Active transcription is required for maintenance of epigenetic memory in the malaria parasite *Plasmodium falciparum*

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SUMMARY

The most severe form of human malaria is caused by the protozoan parasite *Plasmodium falciparum*. The primary antigenic and virulence determinant expressed on the surface of infected red blood cells is PfEMP1, a protein that mediates adhesion and sequestration of the parasites in deep tissue vascular beds. Different forms of PfEMP1 are encoded by different members of the multi-copy *var* gene family. Expression of *var* genes is mutually exclusive, and by switching which gene is expressed, parasites alter both their antigenic and virulence phenotypes. Regulation of *var* gene expression involves gene activation, silencing and cellular memory, and the details of the mechanisms that control this process are not understood. Here we provide evidence that active transcription is required for the maintenance of the cellular memory that marks a specific *var* gene to be stably expressed through numerous cell cycles. Forcing transfected parasites to express increasing numbers of unregulated episomal *var* promoters led to a corresponding down-regulation of the active *var* gene in the parasite's genome, presumably by competing for the transcriptional machinery of the parasite and suggesting the existence of a limited nuclear factor that is required for *var* gene activation. This process allowed us to repress transcription of the active *var* gene without acting through the mechanism that controls mutually exclusive expression, and thus to investigate the role of transcription itself in maintaining epigenetic memory. When the competing episomes were removed, the parasites did not return to their previous *var* gene expression pattern, but rather displayed random *var* gene activation, demonstrating that the epigenetic imprint that controls *var* gene expression had been completely erased and thus linking active transcription to the maintenance of cellular memory.

Keywords

antigenic variation; virulence; transcription; gene expression; epigenetics

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INTRODUCTION

Malaria caused by the parasite *Plasmodium falciparum* is known for its persistent infections as well as its potentially lethal complications. Both of these characteristics are linked to expression of a variable antigen displayed on the surface of infected red blood cells called *Plasmodium falciparum* erythrocyte membrane protein one (PfEMP1)[1]. This protein functions as a surface receptor that mediates binding and cytoadherence of infected cells to various molecules found on the surface of the endothelial cells of the microvasculature. This cytoadherence results in both obstruction of blood flow and a localized immune response that are thought to be responsible for such severe complications as cerebral malaria and pregnancy associated malaria[2]. Each individual parasite is capable of expressing many different forms of PfEMP1, each differing in both its antigenic and cytoadherent characteristics. By switching which PfEMP1 is expressed over the course of an infection, the parasite is able to avoid the antibody response generated by the host to previously expressed forms of PfEMP1[3].

PfEMP1 is encoded by the multi-copy *var* gene family[4–6]. The genome of each parasite contains approximately 60 different *var* genes, each coding for a different form of PfEMP1[7]. The genes are expressed in a mutually exclusive fashion such that only a single gene is transcriptionally active at a time while the remainder of the *var* gene complement is maintained in a transcriptionally silent state[8,9]. This strict regulatory paradigm ensures that only a single form of PfEMP1 is found on the surface of an infected red blood cell, thus the expressed *var* gene determines both the cell's antigenic as well as cytoadherence phenotype. Switches in *var* gene expression result in antigenic variation by changing the form of PfEMP1 exposed to the immune system, thereby allowing parasites to maintain a persistent, long-term infection that is difficult to clear. Proper regulation of *var* gene expression requires recognition of all *var* genes as members of a single family as well as coordinated activation and silencing of individual genes to maintain mutually exclusive expression in the event of a switch. In addition, a form of cellular memory allows parasites to display a relatively low *var* gene switching rate over the course of many cell cycles, thus preventing premature expenditure of the antigenic repertoire[10,11]. This low switching rate reflects the fact that once activated, a *var* gene tends to remain active, suggesting the possibility that the act of transcription itself might help to reinforce the maintenance of the epigenetic marks that control cellular memory. Consistent with this hypothesis, it has recently been shown in other eukaryotic systems that certain chromatin modifying proteins directly associate with RNA polymerase II and exert their influence on chromatin structure during transcriptional elongation, thus linking transcription to chromatin modification and epigenetic memory[12,13]. However, the molecular basis of *var* gene recognition, silencing and activation, as well as the how switching is coordinated, remain obscure.

Each *var* gene contains two separate promoters, one upstream of the first exon that is responsible for expression of the PfEMP1 encoding mRNA and a second found within a conserved intron that separates the two exons of each gene[14]. This intron promoter drives expression of a "sterile" noncoding transcript and has been implicated in *var* gene silencing[6]. We have recently found that "unpairing" a *var* upstream promoter from its adjacent intron promoter renders it constitutively active and unrecognized by the mechanism controlling mutually exclusive expression[15,16]. This resulted in the unusual situation of individual parasites expressing two *var* promoters simultaneously. This observation raised the question as to whether there is any limit to how many *var* promoters an individual parasite nucleus can express at one time, and whether expressing high numbers of unregulated *var* promoters has any effect on the expression of the endogenous, properly regulated *var* genes.

Here we show that by forcing parasites to express increasing numbers of episomal "unpaired" *var* promoters, it is possible to down-regulate and ultimately silence all endogenous *var* genes in the parasite genome. This ability to specifically repress *var* gene transcription without affecting the rest of the genome or the viability of the parasites provided us with the opportunity to investigate the role that active transcription plays in maintaining *var* gene epigenetic memory. When parasites were released from *var* transcriptional repression, they again expressed endogenous, chromosomal *var* genes in a mutually exclusive manner, however individual *var* genes were activated at random, indicating that the epigenetic marks regulating cellular memory that had previously controlled *var* gene expression had effectively been erased from the genome. These results have implications for understanding both *var* gene activation as well as the role of active transcription in maintaining cellular memory in malaria parasites.

RESULTS

Forced expression of multiple, unregulated *var* promoters represses the transcription of endogenous *var* genes

Mutually exclusive *var* gene expression requires pairing of the two promoters found in each gene[15,16]. In order to create transgenic parasite lines that simultaneously expressed multiple *var* promoters, it was essential to use "unpaired" *var* promoters that were constitutively active and not recognized by the mechanism that controls mutually exclusive expression. We therefore utilized the plasmid pVBH in which a *var* promoter that is free from the influence of any adjacent promoter activity and not recognized by the mechanism that controls mutually exclusive expression is used to drive expression of the blasticidin S deaminase gene (*bsd*)[16]. This plasmid was used to transfect an NF54 parasite population (called C3) that predominantly expressed a single *var* gene (PFD1005c). Expression of this particular *var* gene is remarkably stable over time due to its low off rate[17]. Replication of episomes in malaria parasites creates multi-copy concatamers[18,19] and we hypothesized that by increasing drug pressure we would be able to select for parasites that carried correspondingly increasing numbers of active *var* promoters on larger multi-copy pVBH concatamers. Parasite were transfected as described [20,21] and initially selected with 2 μg/ml blasticidin. Once a resistant population was established, the parasites were exposed to increasing concentrations of blasticidin (Fig 1A), thus selecting for parasite populations that carried increasing numbers of active episomal *var* promoters. Selection with higher levels of blasticidin resulted in an initial delayed growth phenotype (Fig 1A), however once established, all parasite populations grew at comparable rates. Using Q-RT-PCR it was possible to quantify the exact copy number of the transgenic, episomal *var* promoters in each parasite population. As expected, the number of episomal *var* promoters increased as we increased drug concentration and varied from around a single copy in parasites selected using the lower blasticidin concentration to approximately 18 copies in those selected with the highest dose (Fig 1B). The increased number of promoters resulted in an overall increase in the total expression of *bsd* in the different transgenic lines (Fig 1C) indicating that they are transcriptionally active and express *bsd*.

We then were interested in measuring the level of expression of the endogenous *var* gene (PFD1005c) that was actively transcribed in the original NF54 culture that was transfected. PFD1005c was the only *var* gene transcribed significantly above background in this population of parasites, and this gene had previously displayed a particularly low off rate, remaining stably expressed for over 6 months of continuous culture after cloning[17]. We observed that as we increased the number of active exogenous *var* promoters, the level of transcription of PFD1005c was significantly down-regulated ($P < 0.01$), and in parasites populations that carried large numbers of the active transgenic "unpaired" *var* promoters, the level of expression of PFD1005c was repressed to a level equivalent to a silent *var* gene (Fig

1D). The data suggest that parasites have an inherent capability of expressing up to ~20 *var* promoters simultaneously, nonetheless in a normal situation, the mechanism that controls mutually exclusive expression limits expression to a single gene. However, in the experiment described here, by forcing expression of an increasing number of unregulated, episomal *var* promoters, we have saturated the parasite's ability to actively transcribe *var* genes, leading to repression of the entire genomic complement. It is worth noting that this is unlike previous genetic manipulations that resulted in silencing of the entire *var* gene repertoire[22]. In our previous work, parasites were forced to express a single, properly regulated transgenic *var* promoter, thus silencing of the *var* complement in that instance was achieved by infiltrating the mechanism that controls mutually exclusive expression.

A specific nuclear element is a limiting factor for *var* gene expression

Interestingly, the observed down-regulation of PFD1005c seemed to be *var*-specific and did not affect parasite growth or the expression of housekeeping genes (Fig 1D). To further test the hypothesis that the observed down-regulation is specific for *var* promoters, we performed transient transfection assays in two transgenic cultures that were stably carrying pVBH concatamers. Parasites grown under low blasticidin pressure (2μg/ml) and therefore carrying low numbers of active *var* promoters, and the culture grown under high blasticidin pressure (20μg/ml) and carrying a high number of active *var* promoters, were used in the experiment. Both cultures were transfected separately with the constructs pVLH and pHLH that express the luciferase reporter gene under the control of either a *var* promoter (pVLH) or an hrp3 promoter (pHLH). Transient tranfections were performed as described[21] and luciferase expression was measured for each of the transfected cultures to determine the effect of the number of active transgenic *var* promoters in each parasite on its ability to express luciferase from either an additional *var* promoter or the hrp3 promoter. The expression of luciferase by a *var* promoter was significantly suppressed (~80%, P < 0.01) in the culture that was already saturated with large numbers of active *var* promoters stably carried on the multi-copy pVBH episomes when compared to luciferase expression in the parasite line that carried a small number of episomal *var* promoters (Fig 2). Interestingly, expression of luciferase by the *hrp3* promoter was down-regulated only by 26% in the culture carrying the large numbers of active episomal *var* promoters when compared to its expression in parasites that carried a small number of active episomal *var* promoters. It is not clear if this small reduction is a result of the increased number of active *var* promoters or perhaps a side effect of the higher level of blasticidin in the media. Nonetheless, these results confirmed that the transcriptional repression caused by saturation with episomal *var* promoters has a much stronger effect on *var* genes than on other genes in the genome.

Down-regulation of endogenous *var* gene expression results in erasing of *var* epigenetic memory

Two recent studies showed that regulation of *var* expression involves a type of "molecular memory" that includes specific histone modifications[10,11]. This memory ensures that once activated, a *var* gene remains active in the vast majority of subsequent cell cycles, thus resulting in the relatively low off rate per generation that is necessary to avoid premature expenditure of the *var* gene repertoire. Without this memory, *var* genes would be activated at random after each cell cycle and would not display the measurable switching dynamics observed over time in long-term cultures[17]. We were interested in whether preventing transcription of PFD1005c by forced expression of high numbers of unregulated episomal *var* promoters would influence the epigenetic memory that had marked this gene as the only active *var* gene for over 7 months in the original culture (Fig 3A). To test this, we removed the drug pressure from the transfected culture and let the parasites grow for a month in regular media, providing enough time for the parasites to shed the pVBH episomes. Plasmid rescue and PCR assays verified that the parasites had shed all the episomes. The level of

transcription of each individual *var* gene in the genome was then measured using Q-RT-PCR as described[22,23].

The gene expression assays demonstrated that all *var* genes were silent in the parasites that were growing under high levels of blasticidin pressure (Fig 3B). However, 10 weeks after drug pressure was removed the parasites did not return to their previous *var* gene expression pattern, but rather displayed activation of many *var* genes (Fig 3C). We were unable to detect any preference in *var* gene expression at this point, indicating that activation was likely random. In particular PFD1005c, the *var* gene that was dominant in the original population of parasites as well as in the parasites grown under low doses of blasticidin, was now barely detectable among the actively transcribed genes. Instead, a pattern of low transcription levels from many *var* genes was evident, consistent with a heterogeneous culture in which numerous subpopulations of parasites exist, all expressing different *var* genes. We again monitored the expression pattern 8 weeks later and again could detect transcripts from numerous endogenous *var* genes. At this time point, a subset of genes became more dominant and the detectable levels were higher than the first time point, suggesting active *var* gene expression switching (Fig 3D). In order to verify that this pattern represented true switching we isolated several clones from this culture by limiting dilution and measured the transcription pattern of the whole *var* gene family of these early clones by Q-RT-PCR. We found that each new clone displayed the typical pattern of a single dominant transcript (data not shown), as would be expected for normal *var* gene switching patterns[17] and indicating that they are once again being expressed in a mutually exclusive manner. These results demonstrate that down-regulating transcription of PFD1005c through competition with high numbers of "unpaired" *var* promoters results in this gene switching to the silent state, and that this silent state persists even after removal of the competing "unpaired" promoters. Thus the epigenetic memory that had previously marked PFD1005c as the only active gene had been completely erased.

Unpaired *var* promoters cannot be epigenetically silenced

As shown above, repression of transcription of an active *var* gene through competition with high numbers of "unpaired" promoters resulted in epigenetic silencing of this gene. Previous work had shown that "unpaired" promoters were by default transcriptionally active and unrecognized by the mechanism that controls mutually exclusive expression [15,16,22,24]. We were therefore interested to determine if an "unpaired" *var* promoter that had been integrated into the genome could similarly be repressed by competition with high numbers of episomal "unpaired" *var* promoters, and if so, would it then become epigenetically silenced. To answer this question, we used a transgenic parasite line (D10/inte) in which an "unpaired" *var* promoter expressing the *luciferase* reporter gene had been integrated into the genome at the *hrp2* locus[15]. In these parasites the *var* promoter driving *luciferase* expression (referred to here as *varLUC*) is constitutively active and not recognized by the mechanism that controls mutually exclusive expression. These parasites were transfected with pVBH and selected with 2, 10 and 20μg/ml blasticidin. In this way we were able to generate 3 parasite lines that carried increasing numbers of active episomal "unpaired" *var* promoters (Fig 4A).

Interestingly, while the level of luciferase expression was significantly down-regulated ($P<0.05$) in correlation with the increasing number of active transgenic *var* promoters (Fig 4B) similar to that observed for expression of the endogenous *var* gene PFD1005c, we were not able to reach complete silencing under either 10 or 20μg/ml blasticidin. Under both of these concentrations the levels of expression of the intact PFD1005c gene were extremely low (Figure 1), suggesting it had switched to the silenced state. In contrast, the *varLUC* gene was never completely silenced and continued to display a low level of expression. 5 weeks after blasticidin pressure was removed and the parasites had shed most of the episomes, luciferase expression levels returned to levels similar to that observed before transfection. These

results show that high numbers of competing episomal "unpaired" *var* promoters can significantly repress the expression of the chromosomal "unpaired" *var* promoter, however, they also demonstrate that unlike PFD1005c, this *var* promoter rapidly reverts to the active state once the competing promoters are removed, suggesting that it is unable to assume an epigenetically silent state. These data provide further support for a model of *var* gene regulation that depends on a cooperative interaction between *var* upstream promoters and the promoter found within *var* introns.

DISCUSSION

The ability to compete for a limiting factor and thus artificially repress transcription of an active *var* gene as described here provided a unique opportunity to investigate the role of transcription in maintaining a *var* gene in the epigenetically determined "on" state. The importance of transcription in establishing and maintaining cellular memory has been well documented in fruit fly development [25]. Recent work in several model systems has demonstrated that the carboxy-terminal domain of RNA polymerase II can associate with histone modifying enzymes, specifically histone methyltransferases, and thus modify the chromatin structure of a gene during transcription elongation[12,13]. This process can therefore "mark" genes that have been recently transcribed. It is possible that a similar mechanism is utilized by malaria parasites to mark the active *var* gene, thus resulting in a positive feedback loop ensuring that once activated, a particular *var* gene remains active for many cell cycles. Such a system would contribute to cellular memory and explain why artificially preventing an active *var* gene from being transcribed results in it reverting to the silent state.

Shedding of high-copy number episomes by parasites in which the entire *var* gene complement had been silenced led to random *var* gene activation (Figure 3), suggesting that when no *var* genes are in the epigenetically determined active state, all have an approximately equal probability of being activated. This artificial situation may be analogous to the point in the life cycle when parasites first exit the infected liver after transmission from a mosquito. Such parasites are leaving a stage of development in which *var* genes are presumably not expressed, and thus the entire gene family is thought to be silent. As the merozoites leave the liver and invade circulating RBCs, random *var* gene activation by each individual parasite would ensure an antigenically diverse population, thereby preventing complete clearance by any pre-existing anti-PfEMP1 antibodies and increasing the chances for a successful blood stage infection. Examination of experimental human infections have detected activation of all *var* genes immediately after parasite leave the liver, consistent with this model[26].

Previous work regarding the regulation of *var* gene expression has resulted in the proposal of different, somewhat conflicting models, particularly with regard to the role of the intron in silencing and mutually exclusive expression[15,16,27,28]. The work presented here demonstrates that when working with transgenic lines of parasites, if the concept of mutually exclusive expression (i.e. the idea that only a *single var* promoter is active at a time) is to be accurately assessed, it is imperative that the concatameric nature of replicating episomes be considered. For example, in parasites transfected with the plasmid pVBH, high doses of blasticidin resulted in silencing of all chromosomal *var* genes and limited expression to only the *var* promoters found on the concatameric pVBH episome. Nonetheless, this should not be interpreted as silencing through the mechanism that controls mutually exclusive expression, as the parasites were expressing a very high number of episomal *var* promoters (perhaps as many as 20) and therefore silencing in this case was rather through saturation of the nuclei's ability to transcribe *var* genes. In contrast, we have previously shown silencing through the pathway that controls mutually exclusive expression by utilizing "promoter trapping"[22,29] to ensure that only a single transgenic *var* promoter is

functional. In this instance the transgenic *var* promoter was paired with an intron and therefore recognized by the regulatory mechanism.

The *var* gene family can be divided into several subgroups based on sequence homology and chromosomal position[30,31]. Although sequence similarities between subgroups of promoters have been described[30], no general motif was found to be involved in *var* gene activation. Nonetheless, in the present study we demonstrated that multiple copies of one promoter type (upsC) were able to keep all *var* genes in the genome in a silent state, therefore all *var* promoter types must share requirements for certain limited nuclear factors. The evidence described here suggests that *var*-specific factor or factors are plentiful enough to permit the simultaneous expression of only a limited number of *var* promoters, in this instance ~20. It is conceivable that the limiting factor is simply the space within a specific, subnuclear expression site. Previous work from several laboratories has shown that *var* genes seem to move into a specific sub-nuclear location upon transcriptional activation[16,27,32,33]. The space within this site may have a limited capacity for promoters and might have reached the highest limits of its capacity with the inclusion of multiple copies of episomal *var* promoters that are forced to be active. Thus the observed repression of the endogenous *var* promoter PFD1005c and the transgenic *varLUC* gene might due to the fact that they are simply forced out of the expression site where active *var* gene transcription occurs.

Alternatively, the limiting factor may not be space per se, but a regulatory element or protein complex that resides within this nuclear compartment and is essential for *var* gene activation by interacting with basal transcription factors, chromatin-remodeling factors or histone modifying enzymes. Supporting evidence for this hypothesis is that as the number of competing episomes increased, the endogenous *var* gene PFD1005c did not simply display continuously falling levels of expression, but rather reverted to a completely silenced state, and this state persisted even after the competing episomes were removed, suggesting that the epigenetic memory for this gene was erased. Similar patterns of repression were observed in other eukaryotic systems and shown to involve transcription initiation or suppression through limiting amounts of promoter regulatory elements[34]. A better comprehension of the components that control nuclear architecture, chromatin assembly and epigenetic memory in *P. falciparum* will greatly improve our understanding of the process of antigenic variation as well as parasite gene regulation in general.

MATERIALS AND METHODS

Parasite culture and transfection

All experiments utilized the *Plasmodium falciparum* NF54 line or transgenic derivatives[15,17] cultivated at 5% hematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate, and 0.1 mg/ml gentamicin. Parasites were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen. Parasites were transfected by using "DNA loaded" red blood cells as previously described[21]. Briefly, 0.2cm electroporation cuvettes were loaded with 0.175ml of erythrocytes and 50 μg of plasmid DNA in incomplete cytomix solution. Transient assay with the plasmids pVLH, and pHLH was performed as described[21]. Parasite populations that stably carry the construct pVBH[16] were selected by culturing in the presence increasing concentrations (2–20 μg/ml) of Blasticidin S HCl (Invitrogen, USA). The constructs pVLH[35], pHLH[20] and pVBH[16] have been previously described, however a brief description is provided here. In pVLH and pHLH, expression of a firefly luciferase gene is driven by either the *var7b* promoter (pVLH) or the promoter of the *hrp3* gene (pHLH). pVBH is identical to pVLH, except the luciferase coding region has been replaced by the gene encoding blasticidin S deaminase.

Clonal cultures originating from a single parasite were created by limiting dilution using 96 well microtiter plates as previously described[36]. Individual plates were screened for parasites during media changes on days 21, 25 and 30. Individual parasite cultures were expanded to 20 ml cultures and used for DNA and RNA extraction.

Genomic DNA extraction

Infected RBCs were pelleted by centrifugation at $3000 \times g$. After discarding the supernatant the pellet was divided into 2 microcentrifuge tubes followed by resuspension in 500 µl phosphate buffered Saline (PBS) and 20 µl 10% Saponin. Parasites were pelleted by centrifugation and washed twice with 1000µl PBS. The parasite pellet was then taken up in 200 µl TSE buffer (100 mM NaCl, 50 mM EDTA, 20 mM Tris, pH 8) to which 40 µl of 10% SDS and 20 µl 6M $NaClO_4$ were added. This suspension was placed on a rocker overnight and the DNA extracted with phenol/chloroform the next morning. The DNA was precipitated from the final aqueous phase with ethanol and resuspended in 100 µl sterile dH_2O.

RNA extraction and Realtime RT-PCR for assaying expression of the *var* gene family

RNA extraction and Q-RT-PCR were performed as previously described[22]. Briefly, RNA was extracted from synchronized ring stage parasites 16–18 hours post invasion. RNA extraction was performed with the TRIZOL LS Reagent® as previously described[37]. RNA to be used for cDNA synthesis was purified on PureLink column (Invitrogen) according to manufacturer's protocol. Isolated RNA was then treated with Deoxyribonuclease I® (Invitrogen) to degrade contaminating gDNA. cDNA synthesis was performed from 800 ng total RNA with Superscript II Rnase H reverse transcriptase ® (Invitrogen) with random primers ® (Invitrogen) as described by the manufacturer. For Q-RT-PCR reactions to detect transcription from all *var* genes present in the 3D7 genome, we employed the primer set of Salanti et. al.[23] with the modifications described in[15,17,22]. Copy numbers of transgenes were calculated using Q-RT-PCR of gDNA and comparing the Ct of the transgene to that of the single copy housekeeping gene seryl-tRNA synthetase (PF07_0073) as described[15].

Luciferase assays

Transient assays were performed as described[38]. Luciferase expression by parasites that stably express luciferase was performed as described by Frank et al.[15]. For both assays, the presented luminesence units are the average of triplicates and normalized to 1% parasitemia.

Statistical analysis

All assays were performed at least in triplicates and the average between replicates is presented together with its standard deviation. The data was sorted using Microsoft Excel and analyzed with the Sigma Stat (SPSS Science) statistical software package with significant levels set at $P < 0.05$. The significance of different promoters on the levels of down regulation of luciferase expression in the transient assay was tested using the independent samples t-test. Comparisons of genes expression levels between and within the different parasite population was performed using one way analysis of variance (ANOVA) followed by post hoc testing of the different groups using the least significant (LSD) test (Fisher LSD test).

Acknowledgments

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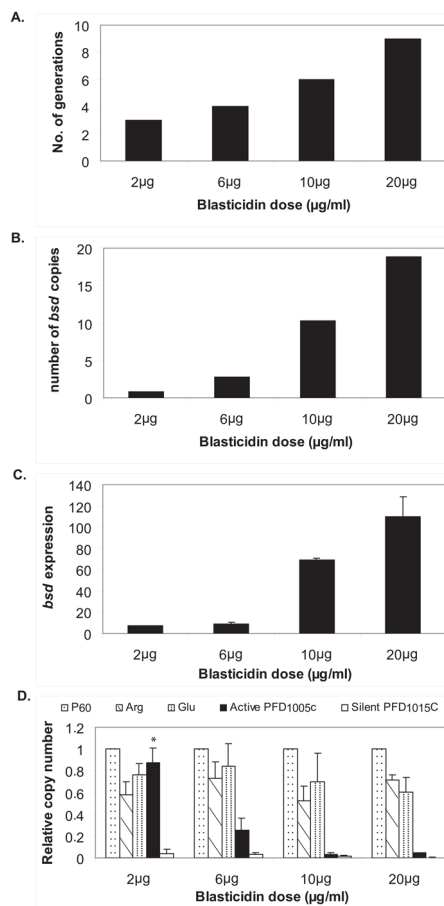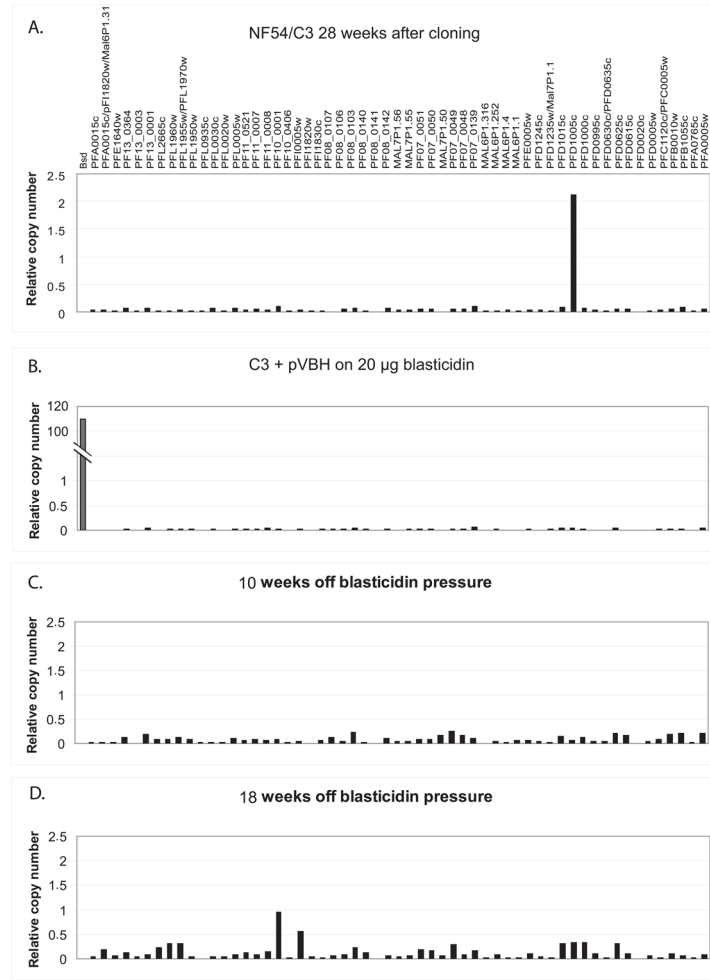
Figure 1.
Transcriptional repression of endogenous *var* genes by forced expression of multiple
episomal *var* promoters. (A) Selection for parasite populations that carry the episome pVBH
using increasing concentrations of blasticidin. The number of generations to reach 5%
parasitemia increases in correlation to the increase in blasticidin concentration. (B) Parasite
populations carry increasing copy numbers of pVBH with respect to blasticidin pressure.
The number of *bsd* copies measured by Q-RT-PCR using gDNA as template is presented for
each of the parasite populations grown under different concentrations of blasticidin. (C) *bsd*
expression is correlated with the number of copies of pVBH. Total *bsd* expression was
measured by Q-RT-PCR using cDNA as template from each of the parasite populations
carrying increasing copy numbers of pVBH. Total expression is presented as relative copy
numbers with respect to the control housekeeping gene seryl-tRNA synthetase (PF07_0073).
(D) Transcriptional repression of the endogenous *var* PFD1005c in parasite populations
expressing high copy numbers of pVBH. Expression levels of the active *var* gene PFD1005c
and the silent *var* gene PFD1015c are presented for each of the parasite populations grown
under different blasticidin concentrations with respect to the 3 control housekeeping genes:
P60 - seryl-tRNA synthetase (PF07_0073), Arg - arginyl-tRNA synthetase (PFL0900c) and
Glu -glutaminyl-tRNA synthetase (PF13_0170), (* significant down regulation, F = 10.49, *P*
< 0.01).
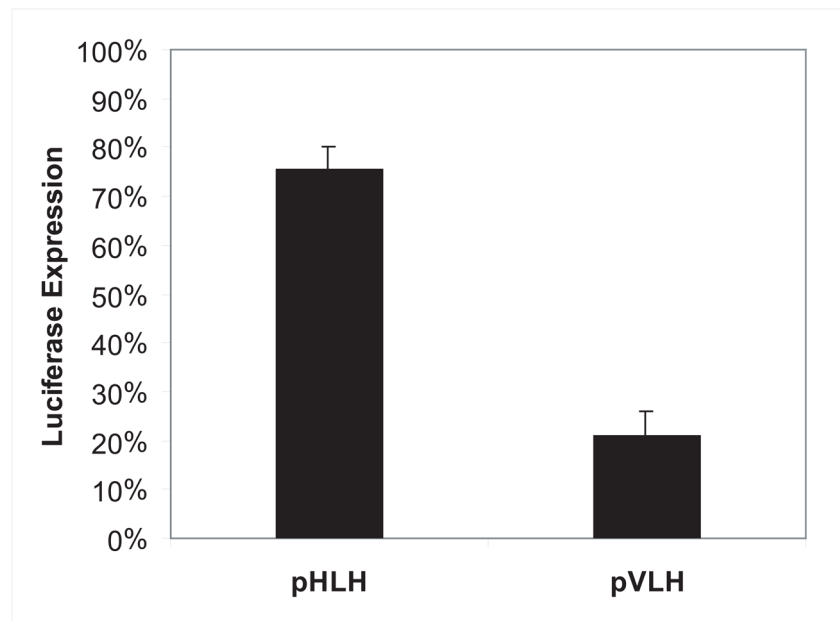
Figure 2.
A specific nuclear element is required for expression of *var* promoters. The histogram shows levels of luciferase expression from parasites transiently transfected with plasmids carrying either a *var* promoter (pVLH) or the unrelated *hrp3* promoter (pHLH). The values show luciferase expression by transgenic parasites that stably a carry high copy number of pVBH episomes (grown under 20 μg/ml blasticidin) as a percentage of that expressed by parasites carrying a low copy number (under 2 μg/ml blasticidin). Error bars represent calculated standard errors.

Figure 3.
Induced repression of an endogenous *var* gene erases its epigenetic memory. Steady state mRNA levels expressed from each *var* gene (labeled by annotation number) as well as *bsd* are shown for each population of parasites. Analysis of the levels of transcription shows that PFD1005c is the dominant gene expressed by parasites in the NF54/C3 population (A). Expression of high copy numbers of pVBH results in silencing of all *var* genes in the family (B). Removal of blasticidin pressure leads to loss of the episomes and a random activation pattern after a period of 10 and 18 weeks (C and D, respectively). The parasite populations in C and D have become heterogenous, with numerous subpopulations present, each expressing a different individual *var* gene.
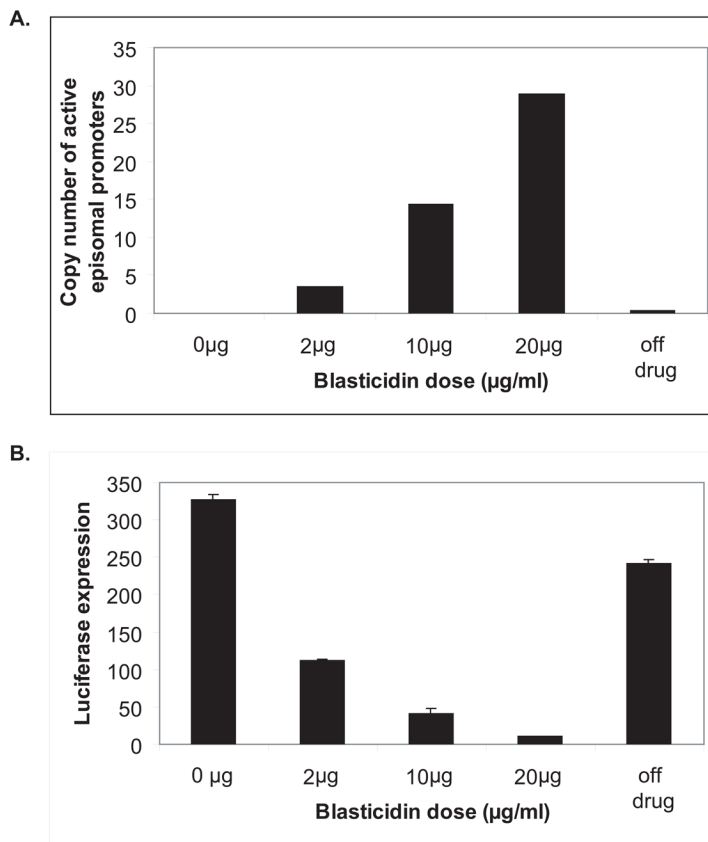
Figure 4.
An unregulated *var* promoter cannot be epigenetically marked for silencing. (A) Selection for *varLUC* parasite populations carrying increasing numbers of pVBH episomes. The number of *bsd* copies measured by Q-RT-PCR is presented for *varLUC* parasites transfected with pVBH and selected on increasing concentrations of blasticidin. Removal of blasticidin pressure resulted in almost complete shedding the pVBH episomes (off drug). (B) Repression of the unregulated *varLUC* promoter does not reach complete epigenetic silencing. The luciferase expression of each of the different parasite populations is presented in luminescence units. Parasite populations in which blasticidin pressure was removed and pVBH episomes shed returned to expressing high levels of luciferase (off drug).