

Alterations in local chromatin environment are involved in silencing and activation of subtelomeric *var* genes in *Plasmodium falciparum*

OnlineOpen: This article is available free online at www.blackwell-synergy.com

Till S. Voss,^{1†} Christopher J. Tonkin,¹
Allison J. Marty,^{1,2} Jennifer K. Thompson,¹
Julie Healer,¹ Brendan S. Crabb¹ and
Alan F. Cowman^{1*}

¹*Division of Infection and Immunity, The Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Australia.*

²*Department of Microbiology, Monash University, Clayton 3800, Australia.*

Summary

***Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the *var* gene family, undergoes antigenic variation and plays an important role in chronic infection and severe malaria. Only a single *var* gene is transcribed per parasite, and epigenetic control mechanisms are fundamental in this strategy of mutually exclusive transcription. We show that subtelomeric *upsB* *var* gene promoters carried on episomes are silenced by default, and that promoter activation is sufficient to silence all other family members. However, they are active by default when placed downstream of a second active *var* promoter, underscoring the significance of local chromatin environment and nuclear compartmentalization in *var* promoter regulation. Native chromatin covering the *SPE2*-repeat array in *upsB* promoters is resistant to nuclease digestion, and insertion of these regulatory elements into a heterologous promoter causes local alterations in nucleosomal organization and promoter repression. Our findings suggest a common logic underlying the transcriptional control of all *var* genes, and have important implications for our understanding of the epigenetic processes involved in the regulation of this major virulence gene family.**

Accepted 2 August, 2007. *For correspondence. E-mail cowman@wehi.edu.au; Tel. (+61) 3 9345 2555; Fax (+61) 3 9347 0852. †Present address: Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, 4051 Basel, Switzerland. Re-use of this article is permitted in accordance with the Creative Commons Deed, Attribution 2.5, which does not permit commercial exploitation.

Introduction

Phenotypic variation is a prerequisite for survival in a competitive and changing environment. In the case of pathogenic microorganisms, this is exemplified by the evolution of antigenic variation of cell surface proteins. Although various fundamentally different strategies for the regulation of antigenic variation have been described in different pathogens (Biggs *et al.*, 1991; Deitsch *et al.*, 1997; Pays *et al.*, 2004; Al-Khedery and Allred, 2006; Dai *et al.*, 2006), most rely on mutually exclusive expression of gene families; that is, only one family member is expressed in a single cell at any given time. By switching to the expression of a different antigenic variant, pathogens escape pre-existing immune responses, increasing the likelihood of chronic infection and successful transmission.

In *Plasmodium falciparum*, responsible for the most severe form of malaria in humans, erythrocyte membrane protein 1 (PfEMP1) undergoes such antigenic variation (Biggs *et al.*, 1991; Roberts *et al.*, 1992). PfEMP1, encoded by the 60-member *var* gene family (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995), is exposed on the surface of infected erythrocytes, where it mediates cytoadherence to host endothelium and to uninfected erythrocytes (Baruch *et al.*, 1996; Gardner *et al.*, 1996; Rowe *et al.*, 1997; Reeder *et al.*, 1999). The resulting sequestration of red cell aggregates in the microvasculature of various organs is linked to severe complications, including cerebral and placental malaria (MacPherson *et al.*, 1985; Carlson *et al.*, 1990; Pongponratn *et al.*, 1991; Berendt *et al.*, 1994; Rowe *et al.*, 1995; Beeson and Duffy, 2005). Hence, due to the combined phenotypes of antigenic variation and cytoadherence, PfEMP1 plays a crucial role in the chronicity of infection and malaria-associated morbidity and mortality.

var gene expression is controlled at the level of transcription and occurs in a mutually exclusive manner (Scherf *et al.*, 1998). They are preceded by one of three major conserved upstream sequences (*upsA*, *upsB*, *upsC*), and a link exists between their chromosomal position and the upstream sequence they possess (Voss *et al.*, 2000; Gardner *et al.*, 2002). *var* genes in similar chromosomal locations were observed to share common

directional orientations (Kraemer and Smith, 2003; Lavstsen *et al.*, 2003). *UpsB* var genes are the most telomere-proximal genes in *P. falciparum* and are transcribed towards the centromere. *UpsA* var genes, and the *var2csa* gene flanked by the unique *upsE* sequence, are located next to and downstream of *upsB* genes and are transcribed towards the telomere. *UpsC* var genes exclusively occur in chromosome-internal clusters. These findings indicated a functional role of var upstream sequences in transcription, and indeed, *upsB* and *upsC* sequences were shown to possess weak promoter activity in transient transfection experiments (Deutsch *et al.*, 1999; Voss *et al.*, 2000). Additionally, sequence- and stage-specific DNA-protein interactions with possible functions in var gene repression and silencing were identified (Voss *et al.*, 2003). The var intron plays an essential role in *upsC* silencing (Deutsch *et al.*, 2001) and, although the underlying mechanisms remain obscure, this finding has been confirmed (Calderwood *et al.*, 2003; Gannoun-Zaki *et al.*, 2005; Dzikowski *et al.*, 2006; Frank *et al.*, 2006). Our recent work suggested that although *upsC* promoters are silenced by default in absence of the intron, silencing is enhanced in presence of this element (Voss *et al.*, 2006). Recently, it has been shown that var gene transcription is controlled at the level of RNA polymerase II-dependent transcription initiation (Kyes *et al.*, 2007).

An important role in global var gene regulation can be attributed to the intranuclear positioning of var genes. Most var genes are located in subtelomeric regions (Gardner *et al.*, 2002) and, as in yeast, *P. falciparum* chromosome ends cluster at the nuclear periphery (Freitas-Junior *et al.*, 2000). Chromosome-central var genes are also located at the nuclear periphery independent of their transcriptional state (Ralph *et al.*, 2005; Marty *et al.*, 2006; Voss *et al.*, 2006). The perinuclear compartment is associated with enhanced transcriptional silencing in yeast and other eukaryotes (Andrulis *et al.*, 1998; Cockell and Gasser, 1999; Gasser, 2001; Misteli, 2004). Likewise, in *P. falciparum* two subtelomeric genes, a chromosomal transgene and *var2csa*, were demonstrated to be reversibly silenced involving locus repositioning (Duraisingh *et al.*, 2005; Ralph *et al.*, 2005). Activation of artificial var loci occurs preferentially at chromosome-end clusters (Voss *et al.*, 2006) and results in silencing of endogenous var gene transcription (Dzikowski *et al.*, 2006; Voss *et al.*, 2006). Together, these observations suggest the existence of a specialized perinuclear var transcription site.

Reversible histone modifications and ATP-dependent nucleosome remodelling provide the framework for the versatile epigenetic control of eukaryotic gene regulation (Jenuwein and Allis, 2001; Moazed, 2001; Rusche *et al.*, 2003). In *P. falciparum*, var gene activation was shown to be associated with histone H4 acetylation (Freitas-Junior *et al.*, 2005) and histone H3K9 trimethylation was linked

to var gene silencing (Chookajorn *et al.*, 2007). Additionally, a role for the *P. falciparum* histone deacetylase silent information regulator 2 (PfSIR2) in var gene silencing has been revealed (Duraisingh *et al.*, 2005; Freitas-Junior *et al.*, 2005). *P. falciparum* chromatin is organized into typical nucleosomal arrays even on transfected episomes (Horrocks *et al.*, 2002), and activation of a silenced subtelomeric transgene was associated with different sensitivities of native chromatin to micrococcal nuclease (MNase) digestion (Duraisingh *et al.*, 2005).

To date, functional investigation of var promoters has focused mainly on the chromosome-central *upsC* type (Deutsch *et al.*, 2001; Calderwood *et al.*, 2003; Gannoun-Zaki *et al.*, 2005; Frank *et al.*, 2006; Voss *et al.*, 2006). Furthermore, while the above findings clearly highlight the important role of var gene regulatory sequences in reversible gene activation and mutual exclusion, the epigenetic processes and factors involved are poorly understood. Here, we investigated the role of subtelomeric *upsB* promoters in var gene regulation, and show that they are silenced by default and are functionally equivalent to chromosome-central *upsC* promoters. We demonstrate that two episomal var gene promoters *in cis* are activated simultaneously, implying that mutual exclusion is not based on single promoter competition but rather on locus activation. Low-resolution chromatin structure analysis identified two MNase-resistant regions in the *upsB* promoter, but revealed no difference in nucleosome positioning between opposite transcriptional states. Interestingly, one of the protected regions contains the previously identified regulatory *SPE2*-repeat array. Targeting of the *SPE2* binding activity to a heterologous promoter induces alterations in nucleosomal organization and promoter repression.

Results

Subtelomeric upsB var gene promoters are epigenetically regulated and silenced by default

In order to test whether *upsB* promoters play a role in the epigenetic regulation of subtelomeric var genes, we transfected constructs pHBupsB, pHBupsB^R and pHBupsB^{RI} containing two drug-resistance cassettes encoding (i) blasticidin deaminase (*bsd*), controlled by the *hsp86* promoter (for stable maintenance of episomes), and (ii) human dihydrofolate reductase (*hdhfr*), driven by an *upsB* promoter (Fig. 1A). A 0.5 kb sequence of rep20 repeats, which naturally occur upstream of subtelomeric *upsB* promoters and favour localization of episomes to perinuclear chromosome-end clusters (O'Donnell *et al.*, 2002), was included in pHBupsB^R and pHBupsB^{RI}. The var gene intron was added in pHBupsB^{RI} because its role in silencing has up to now only been demonstrated for *upsC*

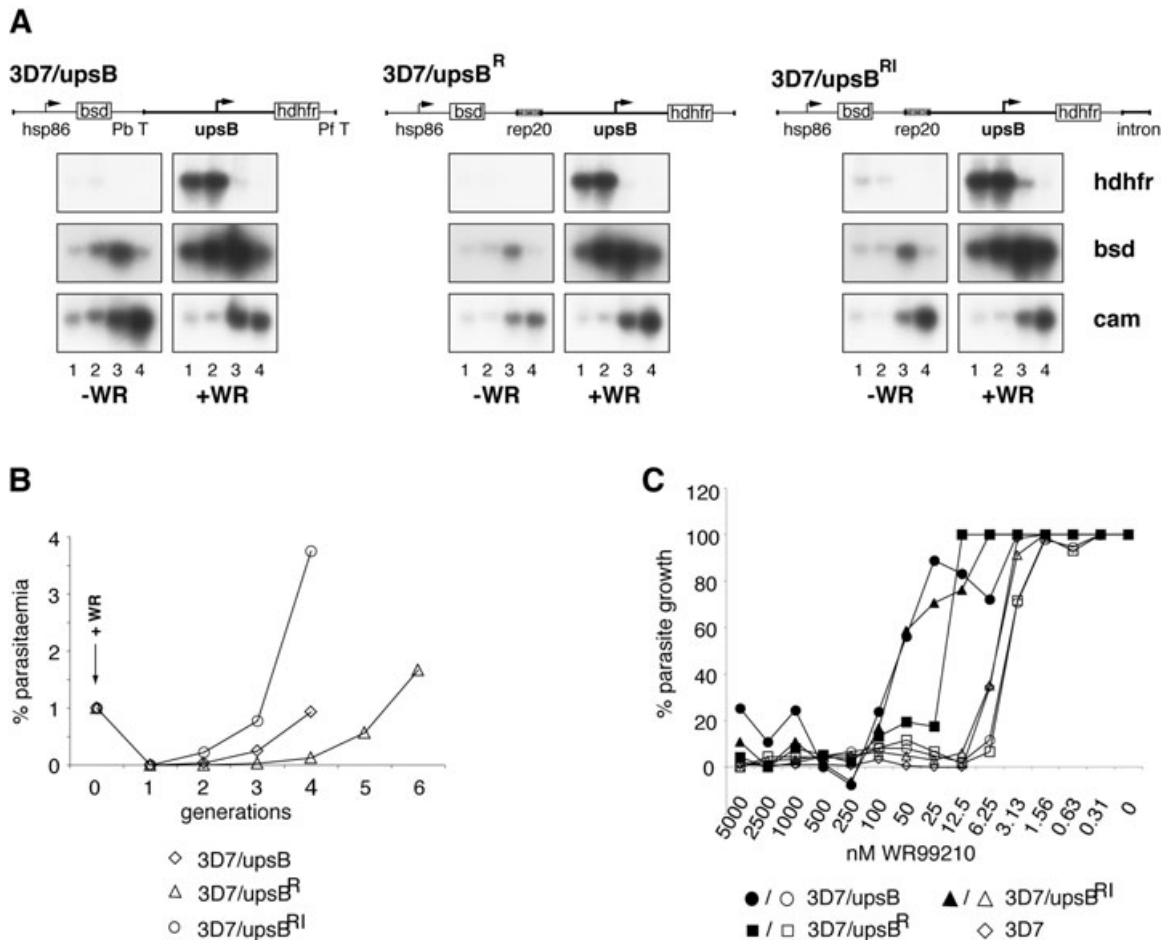


Fig. 1. Epigenetic regulation of *upsB* promoters.

A. Silencing and activation of *upsB*-regulated transcription. Northern blots showing episomal *hdhfr* and *bsd* transcription across the intra-erythrocytic asexual cycle in 3D7/*upsB*, 3D7/*upsB*^R and 3D7/*upsB*^{RI} before (–WR) and after (+WR) WR selection. Transcription of the endogenous *cam* gene serves as a stage-specific loading control. Vector maps are shown above each set of autoradiographs. *hsp86*, heat-shock protein 86 promoter; Pb T, *P. berghei* dhfr-thymidilate synthase terminator; *upsB*, *upsB* upstream sequence; Pf T, *P. falciparum* *hrp2* 3' terminator; rep20, 0.5 kb rep20 repeats; intron, 0.6 kb *var* intron sequence. (1) 0–12 hpi (hours post-invasion); (2) 12–24 hpi; (3) 24–36 hpi; (4) 32–44 hpi.

B. Growth assay. Blasticidin-S-selected parasites were challenged with WR at day 0. Parasite growth in the presence of WR was monitored over the following generations. Assays were repeated twice with the same result.

C. WR sensitivities of 3D7/*upsB*, 3D7/*upsB*^R and 3D7/*upsB*^{RI} before (open) and after (filled) WR selection, and of 3D7 wild-type parasites.

promoters (Deutsch *et al.*, 2001; Calderwood *et al.*, 2003; Gannoun-Zaki *et al.*, 2005; Frank *et al.*, 2006; Voss *et al.*, 2006). After transfection, parasites were selected on blasticidin-S to generate 3D7/*upsB*, 3D7/*upsB*^R and 3D7/*upsB*^{RI}. Northern blot experiments indicated that the *upsB* promoters were silenced by default in all three lines (Fig. 1A). All lines were also sensitive to challenge with WR consistent with the silenced *hdhfr* gene (Figs 1B and C). Continuous growth in the presence of WR, however, selected for WR-resistant populations after 3–6 generations. In these lines, *hdhfr* was transcribed at high levels in ring-stage parasites as a consequence of *upsB* promoter activation (Fig. 1A). The observation that episomal *upsB* promoters maintain the temporal activity profile of endogenous *upsB* promoters (Voss *et al.*, 2003) indi-

cates that the promoter region used is fully functional and contains the required regulatory elements. These data are consistent with what has previously been described for *upsC* promoters.

To derive relative activity values for *upsB* promoters, we determined *hdhfr* transcript levels by densitometry (Figs 2 and S1). *UpsB* promoters produced 61-fold (pHBupsB^R), 37-fold (pHBupsB) and 28-fold (pHBupsB^{RI}) more transcripts after activation compared with the silenced state, similar to the level of *upsC* activation (44-fold). A comparable increase in activity (54-fold) was also measured after integration of pHBupsB^R into the subtelomeric *var* locus PFL0005w (Fig. S2). The silencing emanating from *upsB* promoters spread in *cis*, which was evident from the reduced *hsp86* promoter activity on

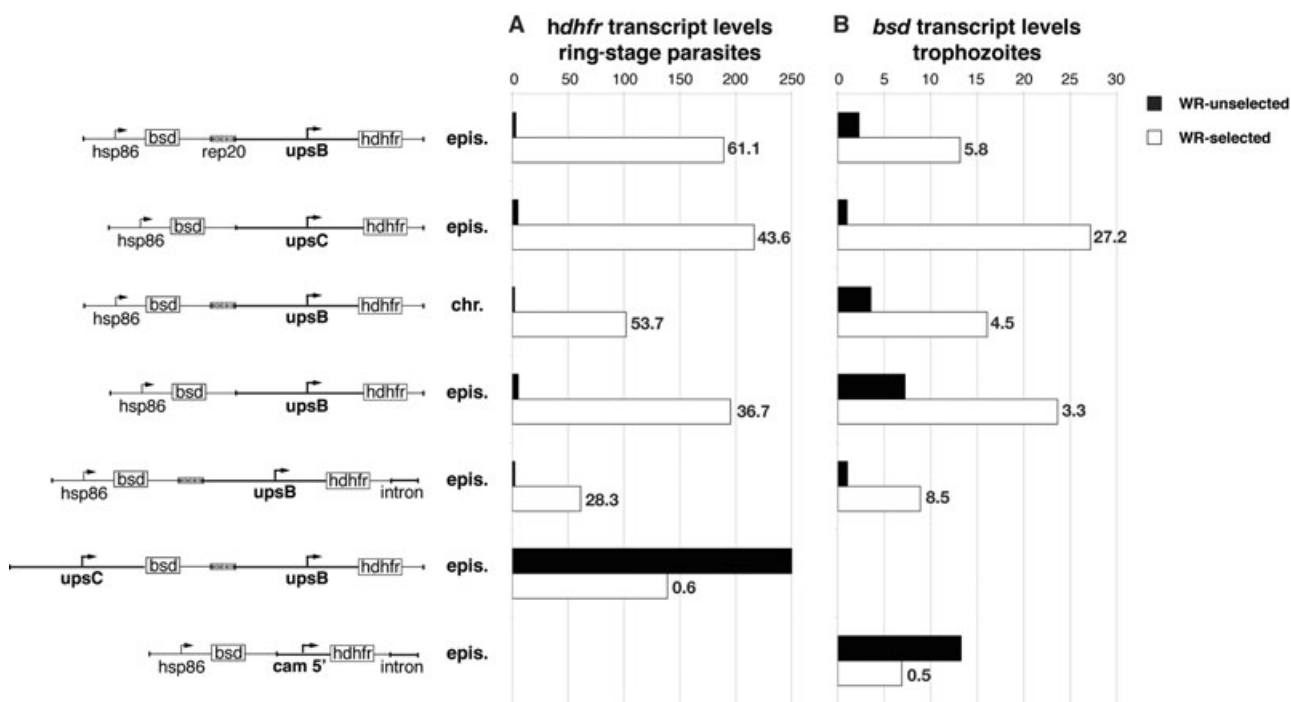


Fig. 2. Analysis of *var* promoter activity by densitometry.

A. Analysis of *hdhfr* transcript abundance in transfected 3D7 ring-stage parasites. Vector maps for each transfected line are shown on the left. The relative *hdhfr* transcript production per promoter before and after selection on WR is displayed (see also *Experimental procedures* and Fig. S1).

B. Analysis of *bsd* transcript abundance in 3D7 trophozoites. The *cam* promoter construct at the bottom of the panel was used as control. The relative *bsd* transcript production per promoter before and after selection on WR is displayed. Numbers behind open bars (WR-selected) indicate the fold increase in steady-state transcripts compared with the default state (WR-unselected) (black bars). epis, episomal; chr, chromosomal.

silenced episomes (Fig. 2). While the intron is not required for *upsB* silencing, it appears to decrease *upsB* activity (Fig. 2), a tendency we also observed with *upsC* promoters (Voss *et al.*, 2006). In contrast to *upsC* promoters, however, *upsB* promoter activation did not occur less frequently in the presence of the intron as demonstrated by the similar sensitivities of 3D7/*upsB*, 3D7/*upsB^R* and 3D7/*upsB^R* parasites to WR challenge (Fig. 1B), suggesting that the *var* intron does not augment silencing of *upsB* promoters.

UpsB promoter activation interferes with mutually exclusive var expression

Our findings reveal striking similarities between the regulation of subtelomeric *upsB* and chromosome-central *upsC* promoters, suggesting that silencing of all *var* genes is generally induced by their *cis*-linked regulatory elements, independent of chromosomal location and/or promoter type. Moreover, activation of artificial *var* loci, both episomal and chromosomal, prevents transcription of endogenous *var* genes, emphasizing the central role of *var* promoters in mutually exclusive *var* gene transcription

(Dzikowski *et al.*, 2006; Voss *et al.*, 2006). However, for *upsB* promoters this effect was only demonstrated for a chromosomal locus (Dzikowski *et al.*, 2006). To test whether activation of artificial *upsB* loci carried on episomes also interferes with mutual exclusion, we investigated the ability of WR-selected 3D7/*upsB^R* parasites to express PfEMP1 and to adhere to the endothelial receptor CD36. Similar to WR-selected 3D7/*upsC^R* parasites (Voss *et al.*, 2006), we find that 3D7/*upsB^R* expressing the *hdhfr*-resistance gene failed to express PfEMP1 (Fig. 3). Consequently, adherence to CD36 of red blood cells (RBCs) infected with WR-selected 3D7/*upsB^R* occurred at an average of 14.4% (13.8% and 15%) compared with unselected parasites. Moreover, fluorescent *in situ* hybridization (FISH) revealed a significant difference ($P < 0.05$) in the colocalization of pHBupsB with telomeric clusters in WR-unselected ($39 \pm 2.8\%$; mean $n = 113$) and WR-selected 3D7/*upsB* parasites ($62 \pm 3.5\%$; mean $n = 102$). This finding indicates that *upsB* promoter activation occurs in perinuclear chromosome-end clusters and provides a further parallel to the regulation of *upsC* (Voss *et al.*, 2006) and *upsE* (Marty *et al.*, 2006) promoters.

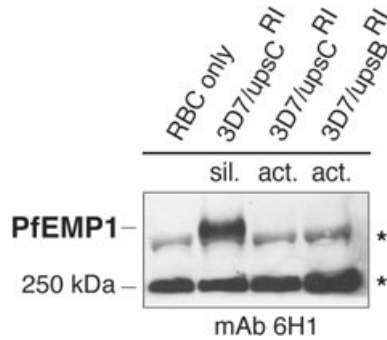


Fig. 3. PfEMP1 is not expressed in *var* knock-down parasites. Detection of PfEMP1 by Western analysis of Triton X-100-insoluble/SDS-soluble membrane fractions from uninfected (control) and iRBCs. The two bands at approximately 250 kDa (asterisks) represent cross-reactive α - and β -spectrin (Cooke *et al.*, 2006). sil., silenced *var* promoter; act., activated *var* promoter.

Two episomal *var* promoters in *cis* are simultaneously active

Because *P. falciparum* episomes are maintained as concatamers of tandemly repeated plasmids (O'Donnell *et al.*, 2001), it was impossible in previous studies to determine whether only a single episomal *var* promoter was activated at any time, or whether instead multiple *var* promoters were active simultaneously on the same DNA molecule. To test this, we designed construct pHBupsCB^R, where two *var* gene promoters, *upsC* and *upsB*, control transcription of the *bsd* and *hdhfr* reporter genes respectively (Fig. 4A). Transfection of pHBupsCB^R followed by selection on blasticidin-S selected for the transgenic line 3D7/upsCB^R. Northern analysis revealed that

upsC promoted transcription of the *bsd* gene in ring-stage and early trophozoite parasites as expected. Strikingly, in this context the downstream *upsB* promoter was fully activated by default, demonstrating that local *var* promoter activation dominates over silencing (Figs 2 and 4A). Consistent with this finding, WR-unselected 3D7/upsCB^R parasites were resistant to WR challenge unlike 3D7/upsB^R parasites, where the *upsB-hdhfr* cassette was silenced (Fig. 1A and 4B). Together, these results prove that multiple episomal *var* promoters *in cis* are active simultaneously. We hypothesize that this is related to the absence of boundary/insulator elements on the plasmids, which are naturally required to prevent simultaneous activation of neighbouring *cis*-linked *var* genes. These findings furthermore suggest that the local chromatin environment is important to determine the transcriptional state of *var* gene promoters.

Evidence for positioned nucleosomes in *var* gene promoters

We recently reported differences in local chromatin structure between the silenced and active states of a subtelomeric transgene in *P. falciparum* (Duraisingh *et al.*, 2005). Here we have investigated whether such alterations are also important in the regulation of *var* gene promoters. Indirect end-labelling of native chromatin in 3D7/upsB^R parasites revealed a pattern of MNase-sensitive sites, which might be indicative for a number of positioned nucleosomes (Fig. 5). A number of MNase-sensitive sites were also preferentially cut in naked plasmid DNA (Fig. S3), and due to the low-resolution mapping strategy

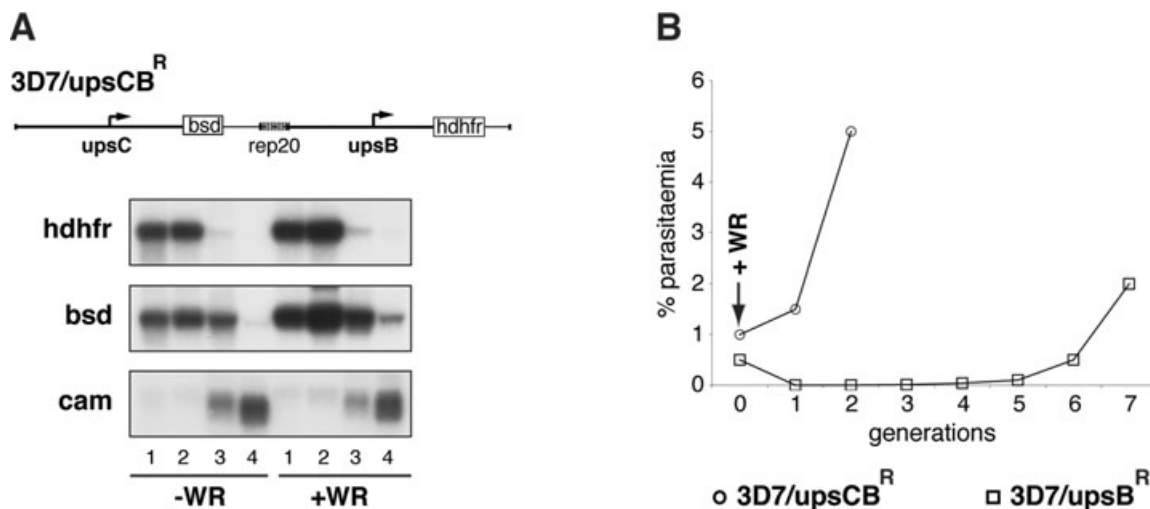


Fig. 4. Simultaneous activation of two *var* gene promoters *in cis*. A. Activity of the *upsC* and *upsB* promoter in 3D7/upsCB^R parasites before (–WR) and after (+WR) WR selection. Northern blots showing episomal *hdhfr* and *bsd* transcription across the intra-erythrocytic cycle. Transcription of the endogenous *cam* gene serves as a stage-specific loading control. The vector map is shown on top. (1) 0–12 hpi; (2) 12–24 hpi; (3) 24–36 hpi; (4) 32–44 hpi. B. Growth assay. Blasticidin-S-selected 3D7/upsCB^R and 3D7/upsB^R parasites were challenged with WR at day 0. Parasite growth in the presence of WR was monitored over the following generations.

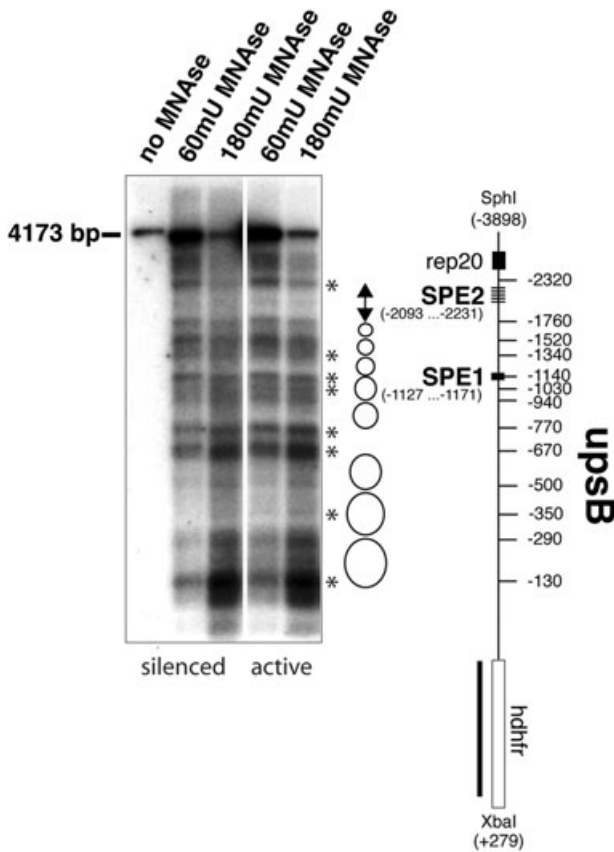


Fig. 5. Low-resolution chromatin analysis of episomal *upsB* promoters. Native chromatin was digested with MNase followed by indirect end-labelling. Southern blots were hybridized with a 257 bp *hdhfr* probe to detect MNase-sensitive sites in the silenced and activated *upsB* promoters on pHBupsB^R. A map of the 4172 bp XbaI/SphI fragment of pHBupsB^R containing the *upsB* promoter is shown on the right. MNase-sensitive sites are highlighted with respect to the *hdhfr* start codon. The positions of the *cis*-acting elements *SPE1* and *SPE2* and the *rep20* repeat region are indicated. The arrow depicts the MNase-resistant region (–1760 to –2320) containing five direct *SPE2* repeats (–2093 to –2231) (Voss et al., 2003). Asterisks identify sites that are also preferentially cut in naked plasmid DNA (Fig. S3). Circles represent a proposed nucleosomal organization.

employed here, it remains unknown whether these sites are truly positioned between adjacent nucleosomes. Two highly accessible sites are located within the region containing the transcriptional start site (–515 to –804) (Voss et al., 2003), followed by a protected area within the 5' untranslated region. A 200 bp region in the promoter (–940 to –1140) contains three consecutive MNase-sensitive sites, positioned directly downstream of the *SPE1* regulatory element (–1127 to –1171) (Voss et al., 2003). A 500 bp region upstream (–1760 to –2320) was highly resistant to digestion even at high enzyme concentrations. This region contains the regulatory *SPE2*-repeat array (–2093 to –2231) (Voss et al., 2003), suggesting that the *SPE2* binding activity may play a key

role in chromatin organization and epigenetic regulation. However, we neither observed significant differences between the silenced and active states of *upsB* (Fig. 5) or *upsC* promoters (data not shown), nor did we detect any effects conferred by *rep20* repeats or the *var* intron on the local chromatin structure of *var* gene promoters (data not shown).

Regulatory SPE2 elements induce local chromatin compaction and repress the activity of a heterologous promoter

We were interested in testing the potential role of *SPE2* elements in chromatin organization and transcriptional regulation. We generated construct pHBcamSPE2^R with three tandem copies of *SPE2* inserted into the heterologous *P. falciparum* calmodulin (*cam*) promoter (Fig. 6A). pHBcam^R (Voss et al., 2006) and pHBcamSPE2m^R, where point mutations in the *SPE2* sequence prevent the interaction with the *SPE2* binding protein (Voss et al., 2003), were used as controls. The three blasticidin-S-resistant transgenic lines 3D7/*cam*, 3D7/*camSPE2*^R and 3D7/*camSPE2m*^R were resistant to WR challenge, indicating that the *cam* promoter was not silenced by insertion of *SPE2* elements (data not shown). However, insertion of *SPE2* strongly affected the temporal activity profile of the *cam* promoter. The activity of the *camSPE2* promoter was restricted to schizont-stage parasites [32–44 h post invasion (hpi)]. In contrast, the wild-type *cam* promoter was active across much of the intra-erythrocytic cycle, and insertion of mutated *SPE2* elements had no effect (Fig. 6A). To obtain evidence that this observation was due to the actual interaction of the *SPE2* binding activity with its cognate binding site, we analysed the chromatin structure in these promoters by indirect end-labelling. The pattern of MNase-sensitive sites in the episomal wild-type *cam* promoter was similar to the pattern observed in a *cam* promoter at a subtelomeric transgene locus (Duraishigh et al., 2005). However, after insertion of the *SPE2* elements at position –833, the two sensitive sites at –890 and –1050 were now protected from MNase digestion (Fig. 6B). Furthermore, these alterations were specific to the *SPE2* elements as insertion of the mutated *SPE2* repeats had no effect (Fig. 6C). These findings are consistent with the MNase resistance of the *SPE2* array in *upsB* promoters, and indicate that the change in temporal *cam* promoter activity is a direct result of *SPE2* binding factor recruitment to the introduced *SPE2* elements.

Discussion

var gene promoters are of central importance in epigenetic regulation and mutual exclusion of *var* gene transcription. Previous studies focused almost exclusively

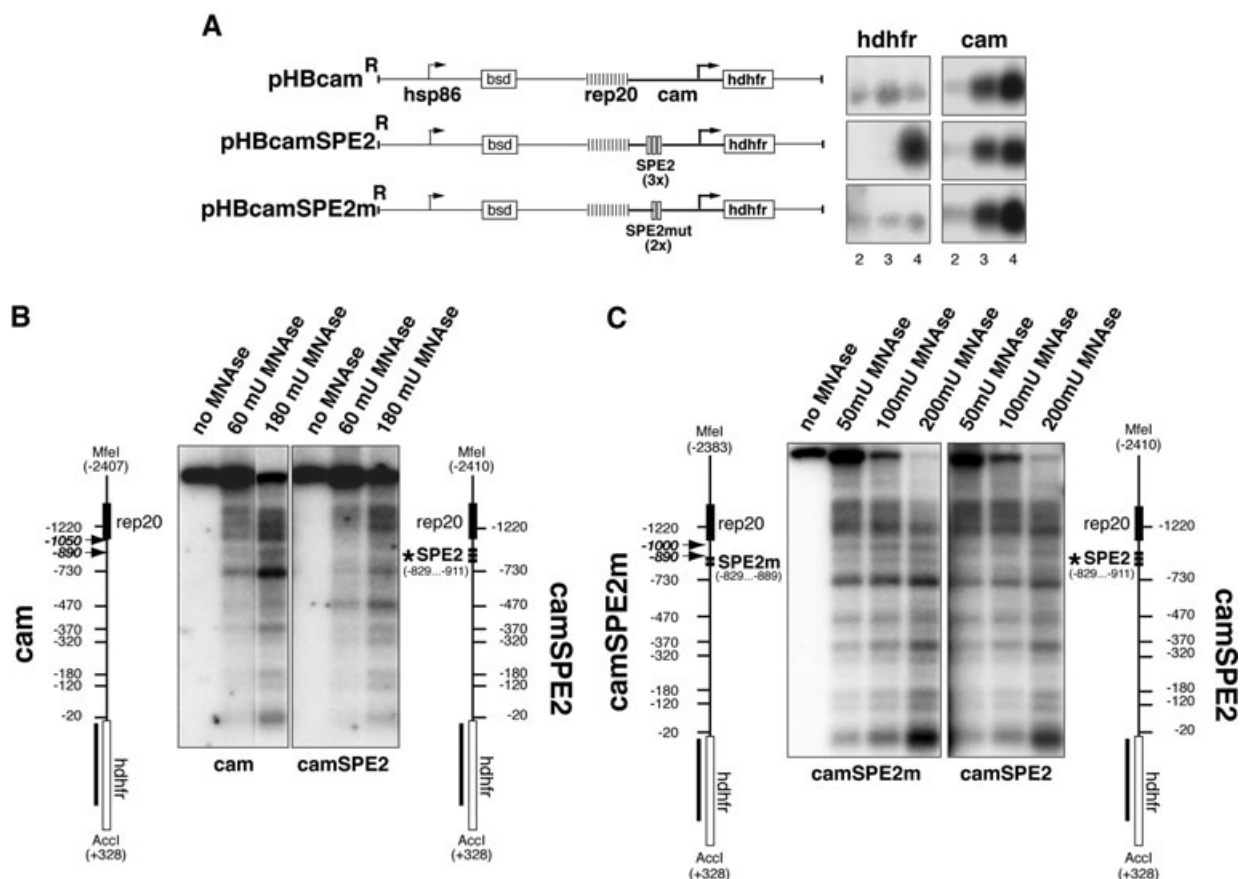


Fig. 6. Insertion of *SPE2* elements into the heterologous *cam* promoter.

A. The effect of *SPE2* insertion on *cam* promoter timing. Plasmid maps are shown on the left. Stage-specific Northern blots to detect *hdhfr* and *cam* (control) transcription is shown on the right. (2) 12–24 hpi; (3) 24–36 hpi; (4) 32–44 hpi.

B. Comparison of the chromatin structure of the episomal *cam* and *camSPE2* promoters. MNase-sensitive sites are indicated with respect to the *hdhfr* start codon. The position of the inserted *SPE2* elements (–829 to –911) in *camSPE2* is shown. *SPE2* insertion causes local protection from MNase digestion (asterisk), and the two MNase-sensitive sites in the *cam* wild-type promoter disappear (arrows).

C. Comparison of the chromatin structure of the episomal *camSPE2* and *camSPE2m* promoters. The positions of the inserted *SPE2* (–829 to –911) and *SPE2m* (–829 to –889) elements are shown. Note that the two MNase-sensitive sites in the *cam* wild-type promoter (–890 and –1050) are retained after insertion of the mutated *SPE2m*.

on the role of *upsC* promoters controlling the expression of chromosome-central *var* genes. Here we show that subtelomeric *upsB* promoters (associated with the largest *var* gene subgroup) display similar functional properties: (1) *upsB* promoters are regulated by epigenetic mechanisms to establish either a silenced (default) or active state; (2) the transcriptional state is inherited over many generations in the absence of selective pressure; (3) *upsB* silencing and activation can spread *in cis*, affecting neighbouring loci; and (4) activation of episomal *upsB* promoters occurs preferentially at chromosome-end clusters at the nuclear periphery and interferes with mutual *var* gene exclusion, preventing expression of PfEMP1.

Several studies investigating *var* promoter function in transiently or stably transfected parasites suggested that *upsC* promoters are activated by default and only silenced in the presence of a *cis*-linked *var* intron (Deitsch *et al.*,

2001; Gannoun-Zaki *et al.*, 2005; Frank *et al.*, 2006). In contrast, in this study we have demonstrated efficient silencing of *upsC* (Voss *et al.*, 2006) and *upsB* promoters in absence of the intron. Our results are consistent, however, with a role of the *var* intron in insulator/boundary function and in controlling the degree of promoter activation particularly for *upsC*. An interesting explanation for this ‘intron controversy’ was recently proposed (Frank and Deitsch, 2006) and is related to the intron’s own promoter activity (Calderwood *et al.*, 2003). Heterologous promoters (including the *hsp86* promoter) were able to functionally replace the role of the intron in *upsC* silencing (Frank and Deitsch, 2006). It was proposed that a *cis*-linked promoter activity, rather than the intron sequence itself, mediates cooperative *var* promoter silencing. This would suggest that the *hsp86* promoter present on all constructs in this and our previous study (Voss *et al.*, 2006) may

have functionally replaced the intron to silence *var* promoters. However, Viebig *et al.* (2005) found that integration into the *var2csa* locus of a *hdhfr*-resistance cassette under control of the calmodulin promoter led to the constitutive activation, rather than silencing, of *upsE*-driven transcription of the truncated *var2csa* gene. Hence, and in our view more likely, differences in transfection vector design and/or the drug selection strategy applied to select for transfected parasites (*hdhfr* versus *bsd*) may explain these contradictory results. Whatever the reason, the exact role of the intron in *var* promoter silencing remains elusive and cannot be determined from the existing information, and other approaches are clearly required to clarify this issue.

var genes are located at the nuclear periphery independent of their transcriptional state, and switches in *var* transcription are linked to perinuclear locus repositioning (Duraisingh *et al.*, 2005; Ralph *et al.*, 2005; Voss *et al.*, 2006). A current model suggests a unique perinuclear domain permissive for transcription of a single *var* locus. Consistent with this hypothesis, drug-induced activation of episomal *var* promoters is sufficient to compete with endogenous *var* transcription (Dzikowski *et al.*, 2006; Voss *et al.*, 2006). However, due to the concatameric structure of episomes in *P. falciparum*, previous studies failed to reveal whether the basis for mutual exclusion is truly related to direct *var* promoter competition. To address this issue, we designed plasmid pHBupsCB^R carrying two *cis*-linked *var* promoter cassettes. In presence of an activated upstream *upsC* promoter, the *upsB* promoter was fully activated by default. This is in striking contrast to pHBupsB^R, where the *upsB* promoter is naturally silenced. Therefore, in a closely *cis*-linked context, two *var* promoters can be active simultaneously and *var* promoter activation is dominant over silencing. This suggests that mutual *var* exclusion involves activation of a chromosomal *var* locus through chromatin alterations transforming the regulatory regions into a transcription-competent state. Co-activation of neighbouring chromosomal *var* genes in their native context is presumably prevented by boundary elements that sequester the spread of active chromatin. While this scenario is consistent with the existence of a perinuclear domain competent for *var* gene transcription, it also highlights that a model in which such a domain would be physically restricted to activate only a single *var* gene is not sufficient to explain mutually exclusive *var* transcription.

Despite the fact that all *var* genes are wired into the same allelic exclusion programme, it is noteworthy that different epigenetic factors appear to be involved in the regulation of different *var* gene subsets. As demonstrated by microarray analysis, the *var2csa* and *upsA* *var* genes were de-silenced in PfSIR2 knock-out parasites, whereas transcription of *upsB* and *upsC* *var* genes was largely

unaffected (Duraisingh *et al.*, 2005). ChIP experiments revealed the presence of PfSIR2 at the silenced *var2csa* gene but not a chromosome-central *upsC* locus (Freitas-Junior *et al.*, 2005). However, when activated, both genes were associated with acetylated histone H4, indicating the involvement of multiple histone deacetylases in *var* gene silencing. Furthermore, recent data have identified an epigenetic mark (H3K9 trimethylation) linked to silencing and transcriptional memory of *upsB* and *upsC* *var* genes (Chookajorn *et al.*, 2007). Together, this is additional evidence for the evolution of the *var* gene family into functional and commonly regulated subsets that might play distinct roles in parasite biology and malaria pathogenesis.

Apart from differential histone modifications, silenced and active states of epigenetically regulated genes are often associated with alterations in nucleosomal organization. In general, silenced genes in *Saccharomyces cerevisiae* heterochromatic regions display an ordered array of regularly spaced nucleosomes and decreased sensitivity to various endonucleases (Richards and Elgin, 2002; Rusche *et al.*, 2003). Similarly, we showed previously that a transgene inserted into subtelomeric heterochromatin in *P. falciparum* displayed altered sensitivity to MNase digestion, suggesting the participation of ATP-dependant nucleosome remodelling enzymes in the establishment of the silenced and active states. Our analysis of the chromatin structure in *var* gene promoters did not detect significant differences between opposite transcriptional states. Furthermore, silenced and active *upsC* chromatin displayed an identical MNase-sensitivity pattern in both the absence and presence of a downstream intron (data not shown). It is possible that opposing transcriptional states of *var* promoters are indeed associated with alterations in translational or rotational nucleosome positioning but remained undetected by the low-resolution mapping approach used here, or that altered susceptibility to endonucleases other than Mnase, such as DNase I or restriction enzymes, exists. However, lack of altered chromatin sensitivity to endonucleases was also observed in Polycomb group-dependent silencing in *Drosophila melanogaster* (Pirrotta and Gross, 2005) and in nuclear hormone receptor-mediated gene regulation (Urnov and Wolffe, 2001). Moreover, many studies in yeast compared the chromatin structure of loci silenced in wild-type cells and activated in mutant strains lacking essential heterochromatin components.

The pattern of MNase-sensitive sites is consistent with a mainly nucleosomal organization of *upsB* chromatin and a number of positioned nucleosomes. The sensitive site at -1140 approximates the position of the regulatory *SPE1* element, indicating that this motif may be located and accessible between two adjacent nucleosomes.

Most noteworthy, however, is the highly resistant region between positions -1760 and -2320 containing five tandem repeats of the *SPE2* motif. The *SPE2* binding factor(s) bind cooperatively and with high avidity to their cognate binding sites (Voss *et al.*, 2003), suggesting a role in chromatin organization. The effects achieved by insertion of *SPE2* into the context of the *cam* promoter clearly support this hypothesis: introduction of *SPE2* leads to a local reorganization and compaction of chromatin. As a result, the modified *cam* promoter was rendered completely repressed throughout most of the intraerythrocytic cycle and was only active during the schizont stage. The fact that soluble *SPE2* binding activities are absent in nuclear extracts from ring- to early schizont-stage parasites (Voss *et al.*, 2003) indicates that the entire pool of this factor is tightly associated with their target sites. We therefore speculate that targeted recruitment of the *SPE2* binding activity to the *cam* promoter causes repression/silencing until the intraerythrocytic replication cycle. During this stage, a window opens where repeated replication cycles provide newly synthesized expression cassettes that can be active in absence of soluble *SPE2* binding protein. Following expression of the *SPE2* binding activity late in the cycle, repressive complexes are re-established. However, it appears that the *SPE2* binding factors remain associated with *upsB* promoters even after activation (Fig. 5) and *upsB* promoters are still silenced after removal of the *SPE2* repeats (Fig. S1). Together this suggests that while the role of this interaction in its native context may involve the establishment and maintenance of *upsB* silencing, it is neither sufficient for *upsB* silencing, nor is its inhibition necessary for activation.

In summary, our findings suggest that, despite the marked differences in *upsC* and *upsB* promoter sequence and the absence of detectable common regulatory motifs, the regulation of the entire *var* gene repertoire obeys the same overall logic. We hypothesize that each *var* gene locus represents an individual transcription unit equipped with functional promoter and intron elements. Regional chromatin modifications are initiated at *cis*-acting regulatory motifs and progress locally to mediate both *var* gene silencing and activation.

Experimental procedures

Parasite cultures, transfection and drug response assays

Plasmodium falciparum 3D7 parasites were cultured as described previously (Trager and Jensen, 1978). Growth synchronization was achieved by repeated sorbitol lysis (Lambros and Vanderberg, 1979). Transfection and drug sensitivity assays were performed as described (Voss *et al.*, 2006).

Transfection constructs

pHBupsB^R was generated by replacement of the BglII/BamHI *cam* promoter in pHBcam^R (Voss *et al.*, 2006) with the 2621 bp *upsB* promoter amplified from pCAT4A3 (Voss *et al.*, 2000). pHBupsB^{RI} was derived in the same way from pHBcam^{RI}. pHBupsB lacks the 0.5 kb rep20 repeat sequence in pHBupsB^R and was generated by digestion of pHBupsB^R with PstI/XcmI, deleting the rep20 repeat region including 140 bp of the 5' end of the *upsB* promoter up to the XcmI site (-2481), followed by ligation of a 140 bp PstI/XcmI PCR fragment amplified from pCAT4A3 to restore the full-length *upsB* promoter. The *hsp86* promoter in pHBupsB^R was excised with KpnI and BstBI and replaced with the full-length *upsC* promoter to generate pHBupsCB^R. *SPE2* var gene promoter elements, and mutated *SPE2* elements (*SPE2m*) (Voss *et al.*, 2003) were cloned as blunt-ended double-stranded oligonucleotides into the EcoRV site (-831 bp) of the *cam* promoter in pLT-3 (A. Maier, unpublished). Modified *cam* promoters were amplified from, and used to replace, the wild-type *cam* promoter in pHBcam^R to generate pHBcamSPE2^R and pHBcamSPE2m^R. pHBcamSPE2^R carries three wild-type head-to-tail copies of *SPE2*, and pHBcamSPE2m^R carries two mutated copies of *SPE2* (*SPE2m*).

Northern analysis

Total RNA was isolated at four time points across the 48 h intra-erythrocytic cycle (time point 1, 0–12 hpi; time point 2, 12–24 hpi; time point 3, 24–36 hpi; time point 4, 32–44 hpi) from parasites synchronized three times. RNA isolated from equal numbers of nuclei for each sample was loaded (time points 1 and 2, 10⁷ parasites; time point 3, 0.75 × 10⁷ parasites; time point 4, 0.25 × 10⁷ parasites). Northern blots were hybridized with [α -³²P]dATP-labelled probes detecting *hdhfr*, *bsd*, *cam*, *misp8* and *hsp86* transcription (Figs 1, 4 and S1). Densitometry was used to quantify transcript levels (Fig. S1). To account for differences in RNA loading, the abundance of *hdhfr* transcripts was adjusted by comparison with the endogenous ring stage-specific *misp8* transcription. *bsd* transcription in trophozoites was adjusted by comparison with endogenous *hsp86* transcription. Values were further normalized for plasmid copy numbers (Fig. S1). Thus, the results shown in Fig. 2 represent relative amounts of transcript produced by a single promoter in each transfected line before and after selection on WR. Figures 1, 4 and S1 show results obtained from single blots each.

Southern analysis

For plasmid copy number determination, gDNA was digested with EcoRV/PvuII. Copy number was determined by densitometry after hybridization with the [α -³²P]dATP-labelled *hrp2* 3'UTR probe comparing the signal intensities derived from the endogenous single-copy *pfhpr2* locus (8317 bp) with the plasmid-encoded *hrp2* 3'UTR sequences. Integration of episomes into chromosomal DNA was mapped by hybridization with the *hdhfr* probe and an 846 bp fragment derived from the 5' end of PFL0005w (+544 to +1390) to HindIII/XhoI-digested gDNA.

Cytoadherence of infected RBCs (iRBCs), Western analysis and FISH

Cytoadherence of parasitized erythrocytes to CD36 was analysed as described (Beeson et al., 1999). RBC pellets were used to extract PfEMP1 into the Triton X-100-insoluble, SDS-soluble fraction as described (van Schravendijk et al., 1993). Aliquots (5×10^6 RBCs, 4% trophozoites) were resolved on a reducing 5% polyacrylamide gel and Western blots probed with mouse monoclonal anti-PfEMP1 antibody 6H1 diluted 1:200 (Duffy et al., 2002). FISH analysis was performed using ring-stage parasites as presented elsewhere (Voss et al., 2006), using telomere-associated repeat element 4 and pGEM probes for the detection of chromosome-end clusters and the plasmid backbone respectively.

Indirect end-labelling

Parasites transfected with *var* promoter constructs were analysed at the ring stage (approximately 7.5 ml packed iRBCs, 5% parasitaemia). Parasites transfected with *cam* promoter constructs were harvested at the trophozoite/schizont stage (1.5 ml packed iRBCs, 5% parasitaemia). Parasites were released by saponin lysis of iRBCs, washed twice in ice-cold PBS and permeabilized in 1 ml of ice-cold chromatin digestion buffer [20 mM Tris-HCl (pH 7.5), 15 mM KCl, 60 mM NaCl, 1 mM CaCl₂, 5 mM MgCl₂, 300 mM sucrose, 0.4% NP-40] for 5 min on ice. F200 μ l aliquots were equilibrated at 37°C for 2 min and digested for 3 min at 37°C by adding 5 μ l of MNase dilutions (MBI Fermentas) at 10–80 mU μ l⁻¹. Reactions were stopped with 40 μ l of stop buffer (100 mM EDTA, 4% SDS) and 66 μ l of 2.5 M NaCl. Samples were treated with 100 μ g proteinase K and 50 μ g RNase A for 2–4 h at 50°C. Digested chromatin was extracted twice with phenol/chloroform, precipitated with 2.5 vols 100% ethanol and resuspended in 30 μ l TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Control digests using purified plasmid DNA were performed as above. For indirect end-labelling, samples were digested to completion with restriction enzymes, resolved on 1–1.4% agarose gels, blotted onto Hybond-XL membranes (Amersham) and probed with a [α -³²P]dATP-labelled *hdffhr* probe.

Acknowledgements

We are grateful to Christian Flueck for critically reviewing the manuscript. This work was supported by the National Health and Medical Research Council (NHMRC) and the Wellcome Trust. A.F.C. and B.S.C. are International Fellows of the Howard Hughes Medical Institute. T.S.V. is supported by a fellowship from the Swiss National Science Foundation. C.J.T. is supported by a NHMRC Peter Doherty Fellowship. A.J.M. is supported by an Australian Postgraduate Award.

References

Al-Khedery, B., and Allred, D.R. (2006) Antigenic variation in *Babesia bovis* occurs through segmental gene conversion of the *ves* multigene family, within a bidirectional locus of active transcription. *Mol Microbiol* **59**: 402–414.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. (1998) Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394**: 592–595.

Baruch, D.I., Pasloske, B.L., Singh, H.B., Bi, X., Ma, X.C., Feldman, M., et al. (1995) Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* **82**: 77–87.

Baruch, D.I., Gormely, J.A., Ma, C., Howard, R.J., and Pasloske, B.L. (1996) *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc Natl Acad Sci USA* **93**: 3497–3502.

Beeson, J.G., and Duffy, P.E. (2005) The immunology and pathogenesis of malaria during pregnancy. *Curr Top Microbiol Immunol* **297**: 187–227.

Beeson, J.G., Brown, G.V., Molyneux, M.E., Mhango, C., Dzinjalama, F., and Rogerson, S.J. (1999) *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. *J Infect Dis* **180**: 464–472.

Berendt, A.R., Ferguson, D.J., Gardner, J., Turner, G., Rowe, A., McCormick, C., et al. (1994) Molecular mechanisms of sequestration in malaria. *Parasitology* **108** (Suppl.): S19–S28.

Biggs, B.A., Gooze, L., Wycherley, K., Wollish, W., Southwell, B., Leech, J.H., and Brown, G.V. (1991) Antigenic variation in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* **88**: 9171–9174.

Calderwood, M.S., Gannoun-Zaki, L., Wellems, T.E., and Deitsch, K.W. (2003) *Plasmodium falciparum var* genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J Biol Chem* **278**: 34125–34132.

Carlson, J., Helmsby, H., Hill, A.V., Brewster, D., Greenwood, B.M., and Wahlgren, M. (1990) Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet* **336**: 1457–1460.

Chookajorn, T., Dzikowski, R., Frank, M., Li, F., Jiwani, A.Z., Hartl, D.L., and Deitsch, K.W. (2007) Epigenetic memory at malaria virulence genes. *Proc Natl Acad Sci USA* **104**: 899–902.

Cockell, M., and Gasser, S.M. (1999) Nuclear compartments and gene regulation. *Curr Opin Genet Dev* **9**: 199–205.

Cooke, B.M., Buckingham, D.W., Glenister, F.K., Fernandez, K.M., Bannister, L.H., Marti, M., et al. (2006) A Maurer's cleft-associated protein is essential for expression of the major malaria virulence antigen on the surface of infected red blood cells. *J Cell Biol* **172**: 899–908.

Dai, Q., Restrepo, B.I., Porcella, S.F., Raffel, S.J., Schwan, T.G., and Barbour, A.G. (2006) Antigenic variation by *Borrelia hermsii* occurs through recombination between extragenic repetitive elements on linear plasmids. *Mol Microbiol* **60**: 1329–1343.

Deitsch, K.W., Moxon, E.R., and Wellems, T.E. (1997) Shared themes of antigenic variation and virulence in bacterial, protozoal, and fungal infections. *Microbiol Mol Biol Rev* **61**: 281–293.

Deitsch, K.W., del, P.A., and Wellems, T.E. (1999) Intra-cluster recombination and *var* transcription switches in

- the antigenic variation of *Plasmodium falciparum*. *Mol Biochem Parasitol* **101**: 107–116.
- Deitsch, K.W., Calderwood, M.S., and Wellems, T.E. (2001) Malaria. Cooperative silencing elements in var genes. *Nature* **412**: 875–876.
- Duffy, M.F., Brown, G.V., Basuki, W., Krejany, E.O., Noviyanti, R., Cowman, A.F., and Reeder, J.C. (2002) Transcription of multiple var genes by individual, trophozoite-stage *Plasmodium falciparum* cells expressing a chondroitin sulphate A binding phenotype. *Mol Microbiol* **43**: 1285–1293.
- Duraisingh, M.T., Voss, T.S., Marty, A.J., Duffy, M.F., Good, R.T., Thompson, J.K., et al. (2005) Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in *Plasmodium falciparum*. *Cell* **121**: 13–24.
- Dzikowski, R., Frank, M., and Deitsch, K. (2006) Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. *PLoS Pathog* **2**: e22.
- Frank, M., and Deitsch, K. (2006) Activation, silencing and mutually exclusive expression within the var gene family of *Plasmodium falciparum*. *Int J Parasitol* **36**: 975–985.
- Frank, M., Dzikowski, R., Costantini, D., Amulic, B., Berdough, E., and Deitsch, K. (2006) Strict pairing of var promoters and introns is required for var gene silencing in the malaria parasite *Plasmodium falciparum*. *J Biol Chem* **281**: 9942–9952.
- Freitas-Junior, L.H., Bottius, E., Pirrit, L.A., Deitsch, K.W., Scheidig, C., Guinet, F., et al. (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*. *Nature* **407**: 1018–1022.
- Freitas-Junior, L.H., Hernandez-Rivas, R., Ralph, S.A., Montiel-Condado, D., Ruvalcaba-Salazar, O.K., Rojas-Meza, A.P., et al. (2005) Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* **121**: 25–36.
- Gannon-Zaki, L., Jost, A., Mu, J., Deitsch, K.W., and Wellems, T.E. (2005) A silenced *Plasmodium falciparum* var promoter can be activated in vivo through spontaneous deletion of a silencing element in the intron. *Eukaryot Cell* **4**: 490–492.
- Gardner, J.P., Pinches, R.A., Roberts, D.J., and Newbold, C.I. (1996) Variant antigens and endothelial receptor adhesion in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* **93**: 3503–3508.
- Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., et al. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498–511.
- Gasser, S.M. (2001) Positions of potential: nuclear organization and gene expression. *Cell* **104**: 639–642.
- Horrocks, P., Pinches, R., Kriek, N., and Newbold, C. (2002) Stage-specific promoter activity from stably maintained episomes in *Plasmodium falciparum*. *Int J Parasitol* **32**: 1203–1206.
- Jenuwein, T., and Allis, C.D. (2001) Translating the histone code. *Science* **293**: 1074–1080.
- Kraemer, S.M., and Smith, J.D. (2003) Evidence for the importance of genetic structuring to the structural and functional specialization of the *Plasmodium falciparum* var gene family. *Mol Microbiol* **50**: 1527–1538.
- Kyes, S., Christodoulou, Z., Pinches, R., Kriek, N., Horrocks, P., and Newbold, C. (2007) *Plasmodium falciparum* var gene expression is developmentally controlled at the level of RNA polymerase II-mediated transcription initiation. *Mol Microbiol* **63**: 1237–1247.
- Lambros, C., and Vanderberg, J.P. (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65**: 418–420.
- Lavstsen, T., Salanti, A., Jensen, A.T., Arnot, D.E., and Theander, T.G. (2003) Sub-grouping of *Plasmodium falciparum* 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J* **2**: 27.
- MacPherson, G.G., Warrell, M.J., White, N.J., Looareesuwan, S., and Warrell, D.A. (1985) Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol* **119**: 385–401.
- Marty, A.J., Thompson, J.K., Duffy, M.F., Voss, T.S., Cowman, A.F., and Crabb, B.S. (2006) Evidence that *Plasmodium falciparum* chromosome end clusters are cross-linked by protein and are the sites of both virulence gene silencing and activation. *Mol Microbiol* **62**: 72–83.
- Misteli, T. (2004) Spatial positioning; a new dimension in genome function. *Cell* **119**: 153–156.
- Moazed, D. (2001) Common themes in mechanisms of gene silencing. *Mol Cell* **8**: 489–498.
- O'Donnell, R.A., Preiser, P.R., Williamson, D.H., Moore, P.W., Cowman, A.F., and Crabb, B.S. (2001) An alteration in concatameric structure is associated with efficient segregation of plasmids in transfected *Plasmodium falciparum* parasites. *Nucleic Acids Res* **29**: 716–724.
- O'Donnell, R.A., Freitas-Junior, L.H., Preiser, P.R., Williamson, D.H., Duraisingh, M., McElwain, T.F., et al. (2002) A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. *EMBO J* **21**: 1231–1239.
- Pays, E., Vanhamme, L., and Perez-Morga, D. (2004) Antigenic variation in *Trypanosoma brucei*: facts, challenges and mysteries. *Curr Opin Microbiol* **7**: 369–374.
- Pirrotta, V., and Gross, D.S. (2005) Epigenetic silencing mechanisms in budding yeast and fruit fly: different paths, same destinations. *Mol Cell* **18**: 395–398.
- Pongponratn, E., Riganti, M., Punpoowong, B., and Aikawa, M. (1991) Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. *Am J Trop Med Hyg* **44**: 168–175.
- Ralph, S.A., Scheidig-Benatar, C., and Scherf, A. (2005) Antigenic variation in *Plasmodium falciparum* is associated with movement of var loci between subnuclear locations. *Proc Natl Acad Sci USA* **102**: 5414–5419.
- Reeder, J.C., Cowman, A.F., Davern, K.M., Beeson, J.G., Thompson, J.K., Rogerson, S.J., and Brown, G.V. (1999) The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. *Proc Natl Acad Sci USA* **96**: 5198–5202.
- Richards, E.J., and Elgin, S.C. (2002) Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108**: 489–500.
- Roberts, D.J., Craig, A.G., Berendt, A.R., Pinches, R., Nash, G., Marsh, K., and Newbold, C.I. (1992) Rapid switching to

- multiple antigenic and adhesive phenotypes in malaria. *Nature* **357**: 689–692.
- Rowe, A., Obeiro, J., Newbold, C.I., and Marsh, K. (1995) *Plasmodium falciparum* rosetting is associated with malaria severity in Kenya. *Infect Immun* **63**: 2323–2326.
- Rowe, J.A., Moulds, J.M., Newbold, C.I., and Miller, L.H. (1997) *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* **388**: 292–295.
- Rusche, L.N., Kirchmaier, A.L., and Rine, J. (2003) The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. *Annu Rev Biochem* **72**: 481–516.
- Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatar, C., Pouvelle, B., *et al.* (1998) Antigenic variation in malaria: *in situ* switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*. *EMBO J* **17**: 5418–5426.
- van Schravendijk, M.R., Pasloske, B.L., Baruch, D.I., Handunnetti, S.M., and Howard, R.J. (1993) Immunochemical characterization and differentiation of two approximately 300-kD erythrocyte membrane-associated proteins of *Plasmodium falciparum*, PfEMP1 and PfEMP3. *Am J Trop Med Hyg* **49**: 552–565.
- Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., *et al.* (1995) Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**: 101–110.
- Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herrfeldt, J.A., Peterson, D.S., *et al.* (1995) The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**: 89–100.
- Trager, W., and Jensen, J.B. (1978) Cultivation of malarial parasites. *Nature* **273**: 621–622.
- Urnov, F.D., and Wolffe, A.P. (2001) A necessary good: nuclear hormone receptors and their chromatin templates. *Mol Endocrinol* **15**: 1–16.
- Viebig, N.K., Gamain, B., Scheidig, C., Lepolard, C., Przyborski, J., Lanzer, M., *et al.* (2005) A single member of the *Plasmodium falciparum var* multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *EMBO Rep* **6**: 775–781.
- Voss, T.S., Thompson, J.K., Waterkeyn, J., Felger, I., Weiss, N., Cowman, A.F., and Beck, H.P. (2000) Genomic distribution and functional characterisation of two distinct and conserved *Plasmodium falciparum var* gene 5' flanking sequences. *Mol Biochem Parasitol* **107**: 103–115.
- Voss, T.S., Kaestli, M., Vogel, D., Bopp, S., and Beck, H.P. (2003) Identification of nuclear proteins that interact differentially with *Plasmodium falciparum var* gene promoters. *Mol Microbiol* **48**: 1593–1607.
- Voss, T.S., Healer, J., Marty, A.J., Duffy, M.F., Thompson, J.K., Beeson, J.G., *et al.* (2006) A *var* gene promoter controls allelic exclusion of virulence genes in *Plasmodium falciparum* malaria. *Nature* **439**: 1004–1008.

Supplementary material

This material is available as part of the online article from:
<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05899.x>
 (This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.