Identification of a *cis*-acting DNA–protein interaction implicated in singular *var* gene choice in *Plasmodium falciparum*

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**Summary**

*Plasmodium falciparum* is responsible for the most severe form of malaria in humans. Antigenic variation of *P. falciparum* erythrocyte membrane protein 1 leads to immune evasion and occurs through switches in mutually exclusive *var* gene transcription. The recent progress in *Plasmodium* epigenetics notwithstanding, the mechanisms by which singularity of *var* activation is achieved are unknown. Here, we employed a functional approach to dissect the role of *var* gene upstream regions in mutually exclusive activation. Besides identifying sequence elements involved in activation and initiation of transcription, we mapped a region downstream of the transcriptional start site that is required to maintain singular *var* gene choice. Activation of promoters lacking this sequence occurs no longer in competition with endogenous *var* genes. Within this region we pinpointed a sequence-specific DNA–protein interaction involving a *cis*-acting sequence motif that is conserved in the majority of *var* loci. These results suggest an important role for this interaction in mutually exclusive locus recognition. Our findings are furthermore consistent with a novel mechanism for the control of singular gene choice in eukaryotes. In addition to their importance in *P. falciparum* antigenic variation, our results may also help to explain similar processes in other systems.

**Introduction**

Many unicellular pathogens use antigenic variation to escape adaptive immune responses in the host. The widespread occurrence of this strategy in evolutionary distant species underscores its key role in pathogen survival and spreading. While the underlying control pathways are highly diverse in different systems, both mechanistically and in terms of complexity, antigenic variation is defined by two basic concepts. First, the antigens are encoded by gene families, the members of which are expressed in a mutually exclusive manner. Second, switches in the expression of individual members lead to antigenic variation of surface-exposed antigens. In several medically important pathogens such as *Borrelia* spp., *Neisseria* spp., *Giardia lamblia*, *Plasmodium falciparum* and *Trypanosoma brucei*, this paradigm of clonal phenotypic variation reaches a remarkable yet poorly understood level of sophistication (Deitsch et al., 2009; Dzikowski and Deitsch, 2009; Morrison et al., 2009; Prucca and Lujan, 2009).

The apicomplexan parasite *P. falciparum* causes several hundred million malaria cases and close to one million deaths annually (World Health Organization, 2010). Malaria-associated morbidity and mortality is a result of the intra-erythrocytic developmental cycle (IDC) where repeated rounds of parasite invasion into red blood cells (RBCs) are followed by intracellular maturation and replication. During this stage of infection parasites expose the major virulence factor *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the RBC surface (Leech et al., 1984). This highly polymorphic antigen, encoded by the 60-member *var* gene family, undergoes antigenic variation to facilitate chronic infection and transmission (Biggs et al., 1991; Roberts et al., 1992; Smith et al., 1995; Su et al., 1995; Gardner et al., 2002). Furthermore, PfEMP1 mediates sequestration of infected RBC aggregates in the microvasculature of various organs and is thus directly responsible for severe outcomes, including cerebral and placental malaria (MacPherson et al., 1985; Pongponratn et al., 1991; Baruch et al., 1996; Gardner et al., 1996; Reeder et al., 1999; Beeson and Duffy, 2005).

*var* genes are transcribed by RNA polymerase II (RNA polII) in ring-stage parasites during the first half of the IDC (Scherf et al., 1998; Dzikowski et al., 2006; Kyes et al., 2006;...
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...upstream activating sequence (UAS) that is essential for var promoter activation. Notably, we also identified a region downstream of the transcriptional start site (TSS) and demonstrate an important role for this element in mutually exclusive promoter recognition. In absence of this sequence var promoters are fully active but, unlike wild-type promoters, do not compete with endogenous var gene transcription. Within this region we identified a 47 bp motif that interacts in a sequence-specific manner with an unknown nuclear protein. Together, our results show for the first time that the complex regulation of mutually exclusive var gene transcription involves functional cis-acting modules with intrinsic and position-dependent activities. They are furthermore consistent with a novel mechanism in sustaining singular gene choice in eukaryotes.

Results

Functional var promoter mapping by bi-directional deletion analysis

To identify regulatory var promoter elements we employed a system suitable to analyse promoter activity in stably transfected parasites. All reporter constructs are based on the parental plasmid pBC (Fig. 1A) where the bacterial deaminase (bsd) resistance cassette selects for stable episomes. A 2.5 kb var upsC upstream sequence (PFL1960w) controls transcription of the dual reporter encoding human dihydrofolate reductase fused to green fluorescent protein (hdhfr-gfp). A var gene intron element is located downstream of the hdhfr-gfp cassette to account for its role in var gene regulation. A telomere-associated repeat element 6 sequence (TARE6/rep20) is included for improved plasmid segregation (O’Donnell et al., 2002). In such a context, homogenous populations carrying active upsC promoters are obtained via selection with the antifolate drug WR99210 (WR) (Voss et al., 2006; 2007).

To identify elements involved in promoter activation and mutual exclusion we sequentially truncated the upsC upstream sequence from either the 5′ or 3′ end (Fig. 1B). We chose this bi-directional approach to identify possible functional regions both up- and downstream of the putative TSS. Based on a multiple upsC sequence alignment and the previous experimental mapping of an upsC TSS we expected the TSS of PFL1960w at position −1167 (Deitsch et al., 1999; Voss et al., 2000). Transfected parasites were challenged with WR and resistant populations were obtained for all but one cell line, 3D7/pBC3 (Fig. 1B). Several attempts to select for WR-resistant 3D7/pBC3 parasites failed showing that the region between −1656 to −1217 comprises an important UAS and/or the core promoter. To test if any of the deletions affected promoter...
strength we determined relative \( \text{h} \text{dhfr-gfp} \) transcript levels in ring-stage parasites by quantitative reverse transcriptase PCR (qRT-PCR). As shown in Fig. 1B, transcript levels in 3D7/pBC1 and 3D7/pBC2 were similar to those in 3D7/pBC indicating that the sequence upstream of \(-1656\) does not contribute to \text{var} promoter activity. The promoter in pBC5, lacking \(491\) bp of the \(5^\prime\) UTR, was also fully active. In contrast, the truncation encompassing bps \(-1057\) to \(-1\) in pBC4 caused a significant reduction in steady-state transcript levels. Hence, this approach identified two regulatory regions, located upstream and downstream of the putative TSS, respectively, which fulfil important roles in \text{var} promoter function.

**Functional identification of an autonomous upsC upstream activating sequence**

To learn more about the nature of the putative UAS we set out to analyse its function in the context of a minimal heterologous promoter. We decided to use the knob-associated histidine rich protein (\text{kahrp}) gene promoter for three reasons. First, the TSS of this gene has been mapped to \(849\) bp upstream of the ATG (Lanzer et al., 1992). Second, similar to \text{var} genes the timing of \text{kahrp} transcription peaks in ring-stage parasites. Lastly, the \text{kahrp} locus is not enriched in H3K9me3/PfHP1 (Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009), which is an important consideration in order to avoid heterochromatin-mediated masking of autonomous cis-acting activities. Hence, we generated plasmid \(\text{pBKmin-RI}\) where bps \(-1115\) to \(-1\) of the \text{kahrp} upstream sequence control transcription of the \(\text{h} \text{dhfr-gfp}\) reporter (Fig. 2A). Parasites carrying \(\text{pBKmin-RI}\) episomes were readily obtained after transfection. Notably, the disposition of this plasmid to integrate into the endogenous \text{kahrp} locus allowed us to measure \(\text{Kmin}\) activity also in a chromosomal environment. This integration event essentially causes a promoter swap where \(\text{Kmin}\) drives expression of the endogenous \text{kahrp} gene and the endogenous \text{kahrp} promoter controls transcription of the \(\text{h} \text{dhfr-gfp}\) reporter (Figs 2B and S1). Compared with the endogenous full-length \text{kahrp} promoter, the episomal and chromosomal minimal promoters displayed a 300-fold and 1000-fold reduced activity respectively (Fig. 2C). Hence, \(\text{Kmin}\) clearly fulfilled the requirements for a minimal promoter.

We cloned two overlapping fragments containing the putative upsC UAS upstream of \(\text{Kmin}\) to create upsC-\(\text{Kmin}\) hybrid promoters (\(\text{pBC1Kmin}\) and \(\text{pBC2Kmin}\)) (Figs 2D and S1). The region downstream of the upsC TSS encompassing bps \(-463\) to \(-20\), which has no effect on upsC...
promoter activity (Fig. 1B), was used as negative control (pBC3Kmin). qRT-PCR analysis revealed that upsC fragments C1 (−1679 to −1200) and C2 (−1401 to −727) consistently activated Kmin to a similar extent in both the episomal and chromosomal context whereas fragment C3 had no effect. Furthermore, neither the var intron nor the rep20 element altered Kmin activity. Together, these findings corroborate the results obtained with the upsC deletion constructs and are consistent with the presence of a var UAS located between bps −1401 and −1217. The fact that this element activates transcription from a heterologous minimal promoter suggests an autonomous, context-independent function in activating RNA polII-mediated transcription.

Here, we investigated the functional region downstream of the putative TSS that is defined by plasmids pBC4 and pBC5 (−1057 to −491). Deletion of this region caused a substantial reduction in steady-state transcripts (Fig. 1B), suggesting it may contain important activating sequences. Northern blot analysis confirmed the reduced abundance of steady-state transcripts in 3D7/pBC4 compared with 3D7/pBC and 3D7/pBC5 (Fig. 3). An independent time-course experiment confirmed these results and excluded the possibility of altered transcriptional timing and/or transcript accumulation in 3D7/pBC4 parasites (Fig. S2).
which the unrelated ring stage-specific mahrp1 promoter controls dhfr-gfp transcription, expressed PIEMP1 at normal levels, whereas parasites of the positive control line 3D7/pBC exhibited the expected PIEMP1 knockdown phenotype (Fig. 4A). PIEMP1 expression was also abolished in 3D7/pBC2 showing that the region ranging from −2488 to −1656 bps upstream of the start codon is not important for mutually exclusive locus recognition. In contrast, 3D7/pBC4 and 3D7/pBC5 parasites expressed PIEMP1 at levels similar to the 3D7/pBM-negative control line. Interestingly, both truncated promoters lack the same 491 bp sequence downstream of the TSS suggesting that this region carries sequence information important for mutually exclusive locus recognition.

To map this region more precisely we cloned three additional truncated upsC sequences in pBC6, pBC7 and pBC8 (Fig. 4B). Similar to the full-length promoter in 3D7/pBC, 3D7/pBC8 parasites failed to express PIEMP1 demonstrating that the pBC8 promoter was activated in a mutually exclusive manner. In contrast, 3D7/pBC6 and 3D7/pBC7 expressed PIEMP1 at levels similar to two negative controls (WR-selected 3D7/pBM and unselected 3D7/pBC) showing that these truncated promoters were not subject to mutually exclusive recognition as already observed for 3D7/pBC5. Together, this series of experiments pinpointed a putative 101 bp mutual exclusion element (MEE) (bps −316 to −215) that drives the upsC promoter into mutually exclusive activation; in absence of the MEE promoters escape this restriction and are activated in parallel to endogenous var gene transcription.

The mutual exclusion element interacts specifically with an unknown nuclear factor

The proposed function of the MEE in mutually exclusive activation may be directly linked to the specific recruitment of an unknown regulatory factor. We therefore tested three overlapping fragments (MEE1–MEE3) in electromobility shift assays (EMSA) using parasite nuclear extracts. Whereas MEE1 and MEE3 showed no sign of specific binding (data not shown), the central 47 bp MEE2 fragment formed a DNA–protein complex that was specifically competed by an excess of homologous competitor only (Fig. 5A). To characterize this interaction in more detail we performed competition EMSAs using a set of mutated MEE2 sequences (Fig. 5B). As expected, scrambled MEE2 failed to compete under-scoring the sequence-specificity of this interaction. Four out of six fragments carrying consecutively mutated 8mers (MEE2-mut2/-mut3/-mut5/-mut6) competed with similar efficiency as the MEE2 wild-type sequence (Figs 5B and S3A). In contrast, MEE2-mut4 failed to compete even at a 100-fold molar excess, and MEE2-mut1 competed with intermediate efficiency. Hence, we conclude that the 8 bp

A regulatory region downstream of the TSS is involved in mutually exclusive var gene expression

Transgenic parasites carrying activated full-length var promoters do not transcribe endogenous var genes and fail to express PIEMP1 (Dzikowski et al., 2006; 2007; Voss et al., 2006; 2007; Chookajorn et al., 2007; Howitt et al., 2009; Witmer et al., 2012). This implies that mutually exclusive locus recognition may be mediated by cis-acting regulatory sequence elements located in var gene upstream regions. To test this hypothesis and to identify such functional elements we investigated if any of the activated truncated promoters escaped mutually exclusive activation. The negative control line 3D7/pBM, in which the unrelated ring stage-specific mahrp1 promoter controls dhfr-gfp transcription, expressed PIEMP1 at normal levels.
ATAGATTA sequence mutated in MEE2-mut4 represents a core motif necessary for this specific interaction, whereas the 8mer sequence at the 5' end of MEE2 may have ancillary function in complex formation.

Next, we asked if the MEE2 element also occurs upstream of other var genes. We inspected all var upstream sequences (−600 to −1 relative to the start ATG) and identified a perfect or slightly deviated MEE2 core motif with the consensus sequence (A/T)(A/T)(A/T)GA(T/A)TA in 44 (73%) out of all 60 var genes. Strikingly, in all but four cases this motif (i) is conserved in terms of orientation and position relative to the ATG start codon, (ii) is embedded in an overall highly similar sequence context including a characteristic poly-dT stretch, and (iii) occurs in upsB-, upsC-, upsB/C- and upsB/A-type var genes (Fig. S4). The remaining four core motifs were found in one upsB/C and three upsA-type upstream sequences but they did not share these characteristics; they occurred in a different sequence context and relative position/orientation. In EMSA experiments, the MEE2-like motif derived from another upsC var gene (PF07_0048), in which six nucleotide positions are changed compared with MEE2 including one substitution in the core motif, competed as efficiently as the wild-type MEE2 motif (Fig. 5B). Similarly, the element found upstream of an upsB-type var gene (PFL0005w), in which 19 positions are altered including two in the core motif, competed albeit with lower efficiency (Fig. S3B). In contrast, competitors derived from a var upsA (PF12135w) and a var-unrelated rif (PFB0035c) upstream region, in which an AT(A/T)GATTA core motif is present at the same relative position as in MEE2, failed to inhibit formation of the MEE2–protein complex (Fig. S3C).

Together, our results show that the MEE2-interacting factor (MIF) also binds to related motifs found in a large proportion of var upstream regions. Interestingly,
however, MIF does not bind to unrelated sequences that contain a perfectly conserved 8 bp MEE2 core motif. Hence, this core motif is necessary but not sufficient for binding and the local \textit{var} upstream sequence context plays an important role in mediating stable and sequence-specific complex formation.

\section*{Discussion}

The importance of mutually exclusive transcription of gene families is exemplified by antigenic variation in unicellular pathogens as a prime strategy to secure survival and transmission. In \textit{T. brucei}, the causing agent of African sleeping sickness, mutually exclusive transcription of variant surface glycoprotein genes is carried out by an extranucleolar RNA-poll-containing body (Navarro and Gull, 2001). Another paradigm of mutual exclusion is that of singular odorant receptor (OR) gene choice in individual olfactory neurones in mammals (McClintock, 2010). Here, exclusive transcription of one out of over a thousand OR genes involves regulatory DNA elements both upstream and in the coding regions (Qasba and Reed, 1998; Vassalli \textit{et al.}, 2002; Lomvardas \textit{et al.}, 2006; Fuss \textit{et al.}, 2007; Nguyen \textit{et al.}, 2007), and a negative protein feedback mechanism (Serizawa \textit{et al.}, 2003; Lewcock and Reed, 2004; Shykind \textit{et al.}, 2004). In addition, and in remarkable analogy to mutually exclusive \textit{var} regulation, Lomvardas and colleagues recently described a func-
tional association of H3K9me3 and H3K4me3 with silenced and active OR loci respectively (Magklara et al., 2011). These important discoveries notwithstanding, we still lack detailed knowledge as to how mutually exclusive transcription is achieved in any system. In this study, we developed and successfully applied a complementary functional approach to study mutual exclusion in *P. falciparum var* gene transcription. For the first time, we identified cis-acting entities as important mediators of var gene activation and singular gene choice.

Var gene transcription is mediated by RNA polII and occurs stage-specifically by activation in ring-stage parasites and subsequent repression or poising during the rest of the IDC (Kyes et al., 2007; Lopez-Rubio et al., 2007). Here, we identified a UAS element essential for upsC promoter activation. The position of this element upstream of the natural TSS, and the competence to activate transcription from a heterologous promoter, are attributes inherently associated with the role of UAS elements in transcriptional activation (Levine and Tjian, 2003). Our results are therefore consistent with the sequence-specific recruitment of a transcriptional activator by the UAS to orchestrate the assembly of the pre-initiation complex (PIC) and/or to activate RNA polII-dependent transcription. Interestingly, the fact that this element functions autonomously in a euchromatic context implies a ubiquitous rather than spatially restricted distribution of the transcriptional activator involved, which somewhat precludes a restricted role for this factor in mutually exclusive var activation.

The current model of mutually exclusive var transcription postulates the existence of a physically restricted perinuclear zone dedicated to the expression of a single var gene (Duraisingham et al., 2005; Ralph et al., 2005; Voss et al., 2006; 2007; Dzikowski et al., 2007; Lopez-Rubio et al., 2009). Activation requires entry into this zone with concomitant substitution of the formerly active locus, linked to the removal of H3K9me3/PfHP1 and deposition of H3K9ac and H3K4me2/3 marks predominantly along the region downstream of the TSS (Lopez-Rubio et al., 2007; Perez-Toledo et al., 2009). We identified a deletion downstream of the TSS as the common denominator of all four promoter variants that escaped mutually exclusive activation. Unlike full-length promoters, activation of promoters lacking this region did not occur at the expense of, but in parallel to, the transcription of an endogenous var gene. Notably, this deletion did not alter the relative activity of the promoter showing that the processes of promoter activation and mutually exclusive recognition are uncoupled from each other. The specific binding of a nuclear factor or complex (MIF) to a cis-acting sequence motif present in this region (MEE2) corroborates this hypothesis and suggests an important role for this DNA–protein interaction in mutually exclusive promoter activa-

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regulatory system is important for our understanding of immune evasion and virulence of *P. falciparum* and other pathogens. Furthermore, our results will also help to understand conceptually similar processes in other organisms.

**Experimental procedures**

**Parasite culture and transfection**

*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). Transfections were performed as described (Voss et al., 2006). Parasites were selected on 2.5 µg ml−1 blasticidin-S-HCl and 4 nM WR99210. Transfection constructs are described in supporting experimental procedures.

**Quantitative reverse transcription PCR**

gqPCR was performed on reverse transcribed total RNA and gDNA isolated from synchronous parasite cultures. A detailed protocol, relative transcript calculation and primer sequences are provided in supporting experimental procedures and Table S1.

**Southern and Northern blot analysis**

gDNA was digested with appropriate restriction enzymes over-night and separated in 0.5× TBE-buffered 0.7% agarose gels. Total RNA was isolated from saponin-released parasites using TriReagent (Ambion). RNA was glyoxylated for 1 h at 55°C in five volumes glyoxal reaction mixture and electrophoresis was performed using 1× BPTE-buffered 1.5% agarose gels (Sambrook and Russell, 2001). Blots were probed with 32P-dATP-labelled *dhfr*, *kahrp* and *hsp86* PCR fragments. Membranes were stripped by boiling in 0.1% SDS for 15 min in between hybridizations.

**Western blot analysis**

Detection of hDHFR-GFP and GAPDH (loading control) was performed on whole-cell lysates. Primary antibody dilutions were: mouse anti-GFP (Roche Diagnostics, 1181446001), 1:500; mouse anti-GAPDH 1-10B (kind gift of Claudia Daubenberger), 1:20 000. PfEMP1 was extracted from trophozoite-infected RBC pellets (Triton X-100-insoluble/SDS soluble fraction) as described (van Schravendijk et al., 1993). Extracts were separated by SDS-PAGE using 5% polyacrylamide gels using Tris-glycine or Tris-acetate buffers. PfEMP1 was detected using the monoclonal mouse anti-PfEMP1 antibody 1B/6H-1 (Duffy et al., 2002), 1:500.

**Electromobility shift assay**

High-salt nuclear extracts and EMSAs were prepared and carried out as described (Voss et al., 2002) with the following modifications. Proteins were extracted with 500 mM KCl and incubated
with 20 fmol of radiolabelled probe in 1× EMSA buffer in presence of 200 ng of poly(dA·dT) as non-specific competitor. Complementary oligonucleotide sequences used to generate double-stranded probes and competitors are listed in Table S1.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Supporting experimental procedures**

**Fig. S1.** Southern analysis of gDNA isolated from parasites presented in Fig. 2.

A. Autoradiographs of Southern blots showing episomal maintenance or plasmid integration into the endogenous kahrp locus in 3D7/pBKmin and 3D7/pBKmin-RI. gDNA was digested with BglII and HindIII. Blots were probed with a radiolabelled kahrp fragment. E, episomal; I, integrated.

B. Autoradiographs of Southern blots showing epissomal maintenance or plasmid integration into the endogenous kahrp locus in 3D7/pBC1min, 3D7/pBC2min, and 3D7/pBC3min. gDNA was digested with BglII and HindIII. Blots were probed with a radiolabelled kahrp fragment.

C. Schematic map of the endogenous kahrp locus.

D–F. Schematic maps of the integration events in 3D7/pBKmin (D), 3D7/pBC1min and 3D7/pBC2min (E) and 3D7/pBC3min (F). BglII and HindIII restriction sites and length of the corresponding fragments are indicated.

**Fig. S2.** Transcriptional initiation form an alternative upsC upstream TSS. The promoters in pBC and pBC4 are schematically depicted on top. Semi-quantitative analysis of protein and transcript abundance by Western and Northern blot in a time-course experiment. Total protein and RNA were harvested simultaneously from synchronized 3D7/pBC and 3D7/pBC parasites at three consecutive time points during intra-erythrocytic development (ring stages, 8–18 hpi; late ring stages/early trophozoites, 16–26 hpi; late trophozoites/early schizonts, 24–34 hpi). Expression of dhFR-GFP and GAPDH (loading control) was detected with anti-GFP and anti-GAPDH antibodies respectively (upper panels). Steady-state dhfr-GFP and hsp86 (loading control) transcripts were detected using radiolabelled hsfhr and hsp86 probes respectively.

**Fig. S3.** Competition EMSAs. All EMSAs were carried out using radiolabelled MEE2 and parasite nuclear extract.

A. Mutational analysis of MEE2. Competition was carried out in presence of a 25- and 100-fold molar excess of unlabelled DNA. The nucleotide sequences of wild-type and mutated MEE2 elements are indicated on the right. The ATAGATTA core motif is underlined. Mutated 8mers are highlighted in red.

B. Competition of the MEE2 complex by a MEE2-related upsB sequence element. Competition was carried out in presence of a 25-, 100-, 250- and 500-fold molar excess of unlabelled DNA. The nucleotide sequences of wild-type and scrambled MEE2 and the MEE2-related upsB element are indicated on the right. The ATAGATTA core motif is underlined. The differences in the upsB-derived motif compared with MEE2 are highlighted in red.

C. The ATAGATTA core motif is not sufficient for complex formation. Competition was carried out in presence of a 25-, 100- and 500-fold molar excess of unlabelled DNA. The ATAGATTA core motif is underlined. The nucleotide sequences of wild-type and scrambled MEE2 and two unrelated sequence elements that contain the ATAGATTA core motif are indicated on the right.

**Fig. S4.** The MEE2 core motif occurs in a conserved position upstream of 44 var genes.

A. The schematic shows the presence and relative position of the (A/T)(A/T)(GA)(A/T)TA consensus sequence found upstream of 44 var genes. This motif forms the core of the 47 bp MEE2 element that is bound by a nuclear factor in a sequence-specific manner (see Figs 4 and S3). Red boxes indicate the position of the motif in each upstream region. Numbers on the right represent the position of the first nucleotide of the motif relative to the translation initiation ATG. Gene accession numbers were retrieved from PlasmoDB version 7.2 (http://www.plasmoDB.org) and are indicated on the left. The colour code clusters var genes
into the different var gene subgroups upsA, upsB, upsC, upsE, upsB/C and upsB/A (Lavstsen et al., 2003).

B. Alignment of MEE2-related sequences that are centred around the (A/T)(A/T)(A/T)GA(A/T)TA core consensus element in 44 var upstream regions. The original MEE2 motif identified upstream of the upsC var gene PFL1960w is shown as the first sequence in the alignment. The local context of the MEE2-related core motifs shows a high level of sequence similarity that includes a prominent upstream poly-dT stretch. Gene accession numbers are indicated on the left and are colour-coded as in Fig. S4A.

Orientation of the motif is indicated on the right (+, upper strand; −, lower strand). The red bar on top highlights the position of the core motif.

**Table S1.** All primers used in this study are listed. Restriction sites are indicated in bold.

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