Var Gene Promoter Activation in Clonal Plasmodium falciparum Isolates Follows a Hierarchy and Suggests a Conserved Switching Program that Is Independent of Genetic Background

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Antigenic variation of Plasmodium falciparum is mediated by a mutually exclusive expression mechanism that limits expression to an individual member of the multicopy var gene family. This process determines the antigenic and adhesive phenotype of the infected red blood cell. Previously, we showed that var gene switching is influenced by chromosomal position. Here, we address whether var gene transcription follows a general conserved pattern in long-term laboratory parasites and in recently culture-adapted field parasites. Activation of the var gene family was monitored in biological replicates in each parasite isolate every 3–5 generations for up to 3 months. We used transgenic parasites carrying a drug-selectable marker at a defined var locus to characterize var gene activation after the exclusive expression of the transgene. Transgenic parasites exhibited a repeatable hierarchy of var gene activation and a fluctuating transcriptional activity of the transgenic var locus. Transcriptional profiling of wild-type laboratory and field parasites showed a universal bias toward transcription of UpsC var genes and a fluctuating transcriptional activity of the dominant var promoter. The data suggest the existence of an intrinsic var gene transcription program that is independent of genetic background.

Plasmodium falciparum is a serious human pathogen responsible for the death of ~1 million persons annually, mainly pediatric patients. The pathogenicity of P. falciparum is related to the phenomenon of cytoadhesion, which involves specific surface receptors, P. falciparum erythrocyte membrane protein 1 (PfEMP1) [1]. PfEMP1 are the main surface antigens of the infected red blood cell. As children in countries of endemicity develop increasing antibody titers against PfEMP1, they develop a poorly understood clinical immunity that results in a gradual decrease in the severity of P. falciparum infection [2]. However, permanent variation of the presented type of PfEMP1 [3] leads to ineffectiveness of the immune response enabling a chronic infection in the human host. PfEMP1 proteins are encoded by the var multigene family, a group of ~60 genes [4, 5, 6]. A mutually exclusive expression mechanism limits var gene transcription to a single var gene in the late-ring stage of the parasite life cycle, while keeping all other var genes transcriptionally silent [3, 7, 8]. The var genes are positioned either in head-to-head orientation in the
MATERIAL AND METHODS

Parasite Lines and Culture
The clonal transgenic DCJ and C5G12 and the bulk NF54 parasites are described elsewhere [22, 34]. The field isolate MOA was obtained from an asymptotically infected adult Gabonese and culture adapted for 25 generations. The study protocol was approved by the Ethics Committee of the Albert Schweitzer Hospital, Lambaréné, Gabon [35]. MOA clones (MOA-D2, MOA-D5, and MOA-C3) were generated by limiting dilution [36]. Parasites were cultivated as reported elsewhere [34]. Transgenic parasites were selected with either 20 or 5 μg/mL of blasticidin hydrochlorid final concentration (Sigma). Transcriptional profiling of DCJ after selection with 20μg/mL of blasticidin was performed in pentaplicates. Transcriptional profiling in all other clonal parasite lines was performed in duplicates.

RNA Extraction and cDNA Synthesis

Ring-stage parasites were 5% sorbitol synchronized twice in 8 hours according to standard procedures. RNA extraction followed the second synchronization. Before RNA harvesting, exclusive ring-stage and parasite count were determined by counting ~500 erythrocytes per smear. RNA was extracted every 3–5 generations at ring stage parasitemias of 1%–3% (4–10 hours after invasion). RNA extraction and cDNA synthesis were conducted as described elsewhere [22].

var Gene Transcriptional Analysis in NF54/3D7 Laboratory Isolates

For var gene transcriptional profiling, we used the primer set of Salanti et al [37] with the modifications described by Dzikowski et al [34] and Frank et al [22]. We replaced the double primer for PFC0005w/PFC1120c with 2 primer pairs: for PFC0005w, 5’ AACGGACGAAATATGGTAGT 3’ and 5’ GTC GTTGTAAACCATTCTTATG 3’; for PFC1120c, 5’ CAGCTAGTGGAGTAGAAG 3’ and 5’ TGAAGTTCTTATGGGTT 3’. We added MAL8P1.220 5’ ATAGAAAACGCTAC- GAAGG 3’ and 5’ ATGGTTTTGAGCTTGAGAT 3’ and a second primer for the blasticidin gene: 5’ AGTGAAGGACA GTGATGGAC 3’ and 5’ ATTGGTTTTGAGCTTGAGAT 3’. All bsd expression levels were measured with both primer pairs.

Quantitative reverse-transcription polymerase chain reactions (qRT-PCRs) were prepared at a final volume of 20 μL with use of SensiMix Plus SYBR (Quantace QT605-05) and a final primer concentration of 0.25 μmol/L and were measured using a Corbett Research Rotorgene 3000 (95°C for 3 minutes, 95°C for 15 seconds, 50°C for 30 seconds, and 68°C for 30 seconds, followed by 40 cycles at 68°C for 1 minute).

We determined correction factors for each primer pair on gDNA (NF54) with respect to the housekeeping gene fructose biphosphate aldolase (PF14_0425). Each delta cycle threshold (ΔCt) was assessed by subtracting the corrected Ct of an individual primer from the Ct of the housekeeping gene arginyl-tRNA synthetase (PFL0900c). Transcription at each var locus was calculated in relative copy numbers: copy no \( = 2^{-\Delta Ct} \)
Figure 1. Transcriptional profiling of the var gene family in transgenic parasites. Clonal NF54 parasites were genetically modified by stable transfection of var knock-down plasmids as described elsewhere [34]. This resulted in the transgenic lines DCJ and C7G12. A, DCJ was obtained by transfection of a plasmid that uses the recombinant promoter region of the telomeric UpsB var gene PFB1055c to express the resistance gene blasticidine deaminase (bsd). Homologous recombination through a double cross-over event replaced exon1 of PFB1055c with a single copy of bsd, resulting in the telomeric var transgene PFB1055c-bsd. B, C7G12 was obtained by transfecting a plasmid that uses the recombinant promoter region of the central UpsC var locus PFL1960w to express bsd and the var intron to express a second selectable marker human dihydrofolate reductase (hdhfr). This construct integrated in a single cross-over event in the promoter region of PFL1960w in a concatameric fashion, resulting in the central var transgene PFL1960w-bsd. For experimental details on prior transcriptional studies, please see references Dzikowski et al 2006 [34] and Frank et al 2007 [22]. Transcriptional analysis was performed every 5 generations to characterize the switching pattern. var Transcription was observed in transgenic parasites after release of drug pressure when var genes switch freely after bsd-forced expression of the transgene. C, D, A clonal DCJ culture was selected with either 20 or 5 l g/mL of blasticidine and subsequently released from drug pressure and for transcriptional analysis. Transcriptional profiling was performed in quintuplicates and duplicates for DCJ20µg and DCJ5µg, respectively. All biological replicates were released from drug pressure simultaneously. RNA was sampled from each replicate every 3–5 generation of parasite growth. Switching was monitored as described in Materials and Methods. E, It had been previously described that central transgenic var locus PFL1960w-bsd has a very low switch rate [22]. Therefore, C7G12 was grown without drug pressure for a period of 64 generations, and subsequently, transcriptional profiling was performed in duplicates.
(User bulletin 2, Applied Biosystems, http://www.appliedbiosystems.com). To enable comparison across experiments, we quantified individual var gene transcription levels as percentage copy number of the total sum of all individual var gene signals. Long-term transcriptional analysis in NF54 bulk cultures was also monitored with var promoter specific primers (see below).

**var Gene Transcriptional Profiling in Field Isolates and NF54 Bulk Culture**

In the MOA cultures, we used promoter-specific primers A1, A2, B1, B2, C1, and C2 [26]. For primer evaluation, we compared expression analysis with promoter specific and var gene specific primers on cDNA of clonal NF54 parasites. The sum of the signal of the gene-specific primers belonging to one promoter group was expressed as the percentage of the total var gene signal. This was compared with the percentage of the promoter-specific primer of the total promoter signal. Both methods captured the same proportion of the total var signal. In addition, we compared amplification rates of the promoter specific primers on gDNA of NF54 and MOA bulk cultures in triplicates. A1, B2, and C2 consistently amplified the same proportion of the total var promoter signal in both genetic backgrounds and were chosen for transcriptional profiling. An unselected MOA bulk and NF54 bulk culture were sampled for 170 generations and 80 generations, respectively, with the promoter-specific primers.

For analysis of specific var gene expression in the MOA clones, the active var locus was identified by cloning cDNA var fragments obtained using PCR with universal primers [38] followed by sequencing. We identified only a single dominant message in all 3 clones. The sequences can be downloaded at the National Center for Biotechnology Information GenBank database under the accession number JF346755-JF346757. Gene-specific primers were designed for the dominant sequence and were evaluated for qRT-PCR on serial DNA dilutions, followed by sequencing. Primer sequences for MOA-D5 are 5′ TCTATATTTGGAATAGAA 3′ and 5′ CATCCGCGACTCTCTCC 3′; for MOA-C3 are 5′ TCTGTCTTGGTGTTAACC 3′ and 5′ GGTGATCTTCAGATTCGA 3′, and for MOA-D2 are 5′ GATAATGCAAAAGCTGCAATT 3′ and 5′ TCTGTTTCTTGGTTATACC 3′. The gene-specific primers for MOA-D5 amplified DNA with the same efficiency as the housekeeping gene arginyl-tRNA synthetase (PFL0900c). The clone MOA-D5 was propagated for 37 generations in biological replicates, and transcriptional profiling was conducted as described above.

**Growth Quantification of Transgenic Parasites**

C7G12 transgenic parasites that had been propagated without blasticidine pressure were reselected with 20 μg/mL blasticidine (in biological replicates), and growth was compared with C7G12 parasites that had been propagated continuously under 20 μg/mL blasticidine. Starting parasitemia was 0.5% in all cultures. Parasitemia was quantified for 8 generations.

**Statistical Analysis**

We used JMP software, version 5.1, to assess means, mean error bars, and standard deviation in Figures 3 and 6 and Supplementary Figure 1.

**RESULTS**

**var Gene Activation in Transgenic Parasites Follows a Repeatable Hierarchy and Reveals a Fluctuating Transcriptional Activity of Telomeric and Central var Promoters.**

To investigate whether var gene switching follows a predetermined hierarchy, we used the transgenic var knock-down parasites lines DCJ and C7G12. In these transgenic parasites, selection with blasticidine (bsd) generates a population of parasites that exclusively expresses the transgenic var locus (Figure 1A and B). Release from drug pressure allows these parasites to switch freely to alternative endogenous var genes. We initially selected clonal DCJ with 20 μg/mL blasticidine (DCJ20μg), to exclusively express the recombinant UpsB var locus PFB1055c-bsd. The blasticidine-selected parasites were divided in 5 biological replicates (DCJ20μg-1 - DCJ20μg-5). These 5 cultures were released from drug pressure simultaneously and were grown without drug pressure for 35 generations (Figure 1C). Transcription was monitored by gene-specific primers with use of qRT-PCR every 3–5 generations. Immediately after release from drug pressure, we observed a preferential activation of individual var loci in all 5 biological replicates (Figure 2). During the drug-free culture period, additional activation of specific members of the var gene family occurred in all biological replicates (Figure 2), suggesting a conserved switching hierarchy. Longitudinal analysis of the most actively transcribed var genes in the 5 individual cultures revealed similar expression levels in all biological replicates throughout the entire observation period of 35 generations (Supplementary Figure 1). Of note, the transgenic locus PFB1055c-bsd was continuously expressed throughout the entire experiment. Interestingly, the expression of the PFB1055c-bsd transgene initially decreased and subsequently appeared to increase despite the absence of selective pressure (Figure 3A). Transcriptional analysis of the individual endogenous var genes revealed fluctuating levels of transcription that were independent of the PFB1055c-bsd transcriptional signal (Supplementary Figure 1), suggesting a coordinated switching activity among the endogenous var genes.

To assess whether the switching rate of the transgenic locus PFB1055c-bsd was intrinsic or possibly influenced by the strength of drug selection, we decreased selection pressure to 5 μg/mL and repeated the experiment in duplicates (DCJ5μg-1, DCJ5μg-2) (Figure 1D). This revealed that the same endogenous var loci were preferentially activated, again supporting a conserved
Figure 2. Transcriptional analysis of the var gene family reveals preferential transcription of the same var loci across biological replicates. The transgenic parasite strain DCJ (Figure 1A and 1C) was cultured with 20 μg/mL blasticidine. Extensive blasticidine pressure forces the parasites to transcribe the UpsB var transgene PFB1055c-bsd for survival. Five biological replicates (DCJ20μg - 1 – DCJ20μg – 5) were released from drug pressure simultaneously, and free var gene switching from the transgenic var locus PFB1055c-bsd was monitored for 35 generations. Shown are expression profiles of the complete var gene repertoire measured by qRT-PCR after 17 generations of drug-free growth. Transgenic parasites are of 3D7/NF54 genetic background enabling transcriptional profiling at each individual var locus with gene specific primers [37]. The 3 panels represent transcriptional profiles of the var gene family of 3 representative biological replicates. var loci are listed on the x-axis and sorted according to promoter types. Black:
hierarchy when switching starts at the transgenic locus PFB1055c-bsd (Figure 3B). Strikingly, the fluctuation of PFB1055c-bsd expression was even larger than in the first experiment.

We had previously observed that var promoters of central UpsC var genes have extremely low switching rates. However, these observations were made with RNA sampling occurring every 35 generations and, thus, were not able to measure short-term changes in transcriptional activity. We therefore hypothesized that central UpsC var promoters might undergo a similar fluctuating expression pattern as observed for the telomeric UpsB var promoters. To address this question, we performed short-term transcriptional profiling in the C7G12 parasite line (Figure 1B and 1E) that had already been cultured for 64 generations without blasticidin pressure, but continued to transcribe the UpsC var transgene PFL1960w-bsd (C7G12-1, C7G12-2) (Figure 3C). We found a fluctuating bsd signal with reciprocal changes in the transcription of endogenous var genes. To confirm that the relatively small endogenous var signal of the C7G12 parasite line truly represented switching, we reapplied blasticidin pressure to C7G12 parasites that had been grown without blasticidin and compared the growth curve to a C7G12 unselected parasites had switched away from the transgenic locus PFL1960w-bsd and, thus, died under blasticidin pressure (Figure 4).

To exclude the possibility that sampling time differences were responsible for the differences in transcriptional signal, we evaluated whether different sampling times during the ring stage could affect the transcriptional signal. This revealed that the sampling time did not affect the relative expression levels of the individual var loci (data not shown).

Taken together, we observed a repeatable var gene switching hierarchy in parasites carrying telomeric and central transgenic var promoters. Surprisingly, in all individual experiments, the transcriptional signal from the transgenic var loci did not decrease at a constant rate but rather displayed a fluctuating level of activity.

Field Isolates and NF54 Parasites Preferentially Transcribe UpsC Promoters Under Nonselective Tissue Culture Conditions

To verify that the observed transcriptional profiles were not simply a phenomenon of clonal transgenic parasites, we extended our investigations to wild-type NF54 parasites and a field isolate (MOA). To allow comparative analysis of var gene expression in the 2 different genetic backgrounds of which only the NF54 genome is sequenced, we performed transcriptional profiling with UpsA-, UpsB-, and UpsC-specific primers previously developed by Rottmann et al [26] (see Materials and Methods for validation experiments). The UpsC signal was clearly the dominant transcriptional signal at all times in both genetic backgrounds (Figure 5A and 5B), strongly suggesting that, under nonselective conditions, there is a universal bias toward transcription of UpsC var genes. However, the field isolate also exhibited a constant UpsA signal that was not present in the NF54 genetic background.

To characterize the transcription of the complete var gene family in the NF54 background, we applied short-term transcriptional profiling. This revealed that the most active var locus PF08_103 (UpsB/C) displayed a fluctuating transcriptional activity, accompanied by reciprocal changes in the transcriptional activity of PFD0615c (UpsC) (Figure 5C).

Transcriptional Profiling of Clonal Field Isolates Shows a Bias Toward UpsC Expression and Fluctuating Promoter Activity

To characterize the transcriptional profile of an individual UpsC var locus of the MOA field isolate, we generated 3 individual clones by limiting dilution referred to as MOA-D5, MOA-D2, and MOA-C3. We identified the dominant transcript in the 3 clones by cDNA DBL cloning, herewith confirming that the isolates were indeed clonal and expressing an individual var gene. Transcriptional analysis with gene-specific primers showed that each clone was expressing an individual UpsC var locus (Figure 6A). To investigate whether an individual UpsC locus from a field isolate also displayed a fluctuating transcriptional activity, we conducted a high-frequency transcriptional profiling in biological replicates of clone MOA-D5. The transcriptional activity of the transcribed var locus was very high at the first time of transcriptional analysis, immediately after isolation of the clone from limiting dilution. Subsequently, the transcriptional signal decreased but increased again after 25 generations, again displaying a fluctuating transcriptional activity.

DISCUSSION

In this article, we try to address the question whether var gene expression in P. falciparum follows a distinct profile across different genetic backgrounds. In particular, we compare var
Figure 3. Activation of var genes in transgenic parasites carrying telomeric and central var transgenes follows a repeatable hierarchy and reveals a fluctuating pattern of transgene expression (bsd). All var genes with an expression activity of >2% of the total var signal are individually displayed and indicated in the legends. All var genes with expression levels below this level are collectively represented as “rest.” The height of the individual data points represents their fraction of the total var signal in percent. The legend represents the most actively transcribed individual var loci at the time point with the highest endogenous var gene signal (DCJ20µg: generation 17, DCJ5µg: generation 14, C7G12: generation 70). var genes are sorted in descending order.
in both experiments the expression level of the transgene initially decreases but subsequently increases despite the absence of selective pressure.

Switching data after drug release of the C7G12 cell line from 20 pooled data of DCJ20

Figure 3 continued. order according to their transcriptional signal. Promoter types are indicated for each var gene. The x-axis represents the different sampling points throughout the course of the experiment. Each graph represents the pooled data for the individual experiment. The first column in each panel represents the transcriptional profile of the respective cell lines under blasticidin selection (DCJ20μg, DCJ5μg, C7G12 20μg). All var gene transcriptional profiles after the release from drug pressure were conducted in biological replicates (see Materials and Methods). A. Displayed are the pooled data of DCJ20μg switching after growth under 20μg/ml blasticidin and subsequent release from drug pressure (Figure 1C). DCJ carries the UpsB transgene PFB1055c-bsd in the telomeric region on chromosome 2. Note that the same loci are most actively expressed throughout the entire experiment (also see Supplementary Figure 1A and 1B). B. Switching after selection of DCJ5μg with 5 μg/ml of blasticidin and subsequent release from drug pressure (Figure 1A and 1D). Note that the 5 most actively transcribed endogenous var genes are the same in the DCJ20μg and DCJ5μg experiments. In both experiments the expression level of the transgene initially decreases but subsequently increases despite the absence of selective pressure. C. Switching data after drug release of the C7G12 cell line from 20μg/ml blasticidin pressure. C7G12 carries the transgenic UpsC var locus PFL1960w-bsd. Note that the sampling points are between generation 64 and 78 of drug free growth. The extended drug-free culture period refers to the fact that UpsC loci show decreased switching activity compared with UpsB loci [22]. Note that the hierarchy of transcribed endogenous var loci is different from the DCJ cell line. The transgenic promoter of PFL1960w-bsd again displays a fluctuating transcriptional signal.
that at least 2 transcriptional patterns are shared in both genetic backgrounds.

First, we confirm a preferred transcription of UpsC var loci in clonal field isolates in vitro—a phenomenon shown before for unselected wild-type laboratory strains. How can long-term expression of UpsC var genes be beneficial for a parasite that has to evade the human immune response? Possibly, the binding phenotype of the expressed PfEMP1 is of critical importance. UpsC and UpsB var genes are known to bind to CD36. Maintenance of UpsC expression might prolong adhesion in the deep capillary beds and, thereby, delay elimination in the spleen. However, expressing an individual var gene for prolonged periods implies a very high probability of removal. Our second observation of fluctuations in promoter activity may provide a solution to this problem. This pattern of var gene transcription enables the constant generation of small subpopulations of parasites expressing different variants and, thus, secures a population of parasites that can escape the dominant immune response. We observed fluctuations in var promoter activity in transgenic parasites, wild-type NF54 parasites, and culture-adapted field isolates. All experiments were performed in highly synchronized parasite cultures to minimize the influence of cell cycle progression on transcriptional profiles [41]. In addition, we assessed the phenotypic importance of transcriptional

Figure 5. Long-term in vitro var gene transcription of unselected wild-type field parasites and unselected NF54 parasites displays a universal bias towards UpsC transcription. We observed promoter-type expression in long-term tissue cultures in the unselected nonclonal field isolate MOA and the laboratory strain nonclonal NF54. In each Plasmodium falciparum strain, the var repertoire is completely different from the sequenced 3D7/NF54 genome strain. The only common feature is the promoter types. A qRT-PCR protocol using formerly generated promoter-specific primers [26] was established to compare promoter type expression in field and laboratory isolates. A, B, and C on the x-axis refers to var promoter types measured by promoter-specific primers with qRT-PCR. The y-axis represents the transcriptional signal. For panels A and B, this represents the var promoter-specific signal in relative copy numbers. A, var promoter transcription in the nonclonal field isolate MOA. B, var promoter transcription in the NF54 isolate. Note that in both genetic backgrounds the UpsC signal is the predominant signal at all times. Panel C represents transcription of the 10 most active genes in the same NF54 culture over a period of 45 generations of continued growth. The height of the individual data points represents their fraction of the total var signal in percent. All other var genes are summarized as “rest.” The x-axis represents the different sampling points throughout the course of the experiment. Note that PF08_0103 (Ups B/C) displays a fluctuating transcriptional activity that is accompanied by reciprocal changes in the transcription of the locus PFD6015c (Ups C).
fluctuations by reapplying drug pressure to transgenic parasites. This resulted in death of a significant portion of the parasite population, suggesting that decreases in transcriptional signal correspond to true switching activity. What could be the possible mechanism for the increase in transcriptional signal in the absence of selection? From our data, it is not possible to determine whether an increase in transcriptional signal is attributable to overexpression of the transgene in some parasites or to switching back to the transgene of a population of parasites. However, Recker et al [42] recently suggested a switching model in which parasites switch away from the dominant var locus but subsequently switch back. This model would thus explain the observed fluctuations in promoter signal as parasite populations that initially switch away and subsequently switching back to an individual var locus.

The presence of conserved transcriptional patterns across different genetic backgrounds suggests that the immune system is likely seeing subgroups of var genes in a sequential manner; thus, immunity would develop over time against the most readily expressed variants. Recent publications reported fluctuations by reapplying drug pressure to transgenic parasites. This resulted in death of a significant portion of the parasite population, suggesting that decreases in transcriptional signal correspond to true switching activity. What could be the possible mechanism for the increase in transcriptional signal in the absence of selection? From our data, it is not possible to determine whether an increase in transcriptional signal is attributable to overexpression of the transgene in some parasites or to switching back to the transgene of a population of parasites. However, Recker et al [42] recently suggested a switching model in which parasites switch away from the dominant var locus but subsequently switch back. This model would thus explain the observed fluctuations in promoter signal as parasite populations that initially switch away and subsequently switching back to an individual var locus.

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The presence of conserved transcriptional patterns across different genetic backgrounds suggests that the immune system is likely seeing subgroups of var genes in a sequential manner; thus, immunity would develop over time against the most readily expressed variants. Recent publications reported
a sequential antibody response during host life [31, 32] and a biased var gene transcription in vivo [30]. Both findings support the existence of a conserved switching program in parasites. However, it is currently unclear whether the expression profile during natural infection is predominantly shaped by selection [43] or by changes in transcriptional activity [34]. In view of the prolonged expression of individual var loci, our data may favor a model in which strong cross-reactive immune responses can sustain a chronic infection and, thus, contribute to antigenic variation [43]. In summary, our data suggest that transcription of individual var loci is quite constant but that a small fraction of the population switches away from the predominant locus. In this scenario, the bulk of the population would be removed by the immune system, whereas the small population of parasites that switched away would ensure the persistence of infection.

**Supplementary Data**

Supplementary data are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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