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

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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.

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 (Deitsch 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 (Deitsch et al., 2001) and, although the underlying mechanisms remain obscure, this finding has been confirmed (Caldwerd 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 eucharyotic 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 (Deitsch 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, pHBupsBR and pHBupsBR

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 pHBupsBR and pHBupsBR

The var gene intron was added in pHBupsBR

because its role in silencing has up to now only been demonstrated for upsC

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promoters (Deitsch 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/upsBR and 3D7/upsBRI. 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) indicates 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), 37-fold (pHBupsB) and 28-fold (pHBupsB) 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 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

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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/upsBR and 3D7/upsBRI 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/upsBR and 3D7/upsBRI before (open) and after (filled) WR selection, and of 3D7 wild-type parasites.

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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/upsBRI and 3D7/upsB-RI 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/upsBRI parasites to express PfEMP1 and to adhere to the endothelial receptor CD36. Similar to WR-selected 3D7/upsCRI parasites (Voss et al., 2006), we find that 3D7/upsBRI expressing the hdpfr-resistance gene failed to express PfEMP1 (Fig. 3). Consequently, adherence to CD36 of red blood cells (RBCs) infected with WR-selected 3D7/upsBRI 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 ± 2.8%; mean $n = 113$) and WR-selected 3D7/upsB parasites (62 ± 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.
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 pHBupsCBR, where two var gene promoters, upsC and upsB, control transcription of the bsd and hdhfr reporter genes respectively (Fig. 4A). Transfection of pHBupsCBR followed by selection on blasticidin-S selected for the transgenic line 3D7/upsCBR. 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/upsCBR parasites were resistant to WR challenge unlike 3D7/upsBR 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/upsBR 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
employ, 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 pHBCamSPE2R with three tandem copies of SPE2 inserted into the heterologous P. falciparum calmodulin (cam) promoter (Fig. 6A). pHBCamR (Voss et al., 2006) and pHBCamSPE2mR, 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 and 3D7/camsPE2m, 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/camSPE2R and 3D7/camsPE2mR 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 cam:SPE2 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 (Duraisingham 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 sensitivity 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
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 pHBupsCB6 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 pHBupsB8, 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 Polyclamp group-dependent silencing in Drosophila melanogaster (Pirrotta and Gross, 2005) and in nuclear hormone receptor-mediated gene regulation (Urnov and Wolfe, 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 Jenson, 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 was generated by replacement of the BglII/BamHI cam promoter in pHBcam (Voss et al., 2006) with the 2621 bp upsB promoter amplified from pCAT4A3 (Voss et al., 2000). pHBupsB was derived in the same way from pHBcam, pHBupsB lacks the 0.5 kb rep20 repeat sequence in pHBupsB and was generated by digestion of pHBupsB 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 was excised with KpnI and BstBI and replaced with the full-length upsC promoter to generate pHBupsCB. 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 to generate pHBcamSPE2 and pHBcamSPE2m. pHBcamSPE2 harbours three wild-type head-to-tail copies of SPE2, and pHBcamSPE2m harbours 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, 107 parasites; time point 4, 0.25 × 107 parasites). Northern blots were hybridized with [α-32P]dATP-labelled probes detecting hdhr, bsd, cam, mspa 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 hdhr transcripts was adjusted by comparison with the endogenous ring stage-specific mspa 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 phrp2 locus (8317 bp) with the plasmid-encoded hrp2 3′ UTR sequences. Integration of episomes into chromosomal DNA was mapped by hybridization with the hdhr probe and an 846 bp fragment derived from the 5′ end of PFL0005w (+544 to +1390) to HindIII/Xhol-digested gDNA.

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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 PIEMP1 into the Triton X-100-insoluble, SDS-soluble fraction as described (van Schravendijk et al., 1993). Aliquots (5 × 10⁶ RBCs, 4% trophozoites) were resolved on a reducing 5% polyacrylamide gel and Western blots probed with mouse monoclonal anti-PIEMP1 antibody H61 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 μl aliquots were equilibrated at 37°C for 2 min and digested for 3 min at 37°C by adding 5 μl of MNase dilutions (MBI Fermentas) at 10–80 mU μl⁻¹. Reactions were stopped with 40 μl of stop buffer (100 mM EDTA, 4% SDS) and 66 μl of 2.5 M NaCl. Samples were treated with 100 μg proteinase K and 50 μg 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 μl 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 hdhfr probe.

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