

Antigenic variation in malaria: *in situ* switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*

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Members of the *Plasmodium falciparum* var gene family encode clonally variant adhesins, which play an important role in the pathogenicity of tropical malaria. Here we employ a selective panning protocol to generate isogenic *P.falciparum* populations with defined adhesive phenotypes for CD36, ICAM-1 and CSA, expressing single and distinct *var* gene variants. This technique has established the framework for examining *var* gene expression, its regulation and switching. It was found that *var* gene switching occurs *in situ*. Ubiquitous transcription of all *var* gene variants appears to occur in early ring stages. However, *var* gene expression is tightly regulated in trophozoites and is exerted through a silencing mechanism. Transcriptional control is mutually exclusive in parasites that express defined adhesive phenotypes. *In situ* *var* gene switching is apparently mediated at the level of transcriptional initiation, as demonstrated by nuclear run-on analyses. Our results suggest that an epigenetic mechanism(s) is involved in *var* gene regulation.

Keywords: allelic exclusion/antigenic variation/*in situ* activation/silencing/*var* genes

Introduction

Controlled phenotypic variation of surface-exposed antigenic determinants (antigenic variation) is a prime immune evasion mechanism utilized by several pathogenic bacteria and protozoa to maintain a chronic infection in the presence of constant immune pressure exerted by their hosts (Borst and Greaves, 1987; Deitsch *et al.*, 1997). Antigenic variation has been extensively studied in the human pathogens *Neisseria gonorrhoeae*, *Borrelia hermsii* and African trypanosomes, where the clonally variant proteins are encoded by members of large gene families. Switching of expression between members of the corresponding gene families occurs through programmed DNA rearrangements (e.g. gene conversion and reciprocal recombination) moving a transcriptionally silent gene into an active expression site, which in the case of *Borrelia* and African trypano-

somes are telomerically located (reviewed in Borst and Rudenko, 1994; Donelson, 1995; Vanhamme and Pays, 1995).

Plasmodium falciparum, the etiological agent of the most virulent malarial species that infects humans, also uses antigenic variation to maintain a persistent infection (Miller *et al.*, 1994 and references therein). Recent studies have demonstrated that the parasite-encoded erythrocyte membrane protein PfEMP1 is clonally variant. This protein is localized on the surface of the host erythrocyte where it mediates cytoadhesion of the parasitized erythrocytes (P-RBCs) to either uninfected erythrocytes or the lining of the microvasculature to a wide range of cell surface receptors, including CD36, complement receptor 1 and heparan sulfate, amongst others (Baruch *et al.*, 1995; Rowe *et al.*, 1997; Chen *et al.*, 1998). While sequestration of infected erythrocytes within the microvasculature prevents their splenic clearance, the subsequent occlusion of these capillaries is thought to be a prerequisite for cerebral malaria, a lethal complication of *P.falciparum* infection (Miller *et al.*, 1994).

PfEMP1 is encoded by a large multigene family, termed *var*. (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). The majority of the molecule, encoded by exon I of *var* genes, seems to be exposed on the erythrocyte surface and has been implicated in host-cell receptor binding. Exon I codes for 2–5 Duffy-Binding-Like (DBL) domains and a cysteine-rich interdomain region interspersed between DBL1 and DBL2. Apart from short conserved sequence motifs in the first DBL domain (DBL-1), exon I displays considerable sequence diversity between different *var* genes (Su *et al.*, 1995). In comparison, exon II, a putative intracellular domain, is relatively well conserved between different *var* gene variants.

Each parasite contains ~50–150 *var* genes per haploid genome, which are distributed throughout all 14 chromosomes, and are located at chromosome-internal as well as within subtelomeric domains next to the non-coding repetitive element *rep20* (Su *et al.*, 1995; Rubio *et al.*, 1996; Fischer *et al.*, 1997; Hernandez-Rivas *et al.*, 1997; Thompson *et al.*, 1997). From studies correlating *var* gene expression with antigenic and adhesive properties of infected erythrocytes it appears that infected erythrocytes express only a limited number of, probably only one, *var* gene variant at any given time, with *var* gene switching occurring at a rate of ~2% per generation *in vitro* (Roberts *et al.*, 1992; Smith *et al.*, 1995; Rowe *et al.*, 1997; Chen *et al.*, 1998). Although the mechanistic details of *var* gene expression and switching still remain to be elucidated, initial data have suggested that this mechanism is fundamentally different from that of *vsg* gene switching in African trypanosomes. *var* gene expression apparently does not occur from a unique expression site, as expressed *var* genes were mapped to several different chromosomal

loci (Su *et al.*, 1995; Fischer *et al.*, 1997; Hernandez-Rivas *et al.*, 1997). However, the interpretation of these results has been complicated by the fact that they were derived from long-term *in vitro* cultured lines of *P.falciparum* which were heterogeneous with respect to their antigenic and adhesive properties.

Here we have employed the use of a receptor-binding panning assay in order to prepare parasite populations with defined adhesive phenotypes, as characterized by their ability to bind specifically to either CD36, intercellular adhesion molecule 1 (ICAM-1) or chondroitin-sulfate A (CSA). As each selected parasite population was found to express only a single and distinct *var* gene variant, this allowed us to investigate the molecular mechanism of *var* gene expression and switching in an isogenic background. The data presented support the model that *var* gene expression and switching occur *in situ* and are controlled at the transcriptional level through an as yet undefined restriction mechanism.

Results

Isolation of parasite populations with defined adhesive phenotypes

In order to investigate the molecular mechanisms responsible for antigenic variation in *P.falciparum*-infected erythrocytes, we first prepared parasite populations with defined adhesive phenotypes. Cloned Saimiri brain endothelial cell (SBEC) lines which express either CD36 (SBEC-C2), ICAM-1 (SBEC-3A) or CSA (SBEC-17) were used as adhesion receptors for *P.falciparum*-infected erythrocytes in a panning assay. A long-term *in vitro* culture of the *P.falciparum* clone FCR3, which expresses a heterogeneous set of *var* gene variants, was selected by four rounds of panning over each of the SBEC cell lines (Figure 1A). The panning selection procedure resulted in an ~100-fold enrichment for parasites binding to CSA, 30-fold for ICAM-1 and 20-fold for CD36, compared with the initial unselected parasite culture. The parasite populations obtained are termed here FCR3^{CD36}, FCR3^{ICAM-1} and FCR3^{CSA}.

The specificity of the adhesive phenotypes displayed by each of the selected parasite populations was verified as follows: FCR3^{CD36}, FCR3^{ICAM-1} and FCR3^{CSA} parasite populations were each panned over Chinese hamster ovary (CHO) cell lines or transfectants thereof expressing high levels of either human CD36 or ICAM-1 on their surface (Hasler *et al.*, 1993). CSA is naturally expressed on the surface of CHO cells and was removed, where necessary, by incubating CHO cells with chondroitinase ABC prior to the adhesion assay (Rogerson *et al.*, 1995). Each of the parasite populations was found to bind to the CHO cell line expressing the same adhesion receptor as did the corresponding SBEC clone used in the initial panning selection (Figure 1B). In cross-panning experiments, FCR3^{CSA} parasites did not adhere to CD36- or ICAM-1-expressing CHO cells. Correspondingly, nor did FCR3^{CD36} or FCR3^{ICAM-1} parasite populations bind to untransfected CHO cells. However, some cross-adhesive properties, although with reduced binding affinities, were observed for the FCR3^{CD36} and FCR3^{ICAM-1} parasite populations that adhered to ICAM-1- and CD36-expressing CHO cells, respectively (Figure 1B). This phenomenon has previously

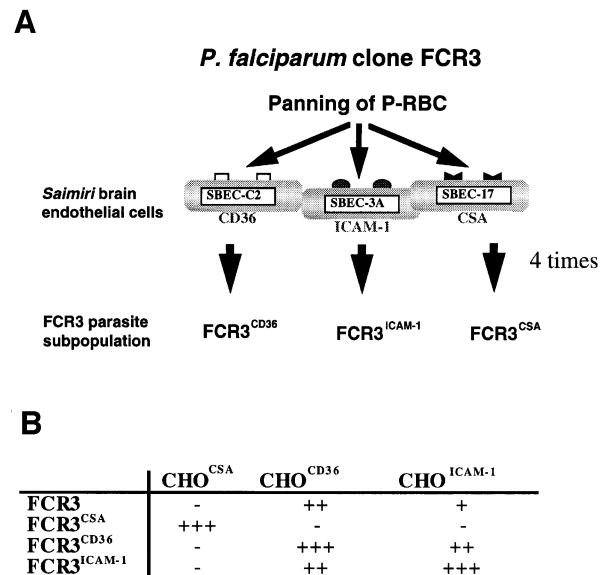


Fig. 1. Selection of parasite subpopulations with distinct adhesive binding properties. (A) Panning of FCR3 parasitized erythrocytes on SBEC expressing either CD36, ICAM-1 or CSA as a parasite adhesion receptor. For this study, we used the cloned parasite line FCR3 that initially had not been selected for any cytoadherence phenotype. After four rounds of panning, parasite binding reached saturation on the surface of the endothelial cell lines. (B) The receptor specificity of the SBEC-selected parasite subpopulations was confirmed using CHO cells expressing either CSA, human CD36 or ICAM-1 on its surface. In the case of CD36 and ICAM-1 transfected CHO cells, the CSA moiety was removed with Case ABC. It is noteworthy that the FCR3^{CSA} selected parasites do not adhere to CD36 or ICAM-1 and vice versa. Parasite cytoadherence is expressed as number of P-RBCs per CHO cell: (-) <0.1; (+) 0.1 to <1; (++) 1 to <5; and (+++) >5.

been observed and is thought to be the result of PfEMP-1 molecules with more than one binding domain (Baruch *et al.*, 1996; Gardner *et al.*, 1996).

Binding of the selected parasite population was sensitive to the addition of antibodies directed against the corresponding adhesion receptor. For example, binding of FCR3^{CD36} parasites to SBEC-C2 expressing CD36 was efficiently inhibited by the CD36-specific monoclonal antibody FA6-152 (70% at 5 µg/ml). Similarly, binding of FCR3^{CSA} parasites to SBEC-17 was abrogated upon the addition of either soluble CSA (>95% at 100 µg/ml) or by pre-treatment of the CSA-expressing cell line with chondroitinase ABC (>95% at 1 U/ml) (data not shown). Control experiments were performed to show that anti-CD36 FA6-152 antibody had no significant effect on the parasite binding to CSA (12% inhibition at 5 µg/ml). Likewise, soluble CSA or chondroitinase ABC did not significantly hinder parasite binding to CD36 (14% inhibition at 100 µg/ml CSA and 10% inhibition at 1 U/ml chondroitinase). These data demonstrate that parasite populations with defined adhesive phenotypes were obtained.

Each gene variant exhibits a defined adhesive phenotype

In order to determine the genetic basis for these adhesive phenotypes, total RNA from each selected parasite population was isolated (immediately after panning over their respective SBEC cell line) and subjected to reverse-

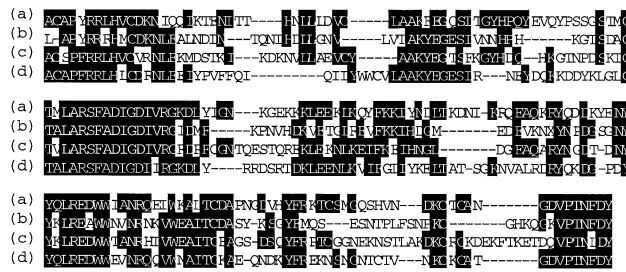


Fig. 2. Predicted amino acid sequence of the DBL-1 region of *var* genes transcribed in FCR3 and selected FCR3 parasites with distinct adhesive phenotypes. Alignment of amino acid sequence deduced from clones (a) *var*^{T3-1}, (b) *var*^{CSA}, (c) *var*^{CD36} and (d) *var*^{ICAM-1}. Shading (with solid black) residues that exactly match the consensus.

transcription-PCR (RT-PCR) amplification using the oligonucleotides *var*A5.2 and *var*B3.2 as primers. These oligonucleotides are degenerate and recognize conserved motifs within the DBL-1 domains of *var* genes. Variability within the DBL1 domains results in PCR products that are specific for the respective *var* gene variant(s) expressed (Smith *et al.*, 1995; Su *et al.*, 1995; Fischer *et al.*, 1997; Hernandez-Rivas *et al.*, 1997). The resulting PCR products, ~500 bp in size, were cloned and 10 clones from each parasite population sequenced. In the case of both FCR3^{CSA} and FCR3^{ICAM-1}, each of the 10 clones analysed contained an identical PCR product. A sequence analysis confirmed that the PCR products obtained were homologous to DBL-1 domains of *var* gene variants, which henceforth are called *var*^{CSA} and *var*^{ICAM-1}. The experiment was independently repeated for the FCR3^{CSA} parasite population, and a set of 40 PCR products analysed. All 40 PCR products were found to be identical, corresponding to the *var* gene variant *var*^{CSA} identified in the previous experiment. For FCR3^{CD36}, nine of the 10 PCR products analysed were identical and homologous to a DBL1 domain of a *var* gene variant henceforth termed *var*^{CD36}. A comparative sequence analysis of the deduced amino acid sequences of the DBL1 domains of *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1} revealed that they are distinct from one another, and also from the *var* gene variant *var*^{T3-1} predominantly expressed in the parental FCR3 population (Hernandez-Rivas *et al.*, 1997; see Figure 2). These data indicate that each parasite population expresses a single and distinct *var* gene variant, which apparently mediates the defined adhesive phenotype responsible for binding to either CSA, ICAM-1 or CD36.

To confirm the conclusion that each selected parasite population expresses a single and distinct *var* gene variant, cross-hybridization experiments were performed. Sequence tags specific for the DBL1 domains of the *var* gene variants *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1} were each hybridized against filters containing either size-fractionated total RNA isolated from FCR3, FCR3^{CSA}, FCR3^{CD36} and FCR3^{ICAM-1}, or the RT-PCR products generated from each of them using the degenerated primers to the DBL-1 domain of *var* genes. It was found that each *var* gene tag hybridized only to the RNA or RT-PCR products of the parasite population from which it was derived, and no other (Figure 3A and B). The Northern analysis revealed that the *var*^{CD36} and *var*^{ICAM-1} transcripts are between 7 and 9 kb in size (Su *et al.*, 1995), typical for *var* genes, whereas the *var*^{CSA} transcript has an unprecedented small

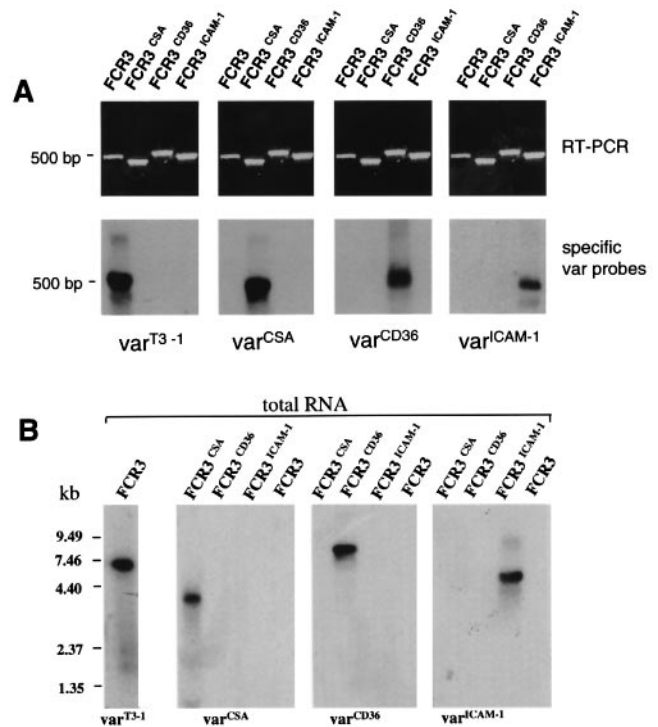


Fig. 3. Transcriptional analysis of *var* genes from FCR3 parasites (trophozoites) with distinct adhesive properties. Total parasite RNA was prepared from the initial unselected FCR3 parasite culture and cytoadherent trophozoites detached directly from endothelial cells (SBEC-C2, -3A and -17). (A) Ethidium-bromide-stained RT-PCR products obtained with the DBL-1 specific primers *var*A5.2 and *var*E3.2 after separation by agarose gel electrophoresis. Signal of the RT-PCR products after probing with *var*^{T3-1}, *var*^{CSA}, *var*^{CD36} or *var*^{ICAM-1} probes and washes under stringent conditions (0.1×SSC, 0.1% SDS, 65°C). (B) The Northern blot of fractionated total RNA of FCR3 parasites. The same blot was hybridized successively with DNA probes *var*^{T3-1}, *var*^{CSA}, *var*^{CD36} or *var*^{ICAM-1}. A unique transcript was detected exclusively in RNA isolated from the homologous parasite.

size of ~4.4 kb, suggesting a *var* gene structure composed solely of one or perhaps two DBL domains. These data support our hypothesis that upon selection for defined adhesive properties, parasite populations are obtained that express a unique and distinct *var* gene variant.

In situ activation of var gene expression

Previous data, derived from parasite populations with heterogeneous antigenic and adhesive phenotypes, have suggested that *var* gene expression, unlike *vsg* gene expression in African trypanosomes, is not linked to a conserved expression site, but rather occurs *in situ*, irrespective of the chromosomal location (Fischer *et al.*, 1997). To verify this hypothesis, we have mapped the chromosomal location of the *var* gene variants identified within the selected parasite populations in which the *var* gene variants *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1} are either transcriptionally active or silent. Agarose blocks containing intact chromosomes from FCR3, FCR3^{CD36}, FCR3^{ICAM-1} and FCR3^{CSA} were prepared, size-fractionated and examined by Southern analysis using specific probes to *var*^{T3-1}, *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1}. The chromosomal location of each *var* gene variant investigated (mapping to chromosomes 3, 10, 13 and 12, respectively) remains unchanged irrespective of its transcriptional status (Figure

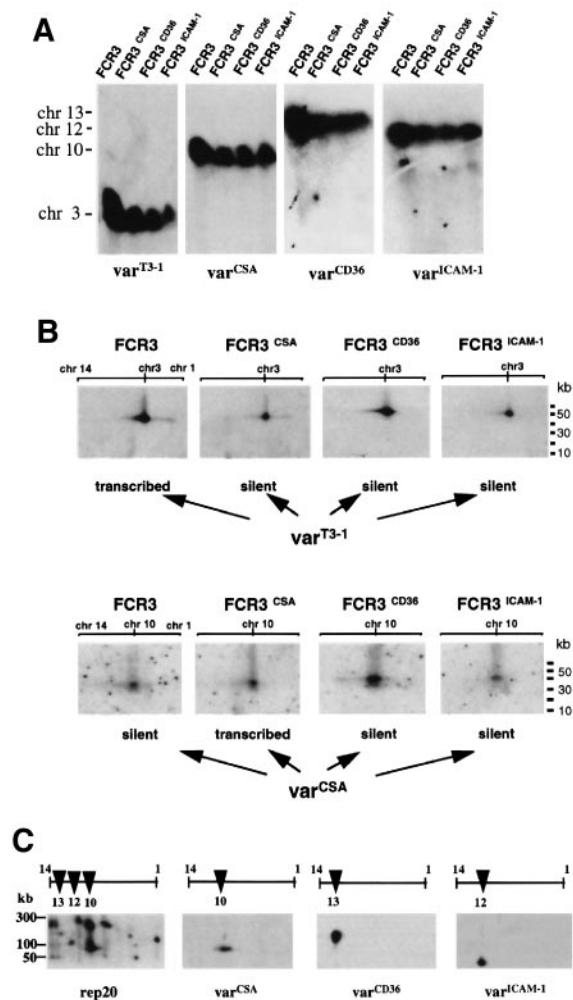


Fig. 4. Genetic analysis of *var* gene expression site switches. Chromosomal location of four *var* genes that are either transcribed or silent. (A) Chromosomes of FCR3, FCR3^{CSA}, FCR3^{CD36} and FCR3^{ICAM-1} parasites were fractionated by PFGE, blotted onto a nylon membrane and hybridized successively with *var*^{T3-1}, *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1}. (B) Fractionation of *Pst*I-digested chromosomes by 2D-PFGE and hybridization of the blotted fragments to *var*^{T3-1} and *var*^{CSA} probes. The membranes were washed under stringent conditions (0.1 × SSC, 0.1% SDS, 65°C). (C) 2-D PFGE of *Apa*I-digested FCR3 chromosomes blotted onto a nylon membrane and hybridized successively to different transcribed *var* genes and the subtelomeric specific probe *rep20*. *var*^{CSA} and *var*^{CD36} expression sites are located in subtelomeric regions next to *rep20* of chromosomes 10 and 13 and *var*^{ICAM-1} is transcribed from a central chromosome region.

4A and B). Fine-scale restriction mapping, using the endonucleases *Apa*I, *Bss*HII, *Sma*I, *Rsa*I, *Dra*I and *Sau*3A, confirmed this finding (data not shown). *var*^{CSA} and *var*^{CD36} consistently map to subtelomeric domains of the chromosomes 10 and 13, respectively, in close proximity to the *rep20* sequence element, whereas *var*^{ICAM-1} always lies within a central domain of chromosome 12 (Figure 4C). Furthermore, no significant DNA rearrangements were observed in the immediate vicinity of the *var* gene variants investigated upon transcriptional activation or silencing. Thus, switching between *var* gene variants appears to occur *in situ*, independent of the chromosomal location, and apparently does not require the transposition of a *var* gene to a specific, possibly telomerically located, expression site.

The modification of nucleotides can play a significant role in the control of gene expression, as exemplified by 5' methylcytosines in higher eukaryotes and β-D-glucosyl-hydroxymethyluracil, base J, in African trypanosomes (Ceder, 1988; Gommers-Ampt *et al.*, 1993). Given that there is evidence for DNA methylation in *Pfalciparum* (Pollack *et al.*, 1991), we explored the possibility that CpG methylation might play a role in differential *var* gene expression. DNA prepared from trophozoites of FCR3, FCR3^{CSA}, FCR3^{CD36} and FCR3^{ICAM-1} was restricted using the methylation-sensitive endonucleases *Hpa*II, *Hha*I, *Rsa*I and *Sau*3A, size-fractionated and hybridized with probes specific for the *var* gene variants *var*^{T3-1}, *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1} (data not shown). The hybridization patterns observed are identical, irrespective of the transcriptional status of the *var* gene variant in each selected parasite population, which would suggest that changes in the methylation pattern play no role in *var* gene switching.

Allelic exclusion of *var* gene expression is transcriptionally regulated

That *var* genes are developmentally expressed has been inferred from studies using heterogeneous parasite populations, in which *var* gene transcripts have been detected only during the early stages of intra-erythrocytic parasite development (Fischer *et al.*, 1997). To define better the onset of *var* gene expression, a FCR3^{CSA} parasite population was selected for CSA binding and the resulting parasites (early schizonts) allowed to reinvade. Total RNA was then isolated 10 h (rings) and 24 h (trophozoites) post-invasion, and the *var* gene expression pattern analysed by RT-PCR using the degenerate oligonucleotide primers to the DBL-1 domain of *var* genes (Figure 5A). A unique PCR product was obtained from the trophozoite stage RNA, which corresponds to *var*^{CSA}, as demonstrated by analysis of 40 independent clones (Figure 5A and B). In comparison, an ill-defined PCR product was obtained from the ring-stage RNA, which, surprisingly, corresponds to a large set of at least 14 different *var* gene variants, as shown by the sequencing of 30 independent clones (Figure 5B). No PCR products were obtained in the absence of reverse transcriptase, indicating that genomic DNA did not contaminate the RNA preparations. The apparent ubiquitous transcription of *var* gene variants in rings, as compared with trophozoites, was confirmed by hybridization analyses in which the respective RT-PCR products were hybridized against filters containing specific sequence tags for 19 different *var* gene variants present in the FCR3 genome (Figure 5C). Sequence tags for *var* gene variants were generated by PCR amplification of genomic DNA using the degenerate oligonucleotides *var*A5.2 and *var*B3.2 as primers. Whereas the probe derived from trophozoite RNA identified the expected single fragment corresponding to *var*^{CSA}, the probe derived from ring RNA hybridized to all sequence tags under stringent hybridization conditions, indicative of the expression of several members of the *var* gene family in ring-stage parasites. Together, these findings suggest that allelic exclusion of *var* gene expression is regulated during parasite development, i.e. transcription of most, if not all, *var* gene variants in early ring stages is restricted to a

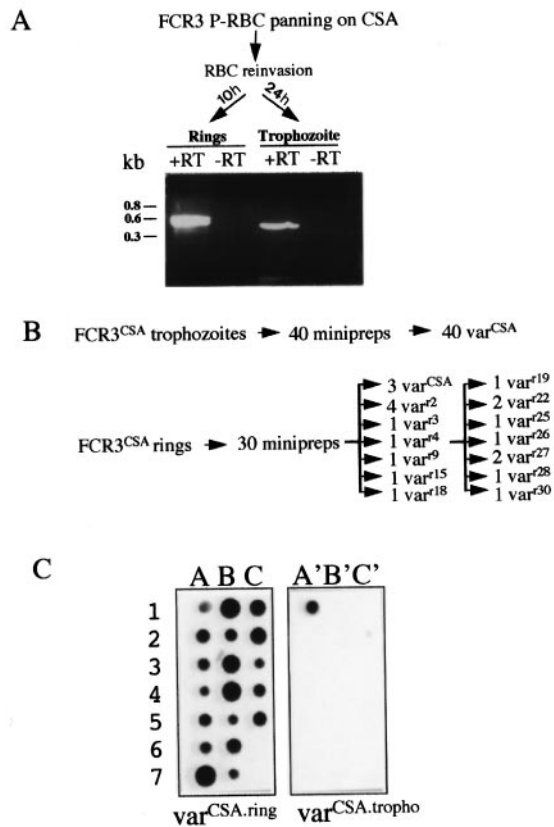


Fig. 5. Transcriptional activity of *var* genes in ring and trophozoite stages. (A) FCR3 trophozoites panned on the endothelial receptor CSA were allowed to reinvade erythrocytes and 10 h (ring stage) or 24 h (trophozoite stage) later total RNA was isolated. RT-PCR products using DBL-1-specific primers A5.2/E3.2 were separated on 1% agarose gel. The omission of reverse transcriptase did not yield any detectable PCR band and treatment with RNase prior to the RT-PCR resulted in no DNA amplification. The RT-PCR products were cloned into the cloning vector pCR2.1. (B) DNA minipreps of randomly chosen clones, 30 from ring and 40 from trophozoite stage RNA, were hybridized to the *var*^{CSA} probe and washed stringently (0.1 × SSC, 0.1% SDS, 65°C). The 40 inserts derived from trophozoite RT-PCR were all positive, and DNA sequence analysis of three of them showed 100% sequence identity to DBL-1 of *var*^{CSA}. Only three of the 30 ring-stage clones hybridized to the *var*^{CSA} probe. Sequencing of the inserts showed that 14 recombinant plasmids contain distinct *var* genes. (C) Dot blot analysis of 19 distinct FCR3-derived DBL-1 *var* gene inserts. Identical blots were probed either with ring or trophozoite RT-PCR *var* DBL-1 products [as shown in (A)] and washed stringently. A1 *var*^{CSA}, A2 *var*^{ICAM-1}, A3 *var*^{CD36}, A4 *var*^{T11-1}, A5 *var*^{T3-1}, A6 *var*^{r30}, A7 *var*^{r9}, B1 *var*^{r18}, B2 *var*^{r2}, B3 *var*^{r3}, B4 *var*^{r4}, B5 *var*^{r15}, B6 *var*^{r18}, B7 *var*^{r19}, C1 *var*^{r22}, C2 *var*^{r25}, C3 *var*^{r26}, C4 *var*^{r27}, C5 *var*^{r28}, C6 pUC18.

single *var* gene variant as the parasite matures into a trophozoite.

The distinct expression of a *var* gene variant in a subsequent non-selected infective cycle would suggest some form of imprinting. To exclude the possibility that our panning procedure causes an aberrant arrest in *var* gene switching, thereby fixing the *var* gene variant expressed, we tested the ability of a selected parasite population to switch to other *var* gene variants. A freshly selected FCR3^{CD36} parasite population was panned over CHO cells expressing CSA, and the *var* gene variant expressed in the resulting parasite population determined. It was found that the resulting parasite population adheres to CSA and expresses the *var* gene variant *var*^{CSA}.

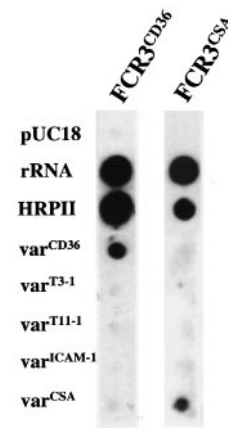


Fig. 6. The transcriptional activity of five distinct *var* genes was examined in parasites with distinct adhesive phenotypes by nuclear run-on analysis. Nascent RNA was prepared from FCR3^{CSA} and FCR3^{CD36} trophozoites and hybridized to the dot blot membrane. The transcriptional activity of the *ribosomal RNA* (*rRNA*) genes and the *histidine rich protein II* gene (*HRPII*) were analysed as a positive control. pUC18 DNA plasmid served as a negative control.

This finding indicates that the original FCR3^{CD36} parasite population had maintained its ability to switch *var* gene expression.

Expression of *var* genes during parasite maturation, presumably resulting from some form of restriction in the expression of other members of the *var* gene repertoire, could be regulated either at the transcriptional or the post-transcriptional level. To address this question, a nuclear run-on analysis was carried out using nuclei prepared from trophozoites of both FCR3^{CSA} and FCR3^{CD36} parasite populations. Radiolabelled nascent RNA, isolated from $\sim 3 \times 10^9$ infected erythrocytes, was hybridized to a filter containing DNA specific for the five *var* gene variants *var*^{T11-1}, *var*^{T3-1}, *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1}, the gene *hrp2*, an *rRNA* subunit gene and the unrelated pUC18 plasmid. Whereas nascent RNA isolated from both FCR3^{CSA} and FCR3^{CD36} trophozoites hybridizes to both *hrp2* and *rRNA*, but not pUC18, the RNA derived from FCR3^{CSA} hybridizes exclusively to the *var*^{CSA} gene variant, and vice versa the FCR3^{CD36} RNA to *var*^{CD36} (Figure 6). This finding clearly demonstrates that *var* gene expression is regulated at the level of transcription.

Discussion

Our data set the framework for a better understanding of the mechanism(s) underpinning antigenic variation in *P.falciparum*. *Var* genes play an important role in the pathogenicity of *P.falciparum*, encoding immunovariant adhesins (PfEMP1) that mediate binding of the host erythrocyte to the endothelial lining of the microvasculature and uninfected erythrocytes. A wide range of different host cell surface molecules, including CSA, ICAM-1 and CD36, serve as receptors for PfEMP1 binding and differences in parasite adhesion properties appear to influence disease outcome (Carlson *et al.*, 1990; Turner *et al.*, 1994; Fried and Duffy, 1996). Studies correlating *var* gene expression patterns with serological and adhesive phenotypes of the infected erythrocyte have suggested that *var* genes are differentially expressed in *P.falciparum*, i.e. a single cell expresses only a limited number of,

possibly only one, *var* gene variant from its repertoire at any given time (Rowe *et al.*, 1997; Chen *et al.*, 1998). Verification of this model, however, has been difficult to obtain due to the heterogeneous nature of parasite populations cultured *in vitro*, which, in the absence of a selective pressure, express many different *var* gene variants at any given time. By employing a receptor panning assay, based on CHO or SBEC cells expressing different receptors for PfEMP1 binding on their surface, we were able to prepare large parasite populations with homogeneous and defined adhesive phenotypes. Genetic analysis of the *var* gene repertoire expressed in each selected parasite population shows that only a single *var* gene variant is expressed, with the exception of those parasites selected for CD36 binding where a second expressed *var* gene variant was detected, albeit at a very low level. No other expressed *var* gene variants were detected in the selected parasite populations in spite of an intensive search employing both RT-PCR and Northern analyses. This finding clearly demonstrates that each individual parasite encodes only a single *var* gene variant.

A comparative sequence analysis revealed that the *var* gene variants expressed in the different FCR3 parasite populations are distinct from one another, an observation confirmed by their different chromosomal locations. Interestingly, independent panning experiments consistently identified that the same *var* gene variant is responsible for a particular adhesive phenotype, irrespective of the adhesive properties of the starting parasite population. For example, the FCR3^{CD36} parasite population selected for CSA binding would always switch back to the same *var*^{CSA} upon selection on CHO cells (data not shown). This finding poses the question of whether each *var* gene variant mediates a defined adhesive phenotype. Although only 13 targets for PfEMP1 binding have thus far been identified (namely, the endothelial receptors thrombospondin (Roberts *et al.*, 1985), CD36 (Ockenhouse *et al.*, 1989), ICAM-1 (Berendt *et al.*, 1989), E-selectin (Ockenhouse *et al.*, 1992), VCAM-1 (Ockenhouse *et al.*, 1992), CSA (Robert *et al.*, 1995; Rogerson *et al.*, 1995), PECAM-1 (Treutiger *et al.*, 1997), the erythrocyte receptors heparan sulfate (Carlson *et al.*, 1992; Chen *et al.*, 1998), bloodgroup sugars A and B (Carlson and Wahlgren, 1992), complement receptor 1 (Rowe *et al.*, 1997), and the serum proteins IgG/IgM and fibrinogen (Scholander *et al.*, 1996), this number is steadily increasing as we investigate this pathogenic adhesive phenomenon in more detail. The parasite clone FCR3 is able to bind to most if not all of these host molecules, and, significantly, expresses a different *var* gene variant to mediate each adhesive phenotype upon selection (Treutiger *et al.*, 1997; Chen *et al.*, 1998; Fernandez *et al.*, 1998; this study).

Although it is possible that each *var* gene variant mediates a defined adhesive phenotype, other interpretations of the data need to be considered. The panning procedure employed in this study may consistently select for that *var* gene variant with the highest affinity for the corresponding receptor. Accordingly, there may be other *var* gene variants in the FCR3 parasite genome that mediate binding to CD36, ICAM-1 or CSA, although with a lower affinity. The ability to bind CD36 has been suggested to be an intrinsic adhesive property of infected erythrocytes, as inferred from binding studies using clinical

isolates (Ockenhouse *et al.*, 1991; Newbold *et al.*, 1997). This property has been attributed to a conserved binding domain of PfEMP1, such as the CIDR domain (Baruch *et al.*, 1995, 1997). That parasite populations selected for CSA binding did not bind CD36, unlike the FCR3^{ICAM-1} parasite subpopulation, may be attributed to the unusual composition of the PfEMP1 variant encoded by *var*^{CSA}. Preliminary results show that it contains only two DBL domains and a region with little homology to the CIDR domain implicated in CD36 binding (unpublished data).

The ability of infected erythrocytes to bind to CSA appears to be an important pathogenic factor in maternal malaria, as previously suggested (Fried and Duffy, 1996). Interestingly, CSA binding is not a general property of all *P.falciparum* strains. A screening of several geographically distinct *P.falciparum* isolates revealed that only some are capable of binding CSA, despite several rounds of selection (data not shown). Moreover, non-CSA binding parasites do not hybridize with the *var*^{CSA} specific probe, whereas parasites capable of binding CSA do, as demonstrated by Southern analysis (data not shown). This finding supports our model that individual *var* genes mediate defined adhesive phenotypes, which, when displayed, may affect the outcome of the disease.

Since all of the three parasite populations are derived from the same parental clone and are interconvertible in terms of their *var* gene variant expressed upon appropriate selection, the opportunity arises of investigating *var* gene switching within isogenic parasites. Mapping the location of expressed and silent *var* gene variants within these isogenic parasite populations clearly indicates that *var* gene expression is not linked to a specific expression site, but rather occurs *in situ*, irrespective of a particular chromosomal location. While the *var*^{CSA} and *var*^{CD36} gene variants are expressed from subtelomeric locations within chromosomes 10 and 3, respectively, *var*^{ICAM-1} is expressed from a central location within chromosome 12. *In situ* activation of *var* genes is supported by previous data which show expression of *var* genes from both subtelomeric and chromosome internal domains of numerous chromosomes (Su *et al.*, 1995; Fischer *et al.*, 1997; Hernandez-Rivas *et al.*, 1997). Thus, the mechanism of *var* gene switching in *P.falciparum* is fundamentally different from switching immunovariant genes in other pathogens (Borst and Greaves, 1987; Deitsch *et al.*, 1997). For example, in African trypanosomes, the active *vsg* gene is transcribed from a specific telomeric expression site, with antigenic variation being habitually mediated through replacing the active *vsg* gene variant with one of the 1000 silent copies present elsewhere in the genome. However, in this parasite antigenic variation can also occur by switching expression between different *vsg* genes, suggesting that some form of allelic exclusion is operating in African trypanosomes as well.

While *var* gene expression is tightly regulated in trophozoites, ubiquitous transcription of all *var* gene variants appears to occur in early ring stages, with no predominance of any one *var* gene variant. It has been speculated that the *var* genes could be controlled at the level of RNA processing by partial degradation of all mRNAs but one (Borst *et al.*, 1995). Although differential RNA degradation would be a plausible interpretation of these data, additional experimental evidence points to a different switching

mechanism. First, relaxed gene transcription in ring stages is not limited solely to *var* genes, rather there appears to be a general pattern of relaxed transcription of many, if not all, *Pfalciparum* genes at this point in the parasite's life-cycle. Specific RT-PCR reactions identified transcription of both gametocyte and sporozoite specific genes in early rings, including the *Pf11-1* and *circumsporozoite* (CS) genes, although at a very low level (data not shown). Secondly, the phenomenon of *in situ var* gene switching is apparently mediated at the level of transcriptional initiation, as demonstrated by nuclear run-on analyses. Radiolabelled nascent RNA isolated from trophozoite parasite populations selected for CSA binding hybridized only to a probe corresponding to the *var*^{CSA} gene variant. Similarly, radiolabelled nascent RNA isolated from trophozoites selected over CD36 identified only the *var*^{CD36} gene variant, and no other. Taken together, these data indicate that each *var* gene is an independent transcription unit in which promoter activity determines the expression status. Activation and silencing of *var* gene promoter activity seems not to involve DNA rearrangements, nor changes in the DNA methylation pattern, as demonstrated by extensive restriction analyses.

Given that maturation of the parasite results in the mutually exclusive expression of a single *var* gene variant, this would indicate that some mechanism is responsible for silencing the activity of all but one *var* gene promoter. It is tempting to speculate that the control of *var* gene switching is mediated through this silencing mechanism, focusing towards a *var* gene variant different from that expressed in the previous cycle, albeit at a low rate. How this effect is brought about remains to be determined. Drawing together our results, we hypothesize that an epigenetic mechanism(s) is involved in *var* gene regulation. It has been demonstrated that transcriptional silencing can be overcome by changes in chromatin states through reversible histone acetylation (Wolffe, 1996). Recently, histone acetylation/deacetylation was described in *Pfalciparum* blood stage parasites (Darkin-Rattray *et al.*, 1996). Thus, chromatin structure analysis might give us a deeper understanding of the factors that regulate the *in situ* activation of the multigene *var* family.

Materials and methods

Parasites and cell lines

Pfalciparum FCR3 parasites were cultivated according to Trager and Jensen (1976). Stable transformants of CHO cells expressing CD36 or ICAM-1 have been described elsewhere (Hasler *et al.*, 1993). Recently, several clones of Saimiri brain microvascular endothelial cells (SBEC) were isolated that express either CD36 (SBEC-C2), ICAM-1 (SBEC-3A) or CSA (SBEC-17) as parasite adhesion receptor (Gay *et al.*, 1995; Robert *et al.*, 1995). Cell culture and P-RBC cytoadherence assays of SBEC were performed as described previously (Gay *et al.*, 1995; Robert *et al.*, 1995). CSA inhibition assays and the removal of chondroitin-sulfate A from SBEC or CHO cells was performed as described (Robert *et al.*, 1995; Rogerson *et al.*, 1995). Inhibition studies of cytoadherence of P-RBCs using anti-CD36 mAb FA6-152 (obtained from Dr L.Edelman, Institut Pasteur, Paris, France) and anti-ICAM-1 mAb 84H10 (Immunotech) were carried out as described previously (Gysin *et al.*, 1997; Pouvelle *et al.*, 1997).

Panning of parasites on endothelial cells

The initial FCR3 parasites showed low binding or no detectable binding to the SBEC cells used in this study. The panning assay was performed

essentially as described previously (Pouvelle *et al.*, 1997). For the isolation of large amounts of adherent parasites, 150 cm² culture flasks containing semiconfluent SBEC were used. Bound parasites were detached using an automatic pipette aid and a 5 ml pipette. Free P-RBCs were centrifuged and used to prepare DNA and total RNA. In some instances, the parasites were allowed to reinvade before the DNA and RNA isolation.

Nuclear run-on analysis

Nuclear run-on analysis on *Pfalciparum* asexual bloodstage parasites was performed as described previously (Lanzer *et al.*, 1994); 0.5 pmol of *Pfalciparum* gene fragments cloned into pUC18 were denatured and transferred onto a nylon membrane (Hybond N+, Amersham) using a dot blot apparatus as described previously (Ausubel *et al.*, 1998). The following *Pfalciparum* genes were used: rRNA (Langsley *et al.*, 1983), HRPII (Wellems and Howard, 1986), FCR3 DBL-1 PCR fragments of *var*^{T3-1} and *var*^{T11-1} (Hernandez-Rivas *et al.*, 1997). *var*^{CSA}, *var*^{CD36} and *var*^{ICAM-1} are described in this work.

Pulsed-field gel-electrophoresis

Pulsed-field gel-electrophoresis (PFGE) and two-dimensional (2-D) PFGE of *Pfalciparum* chromosomes was performed in contour-clamped homogeneous electric field apparatus as described (Hernandez-Rivas and Scherf, 1997).

RT-PCR and Northern analysis

Total RNA was prepared using the Qiagen RNA isolation kit according to the recommendations of the manufacturer. The RNA was treated with DNase free of RNase (Pharmacia) and repurified on the Qiagen RNA columns. Ten micrograms of total RNA was fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N+) and hybridized with random-primer-labelled probes (Hernandez-Rivas *et al.*, 1997). For first-strand cDNA synthesis, 1 µg of total RNA treated with RNase-free DNase was obtained by annealing RNA with oligo (dT)-primers and extending the strand with reverse transcriptase as recommended by the manufacturer (Perkin-Elmer-Cetus). *var*A5.2 and *var*E3.2 were used to amplify DBL domain 1 (Hernandez-Rivas *et al.*, 1997). RT-PCR without the addition of the reverse transcriptase and RNA treated with RNase were used as controls. RT-PCR products were extracted from agarose gels using the Qiagen PCR purification kit.

Southern blot and hybridization

Digested DNA was separated on agarose gels and transferred to Hybond N+ (Amersham) membrane as recommended by the manufacturer. DNA probes were hybridized in 7% SDS, 1% bovine serum albumin (BSA) and 0.5 M NaPO₄ pH 7.4, and washed under stringent wash conditions: 0.1× SSC, 0.1% SDS at 60°C. Dot blot hybridization of various *var* DBL-1 DNA inserts was performed as described (Ausubel *et al.*, 1998) using a dot blot apparatus (Schleicher and Schüll) and 20 ng of DNA per dot on a Hybond N+ (Amersham) membrane.

Accession number information

These sequence data have been submitted to the DDBJ/EMBL/GenBank database under the accession Nos AJ007940, AJ007941 and AF007942.

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