Molecular Cell, Vol. 3, 131–141, February, 1999, Copyright ©1999 by Cell Press

Antigenic Variation in Malaria: a 3′ Genomic Alteration Associated with the Expression of a *P. knowlesi* Variant Antigen

Basima Al-Khedery,† John W. Barnwell,‡ and Mary R. Galinski*†
Department of Medical and Molecular Parasitology
New York University School of Medicine
New York, New York 10010

Summary

Antigenic variation of malaria parasites was discovered in *P. knowlesi*, using a schizont-infected cell agglutination (SICA) assay to detect variant antigens expressed at the surface of infected erythrocytes. Later studies utilizing stable clones, Pk1(A+) and its direct derivative, Pk1(B+)1+, showed that SICA[+] clones express distinct parasite-encoded antigens of ~200 kDa. Here we identify a *P. knowlesi* variant antigen gene and cDNA and demonstrate that it encodes the 205 kDa variant antigen expressed by B+ parasites. This gene belongs to a multigene family, which we term *SICAvar.* Its ten-exon structure with seven cysteine-rich coding modules is unique compared to *P. falciparum var* genes. Further, we highlight a 3′ genomic alteration that we predict is related to *SICAvar* gene switching.

Introduction

Antigenic variation of *Plasmodium* contributes to the chronicity of malaria infections (Brown and Brown, 1965; Brown and Hills, 1974; Marsh et al., 1989; Bull et al., 1998). This phenomenon was first documented in *Plasmodium knowlesi* infections in rhesus monkeys (Brown and Brown, 1965) where the SICA (schizont-infected cell agglutination) assay demonstrated that parasite populations of recrudescing parasitemic waves were characterized by variant phenotypes. The infected erythrocyte membrane components responsible for the agglutination were later identified as the SICA antigens (Howard et al., 1983). Antigenic variation has since been noted in the simian malaria, *P. fragile* (Handunnetti et al., 1987), the rodent malaria, *P. chabaudi* (McLean et al., 1982), and the human malarias, *P. falciparum* (Hommel et al., 1983; Biggs et al., 1992; Roberts et al., 1992) and *P. vivax* (Mendis et al., 1988).

Cloned *P. knowlesi* populations expressing distinct variant antigens (SICA[+]), or not expressing detectable variant antigens (SICA[−]), enabled the demonstration of general features of *Plasmodium* antigenic variation and properties of the SICA antigens (Barnwell et al., 1982). They are synthesized by and transported from the intraerythrocytic parasite, inserted into the erythrocyte membrane, and exposed to the exterior (Howard and Barnwell, 1984a). They are a minor component of the infected red blood cell (IRBC) membrane, detected only by immunoprecipitation of [^{35}S]-methionine metabolic or [^{125}I]–surface labeled antigens (Howard et al., 1983). Further, they are efficiently extracted only in ionic denaturing detergents (Howard and Barnwell 1984b), suggesting an association with the cytoskeleton. This knowledge led to the identification of the *P. falciparum* analogs called erythrocyte membrane protein 1 (PfEMP1) antigens (Leech et al., 1984; Howard et al., 1988). Related molecules have yet to be characterized in other species of *Plasmodium*.

The SICA proteins range from 180 to 225 kDa, and two protein bands are associated with each SICA[+] clone (Howard et al., 1983; our unpublished data). The Pk1(B+)1+ clone (B+) was derived directly in vivo from the Pk1(A+)1+ clone (A+), and the variant antigen phenotypes of both remain stable when passaged in naïve monkeys (Barnwell et al., 1983; Figure 1). The SICA proteins from the A+ and B+ clones are 210/190 kDa and 205/200 kDa, respectively. Antisera against the native proteins only immunoprecipitate the respective antigens and agglutinate erythrocytes infected with parasites with the respective phenotypes.

In vivo experiments suggested that malaria antigenic variation can be induced by variant-specific antibodies and can result from immune selection (Brown, 1973; Brown and Hills, 1974; Barnwell et al., 1983). This contrasts with antigenic variation in other organisms such as *Trypanosoma brucei* or *Borrelia hermsii* where antigenic variation is strictly attributed to antibody selection of minor populations that have switched their variant phenotype (reviewed in Donelson, 1995). In vivo switching in *P. knowlesi* also appears to require the spleen, as new variants do not arise in splenectomized (spx) monkeys, despite the presence of variant-specific antibodies. In fact, upon passage in spx monkeys, the SICA[−] phenotype results, where SICA antigens are not expressed. These data suggest that the spleen has a role in SICA antigen expression as well as switching (Barnwell et al., 1983; Howard 1984). Splenic influence has also been noted in *P. fragile* (Handunnetti et al., 1987) and *P. falciparum* (David et al., 1983; Hommel et al., 1983). Furthermore, SICA[−] parasites revert back to SICA[+] phenotypes in intact monkeys (Barnwell et al., 1983). The rate of parasitemic increase can be identical in nonimmune and immune intact and spx animals, supporting the notion that SICA phenotype changes are not primarily due to selection of minor variants (Brown, 1973; Barnwell et al., 1983; Howard, 1984). As elaborated by Brown and Hills (1974), qualitatively different variant antigen antibody responses occur in *P. knowlesi* infections, some that cause agglutination and induce switching of variant antigen phenotype, and others that have an opsonizing effect with destruction by macrophages. Early in an infection, agglutinating antibodies tend to predominate. The subsequent rise in opsonizing antibodies, with continued production of agglutinating antibodies, affects the level of parasitemia in chronic infections.

*To whom correspondence should be addressed (e-mail: Mary.Galinski@malaria.org).
† Present address: Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, Emory Vaccine Center, Atlanta, Georgia 30329.
‡ Present address: Centers for Disease Control and Prevention, Mailstop F13, 4770 Buford Hwy NE, Atlanta, Georgia 30341.

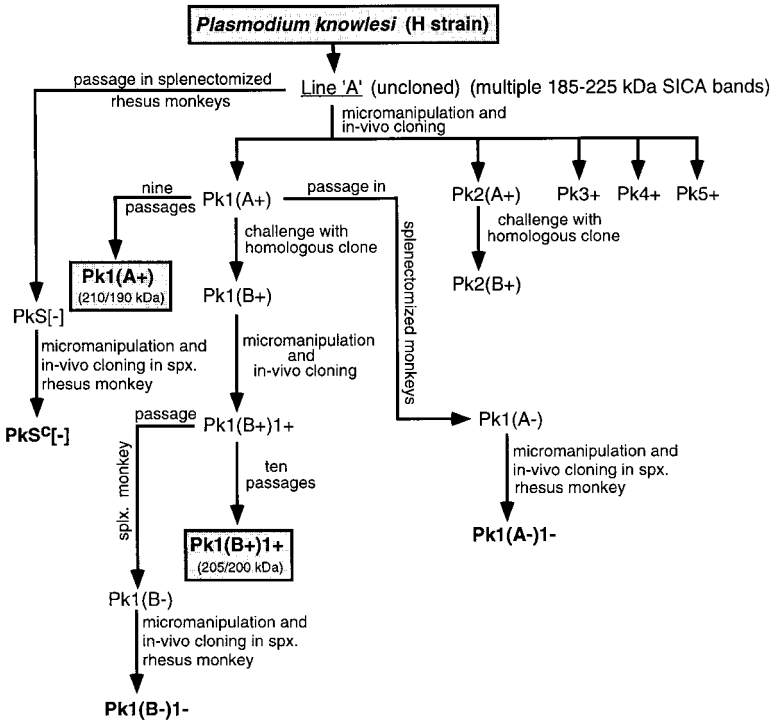

Figure 1. *P. knowlesi* Clone Derivations

Clones were derived by micromanipulation of single SIE from an uncloned line A and subsequent infection of rhesus monkeys as described by Barnwell et al. (1982, 1983). Pertinent to this report, the B+ clone was derived by reinfecting a monkey that had been infected with the A+ clone, with A+ parasites. A single SIE was then picked from the resulting population and used to infect a naïve monkey, giving rise to the B+ clone. As indicated (boxed and shaded areas), these clones continue to express the same protein pairs after many passages in monkeys. However, when these or line A were passaged in spx monkeys, expression of the SICA antigens was lost, resulting in the SICA[−] clones: Pk1(A−)1−, Pk1(B−)1−, and PkSc[−].

Importantly, SICA[−] parasites are less virulent (Barnwell et al., 1983). Thus, paradoxically, SICA antigens aid the development of chronicity, yet also increase virulence. This is so despite the fact that *P. knowlesi* IRBCs have marginal adhesive properties quite different from the virulent human malaria, *P. falciparum*. Maturing *P. falciparum* IRBCs are not found in the circulation, as they become sequestered in various tissues and organs, avoiding hepatic and splenic sites where their destruction could occur (Howard and Barnwell, 1984a). PfEMP1 mediates this by adhering to receptors on vascular endothelium (reviewed in Borst et al., 1995). In contrast, *P. knowlesi* IRBCs do not or only partially recede from the circulation, in which case the IRBCs are mostly found in the sinusoids of the liver and spleen (Miller et al., 1971). These fundamental differences suggest that malarial variant proteins mediate virulence by more than one means, not only via vascular cell adhesion.

The *P. falciparum* genes encoding EMP1 were cloned recently and were shown to belong to a large diverse multigene family called *var* genes (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). Still, today the mechanism(s) that governs *var* gene expression remains elusive. The lack of an in vivo system for *P. falciparum* antigenic variation, use of in vitro systems where clones may undergo rapid switching (Roberts et al., 1992), and the potential for spontaneous deletions in vitro (e.g., Corcoran et al., 1986; Pologe and Ravetch, 1986) that are not necessarily observed in vivo (Scherf and Mattei, 1992) may make analyses of the mechanism(s) particularly challenging in this species.

P. knowlesi offers the advantage of established in vivo and in vitro model systems where variant antigen switching mechanisms can be rigorously evaluated, importantly, in the context of the host environment. Here, we present the *P. knowlesi SICAvar* multigene family, which provides access to the genetic and immunobiological tools needed to conduct such investigations as well as a comparative model for the *P. falciparum var* gene family. We also demonstrate a 3′ difference in the locus of the expressed versus silent *P. knowlesi* 205 kDa *SICAvar* gene and speculate that this alteration is related to the gene switching process.

Results

Identification of the *SICAvar* Multigene Family and Transcripts

Rabbit antisera, 187αPkA and 201αPkB, raised against A+ and B+ schizont-infected erythrocyte (SIE) membranes immunoprecipitate the appropriate variant antigens from SDS extracts: 210/190 kDa proteins from A+ extracts (Figure 2A, lanes U and A) and 205/200 kDa proteins from B+ extracts (Figures 2B and 2C, lanes U and A). They do not, however, recognize the antigens from reduced and alkylated (R/A) extracts (Figures 2A and 2B). Conversely, 538αPkB serum, prepared against electroeluted 205/200 kDa B+ SICA antigens, only recognizes them from the R/A B+ extracts (Figure 2C). A slight retardation is observed when they are R/A prior to immunoprecipitation (Figure 2C, lane R/A, arrows).

Screening of B+ cDNA libraries with adsorbed 538αPkB serum yielded clones 538-6 (from library PkBλZ-4) and 24 (from library PkBλZ-3), which contain inserts of 1.9 and 3.2 kb, respectively, and are identical for 1.2 kb and then diverge (Figure 3A). Downstream of the open reading frames (ORFs), they are A/T rich. Clone 24 also contains a 305 bp intron, suggesting that it represents a minor genomic DNA (gDNA) contaminant or preprocessed mRNA.

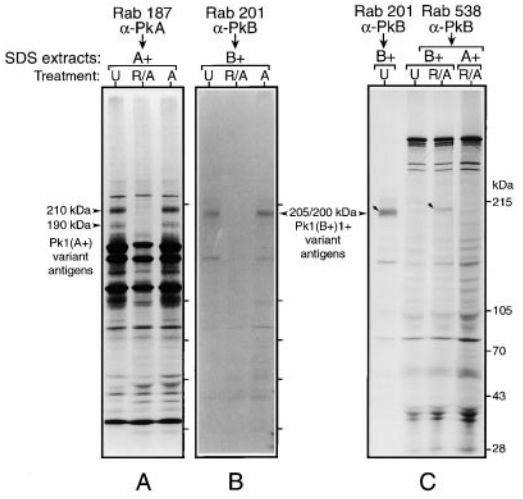

Figure 2. SICA Variant Antigen Epitopes Are Conformational

SDS extracts of [^{35}S]-methionine metabolically labeled A+ and B+ SIE were untreated (U), reduced and alkylated (R/A), or only alkylated (A), and then immunoprecipitated with the designated rabbit (Rab) antisera and analyzed by SDS-PAGE under reducing conditions with protein standards from Bio-Rad.

(A and B) 187α-PkA (partially adsorbed; see Experimental Procedures) and 201α-PkB (extensively adsorbed) recognize their respective variant antigens only in their native conformation (U or A lanes). Samples were run on a 4%–10% continuous gradient.

(C) 538α-PkB serum (unadsorbed) recognizes the B+ variant proteins only from R/A B+ extracts. Arrows indicate the change in migration observed compared to the native protein recognized by the 201α-PkB serum. Samples were run on a 4%–12.5% continuous gradient.

The coding regions hybridize to a large number of fragments of varied intensities on Southern blots of *P. knowlesi* gDNA (Figure 3B) with no obvious differences between the A+ and B+ patterns, but marked differences in the Philippine strain. In contrast, hybridization of the same blot with *P. knowlesi* cDNAs corresponding to known single-copy genes produced single bands (data not shown). These results established that clones 538-6 and 24 belong to a large multigene family. Even under low stringency, these probes did not react with gDNA of the closely related species, *P. cynomolgi* and *P. vivax*, nor with *P. falciparum* or rhesus monkey gDNA (Figure 3B, and data not shown).

Northern blot analyses showed that the same probes hybridize to a minor ~7.0 kb transcript, which is evident in B+, but not A+ parasites (Figure 3C). In contrast, five unrelated genes tested hybridized with equal intensities to transcripts of both the A+ and B+ RNA (data not shown). Furthermore, stage-specific blots yield an intense signal in the ring stage RNA and a very weak signal in later stage RNA (Figure 3D and data not shown).

Clones 538-6 and 24 Encode Portions of the 205 kDa B+ SICAvar Antigen

Rabbit antisera prepared against glutathione S-transferase (GST) fusion proteins related to the coding regions of clones 538-6 (25αr538-6) and 24 (35αr24), but not a control serum, immunoprecipitate [^{35}S]-methionine-labeled SIE proteins of ~200 kDa (Figure 4A, lanes 3–8). The protein immunoprecipitated by the 25αr538-6 serum from untreated and R/A extracts (Figure 4A, lanes 3 and 4) comigrated with the 205 kDa SICA antigen recognized by the 201αPkB serum (Figure 4A, lane 1), and the slower migrating (~215 kDa) denatured B+ SICA antigens recognized by the 538αPkB serum (Figure 4A, lane 2). The 35αr24 serum cross-reacted very weakly with proteins that comigrated with the nondenatured 205/200 kDa antigens (Figure 4A, lane 5) yet reacted strongly with the protein from R/A extracts (Figure 4A, lane 6), which comigrated with that immunoprecipitated by the 25αr538-6 serum (Figure 4A, lane 4).

Taking advantage of the lack of discrimination toward conformation presented by the 25αr538-6 serum, we were able to prove that the protein immunoprecipitated by the fusion protein antisera corresponds to the 205 kDa SICA antigen. Most telling, when presented with untreated B+ SDS extracts, from which the protein recognized by 25αr538-6 serum (Figure 4B, lane 4) had been depleted (see Experimental Procedures), the 201αPkB

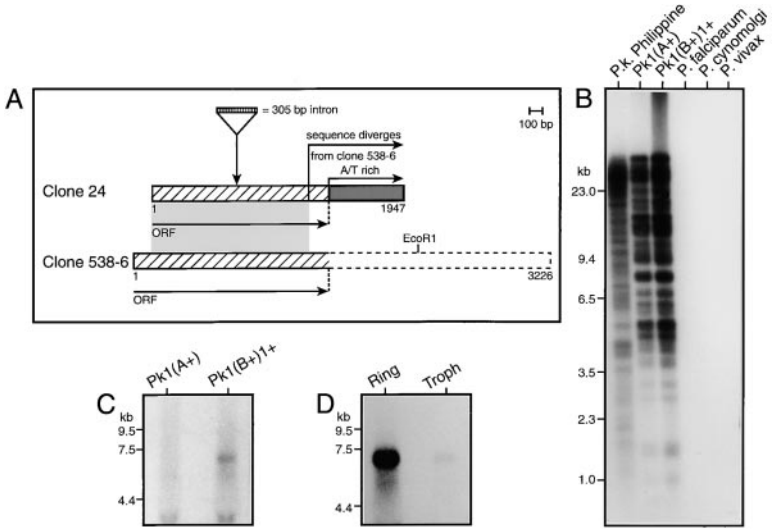

Figure 3. Identification of the *SICAvar* Multigene Family and Transcripts

(A) Schematics of clones 24 (1947 bp) and 538-6 (3226 bp). The ORFs of each are noted by arrows and hash marks. Downstream, both sequences become A/T rich. The intron of clone 24 and the point its sequence diverges from clone 538-6 are also noted.

(B) Southern blot: EcoRI digested gDNA from several *Plasmodium* species probed with clone 24. Lambda HindIII and EcoRI-digested markers (Sigma) are indicated.

(C) Northern blot: middle-to-late trophozoite stage A+ and B+ RNA probed with clone 24.

(D) Northern blot: ring and middle-to-late trophozoite stage RNA of the B+ parasites, probed with clone 24. RNA markers from GIBCO-BRL are shown.

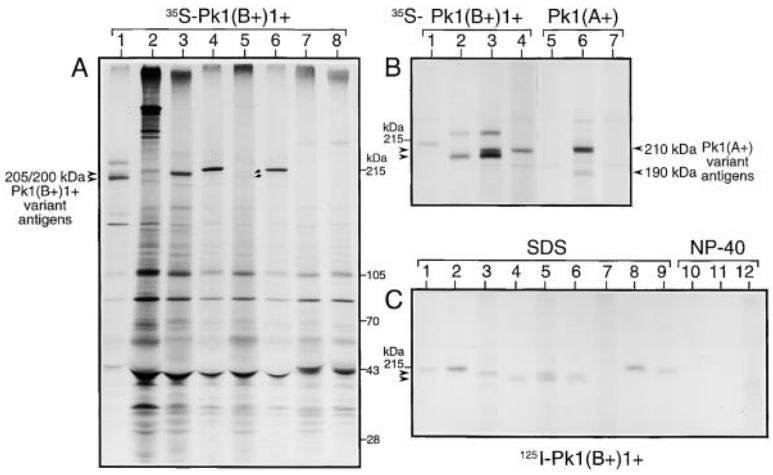

Figure 4. 25α-r538-6 and 35α-r24 Sera Recognize the 205 kDa Variant Antigen from B+ Parasites

Immunoprecipitation of SDS extracts of [35S]-methionine-labeled B+ and A+ SIE (A and B). Immunoprecipitations of sequential NP-40 (lanes 10–12) and SDS (lanes 1–9) extracts of [125I]–surface labeled B+ SIE (C). Protein standards noted are from Bio-Rad. Legend code: lane, protein(s) recognized, antiserum, extract treatment. R/A, reduced and alkylated. U, untreated.

(A) Lane 1, 205/200 kDa, 201αPkB, U. Lane 2, retarded 205/200 kDa, 538αPkB, R/A. Lane 3, 205 kDa, 25αr538-6, U. Lane 4, ~215 kDa, 25αr538-6, R/A. Lane 5 (arrowheads), 205/200 kDa, 35αr24, U. Lane 6, ~215 kDa, 35αr24, R/A. Lanes 7 and 8, control rabbit serum, U and R/A, respectively. Samples were run on a 4%–12.5% continuous SDS-PAGE gradient gel under reducing conditions.

(B) Lane 1, ~215 kDa, 25αr538-6, R/A of 205/200 kDa proteins immunoprecipitated by 201αPkB. Lane 2, 200 kDa, 201αPkB, U, and depleted of the 205 kDa protein by preincubation with 25αr538-6. Lane 3, 205/200 kDa (arrowheads on the left); 201αPkB, U. Lane 4, 205 kDa, 25αr538-6, U. Lane 5, no signal, 25αr538-6, U. Lane 6, 210/190 kDa (arrows on the right), 187αPkA, U. Lane 7, no signal, 35αr24, U. Samples were run on a 5% SDS-PAGE gel under reducing conditions.

(C) Lane 1, ~215 kDa, 25αr538-6, R/A of 205/200 kDa proteins immunoprecipitated with 201αPkB (see Experimental Procedures). Lane 2, ~215 kDa, 25αr538-6, R/A. Lane 3, 205 kDa, 25αr538-6, U. Lane 4, 200 kDa, 201αPkB, U, and depleted of the 205 kDa protein by preincubation with 25a538-6. Lane 5, 205/200 kDa, 201αPkB, U. Lane 6, 205/200 kDa, 201αPkB, U, and preincubated with 35αr24. Lane 7, no signal, 35αr24, U. Lane 8, ~215 kDa, 35αr24, R/A. Lane 9, ~215 kDa, 35αr24, R/A of 205/200 kDa proteins immunoprecipitated with 201αPkB. Lane 10, no signal, 35αr24, U. Lane 11, no signal, 25a538-6; lane 12, no signal, 201αPkB, U. Samples were run on a 5% SDS-PAGE gel under reducing conditions.

serum immunoprecipitated the 200 kDa SICA antigen (Figure 4B, lane 2), but not the 205 kDa protein, which it usually also recognizes. Also, after the irreversible denaturation of the proteins immunoprecipitated by the 201αPkB serum from untreated extracts (a duplicate reaction of that in Figure 4B, lane 3) and their immunoprecipitation with the 25αr538-6 serum, a band with the characteristic retardation of the R/A SICA antigens was observed (Figure 4B, lane 1). Furthermore, neither of the fusion protein antisera immunoprecipitated the 210/190 kDa variant antigens (Figure 4B, lane 6) from A+ SDS extracts (Figure 4B, lanes 5 and 7). Importantly, identical results were obtained with SDS extracts from [125I]–surface labeled B+ SIE (Figure 4C and data not shown). Direct agglutination tests were performed with antisera 25αr538-6 and 35α24, but as with several anti-PfEMP-1 fusion protein sera (Baruch et al., 1995), these antisera did not agglutinate the IRBCs.

The B+ 205 kDa *SICAvar* Gene Has Ten Exons

Having established that clones 538-6 and 24 encode part of the 205 kDa SICA antigen, we identified the full sequence using 5′ RACE (rapid amplification of cDNA ends) (Frohman et al., 1988) and 3′ end gDNA cloning strategies (Figure 5). To ensure extension of the 205 kDa *SICAvar* gene, over related family members, primers used for reverse transcription (RT) were first tested for their specificity on gDNA. Specific primers, such as gene-specific primer-7 (GSP-7), hybridized to one band in A+ and B+ gDNA digests. A 5′ RACE scheme beginning with GSP-7 yielded a 2.1 kb clone (R-2.1) that shares ~500 bp with clones 24 and 538-6. A nested polymerase chain reaction (PCR) using cp1 and the products of this RACE reaction as starting DNA, yielded a 1.35 kb clone (R-1.35), which overlaps with R-2.1 by 52 bp. However, an ATG start codon was not yet evident, so another 5′ RACE scheme was conducted, beginning with cp1 for the RT reaction. This yielded clone R-2.0, which overlaps with R-1.35 by ~1.0 kb and extends the ORF by 463 bp. A putative start site exists 528 bp from the beginning of R-2.0, preceded by the consensus sequence “ACACC” (Kozak, 1984).

The cDNA composite was verified by analyzing gene-specific PCRs with gDNA as the template, and gDNA Southern blots with GSPs as probes. Primers designed from R-1.35 and R-2.1 were used to amplify and clone P16, which shows identity in its overlapping regions. Also, primers 1F, cp1, and GSP-7, corresponding to sequences from R-2.0, R-2.1, and 538-6, respectively, hybridized, as would be expected from the deduced *SICAvar* physical map, to the same EcoRI and HpaI fragments when used sequentially as probes on digested A+, B+, and H strain gDNA (Figure 6). The 1F and cp1 primers also hybridized to the same band in the HindIII-digested gDNA. Furthermore, an antiserum against sequence encoded in R-2.0 (36αrR2.0) immunoprecipitated the 205 kDa B+ antigen from native and denatured extracts and efficiently competed with 25αr538-6 and 201αPkB sera, and an antiserum against the cytoplasmic domain (17αrCyto) was highly reactive with the 205 kDa antigen (data not shown).

The *SICAvar* sequence downstream of clone 24 was obtained from a 13 kb gDNA clone (λD13.0), identified by screening a B+ λDASH II library with GSP-7. This insert contains 6 kb and 7 kb XbaI fragments, which were subcloned into pBluescript II. Analysis of the 6 kb fragment indicated that its 5′-most 4 kb represents an

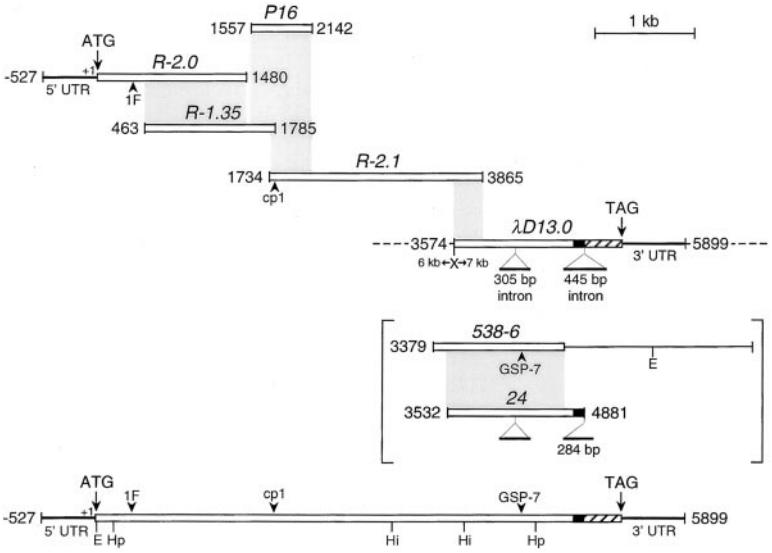

Figure 5. Composite of Clones Encoding the 205 kDa SICAvar from B+ Parasites

cDNA and gDNA clones, including clones 538-6 and 24 (brackets), are aligned to depict the complete coding sequence of the 205 kDa SICAvar from B+ parasites. Open boxes represent the sequence encoding the extracellular domain; the black box, the TM segment; and the hatched box, the cytoplasmic domain. The positions of the clones relative to the full sequence are noted by base pair numbers. Shaded areas highlight regions of identity. The 1F, cp1, and GSP-7 primers, start (ATG) and stop (TAG) codons, and restriction enzyme sites (E, EcoRI; Hp, HpaI; Hi, HindIII; and X, XbaI) are also noted.

artifact; however, the remaining 2 kb is identical to sequence in clones R-2.1, 24, and 538-6. The 7 kb XbaI clone then has 1896 bp identical with clone 24 (Figure 5). Two introns are evident here, the 305 bp intron identified previously in clone 24 and a 445 bp intron whose 5′-most 284 bp are identical to the A/T-rich sequences at the end of clone 24. This intron is followed by an ORF of 395 bp, which we predicted from the total gene structure and size, defines the final exon of this *SICAvar* gene. This is now corroborated with additional data on other *SICAvar* genes (unpublished data). This sequence ends in a TAG stop codon and is followed by a noncoding A/T-rich region. 3′ RACE reactions to obtain the corresponding *SICAvar* cDNA were attempted but proved unsuccessful. Whether the unique sequence at the 3′ end of clone 538-6 represents an artifact or a structure relevant to the process of antigenic variation is being evaluated.

Genomic DNA fragments corresponding to the cDNA of clones R-2.0 through R-2.1 were obtained by PCR. Seven primer pairs were used to amplify overlapping regions from B+ gDNA. All but one product (~3.7 kb) were cloned and sequenced. Except for a few differences, probably introduced by PCR, the coding sequences are identical to those obtained by 5′ RACE. However, six more introns were identified (Figure 6B), bringing the total number to nine. Each contains typical 3′ pyrimidine tracks and consensus splice sites. The sizes of the gDNA fragments hybridizing in Figure 6A also support the presence of these introns. The 3.7 kb PCR product that resisted cloning despite various attempts contains the third and fourth introns. Comparison of this product with the corresponding one amplified from cDNA indicates that intron 3 is ~3.0 kb (data not shown). Its position and that of the fourth intron were determined by end sequencing of the 3.7 kb product.

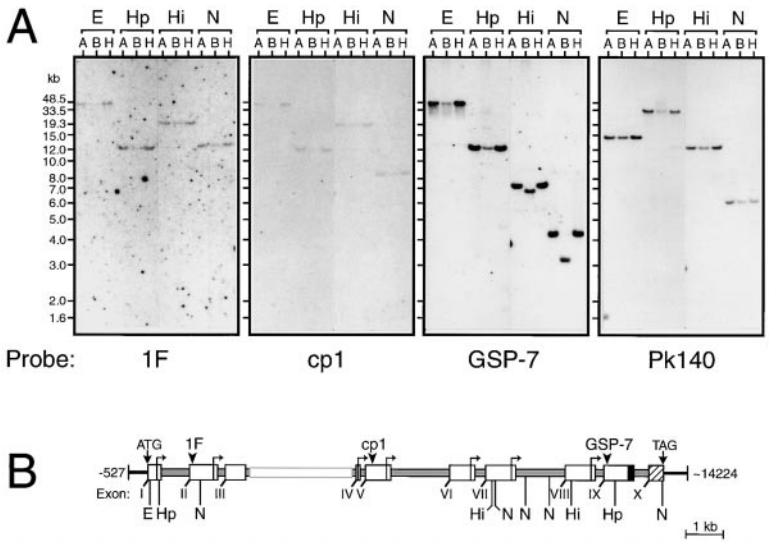

Figure 6. The Expressed B+ 205 kDa SICAvar Gene Has Ten Exons and a 3′ Genomic Alteration

(A) Southern blots: gDNA from A+ [A], B+ [B], and uncloned H strain [H] parasites, digested with EcoRI (E), HpaI (Hp), HindIII (Hi), and NciI (N), and hybridized sequentially with 1F, cp1, and GSP-7 probes, indicated in (B), and the Pk140 probe under stringent conditions. The DNA markers noted are from GIBCO-BRL.

(B) Schematic of the B+ 205 kDa *SICAvar* gene. Wide boxes correspond to exons I–X; the black box, the TM coding segment; and the hatched box, the cytoplasmic coding domain. The narrow gray boxes represent introns, and the thin black lines 5′ and 3′ UTR. The ~3 kb third intron (see text) is depicted by an open gray-lined box. Bent arrows mark the beginning of the CRDs. Restriction sites are: E, EcoRI; Hp, HpaI; Hi, HindIII; N, NciI. The positions of the 1F, cp1, and GSP-7 probes used in (A), and start (ATG) and stop (TAG) codons, are also noted.

The *SICAvar* gene (~14 kb) is at least twice the size of its ~7 kb transcript (Figure 3C).

Analysis of the 205 kDa B+ SICAvar Protein

The deduced B+ 205 kDa *SICAvar* ORF is 5271 bp and encodes a protein of 1759 amino acids (aa) with a calculated mass of 201 kDa. The putative start methionine is followed by 5 positive aa and a weakly hydrophobic 14 aa segment, which may function as a signal peptide. The protein is otherwise hydrophilic, except for a 30 aa segment 134 aa from the carboxyl terminus, which we predict defines a transmembrane (TM) domain (Figure 7). The isoelectric point (pI) of the predicted external part of this protein is ~7.65 with a cysteine content of 4.5%, whereas the presumed cytoplasmic region (133 aa) has a pI of ~5.15 and 0.75% cysteines.

The SICAvar protein sequence has seven cysteine-rich domains (CRDs) that range from 205 to 235 aa and make up most of the protein (Figure 7). These modules contain ten conserved cysteines but are otherwise quite varied, the highest homology being around cysteine 5. Interestingly, the *SICAvar* introns are in a defined region near the beginning of each CRD coding region (Figures 6B and 7A). CRD#2 is also interrupted by a second intron. The ninth and final intron lies between the sequence coding for the TM and cytoplasmic domains at a position similar to the intron in the two-exon structure of *P. falciparum var* genes.

To date, no significant similarities to the *SICAvar* coding regions have been detected in database searches. However, the DNA sequence after the stop codon is similar to malarial subtelomeric DNA (Vernick and McCutchan, 1988; Dore et al., 1990), suggesting a similar location for this *SICAvar* gene. Structural protein motifs suggestive of function were not identified, but potential serine/threonine protein kinase phosphorylation sites are present in the cytoplasmic domain.

The 3′ End of the 205 kDa Gene Differs in the B+ Clone, Where Its Protein Is Expressed, Compared to the A+ Clone, Where It Is Not Expressed

The gDNA of A+ and B+ parasites has proven to be identical when examined with probes from five different genes in extensive fingerprint analyses (data not shown); likewise, the ethidium bromide staining patterns from over ten restriction enzyme digests tested and the *SICAvar* hybridization patterns in Figure 3B appear identical. Similarly, several 205 kDa *SICAvar* GSPs and a probe corresponding to a *P. knowlesi* merozoite surface protein, Pk140 (Hudson et al., 1988) gave identical fingerprint patterns (Figure 6A). In stark contrast, GSP-7, designed from an area near the 3′ end of the 205 kDa *SICAvar* gene, detected a major difference between the parental H strain and A+ gDNA, and the B+ gDNA (Figure 6A, panel 3). Whereas this probe hybridizes to an ~3.0 kb NciI fragment in B+ gDNA, as would be expected based on the restriction map (Figure 6B), the NciI fragment detected in A+ and the H strain gDNA is ~4.2 kb. Similarly, the HindIII fragment is larger in A+ and H strain gDNA. However, the difference between the HindIII fragments is less, only being ~0.5 kb. Mapping with other enzymes also revealed further anomalies in this region (data not shown).

Discussion

A major breakthrough in understanding malaria antigenic variation came when several laboratories reported the identification of a large gene family responsible for the variation of parasite proteins exposed at the surface of *P. falciparum*–infected erythrocytes (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). Further analyses of the *P. falciparum var* genes have since been conducted (Rubio et al., 1996; Fischer et al., 1997; Hernandez-Rivas et al., 1997; Thompson et al., 1997); however, the mechanism(s) governing switching of variant types has not been readily forthcoming and will likely prove to be quite unique compared to known mechanisms in other organisms (reviewed Deitsch et al., 1997). Complementary studies in model systems should yield insights into the associated immunobiological and genetic processes, as they are likely to share features, but until now, no other *Plasmodium* variant antigen gene family has been described.

The *P. knowlesi*/rhesus monkey model is particularly well suited for in vivo and in vitro investigations of malaria antigenic variation and may provide novel insight into this phenomenon, particularly with regards to in vivo mechanisms where the host environment appears to modulate the process. Thus, we set out to compare the expressed and silent variant antigen genes of the *P. knowlesi* A+ and B+ clones. One goal was to investigate what gDNA or RNA alteration(s), if any, may have occurred in the switch from the expression of the 210/190 kDa antigens of the parental A+ parasites to the expression of the 205/200 kDa antigens in the B+ progeny.

Our evidence shows that clones 538-6 and 24 encode a portion of the 205 kDa antigen and belong to a large multigene family: antisera against their fusion proteins immunoprecipitate the 205 kDa protein from both [^{35}S]-metabolic and [^{125}I]–surface labeled B+ extracts, competitive adsorption experiments show that these antisera are specific for the 205 kDa protein, and Southern analyses highlight numerous *P. knowlesi* DNA fragments.

The complete coding sequence for the 205 kDa gene was deduced from multiple cDNA and gDNA clones. Specific primers were identified to initiate RACE reactions, screen a λDASH II gDNA library, conduct confirmatory PCR and RT-PCR reactions with gDNA and cDNA, and to probe gDNA Southern blots to provide proof that all cloned sequences are in fact part of the 205 kDa *SICAvar* gene and not another member of the *SICAvar* family. These data are backed by immunoprecipitations that confirm we are describing an expressed *SICAvar* gene from the perspective of gDNA, RNA, and protein, that is, one that is actually transcribed, translated, and transported to the IRBC surface.

The deduced structure of the 205 kDa SICAvar protein is consistent with the experimental work that originally defined the SICA proteins. It displays a putative signal for transport to the IRBC membrane, a predominant external variant domain, a TM region, and a cytoplasmic domain. The putative signal sequence is not typical, as defined by standard programs, yet as a hydrophobic region has some potential for being a valid signal. The CRDs of this and 22 other SICAvar proteins under investigation (unpublished data) have no resemblance to the

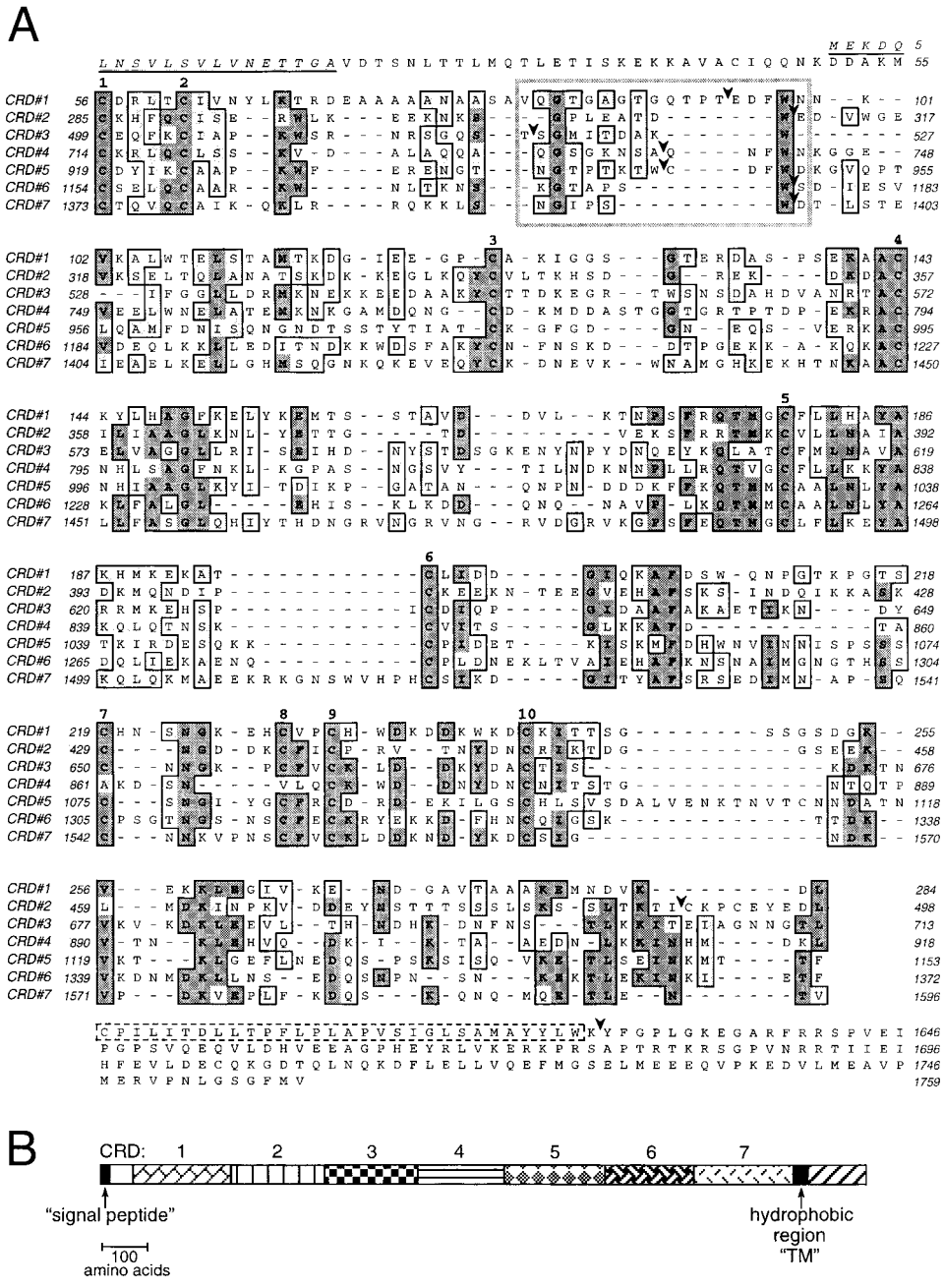

Figure 7. The B+ 205 kDa SICAvar Sequence Has Seven Cysteine-Rich Domains

(A) The deduced sequence of the B+ 205 kDa SICAvar antigen with an alignment of its CRDs (MacVector clustal program, Oxford Molecular). Identical residues are shaded dark gray, and conserved residues in light gray. The conserved cysteines are numbered 1–10. The large stippled box highlights the regions (arrowheads) where introns interrupt the CRD coding sequences. The potential signal peptide is double underlined, and the TM segment is boxed within dashed lines, followed by an arrowhead marking the place of the last intron. An arrowhead also marks the second intron in the CRD#2 coding region. Gaps (−) were introduced or removed to achieve the best alignment.

(B) Bar diagram of the B+ 205 kDa SICAvar. The putative signal peptide, seven diverse CRDs, TM, and cytoplasmic regions are depicted.

cysteine-rich Duffy binding-like (DBL) domains encoded by the EMP1 *var* genes. Their general similarity to each other, however, could account for the limited cross-reactivity sometimes observed with SICAvar antisera. The var DBL domains were named after similar motifs from merozoite proteins that bind to erythrocyte invasion receptors (Adams et al., 1992). In turn, it was proposed that the *P. falciparum* var DBLs might be critical for cytoadhesion and sequestration. Since then, DBL domains have been shown to mediate rosette formation (Rowe et al., 1997; Chen et al., 1998a). The CRDs of the SICAvar proteins also bear no similarity to the cysteine-rich interdomain region (CIDR) of the PfEMP-1 proteins, which is responsible for adhesion of IRBCs to the CD36 receptor on endothelium (Baruch et al., 1997). In this context, it is not surprising that the SICAvar CRDs are

not similar, since erythrocytes infected with *P. knowlesi* do not exhibit cytoadhesion in the same profound way that erythrocytes infected with *P. falciparum* do. These data, however, pose the question as to what transcending role(s) the variant proteins might play at the surface of IRBCs, regardless of whether the infecting species becomes sequestered.

The cytoplasmic domain of the 205 kDa SICAvar (132 aa) and PfEMP-1 antigens (~400–450 aa) do not have sequence in common; however, the 205 kDa SICAvar cytoplasmic domain serum (17αrCyto) reacts with other SICA proteins, indicating similarity within this family. The acidic PfEMP-1 cytoplasmic domains may anchor to the IRBC membrane via ionic interactions with the basic aa of the knob-associated histidine-rich protein (KAHRP) (reviewed in Deitsch and Wellems, 1996). Similarly, the SICAvar cytoplasmic domain may bind to RBC cytoskeletal proteins. Potential serine and threonine phosphorylation sites also suggest that the variant protein could participate in signaling events or have regulated interactions with the cytoskeletal matrix. Antibody interaction with the external domains of the variant proteins alters their interaction with the cytoskeleton, allowing them to be extracted in nonionic detergents. If agglutinating antibodies in fact modulate switching of SICA [+] types (Brown, 1973; Brown and Hills, 1974; Barnwell et al., 1983), perhaps they do so by triggering a signal(s) at this interface that is relayed to the nucleus. Now that SICA antibody and gene probes are available, it will be feasible to reassess earlier findings at the molecular and cell biological levels.

The relationship of the 205/200 kDa protein doublet has been open to speculation. The consistent detection of two bands from the B+ and other clones has been thought to represent reproducible degradation, processing, a heterodimer, or that each clone expresses two variant antigens. It now appears that the B+ doublet represents two distinct proteins, encoded by two genes. A variant antigen doublet has also been described in clones of the related apicomplexan parasite, *Babesia bovis*, which is comprised of two distinct proteins (reviewed in Allred, 1998).

The *SICAvar* ten-exon structure contrasts sharply with the *P. falciparum var* gene two-exon structure (Su et al., 1995) and is also unique compared to most known *Plasmodium* genes, which have no introns, or a few short introns at most. It is particularly striking how the beginning of each SICAvar CRD coding region is interrupted by an intron. This setup may provide the parasite with another means to generate *SICAvar* diversity, since the introns with their higher A/T content may allow for increased recombination and exon shuffling (Gō and Nosaka, 1987). This, along with the ability to switch expression among gene family members, provides the potential for an extra large repertoire of variant genes within natural *P. knowlesi* populations.

P. falciparum var gene studies have not yet indicated how switching actually occurs. It is known that *var* genes can be found singly or clustered, are located on most or all chromosomes and are not restricted to specific locations, do not appear to undergo duplication and transposition events as in other organisms such as trypanosomes (reviewed in Borst et al., 1998), and do not display any readily apparent rearrangements, deletions, or insertions when transcribed (Smith et al., 1995; Su et al., 1995; Rubio et al., 1996; Fischer et al., 1997; Hernandez-Rivas et al., 1997; Thompson et al., 1997).

We are investigating the transcriptional, gDNA, and epigenetic factors that might govern expression of the *SICAvar* genes. Here we show that the B+ 205 kDa *SICAvar* gene is expressed as an ~7.0 kb transcript. Importantly, this transcript was detected in trophozoite stage RNA of B+ but not A+ parasites. The protein is detectable in mature trophozoites and in schizonts. However, abundant transcripts are present much earlier in ring stage parasites. Borst et al. (1995) speculated that malaria parasites might constitutively express all *var* transcripts representing their multigene family and then degrade those not to be translated. Our initial analyses and recent studies on *P. falciparum var* gene transcript expression (Chen et al., 1998b; Scherf et al., 1998) could support a hypothesis along these lines. Given these data and our gDNA data showing a 3′ genetic alteration, we speculate that this alteration might be a means to "tag" the 3′ end of the variant gene whose transcript is to be translated. Such a mechanism would begin at the DNA level yet be compatible with possible transcriptional or posttranscriptional control mechanisms.

As for *P. falciparum var* genes (Smith et al., 1995; Su et al., 1995), we did not readily detect genome alterations that could correlate with *SICAvar* gene expression. However, upon hybridization of selected GSPs to a series of gDNA digests, a localized 3′ alteration in the expressed 205 kDa *SICAvar* gene was pinpointed. The 3′ region of the 205 kDa *SICAvar* gene is clearly different in the B+ parasites where the protein is expressed, compared to the A+ or wild-type H strain parasites, where it is not. In sharp contrast, upstream *SICAvar* probes gave identical hybridization patterns, as did six unrelated probes. Large deletions are known to occur in *Plasmodium* cultures (e.g., Corcoran et al., 1986; Pologe and Ravetch, 1986; Scherf and Mattei, 1992), and one might argue that what we are observing could reflect a rare chance in vivo deletional event unrelated to the switching process. However, aside from the fact that in all other analyses performed, A+ and B+ gDNA appear to be identical, it seems highly unlikely that a chance event would lead to the structure shown in Figure 6B, which appears to be a bonafide *SICAvar* gene. We have recently cloned the ~4.2 kb NciI fragment corresponding to the 3′ region of the 205 kDa *SICAvar* gene from A+ gDNA. Comparative analyses to date show that it is identical to the 205 kDa gene sequence from the B+ gDNA for 2.34 kb but then differs, beginning within intron IX preceding the cytoplasmic domain, yet it still retains structural features and homology typical of *SICAvar* genes. A potential deletion involving a putative *SICAvar* gene downstream from the 205 kDa *SICAvar* locus, whereby its 3′ end would replace the actual 3′ end of the 205 kDa *SICAvar* gene, would entail an estimated 10–15 kb loss of DNA and would be readily detected on Southern blots. To the contrary, we show that all three *SICAvar* GSPs recognize an ~45 kb EcoRI fragment in the gDNA of all three parasite populations.

Additional investigations are needed to confirm the

nature of the 3′ genetic alteration we are observing and to determine if similar alterations are associated with the expression of other *P. knowlesi SICAvar* genes. In line with our discussion above, this type of alteration could serve the purpose of "tagging" selected *SICAvar* genes so that their transcripts are targeted for translation. Further investigation will either confirm or void our speculation on this potential mechanism for controlling SICAvar protein expression and, perhaps by analogy, PfEMP1 expression.

Experimental Procedures

Parasites

P. knowlesi (H or Philippine strain) was propagated as in Barnwell et al. (1983). The *P. cynomolgi* Mulligan, *P. falciparum* FVO, and *P. vivax* Belem strains were cultivated as is typical for each (Trager and Jensen, 1976; Galinski et al., 1992).

IRBC Proteins and Antisera

The 187αPkA and 201αPkB sera were raised against IRBC membranes of A+ and B+ SIE prepared as in Aley et al. (1984) and emulsified with Freund's adjuvant. New Zealand white rabbits were immunized 3× subcutaneously with intervals of 3–4 weeks. Antisera were collected 2 weeks later. The 538αPkB serum was raised similarly against electroeluted 205/200 kDa B+ protein.

To enrich for α-PkB SICA antigen reactivities, sera aliquots were incubated with PBS-washed rhesus monkey RBCs, then passed over Affi-Gel 10/15 (Bio-Rad) coupled with SDS extracts of uninfected rhesus monkey RBC, A+ SIE, and SICA[−] SIE. Antisera used for immunoscreening were also adsorbed on formalin-fixed *Escherichia coli*, Affi-Gel 10/15 conjugated with soluble proteins from *E. coli* sonicates, and nitrocellulose containing wild-type λZAP II plaques. The 187αPkA serum was only adsorbed with SICA[−] SIE extracts.

GST Fusion Proteins and Antisera

GST fusion proteins, r538-6, r24, and rR-2.0, were prepared in the pGex-4T-3 vector (Pharmacia). The 2.2 kb EcoRI fragment of pBluescript clone 538-6 was subcloned in-frame using the 5′ BamHI and 3′ SalI vector sites, and the inserts of clones 24 and R-2.0 were subcloned directly into EcoR1. The fusion proteins of these clones correspond to aa 1127–1573, 1178–1397, and 7–493 (Figure 7), respectively. To obtain the rCyto protein, exon X was amplified from the 7.0 kb XbaI fragment of clone λD13.0 and ligated into pGEX-1λT (Pharmacia).

Fusion proteins were obtained by induction with 0.1 mM IPTG at 18°C for 24 hr in SURE cells or *Epicurian coli* BL21 (Stratagene), and purified using glutathione-Sepharose CL-4B. r538-6 and r24 were used in the bead-bound form for immunizations. Soluble rR-2.0 was used for two immunizations, followed by a boost of electroeluted rR-2.0. The 17αrCyto serum was raised against electroeluted protein. Antisera were heat inactivated and adsorbed with extracts of GST-expressing *E. coli* coupled to Affigel 10/15, formalin-fixed bacteria, and human O^+ erythrocytes.

Radiolabeling, Extraction, and R/A of Parasite Proteins

Parasites were metabolically labeled in cysteine/methionine-deficient RPMI-1640 (ICN Biomedicals) with 10% human AB^+ serum, 100 μCi/ml trans-[^{35}S]-methionine/cysteine (ICN Biomedicals), and 10% v/v regular RPMI-1640 added. IRBCs, harvested at the 2–4 nucleated stage and purified on Percoll, were surface iodinated as in Howard et al. (1983). NP-40/SDS extracts were prepared by resuspending the SIE pellet in ice-cold NP-40/PBS/PI (1% NP-40/PBS with protease inhibitors: 1 mM PMSF; 0.1 mM each of TPCK, TLCK, leupeptin, chymostatin, and antipain; 10 μM EP-64; and 1 μM pepstatin A), and incubating for 30 min on ice, with occasional vortexing. The extracts were clarified at 18,000 × g for 30 min at 4°C, and the supernatants saved. The pellet was washed 1× with 1% NP-40, extracted with 1% SDS/PBS for 10 min at RT, and clarified at RT. The supernatant was diluted 10× with NP-40/PBS/PI. For every 2–3 × 10^8 SIE, 1 ml 1% NP-40 and 100 μl 1% SDS were used.

Reduction/alkylation was performed while extracting the SIE pellet in 1% SDS/Tris-buffered saline (TBS) (pH 8.0) with 10 mM dithiothreitol at 4°C for 1 hr followed by 50 mM iodoacetamide at RT in the dark for 15 min. Treated extracts were diluted 10× with NP-40/PBS/PI and dialyzed in PBS.

Immunoprecipitations

Radiolabeled antigens were immunoprecipitated by standard methods. For Figure 4B, lane 1, and Figure 4C, lanes 1 and 9, following depletion of B+ extracts with 201αPkB serum, the beads were washed 2× with TBS (pH 8.0), incubated with 50 μl 1% SDS at RT for 15 min, and R/A as above. Nine volumes of NP-40/PBS/PI were added to the supernatant, dialyzed overnight (ON) at 4°C against PBS, and then incubated with 100 μl of protein A–Sepharose for 2 hr at 4°C. The supernatant was then immunoprecipitated with 25α-r538-6 or 35αr24 sera. In Figure 4B, lanes 2 and 4, and Figure 4C, lanes 3 and 4, the 205 kDa antigen was first depleted from B+ extracts by immunoprecipitation with 25α-r538-6 serum, and then the supernatant was incubated ON at 4°C with 100 μl of fresh protein A–Sepharose before subsequent immunoprecipitation with 201αPkB serum. Immunoprecipitates were analyzed by standard SDS-PAGE and fluorography.

Southern Blots

Plasmodium gDNA was isolated as in Galinski et al. (1992). Restricted gDNA (1–3 μg) was fractionated on 0.6% horizontal TAE agarose gels for ~18 hr at 25 v. Prehybridization, hybridization, and washes for blots analyzed with [α-^{32}P]dATP random-primed DNA were performed as in Galinski et al. (1992). For oligonucleotide probes, prehybridization was carried out for ~5 hr at 50°C in 6× SSC, 5× Denhardt's, 50 mM sodium phosphate, 0.5% SDS, 50 μg/ml salmon sperm DNA, and 20 μg/ml yeast tRNA. Overnight hybridization, minus Denhardt's, was performed 5°C below the probe's Tm. Washes included: 2× for 5 min in 6× SSC/0.5% SDS at 42°C and 1× for 3 min in 1× SSC/0.5% SDS at the hybridization temperature. Oligonucleotides were labeled as in Quijada et al. (1997) and used at 1 × 10^6 − 1 × 10^7 cpm/ml. Probes were removed with boiling 0.1× SSC/0.1% SDS.

RNA

Stage-specific RNA was isolated from rhesus monkey IRBCs using kits from Stratagene and Ambion. Northern blots, using 30 μg of total RNA/lane, were produced as in Galinski et al. (1992). For 5′ RACE, total RNA (10–20 μg) from ring and early-to-middle trophozoites was treated with 100 U/ml of RNase-free DNaseI (Ambion), and the efficiency was assessed by PCR analysis of RNase H–treated samples, using GSPs flanking the 305 bp intron (Figure 5). RACE was performed on 3 μg total RNA using GIBCO-BRL's version 2.0 system with RT at 50°C for 90 min. PCRs were carried out using 2.5 μl of oligo-d(C)-tailed cDNA and 200 nM each of the abridged anchor primer and nested antisense GSP. Reactions totaled 50 μl with 2.5 U of Expand DNA polymerase mix (Boehringer Mannheim), 1.75 mM $MgCl_2$ and 350 μM of each dNTP, and products were cloned into the pCR3.1 "TA" vector (Invitrogen).

Libraries

Two cDNA libraries representing middle trophozoites to early schizonts were constructed using the SuperScript choice system (GIBCO-BRL), with a few substitutions: library PkBλz-3 was made by oligo-d(T) priming of total RNA (7.5 μg) and library PkBλz-4 by random hexamer priming of oligo-d(T)-purified mRNA. After ligation of EcoRI/NotI adaptors (Pharmacia) and size fractionation, the cDNA was cloned into EcoRI/CIAP λZAP Express (PkBλz-3) or λZAP II (PkBλz-4), packaged using Gigapack II Gold extracts (Stratagene), and amplified using XL1-blue MRF′ and XL1-blue cells, respectively. Immunoscreening, plaque purification, and in vivo excisions were conducted as recommended (Stratagene).

The B+ λDASH II mung bean nuclease library was prepared as in Galinski et al. (1992) using 30% formamide. Hybridization with GSP-7 at 62°C was performed as described above. The filters were washed 3× for 15 min in 6× SSC/0.5% SDS at 42°C, and 1× for 3

min in 1× SSC/0.5% SDS at 62°C. Positive phage were purified by screening 2× with GSP-7.

DNA Sequencing and Analysis

Plasmid DNA, isolated by alkaline lysis (Sambrook et al., 1989), was sequenced on both strands (Sanger et al., 1977). Sequenase version 2.0 kits (United States Biochemical/USB) and [^{35}S]dATP (DuPont) were generally used, with the inclusion of pyrophosphatase and terminal deoxynucleotidyl tranferase (USB) (Kho and Zarbl, 1992). The TaqTrack system (Promega) was also used to resolve some compressions. DNA and protein sequence analysis was carried out using Genetics Computer Group (GCG) sequence analysis software (Devereux et al., 1984) and MacVector programs (Oxford Molecular).

Acknowledgments

This research was supported by a Irma T. Hirschl Trust Scholars Award to J. W. B., and a Biomedical Research support grant, NYU Whitehead Fellowship, and NIH/NIAID grant #R01-AI35804 awarded to M. R. G. We also thank Paul Ingravallo, Jingkan Han, and Greg Paré for technical assistance sequencing the *SICAvar* gDNA.

Received August 18, 1998; revised December 8, 1998.

References

Adams, J.H., Sim, B.K., Dolan, S.A., Fang, X., Kaslow, D.C., and Miller, L.H. (1992). A family of erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci. USA *89*, 7085–7089.

Aley, S.B., Barnwell, J.W., Daniel, W., and Howard, R.J. (1984). Identification of parasite proteins in a membrane preparation enriched for the surface membrane of erythrocytes infected with *Plasmodium knowlesi*. Mol. Biochem. Parasitol. *12*, 69–84.

Allred, D.R. (1998). Antigenic variation in *Babesia bovis*: how similar is it to that in *Plasmodium falciparum*? Ann. Trop. Med. Parasitol. *92*, 461–472.

Barnwell, J.W., Howard, R.J., and Miller, L.H. (1982). Altered expression of *Plasmodium knowlesi* variant antigen on the erythrocyte membrane in splenectomized rhesus monkeys. J. Immunol. *128*, 224–226.

Barnwell, J.W., Howard, R.J., Coon, H.G., and Miller, L.H. (1983). Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. Infect. Immun. *40*, 985–994.

Baruch, D.I., Pasloske, B.L., Singh, H.B., Bi, X., Ma, X.C., Feldman, M., Taraschi, T.F., and Howard, R.J. (1995). Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell *82*, 77–87.

Baruch, D.I., Ma, X.C., Singh, H.B., Bi, X., Pasloske, B.L., and Howard, R.J. (1997). Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence. Blood *90*, 3766–3775.

Biggs, B.A., Anders, R.F., Dillon, H.E., Davern, K.M., Martin, M., Petersen, C., and Brown, G.V. (1992). Adherence of infected erythrocytes to venular endothelium selects for antigenic variants of *Plasmodium falciparum*. J. Immunol. *149*, 2047–2054.

Borst, P., Bitter, W., McCulloch, R., Van Leeuwen, F., and Rudenko, G. (1995). Antigenic variation in malaria. Cell *82*, 1–4.

Borst, P., Bitter, W., Blundell, P.A., Chaves, I., Cross, M., Gerrits, H., van Leeuwen, F., McCulloch, R., Taylor, M., and Rudenko, G. (1998). Control of VSG gene expression sites in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *91*, 67–76.

Brown, K.N. (1973). Antibody induced variation in malaria parasites. Nature *242*, 49–50.

Brown, K.N., and Brown, I.N. (1965). Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. Nature *208*, 1286–1288.

Brown, K.N., and Hills, L.A. (1974). Antigenic variation and immunity to *Plasmodium knowlesi*: antibodies which induce antigenic variation and antibodies which destroy parasites. Trans. R. Soc. Trop. Med. Hyg. *68*, 139–142.

Bull, P.C., Lowe, B.S., Kortok, M., Molyneux, C.S., Newbold, C.I., and Marsh, K. (1998). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat. Med. *4*, 358–360.

Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S., and Wahlgren, M. (1998a). Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. J. Exp. Med. *187*, 15–23.

Chen, Q., Fernandez, V., Sundstrom, A., Schlichtherle, M., Datta, S., Hagblom, P., and Wahlgren, M. (1998b). Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature *394*, 392–395.

Corcoran, L.M., Forsyth, K.P., Bianco, A.E., Brown, G.V., and Kemp, D.J. (1986). Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. Cell *44*, 87–95.

David, P.H., Hommel, M., Miller, L.H., Udeinya, I.J., and Oligino, L.D. (1983). Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. Proc. Natl. Acad. Sci. USA *80*, 5075–5079.

Deitsch, K.W., and Wellems, T.E. (1996). Membrane modifications in erythrocytes parasitized by *Plasmodium falciparum*. Mol. Biochem. Parasitol *76*, 1–10.

Deitsch, K.W., Moxon, R.E., and Wellems, T.E. (1997). Shared themes of antigenic variation and virulence in bacterial, protozoal, and fungal infections. Microbiol. Mol. Biol. Rev. *61*, 281–293.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. *12*, 387–395.

Donelson, J.E. (1995). Mechanisms of antigenic variation in *Borrelia hermsii* and African trypanosomes. J. Biol. Chem. *270*, 7783–7786.

Dore, E., Pace, T., Ponzi, M., Picci, L., and Frontali, C. (1990). Organization of subtelomeric repeats in *Plasmodium berghei*. Mol. Cell. Biol. *10*, 2423–2427.

Fischer, K., Horrocks, P., Preuss, M., Wiesner, J., Wunsch, S., Camargo, A.A., and Lanzer, M. (1997). Expression of *var* genes located within polymorphic subtelomeric domains of *Plasmodium falciparum* chromosomes. Mol. Cell. Biol. *17*, 3679–3686.

Frohman, M.A., Dush, M.K., and Martin, G.R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA *85*, 8998–9002.

Galinski, M.R., Medina, C.C., Ingravallo, P., and Barnwell, J.W. (1992). A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. Cell *69*, 1213–1226.

Gō, M., and Nosaka, M. (1987). Protein architecture and the origin of introns. Cold Spring Harb. Symp. Quant. Biol. *52*, 915–924.

Handunnetti, S.M., Mendis, K.N., and David, P.H. (1987). Antigenic variation of cloned *Plasmodium fragile* in its natural host *Macaca sinica*. Sequential appearance of successive variant antigenic types. J. Exp. Med. *165*, 1269–1283.

Hernandez-Rivas, R., Mattei, D., Sterkers, Y., Peterson, D.S., Wellems, T.E., and Scherf, A. (1997). Expressed *var* genes are found in *Plasmodium falciparum* subtelomeric regions. Mol. Cell. Biol. *17*, 604–611.

Hommel, M., David, P.H., and Oligino, L.D. (1983). Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. Antigenic variation, antigenic diversity, and the role of the spleen. J. Exp. Med. *157*, 1137–1148.

Howard, R.J. (1984). Antigenic variation of bloodstage malaria parasites. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *307*, 141–158.

Howard, R.J., and Barnwell, J.W. (1984a). Roles of surface antigens on malaria-infected red blood cells in evasion of immunity. Contemp. Top. Immunobiol. *12*, 127–200.

Howard, R.J., and Barnwell, J.W. (1984b). The detergent solubility

properties of a malarial (*Plasmodium knowlesi*) variant antigen expressed on the surface of infected erythrocytes. J. Cell. Biochem. *24*, 297–306.

Howard, R.J., Barnwell, J.W., and Kao, V. (1983). Antigenic variation of *Plasmodium knowlesi* malaria: identification of the variant antigen on infected erythrocytes. Proc. Natl. Acad. Sci. USA *80*, 4129–4133.

Howard, R.J., Barnwell, J.W., Rock, E.P., Neequaye, J., Ofori-Adjei, D., Maloy, W.L., Lyon, J.A., and Saul, A. (1988). Two approximately 300 kilodalton *Plasmodium falciparum* proteins at the surface membrane of infected erythrocytes. Mol. Biochem. Parasitol. *27*, 207–223.

Hudson, D.E., Wellems, T.E., and Miller, L.H. (1988). Molecular basis for mutation in a surface protein expressed by malaria parasites. J. Mol. Biol. *203*, 707–714.

Kho, C.J., and Zarbl, H. (1992). A rapid and efficient protocol for sequencing plasmid DNA. Biotechniques *12*, 228–230.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. *12*, 857–872.

Leech, J.H., Barnwell, J.W., Miller, L.H., and Howard, R.J. (1984). Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. J. Exp. Med. *159*, 1567–1575.

Marsh, K., Otoo, L., Hayes, R.J., Carson, D.C., and Greenwood, B.M. (1989). Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans. R. Soc. Trop. Med. Hyg. *83*, 293–303.

McLean, S.A., Pearson, C.D., and Phillips, R.S. (1982). *Plasmodium chabaudi*: antigenic variation during recrudescent parasitaemias in mice. Exp. Parasitol. *54*, 296–302.

Mendis, K.N., Ihalamulla, R.I., and David, P.H. (1988). Diversity of *Plasmodium vivax*-induced antigens on the surface of infected human erythrocytes. Am. J. Trop. Med. Hyg. *38*, 42–46.

Miller, L.H., Fremount, H.N., and Luse, S.A. (1971). Deep vascular schizogony of *Plasmodium knowlesi* in *Macaca mulatta*. Distribution in organs and ultrastructure of parasitized red cells. Am. J. Trop. Med. Hyg. *20*, 816–824.

Pologe, L.G., and Ravetch, J.V. (1986). A chromosomal rearrangement in a *P. falciparum* histidine-rich protein gene is associated with the knobless phenotype. Nature *322*, 474–477.

Quijada, L., Moreira, D., Soto, M., Alonso, C., and Requena, J.M. (1997). Efficient 5′-end labeling of oligonucleotides containing self-complementary sequences. Biotechniques *23*, 658–660.

Roberts, D.J., Craig, A.G., Berendt, A.R., Pinches, R., Nash, G., Marsh, K., and Newbold, C.I. (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria. Nature *357*, 689–692.

Rowe, J.A., Moulds, J.M., Newbold, C.I., and Miller, L.H. (1997). *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. Nature *388*, 292–295.

Rubio, J.P., Thompson, J.K., and Cowman, A.F. (1996). The *var* genes of *Plasmodium falciparum* are located in the subtelomeric region of most chromosomes. EMBO J. *15*, 4069–4077.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Press).

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Scherf, A., and Mattei, D. (1992). Cloning and characterization of chromosome breakpoints of *Plasmodium falciparum*: breakage and new telomere formation occurs frequently and randomly in subtelomeric genes. Nucleic Acids Res. *20*, 1491–1496.

Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatur, C., Pouvelle, B., Gysin, J., Lanzer, M. (1998). Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*. EMBO J. *17*, 5418–5426.

Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., Pinches, R., Newbold, C.I., and Miller, L.H. (1995). Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell *82*, 101–110.

Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herrfeldt, J.A., Peterson, D.S., Ravetch, J.A., and Wellems, T.E. (1995). The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. Cell *82*, 89–100.

Thompson, J.K., Rubio, J.P., Caruana, S., Brockman, A., Wickham, M.E., and Cowman, A.F. (1997). The chromosomal organization of the *Plasmodium falciparum var* gene family is conserved. Mol. Biochem. Parasitol. *87*, 49–60.

Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science *193*, 673–675.

Vernick, K.D., and McCutchan, T.F. (1988). Sequence and structure of a *Plasmodium falciparum* telomere. Mol. Biochem. Parasitol. *28*, 85–94.

GenBank Accession Numbers

The accession numbers for the DNA sequences reported here are: cDNA, AF078128; gDNA, 5′UTR to beginning of intron III, AF078129; and gDNA, end of intron III through 3′UTR, AF078130.