit parasite growth in contrast to results of Yuasa et al., (2005) who found an IC_{50} of 35 ± 4.2μM. Differences might be due to the methods applied. We measured the incorporation of ^{3}H-hypoxanthine into the growing parasites while the group of Yuasa monitored parasite growth by counting 1000 RBCs in Giemsa stained thin blood smears. Knockout parasites were a little more tolerant to addition of cGMP analogues. This might be due to the fact that cGMP levels are already increased in the knockout parasites and the parasites are more used to this state. On the other hand the knockout parasites were slightly more sensitive to cAMP analogues. The fact that only minor differences between knockout and wildtype parasites were detected might imply that none of the tested inhibitors works on *Pf*PDE1 alone.

Generally it has to be noted that the expression levels of *Pf*PDE1 are very low in the wildtype parasite and that it is hence questionable if *Pf*PDE1 was the right choice to be deleted.

Role of PfPDE1 in sexual parasite stages

With the creation of a *Pf*PDE1 knockout we have generated a very useful tool to study the function of *Pf*PDE1 in gametocytes or sporozoites, as *Pf*PDE1 mRNA is also found in these stages. The fact that deletion of *Pf*PDE1 is possible in blood-stage parasites strengthens the hypothesis that the main function of *Pf*PDE1 is in sexual forms where cyclic nucleotide signaling has been indicated.

Addition of cAMP to parasite cultures with high parasitaemia or addition of PDE inhibitors enhances gametocyte formation indicating a role for cAMP signaling in gametogenesis but not for cGMP (Kaushal et al., 1980; Brockelman, 1982; Trager and Gill, 1989). Yet, the fact that *Pf*PDE1 is cGMP specific reduces the chances that it plays a role in gametogenesis.

Studies with PDE inhibitors have suggested a role for the cGMP signaling pathway in exflagellation (Martin et al., 1978; Kawamoto et al., 1990; Kawamoto et al., 1993). This process occurs in the mosquito midgut when eight flagellated male gametes emerge from a single infected cell. A role of the cGMP signaling pathway in exflagellation is supported by investigations of the mode of action of xanthurenic acid (XA). XA is a mosquito derived factor that can trigger exflagellation (Billker et al., 1998; Garcia et al., 1998). Upon administration of XA, membrane associated GC activity increases leading to enhanced levels of cGMP (Muhia et al., 2001). A knockout of *Pf*PDE1 might therefore enhance exflagellation.

We have an ongoing collaboration with D. Baker from the London School of Tropical Medicine and Hygiene who is interested in the role of cyclic nucleotide signaling in sexual parasite stages. They are using the *Pf*PDE1 knockout strain described in this thesis to gain more information about the possible role of PDEs in formation of gametocytes and gametes and the further development in the mosquito.

General conclusions and outlook

Very likely *Pf*PDE1 plays no important role in asexual blood-stage parasites. Deletion of *Pf*PDE3 and *Pf*PDE4 seems also to be possible in blood-stage parasites (unpublished data D. Baker). Thus *Pf*PDE2 is most likely the only PDE candidate that is essential in blood-stage parasites. Its high expression in asexual blood-stage parasites strengthens this hypothesis. A deletion of *Pf*PDE2 will reveal if this PDE is essential in *P. falciparum*. If not, the role of PDEs in asexual blood-stage parasites is generally questionable.

One objective of this thesis was to evaluate the suitability of *Pf*PDE1 as a drug target. Because *Pf*PDE1 is not essential in the asexual blood-stage form of *P. falciparum,* it is not a suitable drug target to cure an individual suffering from acute malaria. If its function lies in the formation of gametocytes in humans or gametes in the mosquito, a drug against *Pf*PDE1 might reduce transmission of parasites from human to mosquito by blocking sexual reproduction of the parasite. If *Pf*PDE1 is involved in sporozoite invasion it might be used as a prophylactic drug only.

We generated a useful tool to investigate the role of cyclic nucleotides in sexual parasite stages and investigation of their role in gametogenesis is ongoing.

Although *Pf*PDE1 mRNA has been found in sporozoites (Bahl et al., 2003), nothing is known about signaling processes during this stage. The knockout parasites might thus display a defect during the development in the mosquito, in the course of invasion of hepatocytes, or during liver-stage development. Monitoring the development of these knockout parasites in the mosquito or if possible in hepatocytes might give some insight to cGMP signaling in those stages.

The role of *Pf*PDE1 in the cGMP signaling pathway might further be evaluated by comparing mRNA levels from kinases and cyclases between the knockout and wildtype parasites in different stages.

Chapter 5: General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

Two examples for the use of transfection technology in *Plasmodium falciparum* have been shown in this PhD thesis. In the first part transfection was used to investigate the regulation mechanism of *P. falciparum var* genes by generating transgenic parasites harboring constructs with different *var* gene upstream regions driving luciferase expression. In the second part we generated a Phosphodiesterase 1 (PDE1) knockout parasite and characterized the resulting phenotype.

Although there is no obvious linkage between the two projects it could be speculated that both proteins are implicated in intracellular signaling. *Pf*PDE1 is involved in the cGMP-signaling pathway and *Pf*EMP1 is located in the erythrocyte membrane where it mediates binding to various host cell receptors (summarized in Table 1 of the general introduction). It might be speculated that *Pf*EMP1 is not only mediating cytoadherence and sequestration but it might also serve as a signaling receptor. A potential signal might proceed via the cGMP-signaling pathway and might trigger a switch in *var* gene expression.

Another connection between PDEs and *Pf*EMP1 is their role as a potential drug target or vaccine candidate, both urgently needed for the struggle against malaria. Although it has been shown that PDEs are potential drug targets in Trypanosomes (Zoraghi et al., 2001; D'Angelo et al., 2004; Kunz et al., 2005; Alonso et al., 2006; Diaz-Benjumea et al., 2006), we excluded *Pf*PDE1 from the list of useful targets in *P. falciparum* by the simple fact that our knockout parasite line had the same phenotype and even a higher growth rate than wildtype parasites. It remains to be analyzed if another PDE is a better drug target in *P. falciparum*.

Sera from malaria endemic areas often recognize *Pf*EMP1 and its variants have been associated with either severe or uncomplicated malaria (Kaestli et al., 2004). The fact that *Pf*EMP1 variants of the severe subtype tend to be more immunogenic and to be better recognized than those of the uncomplicated subtype proposes that these *Pf*EMP1 molecules are promising

vaccine candidates potentially able to generate protective immunity against severe disease (Moll et al., 2007). Once *var* gene regulation is understood it might be possible to prevent *var* gene expression or to disrupt the switching mechanism. If only one variant of *Pf*EMP1 could be expressed by a parasite the immune system would have a better chance to produce protective antibodies.

Once cloning of transfection constructs in *E. coli* succeeded, transfection of plasmids and creation of stable transfected parasite lines that harbor the plasmids episomally was successful throughout and took three to eight weeks. Ablation of the *Pf*PDE1 gene took one year from transfection of the plasmid to the generation of a clonal population derived from a single ancestor. Transfection event as such is a fast and reliable method to transfer a plasmid into a parasite but the investments to grow and handle the parasites are immense.

During its lifecycle, *P. falciparum* infects two different hosts and invades various cell types. The parasite has adapted to these numerous surroundings by changing from one life stage to another. In our laboratory we only have the expertise to study the asexual blood stage cycle of the parasite. This 48h cycle consists of the invasive merozoite, the ring, the trophozoite and the schizont stage. In order to compare results from different parasite lines the populations need to be highly synchronous and at the same stage. Consequently, preparation of the parasites for experiments must either be conducted in parallel or consistency of parasite handling has to be guaranteed. The cell cycle of the 3D7 wildtype parasite is not always consistent when cultured *in vitro*. Addition of different plasmids and selection pressure may change cycle duration additionally, making it difficult to treat different parasite lines identically over time. During the cultivation process, parasite lines have time to mutate. Loss of parts of chromosome 9 has been observed *in vitro* in isolates and clones of *P. falciparum* (Shirley et al., 1990). During our studies we observed a deletion in the *var* intron of one construct, and integration of one plasmid into the chromosome. Parasite populations are thus not static but are capable of changing. Transfected parasite lines have hence to be regularly monitored for changes that might influence the outcome of experiments.

Seeing that the establishment of a transgenic parasite line is very labor intensive it is important to thoroughly evaluate targets and carefully design constructs. This was not done before generating the PDE1 knockout parasites since there was no apparent phenotype to be expected in asexual blood stage parasites, as expression levels of *Pf*PDE1 are marginal in blood stage parasites. A possible role of PDE1 would rather be probable in sexual blood stage parasites or in the mosquito stages where expression levels are higher. Moreover, it is important to verify the integrity of constructs before transfection of parasites. The GFP fusion construct designed to study localization of *Pf*PDE1 was not properly analyzed before transfection and subsequently found to lack a promoter driving GFP expression.

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Education

1997 - 2003	University of Basel, Switzerland, major: molecular parasitology
2000	**Practical training** in insect ecology: The influence of both male and female mosquito size of *Anopheles gambiae* on mating success. ICIPE (International Center of Insect Physiology and Ecology) Malaria Vector Program, Mbita Point Research and Training Centre, Kenya. Supervisor: Dr. Bart Knols
2002-2003	**Diploma** in molecular parasitology: Silencing in *Plasmodium falciparum var* genes Molecular Parasitology, Swiss Tropical Institute, Basel, Switzerland. Supervisor: Prof. Dr. Hans Peter Beck
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2002 Annual Congress of the Swiss Society of tropical Medicine and Parasitology (SSTMP), Bern, Switzerland
Talk: Silencing of *Plasmodium falciparum var* genes.

2003 PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Munchenwiler, Switzerland.
Talk: Silencing of *Plasmodium falciparum var* genes.

2004 Interaction seminar of the evolutionary biology group, Basel, Switzerland
Talk: Progress report of my PhD thesis.

2004 Joint meeting of the 'Deutsche Gesellschaft für Tropenmedizin und Internationale Gesundheit' and the Swiss Society of Tropical Medicine and Parasitology, Wurzburg, Germany.
Talk: Silencing of *Plasmodium falciparum var* genes.

2005 Molecular Parasitology Meeting IX, Woods Hole, USA.
Poster: Silencing in *Plasmodium falciparum var* genes - searching for an interaction site for the *var* intron in the *var* upstream region.

2005 PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Ascona, Switzerland.
Talk: Silencing in *Plasmodium falciparum var* genes - searching for an interaction site for the *var* intron in the *var* upstream region.

2005 Annual Congress of the Swiss Society of tropical Medicine and Parasitology (SSTMP), Ascona, Switzerland
Poster: Silencing in *Plasmodium falciparum var* genes - searching for an interaction site for the *var* intron in the *var* upstream region.

2006 Annual workshop of the COST-European Cooperation in the field of Scientific and Technical Research, Dresden, Germany.
Talk: Silencing in *Plasmodium falciparum var* genes - searching for an interaction site for the *var* intron in the *var* upstream region.

2006 Research seminar, Swiss Tropical Institute, Basel, Switzerland.
Talk: Silencing in *Plasmodium falciparum var* genes - searching for an interaction site for the *var* intron in the *var* upstream region

Publications

2003 **Voss TS, Kaestli M, Vogel D, Bopp S, Beck HP.**
Identification of nuclear proteins that interact differentially with *Plasmodium falciparum var* gene promoters
Mol Microbiol. 2003 Jun; 48(6): 1593-607.

2007 **Bopp S, Wentzinger L, Beck HP, Seebeck T.**
Characterization of a phosphodiesterase gene deletion in *Plasmodium falciparum*.
Manuscript in preparation.

2007 **Bopp S, Dzikowsky R, Deitsch K, Beck HP.**
Identification of *var* gene upstream regulation sites
Manuscript in preparation

Laboratory Skills

Designing and cloning of expression vectors in bacteria and *Plasmodium*
Basic immunoassays (Northern, Southern and Western blotting, IFA)
FACS analysis
Luciferase assay
Cultivation and transfection of *P. falciparum* blood-stage forms

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Appendix: Identification of nuclear proteins that interact differentially with *Plasmodium falciparum var* gene promoters

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Identification of nuclear proteins that interact differentially with *Plasmodium falciparum var* gene promoters

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Summary

The *Plasmodium falciparum* virulence factor PfEMP1 is responsible for both antigenic variation and cytoadherence of infected erythrocytes in malaria. Approximately 50 *var* genes per parasite genome code for this highly polymorphic surface protein. We showed recently that chromosome-central and subtelomeric *var* genes are controlled by different promoters. Here, we report that transcriptional repression of *var* genes located in different chromosomal regions occurs by different mechanisms. Subtelomeric *var* gene transcription is repressed 4–8 h before that of chromosome-central *var* genes. Both repression events coincide with the shifted expression of two distinct nuclear proteins binding specifically to conserved sequence motifs, *SPE1* and *CPE*, present in the respective promoter. Furthermore, a reiterated and highly conserved subtelomeric *var* promoter element (*SPE2*) interacts with a nuclear factor exclusively expressed during S-phase. Promoter analysis by transient transfection suggested direct involvement of these interactions in *var* gene repression and silencing, and identified regions implicated in transcriptional activation of *var* genes.

Introduction

Cytoadherence of parasite-infected red blood cells (iRBCs) to host endothelial cells constitutes a major virulence determinant in *Plasmodium falciparum* malaria. This adhesion is mediated through a large parasite-encoded antigen, termed *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is deposited on the surface of infected erythrocytes during the advanced intraerythrocytic cycle (Leech *et al.*, 1984; Biggs *et al.*, 1991; Roberts *et al.*, 1992). PfEMP1-mediated binding of iRBCs to endothelial cells occurs via host surface receptors such as ICAM-1, CD36 and CSA (Baruch *et al.*, 1996; Gardner *et al.*, 1996; Reeder *et al.*, 1999). As a consequence, iRBCs sequester in microvasculatory capillaries of various organs and thus contribute to the severe morbidity and mortality associated with malaria tropica (Pongponratn *et al.*, 1991; Berendt *et al.*, 1994). Sequestration in the brain is involved in cerebral malaria with frequent fatal outcomes (Turner *et al.*, 1994; Newbold *et al.*, 1997), whereas binding to CSA and hyaluronic acid in the placenta poses a risk to both pregnant women and the foetus (Fried and Duffy, 1996; Beeson *et al.*, 2000). PfEMP1 has also been shown to downregulate dendritic cell function (Urban *et al.*, 1999) and to be involved in rosetting (Rowe *et al.*, 1997; Chen *et al.*, 1998a), another determinant of disease severity. Owing to its exposure on the surface of iRBCs, PfEMP1 is recognized by variant-specific antibodies, and these play a pivotal role in protection from clinical disease (Bull *et al.*, 1998). However, antigenic variation of PfEMP1 allows the parasite to escape existing immune responses and, hence, to establish chronic infections.

PfEMP1 is encoded by the *var* gene family, which comprises ≈50 highly diverse genes per haploid genome (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995; Gardner *et al.*, 2002). The majority of *var* genes are located in the subtelomeric region of nearly all chromosomes, but are also found in central clusters on chromosomes 4, 6, 7, 8 and 12 (Rubio *et al.*, 1996; Fischer *et al.*, 1997; Hernandez-Rivas *et al.*, 1997; Thompson *et al.*, 1997; Gardner *et al.*, 2002). We showed recently that, despite the tremendous sequence diversity observed in *var* gene coding regions, *var* gene promoters are highly conserved and exist in two distinct forms (Voss *et al.*, 2000). With the completion of *P. falciparum* genome sequencing, a third type of *var* upstream sequence became apparent, and the three classes have been termed upsA, upsB and upsC (Gardner *et al.*, 2002). The type of promoter associated with a particular *var* gene is

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strongly correlated with its chromosomal location. *var* genes residing in internal domains are associated with the upsC (5B1-type) promoter. The most telomerically located *var* genes are almost exclusively controlled by a upsB (*var*17-type) promoter. Another subset of subtelomeric *var* genes is flanked by the upsA promoter. However, compared with the upsB type, these telomere-associated *var* genes are located towards the centromere but are transcribed towards the telomere. Episomally located upsB and upsC promoters were able to drive reporter gene expression (Deitsch *et al*., 1999; Voss *et al*., 2000), and various studies have shown that *var* genes can be transcribed from both locations (Fischer *et al*., 1997; Hernandez-Rivas *et al*., 1997; Scherf *et al*., 1998; Voss *et al*., 2000). Apparently, every *var* gene represents a single transcriptional unit capable of *in situ* activation. Nevertheless, *var* gene expression occurs in a mutually exclusive manner, i.e. only one *var* gene is actively transcribed while the remaining copies remain silenced (Chen *et al*., 1998b; Scherf *et al*., 1998). Switching in *var* gene expression takes place through *in situ* gene activation (Scherf *et al*., 1998). Mutually exclusive transcription among various expression sites has also been reported to participate in antigenic variation of the variant surface glycoproteins in *Trypanosoma brucei* (reviewed by Borst and Ulbert, 2001). However, the main mechanisms used for antigenic variation in protozoan and bacterial pathogens involve DNA rearrangements such as translocation of silenced genes into active expression sites by duplicative or reciprocal recombination (Borst and Greaves, 1987; Donelson, 1995).

Despite its importance in both parasite survival and virulence, the mechanisms involved in regulation of *var* gene expression remain largely unknown. It was shown by nuclear run-on analysis in monomorphic parasite populations expressing a single PfEMP1 variant that *var* gene transcription is controlled at the transcriptional level (Scherf *et al*., 1998). However, it is still puzzling how parasites activate a single *var* gene while keeping all other copies repressed or silenced. *var* gene promoters maintained on episomes were active irrespective of the state of the endogenous chromosomal promoter (Deitsch *et al*., 1999). In addition, Deitsch *et al*. (2001a) reported that silencing of episomal *var* gene promoter-driven transcription involved the co-operative interaction between the intron and 5′ flanking sequence of *var* genes. Complete silencing, however, was only achieved after plasmid transition through S-phase. Together, these results clearly suggest the involvement of epigenetic mechanisms in regulation of *var* gene transcription.

We were interested in identifying structural and functional elements in different *var* gene promoter types and to gain insight into regulation of *var* gene transcription. We show that repression of subtelomeric and chromosome-central *var* gene transcription during intraerythrocytic development occurs 4–8 h apart. This shifted repression sharply coincided with the expression of two distinct DNA-binding activities specifically interacting with sequence recognition motifs unique to either subtelomeric or central *var* gene promoters. Transient transfection experiments indicated a direct participation of these DNA-binding activities in *var* gene repression and also revealed regions with regulatory activities implicated in transcriptional activation. A third nuclear protein specifically expressed during S-phase interacted with a reiterated sequence motif in subtelomeric promoters. The likely involvement of this activity in silencing of subtelomeric *var* gene transcription is discussed.

Results

EMSA screening of subtelomeric and chromosome-central var gene *promoters*

To investigate *var* gene promoters for the presence of elements possibly involved in the regulation of *var* gene transcription, we tested a series of restriction fragments derived from the promoters of 3D7 *var* genes 4A3 (upsB) and 5B1 (upsC) (Voss *et al*., 2000) in gel retardation assays (Fig. 1). Initially, most of these fragments appeared to interact with nuclear proteins. However, it became evident that there was interference of a non-specific ssDNA-binding protein, which we identified as *P. falciparum* replication protein A (RPA) (Voss *et al*., 2002), with labelled single-stranded (ss) probes. Inclusion of unlabelled ss competitor DNA prevented the formation of these complexes. Thereafter, we detected three nuclear activities specifically binding to *var* gene promoter fragments 5B1s(4), 4A3s(1a) and 4A3s(8) (Figs 2 and 3). Cytosolic control extracts did not contain activities binding to any of these probes. Competition electrophoretic mobility shift assays (EMSAs) clearly revealed the specificity of these interactions. We observed two closely co-migrating complexes for 4A3s(8) and, in the case of 4A3s(1a) and 5B1s(4), even multiple shifted bands were apparent. In competition studies, the intensities of these additional bands decreased and increased concordant to the main complex, indicating partial dissociation of multisubunit protein complexes and/or partial proteolysis of the binding factors, rather than binding of different proteins to the same recognition sequence.

Specificity of var *promoter-binding activities*

To fine map the identified sequence elements, we carried out competition studies using overlapping double-stranded (ds) oligonucleotides. The complex formed with 5B1s(4) was not competed by 5B1s(4)-1, -2 and -4

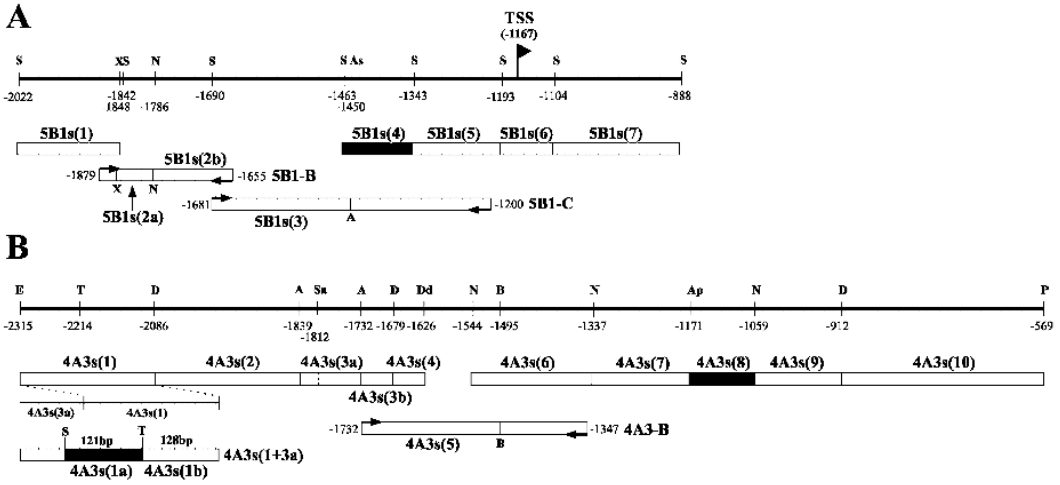


Fig. 1. *var* gene promoter fragments analysed in EMSAs. Fragments bound by nuclear proteins are highlighted in black.
A. Restriction map of the chromosome-central *var* gene 5B1 promoter (upsC) with positions relative to the initiation ATG. The flag depicts the position of the transcriptional start site (TSS) of a central *var* gene (*var7b*) (Deitsch *et al*., 1999).
B. Restriction map of the subtelomeric *var* gene 4A3 promoter (upsB). Fragment 4A3s(1+3a) was an accidental hybrid molecule consisting of 4A3s(3a) ligated in reverse orientation to the 5′ end of 4A3s(1).
Horizontal arrows indicate PCR primers used to generate fragments not present in *var* promoter restriction libraries. A, *Ava*II; Ap, *Apa*LI; As, *Ase*I; B, *Bgl*II; Dd, *Dde*I; D, *Dra*I; E, *Eco*RV; N, *Nde*I; P, *Pac*I; S, *Ssp*I; Sa, *Sau*3AI; T, *Tsp*509I; X, *Xba*I.

(Fig. 2A). Efficient competition occurred only by a 100-fold molar excess of 5B1rec (69 bp). Neither the sense nor the antisense strand of 5B1rec had an effect on complex formation. The addition of 5B1s(4)-3 caused a reduction in signal intensity, indicating that a sequence motif contained within 5B1s(4)-3 alone is not sufficient for binding, but forms part of a recognition element encoded by 5B1rec, which we termed *CPE* (chromosome-central *var* gene promoter element). *CPE* is located at position −1426 to −1357 with respect to the initiation ATG of 5B1 and contains a 4 bp inverted repeat also present in 5B1s(4)-3. We identified *SPE1* (subtelomeric *var* gene promoter element 1) (Fig. 2B) in the promoter of 4A3 encompassing nucleotides −1171 to −1127 by competition of the nuclear activity binding to 4A3s(8) with 4A3s(8)-1. In contrast, neither 4A3s(8)-2 nor the sense and antisense strands of 4A3s(8)-1 interfered with binding.

The specific protein interaction involving 4A3s(1a) was efficiently competed by 4A3s(1b) (data not shown). As expected, a DNA–protein complex formed with 4A3s(1b) could be prevented by the addition of 4A3s(1a) (Fig. 3B). Although both probes were of similar size, the major complexes (C2, C3) formed with 4A3s(1b) showed a clearly reduced mobility compared with complex C1 formed with 4A3s(1a). These observations suggested an interaction of the same nuclear factor with identical or related sequence motifs in both probes, and the binding of multiple proteins to 4A3s(1b). To investigate this possibility in more detail, we performed EMSA competition studies using overlapping ds oligonucleotides covering the entire 4A3s(1) locus. 4A3s(1)-1, -2 and -3 were unable to inhibit complex formation, whereas competitors 4A3s(1)-1.2, -c1, -c2 and -c3 completely prevented binding to both probes (Fig. 3A–C). Sequence comparison revealed the presence of a conserved sequence motif (*SPE2*) in all four competitors consisting of a direct (T/G)GTGC(A/G) repeat spaced by four nucleotides (Fig. 3D). In 4A3s(1)-1, all but the last two nucleotides of this motif are present in ds conformation (see Table 1). Using Klenow enzyme, we generated the complete recognition motif in 4A3s(1)-1 by refilling the 5′ overhang. This procedure transformed 4A3s(1)-1, but not 4A3s(1)-2, into an efficient competitor (Fig. 3E). The introduction of two point mutations in either the first (repmotM1) or the second (repmotM2) repeat of the *SPE2* consensus sequence completely eliminated the ability to prevent protein binding (Fig. 3F). A ds oligonucleotide coding for a degenerate rep20 tandem repeat (Åslund *et al*., 1985) also failed to compete (Fig. 3B). Together, these findings revealed that the *SPE2*–protein interaction occurred in a highly specific manner. The presence of three binding sites in the 4A3s(1b) probe greatly facilitated the binding of additional factors (complexes C2 and C3), and we hardly observed 4A3s(1b) complexes with an electrophoretic mobility corresponding to the binding of one protein (C1), despite the fact that the majority of radiolabelled 4A3s(1b) occurred as free probe. Strikingly,

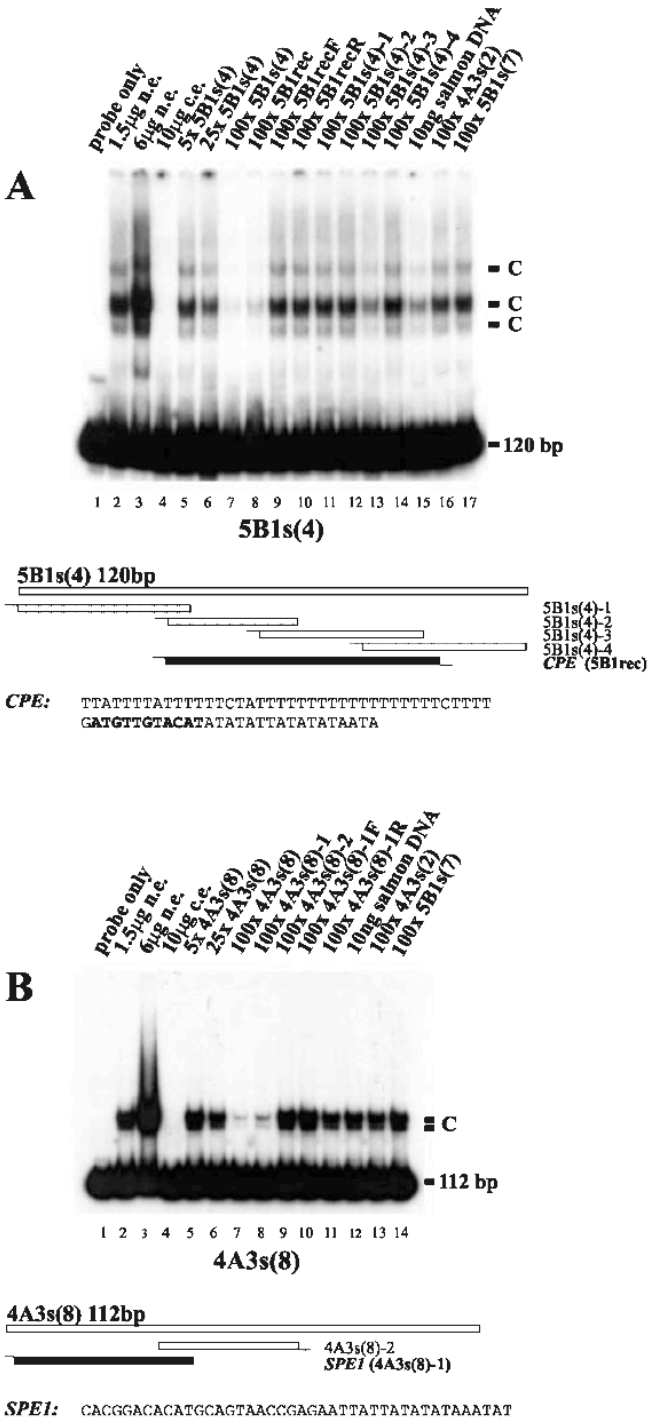
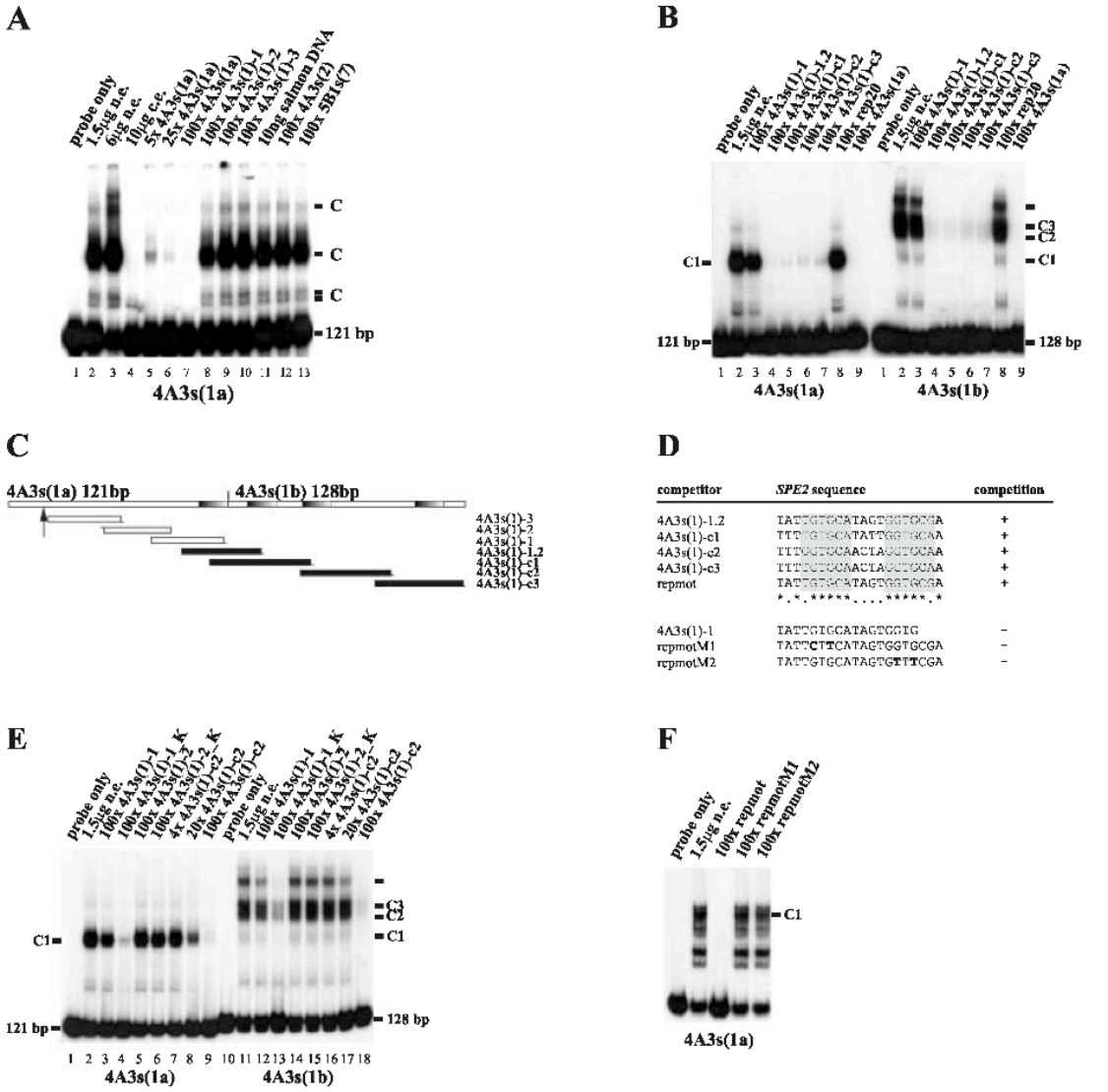


Fig. 2. Competitive gel retardation assays and identification of *var* promoter elements *SPE1* and *CPE*.
A. Fragment 5B1s(4) formed one major and two minor complexes after incubation with nuclear extracts (lanes 2 and 3) but not with cytoplasmic extracts (lane 4). Complex formation in the presence of various specific and non-specific competitors was analysed in lanes 5–17. A scheme depicting overlapping competitor DNAs covering 5B1s(4) and the nucleotide sequence of *CPE* is shown. The inverted 4 bp repeat separated by three nucleotides is highlighted in bold.
B. Fragment 4A3s(8) formed one major and one closely co-migrating minor complex after incubation with nuclear extracts (lanes 2 and 3) but not with cytoplasmic extracts (lane 4). Competition experiments were analysed in lanes 5–14. A scheme depicting overlapping competitor DNAs covering 4A3s(8) and the nucleotide sequence of *SPE1* is shown.
The molar excess of competitors is indicated above each lane. Sheared salmon sperm DNA was added at a 250-fold weight excess. ss oligonucleotide competitors are denoted by F (forward) or R (reverse). n.e., nuclear extract; c.e., cytosolic extract.



g. 3. Interaction of SPE2BP with *SPE2* sequence elements.
. One major and three minor complexes were formed with fragment 4A3s(1a) after incubation with nuclear extracts (lanes 2 and 3) but not with rtoplasmic extracts (lane 4). In lanes 5–13, the effect of various competitors on binding was analysed.
. Radiolabelled 4A3s(1a) and 4A3s(1b) were incubated with nuclear extracts (lanes 2). The complexes formed with 4A3s(1b) (C2 and C3) owed reduced mobility compared with C1 formed with 4A3s(1a). Various competitors were analysed in lanes 3–9. The rep20 competitor onsisted of a degenerate rep20 tandem repeat. 4A3s(1a) was used as a control for competition experiments (lanes 9).
. Competitors 4A3s(1)-1.2, -c1, -c2, -c3 (black) prevented complex formation with radiolabelled probes 4A3s(1a) and 4A3s(1b). Grey shaded ctangles indicate *SPE2* consensus sequences at their relative positions in 4A3s(1). The arrow marks the boundary to 4A3s(3a) in the hybrid olecule 4a3s(1a) (see Fig. 1).
. The *SPE2* sequences present in various competitors are shaded. Point mutations introduced into repmotM1 and repmotM2 are highlighted in old.
. The effect of filling in the 5′ overhangs of 4A3s(1)-1 or 4A3s(1)-2 on *SPE2* binding. Klenow-treated ds oligonucleotides 4A3s(1)-1°K or 4A3s(1)-°K in competition with 4A3s(1a) were analysed in lanes 4 and 6 respectively. Untreated competitors were used in samples 3 and 5. Lanes 12–5 show the effect of the Klenow-treated competitors on binding to radiolabelled 4A3s(1b). Reduction in complex formation through increasing nounts of 4A3s(1)-c2 was analysed in lanes 7–9 and 16–18.
The effect of point mutations in either the first (repmotM1) or the second (repmotM2) repeat present in the *SPE2* consensus sequence (repmot) n protein binding. The faster migrating complexes are most likely caused by proteolysis.

Table 1. PCR primers and oligonucleotides used in EMSA gel retardation assays.

Transfection constructs and PCR fragments	Oligonucleotide name	Used in the generation of	Oligonucleotide sequence
4A3 promoter sequence	4A3-28R	All pCAT4A3 derivatives	CTGTATTACATCAGTGCTTGCTATTTGTTTT**CCTAGG**GCGC
	4A3-2202F	pCAT4A3-2202	GCAT**AAGCTT**GCAACTAGGTGCAACATTTTAC
	4A3-1732F	pCAT4A3-1732 PCR 4A3-B	GCAT**AAGCTT**GGATCTAGTCTTTAGGGTTCCCAT
	4A3-1517F	pCAT4A3-1517	GCAT**AAGCTT**GGATCTATAGCTACTATATAAAGATCTG
	4A3-1373F	pCAT4A3-1373	GCAT**AAGCTT**GTTAAAGAACATATCTGTTCATCAAGGT
	4A3-1210F	pCAT4A3-1210	GCAT**AAGCTT**AAAAATCGAAATGGAAGATAC
	4A3-1114F	pCAT4A3-1114	GCAT**AAGCTT**GGGTTTAGGAATACGTATGCCTTTATG
	4A3-1066F	pCAT4A3-1066	GCAT**AAGCTT**GAGAGAATACATATGCTCTTG
	4A3-804F	pCAT4A3-804	GCAT**AAGCTT**CCTTATGCTACATGATATGTCATA
	4A3-515F	pCAT4A3-515	GCAT**AAGCTT**GAAACATGTATGTTTTTATATGTATGT
	4A3-2621F	pCAT4A3-Δ	TCCT**AAGCTT**ACTAATTTATGTCCTATAGGTACG
	4A3-1180R	pCAT4A3-Δ	CGAT**AAGCTT**TTTTGGGTATCTTCCATTTCG
	4A3-1347R	PCR 4A3-B	CAATTTCTTGTATAGACAAGTAGTTTCC
	4A3-*Hpa*l_R	pVLUC4A3/-Δ	CAGTGCTTGCTATTTGTTTT**CAATTG**CG
5B1 promoter sequence	5B1EABR	All pCAT5B1 derivatives	CTATTG**TTCGAA**ATACTTCGCGTATAATCTCATTATTACACGTACG
	5B1-1879F	pCAT5B1-1879 PCR 5B1-B	GCAT**AAGCTT**GCATCCTATATGTATATTATATACATTCTCTC
	5B1-1681F	pCAT5B1-1681 PCR 5B1-C	GCAT**AAGCTT**CATAATTTCATCATTATTAAAGTAGAGAAA
	5B1-1508F	pCAT5B1-1508	GCAT**AAGCTT**GAAGTATGTATACAAAATAGATG
	5B1-1381F	pCAT5B1-1381	GCAT**AAGCTT**GTACATATATATTATATATAATAC
	5B1-1325F	pCAT5B1-1325	GCAT**AAGCTT**ATATAACAAAAAAAAATTAATATG
	5B1-1228F	pCAT5B1-1228	GCAT**AAGCTT**CATAGAAATGTGGTAGATAATATAGATAGA
	5B1-1086F	pCAT5B1-1086	GCAT**AAGCTT**TATATATGTTCTTCGATTATAATTCTTTT
	5B1-2522F	pCAT5B1-Δ	GCAT**AAGCTT**CCTTATGCTACATGATATGTCATA
	5B1-1483R	pCAT5B1-Δ	CGAT**AAGCTT**CATCTATTTTGTATACATAATTC
	5B1-1655R	PCR 5B1-B	GTATTAAAGTAGTAATAATTTCATCTC
	5B1-1200R	PCR 5B1-C	GTATCTTTACACCATCTATTATATCTATC
	5B1-*Hpa*l_R	pVLUC5B1/-Δ	CACATACATATATCCATATACA**CAATTG**GC
	T7-*Kpn*I	pVLUC5B1/-Δ pVLUC4A3/-Δ	GG**GGTACC**TAATACGACTCACTATAGGG

EMSA competitors and probes	Oligonucleotide name	Competitor	Oligonucleotide sequence
	5B1s(4)-1F	5B1s(4)-1	ATGTTTTTTTATTAATATAATAATCCTTTTTTTATGTTATTTTA
	5B1s(4)-1R		AAAAAAATAATTATATTATTAGGAAAAAAATACAATAAAAT
	5B1s(4)-2F	5B1s(4)-2	ATGTTATTTTATTTTTTCTATTTTTTTTTTTTTT
	5B1s(4)-2R		AATAAAATAAAAAAGATAAAAAAAAAAAAAA
	5B1s(4)-3F	5B1s(4)-3	ATGTTTTTTTTTTTTTTTTCTTTTGATGTTGTACATATATAT
	5B1s(4)-3R		AAAAAAAAAAAAAAAAGAAAACTACAACATGTATATATA
	5B1s(4)-4F	5B1s(4)-4	ATGTACATATATATTATATATAATACATTATATATTATAAT
	5B1s(4)-4R		ATGTATATATAATATATATTATGTAATATATAATATTA
	5B1recF	5B1rec	TGTTATTTTATTTTTTCTATTTTTTTTTTTTTTTTTTCTTTTGATGT TGTACATATATATT
	5B1recR		ATATATAATAAATAAAATAAAAAAGATAAAAAAAAAAAAAAAAAAAG AAAACTACAACATGTATATATAATATATATTATGTA
	4A3s(1)-1F	4A3s(1)-1	CACATTTTTTTTGGTGCGACTTTATTGTGCATAGTGGTG
	4A3s(1)-1R		GTGTAAAAAAAACCACGCTGAAATAACACGTATCACCACGCT
	4A3s(1)-1.2F	4A3s(1)-1.2	CGACTTTATTGTGCATAGTGGTGCGAATTTATACTTTGGTGCA
	4A3s(1)-1.2R		AGTTGCACCAAAGTATAAATTCGCACCACTATGCACAATAAAG TCG
	4A3s(1)-2F	4A3s(1)-2	TAGTGATACCACACATGTGGGAAGACCACACATTTTTTT
	4A3s(1)-2R		ACTATGGTGTGTACACCCTTCTGGTGTGTAAAAAAA
	4A3s(1)-3F	4A3s(1)-3	ATCACACATACGTGGTAATACCACATATATAGTGATACCAC
	4A3s(1)-3R		TAGTGTGTATGCACCATTATGGTGTATATATCACTATGGTGTG
	4A3s(1)-c1F	4A3s(1)-c1	CATAGTGGTGCGAATTTATACTTTGGTGCAACTAGGTGCAACAT TTTACTTTTG
	4A3s(1)-c1R		GTATCACCACGCTTAAATATGAAACCACGTTGATCCACGTTG TAAAATGAAAACACGT
	4A3s(1)-c2F	4A3s(1)-c2	CTTTTGTGCAACTAGGTGCAACTTGATAAACACTGCGATGGAG GTGC

Table 1. *cont.*

EMSA competitors and probes	Oligonucleotide name	Competitor	Oligonucleotide sequence
	4A3s(1)-c2R		AA GAAAACACGTTGATCCACGTTGAACTATTTGTGACGCTAC CTCCACGTTGTA
	4A3s(1)-c3F	4A3s(1)-c3	GGTGCAACATTTTACTTTTGTGCATATTGGTGCAACATTT TACTTTGGTGCA
	4A3s(1)-c3R		CCACGTTGTAAAATGAAAACACGTATAACCACGTTGTAAAAT GAAACCACGTTGA
	repmotF	repmot	CTTTATTGTGCATAGTGGTGCGAATTTATA
	repmotR		GAAATAACACGTATCACCACGCTTAAATAT
	repmotM1F	repmotM1	CTTTATT***CT T***CATAGTGGTGCGAATTTATA
	repmotM1R		GAAATAA***G A A***GTATCACCACGCTTAAATAT
	repmotM2F	repmotM2	CTTTATTGTGCATAGTG***TT T***CGAATTTATA
	repmotM2R		GAAATAACACGTATCAC***A A A***GCTTAAATAT
	4A3s(8)-1F	4A3s(8)-1	TGCACGGACACATGCAGTAACCGAGAATTATTATATATAAATAT
	4A3s(8)-1R		GTGCCTGTGTACGTCATTGGCTCTTAATAATATATATTTATA
	4A3s(8)-2F	4A3s(8)-2	ATATAAATATATATATGTATATTTTGGGTTTAGGAAT
	4A3s(8)-2R		TATATTTATATATATACATATAAAACCCAAATCCTTATGC

Forward (F) oligonucleotides are indicated 5′ → 3′, reverse (R) oligonucleotides 3′ → 5′. Restriction enzyme recognition sites are highlighted in bold. Point mutations introduced into repmotM1 and remotM2 are italicized and in bold.

whereas a 20-fold molar excess of 4A3s(1)-c2 significantly reduced complex formation with 4A3s(1a) (which harbours only one binding site) no effect was observed with 4A3s(1b) (Fig. 3E). We therefore conclude that binding of this factor to *SPE2* elements occurs in a co-operative manner.

To test for cross-reactivity, we used oligonucleotides 5B1rec, 4A3s(8)-1 and repmot, harbouring the recognition motifs *CPE*, *SPE1* and *SPE2*, respectively, as competitors. Figure 4 shows that the three DNA-binding factors represent distinct activities and that central and subtelomeric *var* gene promoters interact with different nuclear proteins. Multiple sequence alignments of *var* gene 5′ flanking sequences retrieved from the NCBI Malaria Genetics and Genomics section (http://www.ncbi.nlm.nih.gov/Malaria/plasmodiumbl.html) showed that the *CPE* element was highly conserved among all seven upsC sequences investigated. Variation was observed in six nucleotide positions and in the length of the poly dT stretches only, whereas the 4 bp inverted repeat was perfectly conserved. Analysis of 12 upsB promoters revealed a perfect conservation of the *SPE1* recognition motif except for two nucleotide positions. *SPE2* elements were present in all upsB sequences analysed and occurred in arrays of 5–18 repeats. The position of *SPE1*, *SPE2* and *CPE* with respect to the ATG start codon of *var* genes is also conserved (data not shown).

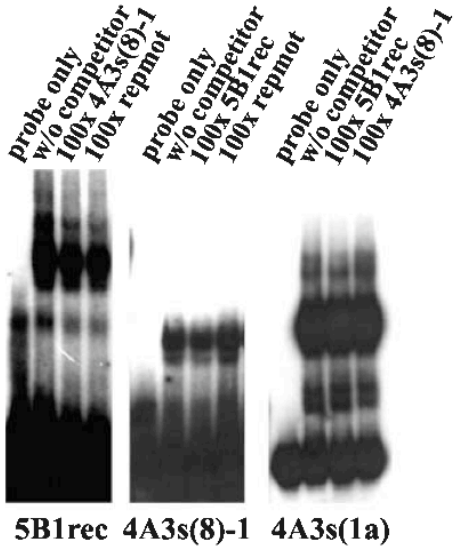


Fig. 4. Cross-competition EMSAs. EMSA showing the absence of cross-competition between *CPE*, *SPE1* and *SPE2* and their cognate binding factors. n.e., nuclear extract.

Stage-specific expression of CPE-, SPE1- *and* SPE2-*binding proteins*

We investigated the expression of the *CPE*-, *SPE1*- and *SPE2*-binding activities (CPEBP, SPE1BP and SPE2BP) across the intraerythrocytic parasite cell cycle (Fig. 5). To standardize this comparative analysis, we used stage-specific protein preparations derived from an equal number of nuclei. SPE1BP first appeared in parasites 16–26 h post invasion (h.p.i.) and increased to maximal levels in parasites older than 24 h.p.i. CPEBP was not detected until 24–34 h.p.i. and was most pronounced in schizonts. SPE2BP, however, was exclusively expressed in late-stage parasites older than 34 h.p.i. The expression of SPE1BP and CPEBP in trophozoites and schizonts suggested a possible involve-

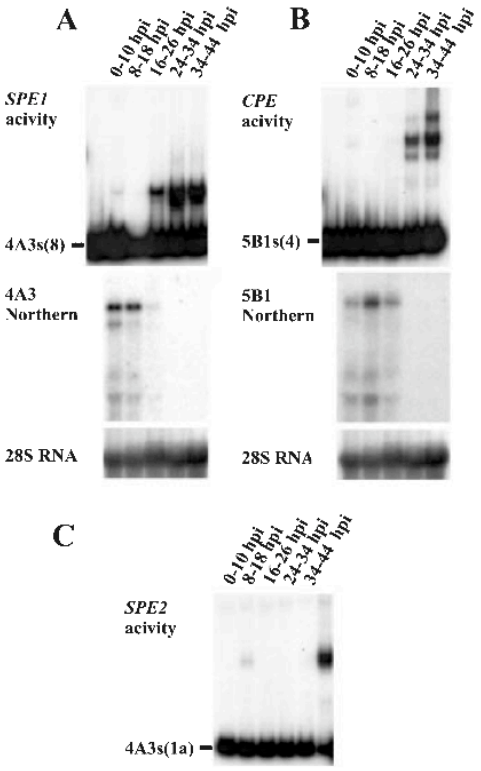


Fig. 5. Stage-specific expression of SPE1BP, CPEBP and SPE2BP in relation to *var* gene transcription. Intraerythrocytic stages are indicated above each lane in hours post invasion (h.p.i.).
A. Top: *SPE1*-binding activity in nuclear extracts derived from synchronized parasite cultures. Middle: upsB-type *var* gene transcription monitored by Northern analysis of total RNA obtained from the same stages. The filter was probed with a radiolabelled PCR fragment encompassing nucleotides –460 to –1 of the 4A3 5′ UTR. Bottom: stage-specific 28S Northern as a control for equal RNA loading.
B. Top: *CPE*-binding activity in nuclear extracts derived from synchronized parasite cultures. Middle: stage-specific Northern analysis of upsC-type *var* gene transcription. The filter was probed with a radiolabelled PCR fragment encompassing nucleotides –514 to – 237 of the 5B1 5′ UTR.
C. Stage specificity of the *SPE2*-binding activity.

ment in *var* gene repression as *var* gene transcription was shown to occur in parasites up to 18–27 h.p.i. only (Kyes *et al*., 2000). We therefore compared stage-specific transcription of upsB- and upsC-type *var* genes with expression of SPE1BP and CPEBP. This was realized by Northern analysis using total RNA isolated from the same stages as for isolation of nuclear proteins. Probes from the conserved 5′ untranslated regions (UTR) of *var* genes 4A3 or 5B1, identifying upsB- and upsC-type *var* transcripts, respectively, clearly showed that transcription of subtelomeric *var* genes is turned off earlier than that of central *var* genes. Concordantly, the shifted cessation of subtelomeric and central *var* gene transcription exactly correlated with the delayed occurrence of CPEBP over SPE1BP, indicating their involvement in repression of *var* gene transcription.

var *gene promoter activity analysis by transient transfection*

We showed recently that both types of *var* gene promoters were active in transient transfection assays (Voss *et al*., 2000). As an alternative approach to identify functional *var* gene promoter regions, we generated nested 5′ deletions in the 5B1 and 4A3 promoters. These fragments were used to replace the full-length promoters in pCAT5B1 and pCAT4A3 respectively. The data presented in Fig. 6A reveal two regions in the 5B1 promoter dominantly affecting promoter activity. Deletion of nucleotides –2522 to –1879 reduced activity to 26%, indicating the presence of *cis*-acting elements involved in the activation of chromosome-central *var* gene transcription. Further nucleotide removal to –1508 had no dramatic effect. Deletion of the sequence from –1508 to –1325, harbouring the entire *CPE* element, caused an additional drop in promoter activity to barely detectable levels (6% compared with pCAT5B1). This region may therefore comprise additional positive regulatory elements and the core promoter. Deletion to –1228 and –1086 completely abolished promoter activity.

In the 4A3 promoter, deletion of the most 5′ 419 bp had no effect on overall promoter activity (Fig. 6B). However, removal of nucleotides –2202 to –1732 (containing the *SPE2* motifs) resulted in a 2.2-fold increase. Further deletion to –1210 again caused a stepwise reduction to the value observed with the full-length constructs. 5′ removal of the *SPE1* motif in pCAT4A3-1114 and pCAT4A3-1066 caused no effect. The large standard deviation in the pCAT4A3-804 sample resulted from an extraordinarily high value obtained in one experiment, which most probably represented an artifact. The average relative activity for this construct obtained in the other three experiments, however, was 28%. Further deletion to –514 completely abolished promoter activity. We therefore conclude that the region encompassing –1066 to –514 contains functional *cis*-acting elements involved in activation and basal transcription of subtelomeric *var* genes.

To investigate any possible effect of *CPE* or *SPE1* on *var* gene repression, we transiently transfected synchronized parasites with pVLUC5B1-Δ and pVLUC4A3-Δ carrying internal deletions encompassing *CPE* and *SPE1* respectively. We used the luciferase gene for these experiments as the long half-life of CAT in eukaryotic cells (50 h) (Thompson *et al*., 1991) makes this reporter unsuitable for the analysis of stage-specific pro-

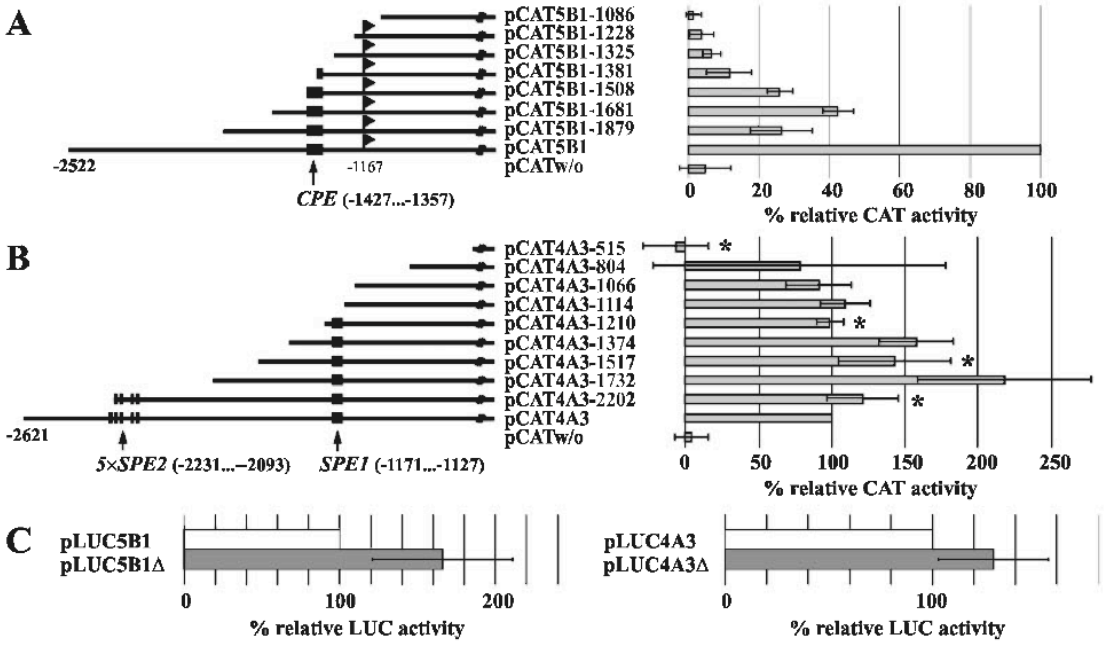


Fig. 6. Analysis of 5B1 and 4A3 *var* gene promoter activities by transient transfection. For transfections using CAT constructs, pCAM5/3 was used as a positive control (Crabb and Cowman, 1996; data not shown). pCATw/o, lacking a promoter, was used as a negative control (Voss *et al.*, 2000). The length of the promoter in each construct is depicted on the left. Activity values are indicated as a percentage of relative CAT or luciferase activity compared with the activity obtained with parental constructs. Experiments have been repeated four times.
A. Nested 5′ deletion analysis of the 5B1 promoter (upsC). The location of *CPE* is indicated by a filled rectangle. The putative TSS is depicted by a flag.
B. 5′ nested 4A3 promoter (upsB) deletion analysis. The positions of *SPE1* and the repeated *SPE2* elements are indicated by rectangles. Experiments have been repeated four times, and CAT activity has been measured three times in each experiment. Constructs marked by an asterisk have been transfected three times only.
C. Effect of internal promoter deletions on promoter activity. In pVLUC5B1-Δ, nucleotides −1483 to −1325 were deleted from the 5B1 promoter including *CPE* (–1427 to −1357). Nucleotides −1180 to −1073 [including *SPE1* (–1171 to −1127)] were deleted from the 4A3 promoter in pVLUC4A3-Δ. Control transfections without plasmid DNA yielded activity values between −14 and 0.

moter activity. Deletion of nucleotides −1483 to −1325 (pVLUC5B1-Δ) resulted in a 1.65-fold average increase in luciferase activity compared with that obtained with the parental plasmid pVLUC5B1. Deletion of *SPE1* in pVLUC4A3-Δ led to a 1.3–fold increase compared with pVLUC4A3 (Fig. 6C). These results indicate an involvement of the interactions of CPEBP and SPE1BP with their cognate regulatory binding motifs in stage-specific repression of chromosome-central and subtelomeric *var* gene expression respectively.

Discussion

Plasmodium falciparum var gene expression is controlled by different yet conserved promoters, and these structural differences correlate with chromosomal location and orientation of *var* genes (Voss *et al.*, 2000; Gardner *et al.*, 2002). Recent findings suggest that *cis*-acting elements mediating *var* gene expression and silencing must be conserved within every *var* gene promoter and that regulation of *var* gene expression is subject to higher level silencing mechanisms.

We identified three highly conserved *var* gene promoter elements interacting with distinct DNA-binding proteins. The *SPE1* and *SPE2* motifs are specifically associated with subtelomeric upsB promoters, and *CPE* is a characteristic sequence element in chromosome-internal promoters (upsC). Complex formation with these elements occurred in a highly sequence-specific manner and involved three different nuclear activities. Genome-wide BLAST analysis (http://www.ncbi.nlm.nih.gov/Malaria) revealed that these elements are exclusively associated with *var* genes. *SPE1*, *SPE2* and *CPE* have no significant similarities to eukaryotic transcription factor binding sites reported in the TRANSFAC database (http://www.transfac.gbf.de). However, the organization of

the *SPE2* motif as two direct 6 bp repeats spaced by four nucleotides is reminiscent of nuclear hormone receptor (NHR) response elements (reviewed by Aranda and Pascual, 2001). Whether this is of any significance for the nature of the *SPE2*-binding protein remains to be elucidated, most of all because NHRs have as yet only been described in metazoans.

var genes are transcribed in parasites up to 18–27 h.p.i. (Kyes *et al.*, 2000). Here, we show for the first time that subtelomeric *var* messages were observed in parasites up to 18 h.p.i. only, whereas chromosome-internal *var* transcripts were still detected in parasites 16–26 h.p.i. This was in perfect correlation with the occurrence of SPE1BP 18 h.p.i. and the 4–8 h delayed expression of CPEBP. These results showed that the structural differences in *var* gene promoters reflect functional and regulatory differences in transcriptional repression of *var* genes located in different chromosomal domains. They further suggested that *SPE1* and its cognate binding factor SPE1BP are directly involved in repression of subtelomeric *var* genes, whereas chromosome-internal *var* gene transcription is repressed 4–8 h later by the interaction of CPEBP with *CPE*.

In transient transfection experiments, internal deletion of stretches harbouring *SPE1* or *CPE* from the subtelomeric or chromosome-internal promoter led to an increase in luciferase activity compared with the wild-type promoters (1.65-fold for *CPE* and 1.3-fold for *SPE1*). These results clearly hint at an involvement of these regulatory elements in *var* gene repression. However, the absence of a pronounced difference in activity between wild-type and mutant promoters indicates that more complex processes are involved. On one hand, and as suggested by others (Scherf *et al.*, 1998; Deitsch *et al.*, 1999), epigenetic mechanisms may be involved in the regulation of *var* gene expression. When working with episomes, such effects may impede the investigation of promoter regulatory processes, as the chromatin configuration of episomal and chromosomal DNA may differ substantially. For example, it has been shown that episomal *var* gene promoters are desilenced irrespective of the transcriptional state of the endogenous promoter (Deitsch *et al.*, 1999). Similar findings have been obtained for the *P. falciparum* gbp130 promoter, in which the loss of developmental restriction of the episomal promoter was attributed to observed differences in chromatin structure between episomal and chromosomal copies (Horrocks and Lanzer, 1999). Furthermore, even if replicated plasmids were properly assembled into chromatin during S-phase, rapid loss of episomes as a result of inefficient segregation (van Dijk *et al.*, 1997; O'Donnell *et al.*, 2001) and continuous uptake of naked plasmid DNA into parasite nuclei during intracellular growth (Deitsch *et al.*, 2001b) would mask the repressive effects of *SPE1* or *CPE*. On the other hand, additional *trans*-acting factors may participate in *var* repression and, thus, total inhibition of repression would only be observed after preventing these interactions as well. Studies using transgenic parasites carrying mutations in *SPE1* and *CPE* in chromosomal *var* promoters are definitely needed to explore further their effect on *var* gene repression.

We identified several regions involved in activation of *var* gene transcription in both promoters. 5′ nested removal of a fragment containing the *CPE* motif from the 5B1 promoter resulted in a decrease in activity to barely detectable levels, suggesting the presence of positive *cis*-acting element(s) and the core promoter within the deleted region. It is therefore not surprising that no repressive effect was observed upon nested deletion of *CPE*. A second region displaying strong activating properties is positioned further upstream (–2522 to –1879) as deletion of this stretch retained only 26% activity. 5′ deletion to –1228 and –1086 (including the putative TSS at –1167) led to a complete abolishment of promoter activity and suggests that the TSS site mapped for *var7b* (Deitsch *et al.*, 1999) is conserved among chromosome-central *var* genes. Deletion analysis of the subtelomeric 4A3 promoter revealed a functional region with activation potential between nucleotides –1066 and –504. Our results also indicate that the TSS of subtelomeric *var* genes is located between nucleotides –804 and –514. We were not able to detect any DNA–protein interactions in the fragments participating in transcriptional activation of *var* genes. This does not imply, however, that such interactions do not exist. Factors involved in transcriptional activation of a single *var* gene are likely to be of low abundance, aggravating their detection in standard gel retardation experiments. Furthermore, we cannot exclude the possibility that the binding conditions used in our experiments were inappropriate for stable complex formation involving other sequences and nuclear proteins.

The interaction of SPE2BP with *SPE2* occurred in a highly sequence-specific manner. By sequence comparison, we found that *SPE2* elements occur in arrays of 5–18 repeats in upsB promoters, and these arrays are located close to the rep20 repeat region. We showed that the presence of multiple reiterated *SPE2* motifs facilitated co-operative protein binding. Degenerate rep20 tandem repeats did not interfere with *SPE2* binding, confirming that *SPE2* arrays represent a defined subtelomeric *var* gene promoter element providing multiple binding sites for a distinct nuclear protein.

We hypothesize that the function of SPE2BP may be to participate in transcriptional silencing of subtelomeric *var* genes. (i) We observed a 2.2-fold increase in promoter activity upon deletion of the *SPE2* repeat array. (ii) It is known that assembly of silent chromatin requires transi-

tion through S-phase in other organisms (Miller and Nasmyth, 1984; Firestein *et al.*, 2000). It was also convincingly shown that silencing of episomal transcription mediated by a *var* gene promoter in *P. falciparum* required plasmid passage through S-phase (Deitsch *et al.*, 2001a). SPE2BP was specifically expressed during S-phase and mitosis in parasites 34–44 h.p.i. and, thus, this activity may be involved in establishing the silenced state of subtelomeric *var* genes in newly developing merozoites. (iii) Perinuclear localization of telomeric clusters facilitates telomeric silencing in *Saccharomyces cerevisiae* (Maillet *et al.*, 1996; Andrulis *et al.*, 1998), and high concentrations of proteins essential for silencing are found in this nuclear compartment (Gotta *et al.*, 1996; Maillet *et al.*, 1996). As in yeast, clusters of *P. falciparum* telomeres locate to the nuclear periphery (Freitas-Junior *et al.*, 2000). Orthologues of most of the yeast proteins involved in telomeric gene silencing (e.g. RAP1, SIR2-4, MLP, Ku-complex) have been identified in the *P. falciparum* genome (Scherf *et al.*, 2001), raising the possibility that this parasite uses similar mechanisms to silence genes close to chromosomal ends. In such a model, the *SPE2* elements could act as silencers interacting with regulatory proteins to initiate nucleation and assembly of silenced chromatin spreading throughout the nearby *var* gene. Co-operative binding of the regulatory factors to reiterated *SPE2* motifs could prove essential in efficient co-repressor recruitment and may be important to establish readily and inherit reliably the silent state of subtelomeric *var* genes. A recent study reported that *var* gene silencing involves co-operative interactions between the *var* intron and 5′ flanking region (Deitsch *et al.*, 2001a). These experiments have only been done with a chromosome-central *var* promoter, and it remains to be tested whether this is also true for subtelomeric *var* promoters. Considering the structural and functional differences in the conserved *var* promoters and a probable different nuclear location of subtelomeric and central *var* genes, it is conceivable that silencing of subtelomeric *var* genes is mediated by different mechanisms.

Our results shed new light on transcriptional regulation of the *var* gene family and inevitably raise the question about the biological significance of the structural and functional differences in *var* gene promoters. The observed differences might only represent an evolutionary result of the spreading of *var* genes throughout the parasite genome where different chromosomal and nuclear domains may impose different overall regulatory mechanisms. But these findings might also reflect distinct functional *var* gene subsets. Variant-specific antibodies play an important role in protection from clinical disease (Bull *et al.*, 1998) and, therefore, antigenic variation is a prerequisite for survival in the human host. We hypothesize that, in naive hosts, expression of chromosome-central *var* genes may prove beneficial for the parasite. Chromosome-central *var* genes reside in stable genomic domains (Pologe and Ravetch, 1988; Lanzer *et al.*, 1993) where only little sequence variation over time is expected to occur. Similarly, upsC-type *var* genes may be quite stable over time because of their inverse orientation and centromere-proximal location compared with upsB-type *var* genes. These genes may code for PfEMP1 variants that confer stable and high-affinity binding of iRBCs to selected host receptors, thus efficiently preventing spleen-dependent killing. In contrast, subtelomeric *var* genes located in highly recombinogenic regions and subject to frequent ectopic recombination events (Freitas-Junior *et al.*, 2000) may have increased antigenic variability, possibly coupled with decreasing binding affinity. In hyperimmune individuals, expression of subtelomeric *var* genes would allow successive exposure of highly variable PfEMP1 molecules to escape existing anti-PfEMP1 responses. Based on agglutination frequencies of field isolates, Bull *et al.* (2000) similarly proposed the existence of a PfEMP1 subset functionally selected for optimal cytoadherence properties in young children that might play an important role in causing severe disease. In older children, immune selection would be more important, displacing optimally cytoadherent variants by those with novel epitopes. Monitoring of *var* gene expression in the field as well as advances in our understanding of PfEMP1–host receptor interactions will be important in testing this hypothesis.

In conclusion, we show for the first time that transcriptional repression of *var* genes located in different chromosomal domains uses different mechanisms. Interactions of different nuclear proteins with distinct promoter elements unique to either subtelomeric or chromosome-internal *var* gene promoters are involved in these processes. Our findings would support programmed *var* gene regulation and open the way for the identification and functional characterization of molecular components engaged in this process. This will be of great value in our understanding of antigenic variation and PfEMP1-mediated virulence.

Experimental procedures

Parasite cultures

Plasmodium falciparum 3D7 parasites were cultured in 100 mm and 150 mm Petri dishes at 5% haematocrit as described previously (Trager and Jensen, 1978) in RPMI medium supplemented with 0.5% albumax (Gibco BRL). Growth synchronization was achieved by sorbitol lysis (Lambros and Vanderberg, 1979).

Parasite nuclear extracts

Parasites were released from RBCs by saponin lysis. Nuclear

proteins were extracted into high-salt buffer from isolated parasite nuclei as described previously (Voss *et al*., 2002).

Subcloning of var *gene 4A3 and 5B1 5′ flanking regions*

The 4A3 5′ flanking region encompassing nucleotides −2312 to −569 was excised from pCAT4A3 (Voss *et al*., 2000) with *Eco*RV and *Pac*I. After digestion of the purified promoter fragment with *Apa*LI, *Ava*II, *Dde*I, *Dra*I and *Nde*I, subfragments were treated with Klenow enzyme to polish 5′ overhangs. Blunt-ended restriction fragments were cloned into pGEM3-Zf(+) (Promega) in a cycle-restriction ligation as described previously (Push *et al*., 1997). The *Sfc*I–*Hin*dIII fragment of the 5B1 promoter encompassing nucleotides −2142 to −248 was excised from pCAT5B1 (Voss *et al*., 2000) and digested further with *Ssp*I. Restriction fragments were cloned as above. After transformation into *Escherichia coli*, individual clones were identified by sequencing. Using this approach, we obtained plasmid clones carrying subfragments 4A3s(1+3a), (2), (3a), (3b), (4)–(10) and 5B1s(1), (4)–(7) (Fig. 1).

Probes and competitor DNA used in EMSAs

Promoter fragments subcloned into pGEM3-Zf(+) were excised with *Bam*HI and *Sac*I and agarose gel purified. Insert 4A3s(1+3a) was digested further with *Tsp509*I and *Sau3*AI to obtain fragments 4A3s(1a) and 4A3s(1b). Fragment 4A3s(5) was obtained by *Bgl*II digestion of the 4A3-B polymerase chain reaction (PCR) product amplified with primers 4A3-1732F and 4A3-1347R (Table 1). PCR fragments 5B1-B and 5B1-C were amplified with primer pairs 5B1-1879F/5B1-1655R and 5B1-1681F/5B1-1200R respectively. 5B1-B was digested with *Xba*I and *Nde*I to yield fragments 5B1s(2a) and 5B1s(2b); 5B1C was digested with *Ase*I to generate 5B1s(3) (Fig. 1). Fragments were radiolabelled with Klenow enzyme by incubating 1 pmol of DNA at 30°C for 20 min in 1 × React2 buffer (Gibco BRL) in the presence of 10 μCi of [α^{32}-P]-dCTP and 50 μM each dATP/dGTP/dTTP. Labelled fragments were excised from 5% nondenaturing polyacrylamide gels and eluted overnight into buffer E (0.1% SDS, 500 mM NH_4 acetate, 1 mM EDTA), followed by precipitation and resuspension in 1 × React3 buffer (Gibco BRL).

Double-stranded (ds) oligonucleotides used as competitors or probes (Table 1) were obtained by annealing equimolar amounts of complementary oligonucleotides in 1′React3 at 95°C for 5 min followed by slow cooling to room temperature. Annealing was confirmed by 20% PAGE analysis. ds oligonucleotide probes 4A3s(8)-1 and 5B1rec were labelled as above and purified using Sephadex G-25 spin columns (Amersham).

EMSA

Crude nuclear proteins (1.5–3 μg) were incubated with 5 fmol of radiolabelled probe in 20 μl of 1 × EMSA buffer [20 mM Hepes, pH 7.8, 60 mM KCl, 0.5 mM EDTA, 2 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 25 μM $ZnCl_2$, 0.1% Triton X-100, 10% glycerol] containing 2 μg pg poly-(dI–dC) as non-specific competitor DNA for 20 min at room temperature. An aliquot of 200 fmol of single-stranded (ss) oligonucleotide 5B1motF (5′-AGAAATGTGGTAGATAATATAGATAGAAAG-3′) was included in every reaction to prevent the formation of nonspecific ssDNA–protein complexes (Voss *et al*., 2002). Binding reactions were analysed on 5% or 6% polyacrylamide gels in 0.5% TBE. For competition experiments, labelled probes were added 10 min after incubation of protein and competitor DNA.

Transfection constructs

Transfection constructs used in this study were derivatives of pCAT4A3 and pCAT5B1. In these plasmids, *var* gene 5′ flanking regions and the *P. falciparum* calmodulin gene 3′ region control the expression of the chloramphenicol acetyltransferase (*cat*) gene (Voss *et al*., 2000). pCAT4A3 was digested with *Hin*dIII and *Bam*HI, and pCAT5B1 was digested with *Hin*dIII to eliminate the *var* gene promoter. 5′ nested deletions of the 4A3 promoter were established by PCR using the primer 4A3-28R in conjunction with the respective 4A3 forward primers (Table 1). 4A3-28R is positioned at −1 with respect to the initiation ATG and harbours a *Bam*HI site; all forward primers carry a *Hin*dIII recognition sequence. PCR products were cloned into the *Bam*HI–*Hin*dIII backbone of pCAT4A3. 5′ nested deletions of the 5B1 promoter were obtained by PCR using 5B1EABR together with the forward 5B1 primers (Table 1). 5B1EABR is located at position −209 with respect to the initiation ATG of *var* gene 5B1 and includes a wild-type *Hin*dIII site. A *Hin*dIII site is also present in all 5B1 forward primers. PCR products were cloned into the *Hin*dIII backbone of pCAT5B1 in sense orientation.

pVLH/int (kindly provided by K. Deitsch), carrying the *var7b* promoter (Deitsch *et al*., 2001a), was used to generate luciferase constructs. The *var* intron present in pVLH/int was excised with *Bam*HI and *Spe*I, and the plasmid was religated (pVLUC) after polishing with Klenow enzyme. The 5B1 promoter, amplified from pCAT5B1 with primers T7-*Kpn*I_F and 5B1-*Hpa*I_R (−108), was ligated into *Kpn*I–*Hpa*I-digested pVLUC, thus replacing the *var7b* promoter (pVLUC5B1). Likewise, the 4A3 promoter [primers T7-*Kpn*I_F and 4A3-*Hpa*I_R (−1)] was introduced to generate pVLUC4A3. To obtain an internal 114 bp deletion in the 4A3 promoter (encompassing *SPE1*), we cloned the *Hin*dIII-digested PCR fragment generated with primers 4A3-2621F and 4A3-1180R (nucleotides −2621 to −1180) into the *Hin*dIII site of pCAT4A3-1066 (pCAT4A3-Δ). The PCR fragment amplified with primers 5B1-2522F and 5B1-1483R was digested with *Hin*dIII and cloned into the upstream *Hin*dIII site of pCAT5B1-1325 to generate pCAT5B1-Δ, carrying an internal deletion of 158 bp (including *CPE*). The 5B1-Δ and 4A3-Δ promoters were amplified as above and cloned into *Kpn*I–*Hpa*I-digested pVLUC to obtain pVLUC5B1-Δ and pVLUC4A3-Δ respectively.

Isolation of parasite total RNA and Northern analysis

Parasite total RNA was isolated and stored as described previously (Kyes *et al*., 2000). For Northern analysis, equal amounts of RNA extracted from synchronized parasite cul-

tures were electrophoretically separated on a 0.8% agarose gel (5 mM GTC) and vacuum transferred to Hybond-XL membranes (Amersham). Subtelomeric *var* transcripts were detected with a 460 bp probe (–460 to –1 of the 4A3 5′ flanking region) amplified with oligonucleotides 17F (5′-GTT TATATATTTTGTAAAATTATAA/TATGAG-3′) and 4A3-28R. Chromosome-central *var* gene transcription was monitored with a 277 bp PCR fragment (–514 to –237) generated with primers 5B1-514F (5′-GCATAAGCTTCCATCACATATAGTAC GACTAAGAAACA-3′) and 5B1EABR. PCR fragments were gel purified and radiolabelled with [α^{32}-P]-dATP and Klenow polymerase using primers 4A3-28R or 5B1EABR respectively. Hybridization was performed at 42°C in UltraHyb buffer (Ambion). The 28S RNA probe was amplified from 3D7 genomic DNA with primers Pf28SF (5′-GTGATGAGAT TGAAGTCAGACG-3′) and Pf28SR (5′-AGTTCAACGAAC CTCTTCTCC-3′).

Parasite transfection and reporter assays

Transient transfection of cultured 3D7 *P. falciparum* ring-stage parasites and CAT assays were performed as described previously (Voss *et al*., 2000). For luciferase assays, saponin-released parasites were lysed in reporter lysis buffer (Promega) at time points according to the occurrence of the respective DNA-binding activities, i.e. 20 h.p.i. for parasites transfected with 4A3 promoter constructs, and 30 h.p.i. for parasites transfected with 5B1 promoter constructs (Fig. 5). The lysate was freeze–thawed once. Luciferase activity in parasite lysates was determined using the luciferase assay system (Promega) and the Autoluminat LB 953 luminometer.

Sequence analysis

Sequence data for *P. falciparum* chromosomes were obtained from The Sanger Centre (http://www.sanger.-ac.uk/Projects/P_falciparum), from The Institute for Genomic Research (http://www.tigr.org), from the Naval Medical Research Centre and from The Stanford DNA Sequence and Technology Centre (http://www-sequence.stanford.edu/group/malaria). Sequencing of *P. falciparum* chromosomes was accomplished as part of the Malaria Genome Project with support from the Wellcome Trust, the Burroughs Wellcome Fund and the US Department of Defense.

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