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Expression of *var* Genes Located within Polymorphic Subtelomeric Domains of *Plasmodium falciparum* Chromosomes

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***Plasmodium falciparum var* genes encode a diverse family of proteins, located on the surfaces of infected erythrocytes, which are implicated in the pathology of human malaria through antigenic variation and adhesion of infected erythrocytes to the microvasculature. We have constructed a complete representative telomere-to-telomere yeast artificial chromosome (YAC) contig map of the *P. falciparum* chromosome 8 for studies on the chromosomal organization, distribution, and expression of *var* genes. Three *var* gene loci were identified on chromosome 8, two of which map close to the telomeres at either end of the chromosome. Analysis of the previously described chromosome 2 contig map and random *P. falciparum* telomeric YAC clones revealed that most, if not all, 14 *P. falciparum* chromosomes contain *var* genes in a subtelomeric location. Mapping the chromosomal location of *var* genes expressed in a long-term culture of the *P. falciparum* isolate Dd2 revealed that four of the five different expressed *var* genes identified map within subtelomeric locations. Expression of *var* genes from a chromosomal domain known for frequent rearrangements has important implications for the mechanism of *var* gene switching and the generation of novel antigenic and adhesive phenotypes.**

Tropical malaria is an infectious disease caused by the protozoan parasite *Plasmodium falciparum*, which invades, and propagates within, human erythrocytes. The morbidity and mortality inflicted by this parasite are associated with the adhesive properties of infected erythrocytes (24, 30, 44). By adhering to the endothelial linings of venular capillaries, the parasite evades elimination by the reticuloendothelial system of the host spleen. Sequestration of infected erythrocytes severely compromises the host, as the subsequent blockage of capillaries results in damage to vital organs (25, 26).

Cytoadherence is mediated by *P. falciparum*-encoded erythrocyte membrane proteins of 250 to 350 kDa, designated PfEMP1 (1, 21, 22). PfEMP1 can bind to several endothelial surface molecules, such as CD36, thrombospondin, and intercellular adhesion molecule 1 (2, 13). PfEMP1 is essential for parasite survival yet renders the surface of the infected erythrocyte immunogenic. *P. falciparum* uses antigenic variation of PfEMP1 (23, 31, 37) to evade the host's immune responses, allowing the parasite to maintain a persistent infection in the presence of constant immune pressure.

PfEMP1 molecules are encoded by *var* genes, a large multigene family (2, 37, 38) that consists of an estimated 50 to 150 members dispersed over several chromosomes (38). Despite their diversity, all *var* genes identified share common structural features (38). The coding region consists of two exons in which spatially conserved sequence motifs exist. The first exon encodes between two and four Duffy binding-like (DBL) domains that are homologous to sequence elements within the *P. vivax* and *P. knowlesi* Duffy antigen-binding protein and the *P. falciparum* EBA-175 erythrocyte-binding protein (28, 38). Exon II encodes a highly charged acidic terminal segment that is cytoplasmically located and conserved among *var* genes (38).

Given the size of a *var* gene, approximately 10 to 12 kb, up to 6% of the *P. falciparum* genome is estimated to contain sequence information for *var* genes (38). However, only a limited number of *var* genes, possibly only one, are expressed per cell at any given time (4, 31). This finding suggests the existence of a mechanism which is responsible for differential *var* gene expression. Precise knowledge of the organization of the *var* gene family and the mechanism of *var* gene switching may aid in the development of a rational antimalarial vaccine.

To initiate studies on the mode of differential *var* gene expression, we have determined the locations of *var* genes along the two completely cloned *P. falciparum* chromosomes 2 and 8. Analysis of these two contig maps, in addition to representative random *P. falciparum* telomeric yeast artificial chromosome (YAC) clones, revealed that *var* genes consistently reside close to the ends of most *P. falciparum* chromosomes. Thus, *var* genes map to a chromosomal domain that is polymorphic and prone to frequent rearrangements. As demonstrated here, subtelomerically located *var* genes are transcriptionally active, which suggests that rearrangements within this highly recombinant domain may contribute to differential *var* gene expression and the generation of *var* gene variants with new adhesive and antigenic phenotypes.

MATERIALS AND METHODS

Parasite culture and RNA isolation. The *P. falciparum* isolates FCR3 (geographic origin, The Gambia), Dd2 (southeast Asia), A2 (The Gambia), D10 (Papua New Guinea), HB3 (Honduras), and 3D7 (unknown) were cultured as described (39). Total RNA was isolated as described previously (7), and mRNA was prepared by using a Stratagene mRNA kit.

YAC libraries and library screening. The chromosome 8 contig was prepared by using YAC libraries prepared from the *P. falciparum* isolates FCR3 (10), Dd2 (6), and 3D7 (12). YAC libraries were transferred to high-density nylon filters and screened by hybridization, using a protocol originally described by Ross et al. (32) and modified by Carmargo et al. (6). The YAC libraries were initially screened by using known chromosome 8-specific probes: pMBP15, λW89, pMBB2, pH9.45, *hrp*II, and *hsp*70-1 (41). The ends of the YAC clones identified were rescued by inverse PCR (40), thereby generating sequence-tagged sites (STS) which were used to rescreen the libraries (18). The PCR conditions were as described previously (18, 35); PCR was performed with a Perkin-Elmer Cetus DNA thermal cycler. STS were cloned into the pCRII vector (Invitrogen) and sequenced by using a Sequenase version 2.0 sequencing kit (United States Biochemical).

Isolation of telomeric YAC clones. The *P. falciparum* YAC libraries used contain telomeric YAC clones that propagate as cotransformants under standard

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growth conditions (6, 10). To isolate these telomeric YACs, the FCR3, Dd2, and 3D7 YAC libraries were plated on selective tryptophan-deficient medium containing uracil (0.05 mg/ml) and 5-fluoro-orotic acid (1 mg/ml) as described previously (10). These conditions select against the presence of those YACs that contain both pYAC 4 vector arms, one encoding the *ura3* gene and one encoding the *trp1* gene. Telomeric YACs lack the *ura3*-encoding vector arm and thus can grow in the presence of 5-fluoro-orotic acid. The resulting YAC clones were shown to be of telomeric origin by hybridization using a probe to the telomere repeat sequences.

PFGE and restriction analysis of chromosomal DNA embedded in agarose. Pulsed-field gel electrophoresis (PFGE) was carried out with a CHEF DR-III apparatus (Bio-Rad) on 1% LE agarose (SeaKem) gels in 0.5× Tris-borate-EDTA at 14°C, using switching conditions described in the figure legends. Isolation of YAC DNA and *P. falciparum* chromosomal DNA and endonuclease restriction within agarose were done as described previously (10).

Hybridization probes for *var* gene mapping. Probes to the *P. falciparum* telomere sequences and to the subtelomeric repeat sequence element rep20 were as described previously (10). The conserved exon II probe of *var* genes (var_c) was a random primer-labeled restriction fragment from the cDNA clone T452 (38). Specific probes for *ebl-1* and *eba-175* are given in references 28 and 36, respectively.

Transcription mapping of *var* genes. Ten micrograms of total RNA or 0.3 to 0.4 μg of mRNA was treated with RNase-free RQ1 DNase (Promega) to ensure that no genomic DNA remained, as confirmed in control PCR experiments (37). The RNA sample was reverse transcribed by using random oligonucleotide primers and Superscript II reverse transcriptase (Gibco-BRL). The resulting cDNA was diluted fourfold and subjected to PCR amplification using either the degenerate UNIEBP primers previously described to identify all transcribed *var* genes (37) or oligonucleotide primers specific for the expressed *var* genes investigated. The sequences of oligonucleotide primers defining expressed sequence tags for the *var* gene variants B, D, and E and for *eba-175* and *PCNA* genes, in 5′-to-3′ orientation, were as follows: var_B, GCGTACATTTTTTGGCAAATG and GGTCTAGAAGAAAAAGTTTGTAG; var_D, TAGCACATGAGAGTCA AACAG and GGTTTAGCAGCAAACTCTTCAAGG; var_E, ATTGAATGTG CTGCTGTGG and GTTGTGTTGGTTTTGTCAC; *eba-175*, GAATCAAGAA TATTGAAACG and TACCCATGATATCACATTCC; and *PCNA*, GTATTC AAATTATGTGGAGC and CGTCATCCATTTTGGGAGC.

For transcription mapping, the PCR products resulting from amplification using the UNIEBP primers were purified over a Chroma 100 spin column (Clontech), and an aliquot was radiolabeled to generate a complex probe specific for all the expressed *var* gene variants in the parasite population at the time of the RNA preparation (var_{ex}).

Nucleotide sequence accession numbers. The accession numbers for the sequences reported in this paper are Y11910 (var_B) and Y11909 (var_E).

RESULTS

Construction of a YAC contig map of the *P. falciparum* chromosome 8. A YAC library derived from the *P. falciparum* line FCR3 was screened with six known chromosome 8 markers, using a hybridization-based strategy developed for the rapid analysis of complex libraries (6, 32). The ends of the YAC clones obtained were rescued, defining new STS which were then used to rescreen the library. This approach yielded only partial coverage of the chromosome, as two small areas of chromosome 8 were not represented in the FCR3 YAC library. YAC clones from two other *P. falciparum* YAC libraries (derived from the clonal lines Dd2 and 3D7) completed the contig.

The chromosome 8 contig map consists of 17 overlapping YAC clones, ranging in size from 40 to 250 kb (Fig. 1). The integrity of each YAC clone was confirmed by comparative restriction analyses, using the restriction endonucleases *Apa*I and *Sma*I, with genomic DNA from the corresponding *P. falciparum* clones (data not shown). In preparing the contig map, 24 new STS markers were obtained, which cover the entire chromosome with an average resolution of approximately 50 kb (Table 1). All STS markers were shown to hybridize back to chromosome 8, to the YAC clone of their origin, and to the neighboring YAC clone (data not shown). The YAC clones at both ends of the contig map, IO1 and 16D9, are telomeric, as indicated by hybridization with an oligonucleotide probe specific for *P. falciparum* telomere repeat sequences (Fig. 2). Additionally, YAC clone VIIID12, derived from *P. falciparum* line Dd2, hybridized with the telomeric probe. In comparison

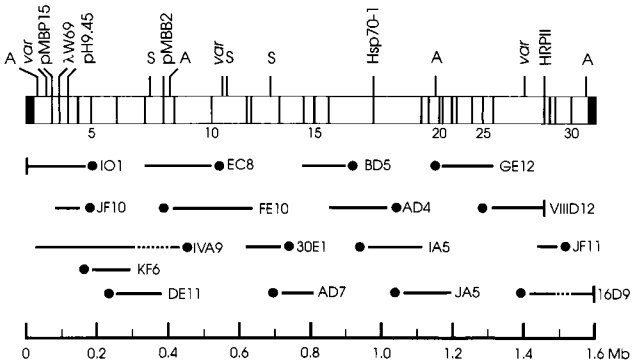

FIG. 1. Representative YAC contig map of *P. falciparum* chromosome 8. The locations of chromosome 8-specific genes, anonymous markers (41), and *var* genes are indicated, as are the cleavage sites of the restriction endonucleases *Apa*I (A) and *Sma*I (S). *P. falciparum* telomere repeat sequences are represented by filled rectangles. All YAC clones are denoted by horizontal lines, with the left arm of each YAC clone represented by a filled circle. YAC clones derived from the *P. falciparum* line FCR3 are indicated by two letters followed by a number. YAC clones VIIID12 and IVA9 are derived from Dd2; YAC clones 30E1 and 16D9 are derived from 3D7. Broken lines denote internal deletions within the YAC clones IVA9 and 16D9. The three telomeric YAC clones IO1, VIIID12, and 16D9 contain only the left vector arm of pYAC4. VIIID12 represents a truncation of chromosome 8 as found in the *P. falciparum* line Dd2. The chromosomal locations of the chromosome 8-specific markers (STS 1 to 30) are sequentially indicated by vertical lines.

with FCR3 and 3D7, Dd2 has a truncated version of chromosome 8 due to a breakage and healing event within the *hrp*II gene (29). No internal YAC clones hybridized with the telomeric oligonucleotide probe. These data indicate that a complete and representative contig map of chromosome 8, which spans the entire 1.6 Mb from telomere to telomere, was obtained.

***var* genes are located within subtelomeric domains.** The YAC contig map of chromosome 8, and the previously described contig map of chromosome 2 (18), provided us with an opportunity to study the distribution and organization of the *var* genes. YAC clones representative of the contig map of either chromosome were size fractionated by PFGE, transferred to a nitrocellulose membrane, and hybridized with a probe to the conserved exon II region of *var* genes (var_c) (38).

Four YAC clones containing *var* genes (IO1, IVA9, FE10, and VIIID12) were identified for chromosome 8 (Fig. 3A). The YAC clones IVA9 and IO1 substantially overlap and contain the same *var* gene locus, as determined by restriction analysis (data not shown). Thus, there appear to be three *var* gene loci on chromosome 8. Two *var* gene loci are located toward either chromosome end and reside on the telomeric YAC clones IO1 and VIIID12 (Fig. 3A). The third *var* locus, on the YAC clone FE10, is located within the internal domain of the chromosome.

The analysis of chromosome 2 identified three YAC clones containing *var* genes: GC6, CG7, and CB4 (Fig. 3B). YAC clones CB4 and CG7 substantially overlap and contain the same *var* gene locus. Thus, there appear to be two *var* gene loci on chromosome 2, mapping to the telomeric YAC clones GC6 and CB4 at either end of the chromosome. Restriction analysis using endonucleases *Hin*cII, *Bam*HI, *Kpn*I, and *Hae*III revealed that the *var* gene loci identified on both chromosome 2 and chromosome 8 contain single-copy genes (data not shown).

Since *var* genes were identified at either ends of chromosomes 2 and 8, we set out to determine if this is generally true for other *P. falciparum* chromosomes. Using a filter hybridization method, we screened three independent YAC libraries

TABLE 1. Chromosome 8-specific STS[a]

STS	Origin	Primer 1	Primer 2	Length (bp)[b]
1	pMBP15	Hybridized		1,800
2	λW69	Hybridized		750
3	pH9.45	Hybridized		2,000
4	JF10 LA	CTTAAGAGATTTACATATATATT	GGAATACACTCATTAATACAC	200
5	KF6 LA	CCCTACACGACGCATATT	GTACTACTACTAACCGTAAAA	420
6	DE11 LA	CACATATATGGATTATTCTTATA	GGTCCACTGGCTCAATTTCTCTG	680
7	EC8 RA	Hybridized		200
8	pMBB2	Hybridized		1,200
9	FE10 LA	AAAGAGAAATCTTTAGTATTATTG	GTATTTTGATCTATAGAATTTATAGAA	550
10	EC8 LA	GGTTACTATAATTATACCTTG	GAGCAGTAGCAGTAGCTGTGG	370
11	30E 1 RA	Hybridized		800
12	FE10 RA	CCAGCAATTTCAGCAGATG	CTCTTAAAAACGGTTCCACC	200
13	30E 1 LA	GTGATAAGAACAAAACATAAAG	CTTATTTATTTCTTATATTTTTCTA	190
14	BD5 RA	GTCACAGATATATTCGATAAAG	CATCTTTTTCATTTAGATATGTAA	220
15	AD7 RA	CAAAATTCTTTCTATCATTTGAA	CTCTAAATAAAGATATAACATATA	450
16	AD4 RA	CTGATATATTAAATAAATAATATTAC	CCATAATGTAATGAAAAGAAAT	380
17	*hsp*70-1	CTGCTGGTGGTGTTATGA	TAACCATTCAAGTATGGTTG	600
18	IA5 RA	ACATTTGTATGTTGCCCTGC	ATCATCTGTTGATATAGAACT	190
19	BG5 RA	AAAAAGAAATTAATAAAGAT	GAATTCGTTAAAATTACA	60
20	FF6 LA	GATATTTATTTAAAATTAAAAGAATT	ACATTTCCAAATAATAAAATATCA	190
21	GE12 LA	CGTCATCTTCTTTAGTGTAT	AGGGGGAAAAAAATAAAATAA	330
22	JA5 RA	GTCAAAAAATATGTATACAACGAA	CATTAACATCATTGTATGAGCAT	280
23	BG5 LA	GATATTAGAAATACAAACAATG	TGATTCAGTTTCTACTGATAA	156
24	JE11RA	ATTAAGTAATATTAGTTTTCATCC	ATTATTTTAACTGAGGATAAAACA	215
25	JE11 LA	TTCCATATGAAAACGAGAAAAA	AAATATATTAAAAAGTTTAATCATC	100
26	GE12 RA	AAGAAAATTATGTAACAATTTG	TTAACTGAGGATAAAACATATTCAA	740
27	*hrp*II	TTCCGCATTTAATAATAACTTGTG	CGTAGGCAATGTGTGGCGGCTTC	918
28	10B5 RA	AACAACTTTCTATAAATTTTCTATAT	GAAACGAGAATGAAAAATATAATT	270
29	JF11 LA	ATTGGACCTTTCTAATTTTTTG	ATAAAATCAAAATACAGAAAGAAA	180
30	10B5 LA	CATAATTATTTATAAGAATTATTTTAT	CTATATATATTCGTTAGTAATTAA	150

[a] The chromosomal locations of the chromosome 8-specific STS are indicated in Fig. 1. The markers were derived from either the left (LA) or right (RA) arm of the indicated YAC clone. The primers characterizing each STS are given in a 5′-to-3′ orientation.
[b] Expected size of the amplification product.

derived from the *P. falciparum* lines FCR3, Dd2, and 3D7 for clones containing *var* genes. The YAC libraries were subsequently hybridized with a probe to the *P. falciparum* telomere repeat sequences. A representation of these data is shown in Fig. 4A. It was found that a high percentage of YAC clones hybridized with both the var_c probe and the telomeric probe (Table 2). For example, when screening the 3D7 YAC library (the average insert size is 70 kb), we found that 45% of the 140 YAC clones that contain a *var* gene are also telomere associated. The probability of encountering a telomere-associated *var* gene rises with the average insert size of the library, as shown for the Dd2 YAC library, which has an average insert size of 150 kb and where 71% of the 188 YAC clones identified with the var_c probe also hybridize with a probe to the telomere repeat sequences (Table 2). To ensure that these *var* genes are indeed located on telomeric YACs, we cured all of the telomeric YAC clones of any possible further cotransformed artificial chromosomes and repeated the analysis. This analysis confirmed that the *var* genes identified were indeed located on telomeric YACs (data not shown).

The origins of several of the telomeric YACs have been identified as chromosomes 2, 3, 4, 8, 11, 12, and 13 (reference 33, this report, and our unpublished data). Experiments discussed below identified two other chromosomes (1 and 7) that contain *var* genes within the subtelomeric domain. These data suggest that most, if not all, *P. falciparum* chromosome ends contain *var* genes.

Subtelomeric *var* genes are expressed in *P. falciparum*. To determine if the expression of a *var* gene is associated with a particular chromosomal location, we mapped the different *var* gene variants expressed in a long-term culture of the *P. falciparum* line Dd2. Total RNA was isolated and subjected to reverse transcription (RT)-PCR amplification using the UNIEBP oligonucleotide primers (28, 37). These primers are degenerate and recognize conserved motifs within the DBL domains of *var* genes and the closely related *eba-175* and *ebl-1*

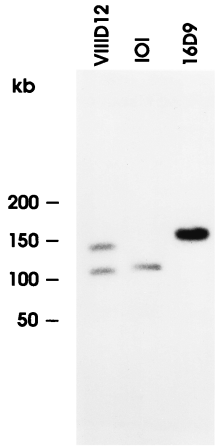

FIG. 2. Characterization of the chromosome 8 telomeric YAC clones. DNAs isolated from the telomeric YAC clones IO1, VIIID12, and 16D9 were embedded in agarose and size fractionated by PFGE (switching time from 5 s to 25 s over 24 h at 5.5 V/cm). Southern blots were hybridized with a radiolabeled oligonucleotide probe specific for the *P. falciparum* telomere repeat sequences $(GGGTTTA)_4$. VIIID12 revealed a second, lower hybridization signal that indicates an internal deletion event which can occur in *P. falciparum* telomeric YAC clones during propagation (11).

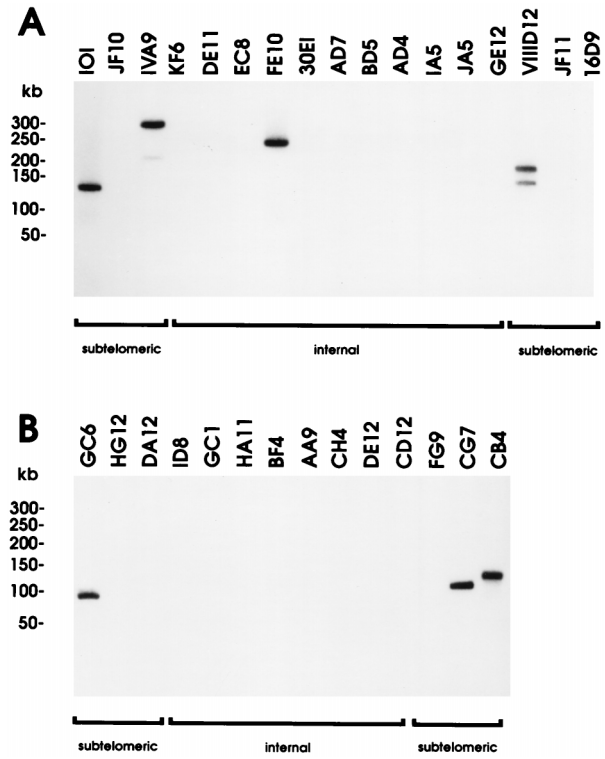

FIG. 3. Mapping of *var* genes within the *P. falciparum* chromosomes 8 (A) and 2 (B). DNA isolated from each YAC clone was embedded in agarose and size fractionated by PFGE (switching time from 5 s to 25 s over 24 h at 180 V). Southern blots containing an ordered array of the minimal representative YAC clones for chromosomes 8 (see Fig. 1 for comparison) and 2 (see reference 18 for comparison) were hybridized with a radiolabeled probe to exon II of the *var* genes. VIIID12 revealed a second, lower hybridization signal which indicates an internal deletion event.

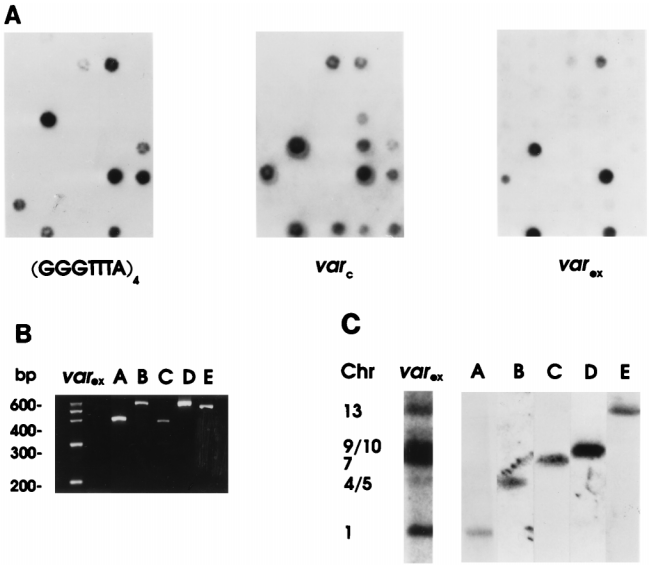

FIG. 4. Mapping of expressed *var* genes within the *P. falciparum* genome. (A) High-density filters containing a YAC library derived from *P. falciparum* strain Dd2 were hybridized with radiolabeled probes to the *P. falciparum* telomere repeat sequences $(GGGTTTA)_4$, the conserved var_c probe, and the complex-expressed var_{ex} probe derived by RT-PCR. A representative section of each of these filters is shown. (B) Ethidium bromide-stained agarose gel showing the complex var_{ex} probe and the isolated PCR products derived from the five expressed *var* genes examined (A to E). The PCR product of 320 bp observed in the var_{ex} lane corresponds to the *ebl-1* gene (data not shown). The 200-bp product was not investigated. (C) Chromosomes (Chr) of the parasite strain Dd2 were size fractionated by PFGE (switching time from 90 s to 300 s over 24 h at 2.8 V/cm followed by a switching time of 300 s to 720 s for 24 h at 2.5 V/cm). Southern blots were hybridized with the radiolabeled complex var_{ex} probe. Subsequently, the filter was hybridized with radiolabeled probes derived from each of the expressed *var* genes examined (A to E).

genes (28). Variability within DBL domains of *var* genes results in unique PCR products (37). Numerous PCR products, ranging in size from 200 to 600 bp, were obtained (Fig. 4B). No RT-PCR products were obtained in the absence of reverse transcriptase or in the presence of RNase A, indicating that genomic DNA did not contaminate the RNA preparation.

The PCR products were radiolabeled to generate a complex var_{ex} probe which was hybridized to a nitrocellulose filter containing the size-fractionated chromosomes of Dd2. At least seven different chromosomes were identified by the complex var_{ex} probe, indicating the expression of *var* genes and the related *ebl-1* and *eba-175* genes (Fig. 4C). To further characterize the expressed var genes, the complex var_{ex} probe was hybridized to nylon filters gridded with the Dd2 YAC library (Fig. 4A). YAC clones containing either the *eba-175* or *ebl-1* gene were subsequently identified by hybridization using probes specific for each gene. Forty-eight YAC clones that hybridized to both the complex var_{ex} probe and the conserved var_c probe remained (Fig. 4A). Given the 10-fold redundancy of the Dd2 YAC library (6), we determined how many different expressed *var* genes were identified. All 48 YAC clones were subjected to PCR amplification using the UNIEBP oligonucleotides as primers. PCR products of five different sizes were readily observed (A to E in Fig. 4B). Sequence analysis confirmed that these PCR products are homologous to DBL domains of other *var* genes (data not shown). However, each PCR product corresponds to a distinct *var* gene variant, mapping to five different chromosomes, 1, 4/5, 7, 9/10, and 13, all of which were also identified by the complex var_{ex} probe (Fig. 4C).

Four of the five expressed *var* genes identified map to telomeric YAC clones (Fig. 5A), suggesting that they lie within subtelomeric domains. This was confirmed by restriction analysis of representative telomeric YAC clones (Fig. 5B and C). All four of these expressed *var* genes map between 20 and 40 kb from the telomere, separated by the subtelomeric repeat sequence element rep20 (Fig. 5B to D). The orientation of the *var* genes varies with respect to the telomeres, as revealed by fine-scale restriction mapping analysis using the conserved exon II var_c probe and a specific probe derived from the DBL domain of exon I of each expressed *var* gene investigated (Fig. 5B to D).

Hybridization of representative YAC clones with the complex var_{ex} probe revealed signals of different intensities (Fig.

TABLE 2. Correlation between chromosome ends and *var* gene location[a]

Strain	No. of YACs	Avg insert size (kb)	No. of YACs containing *var*	Telomere-associated *var* (%)
FCR3	1,300	100	57	65
3D7	3,000	70	140	45
Dd2	1,440	150	188	71

[a] Telomeric YAC libraries derived from *P. falciparum* lines FCR3, 3D7, and Dd2 were analyzed for the presence of *var* genes, using the conserved var_c probe to exon II of *var* genes (38).

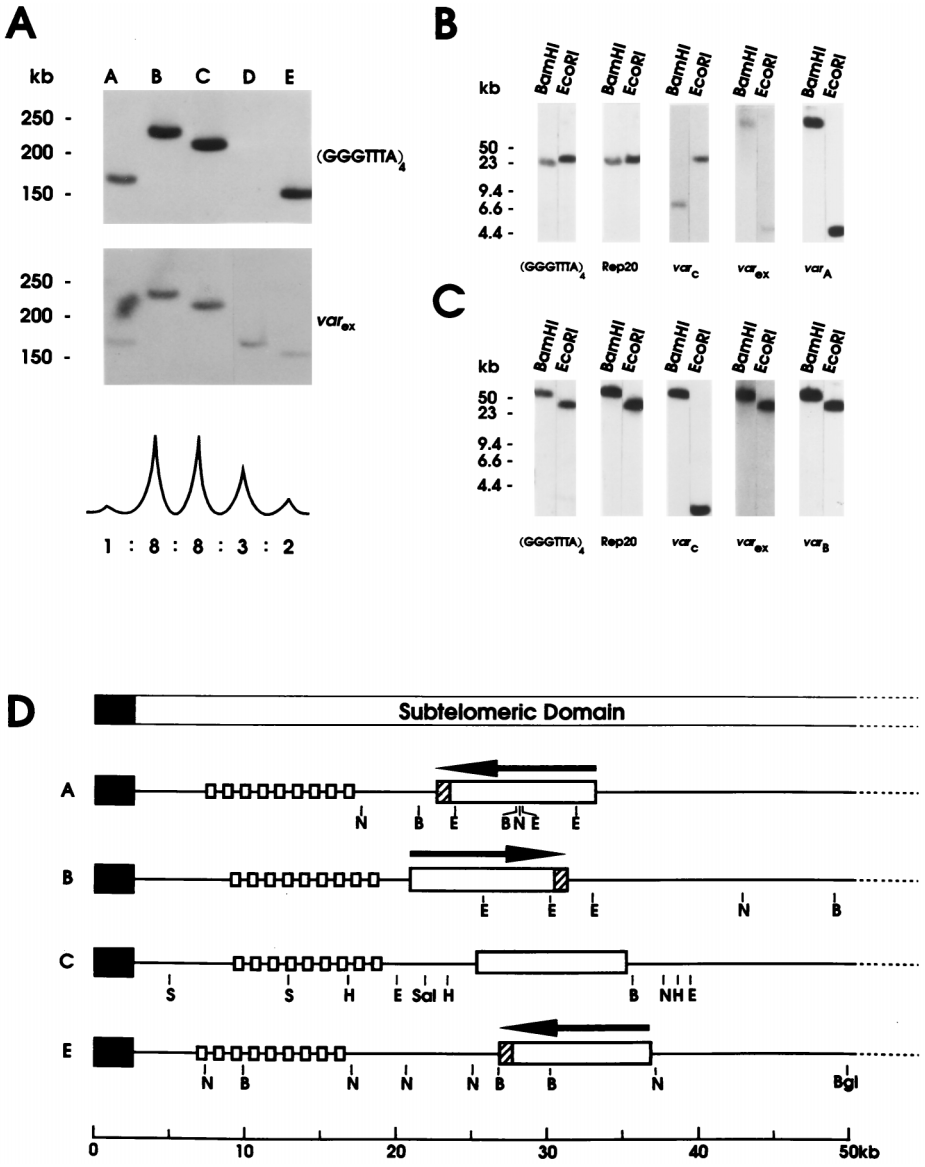

FIG. 5. Mapping of transcribed *var* genes. (A) YAC clones representative of the five expressed *var* genes examined (A to E) were size fractionated by PFGE (switching time 5 s to 25 s over 24 h at 5.5 V/cm), transferred to a nitrocellulose filter, and hybridized with radiolabeled telomere repeat sequences $(GGGTTTA)_4$ and the complex var_{ex} probe. The relative intensities of the var_{ex} hybridization signals are indicated below. (B and C) Restriction analysis of a YAC clone containing the *var* gene variants A (B) and B (C), using the restriction endonucleases *Bam*HI and *Eco*RI. Southern blots were hybridized with radiolabeled telomere repeat sequences $(GGGTTTA)_4$, rep20, the var_c probe, the complex var_{ex} probe, and the specific expression tag probes var_A and var_B. (D) Schematic drawings indicating the chromosomal locations of four telomerically expressed *var* gene variants (A, B, C, and E). *var* genes are indicated by open rectangles, with the conserved exon II domain shown as a hatched box. The direction of transcription is indicated by arrows. Telomeres are shown as filled rectangles, and the subtelomeric repeat sequence element rep20 is represented by small open boxes. Abbreviations: B, *Bam*HI; Bgl, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; S, *Spe*I; Sal, *Sal*I.

5A). These hybridization signals correspond to the subtelomeric *var* genes of interest, as each YAC clone contains only a single-copy *var* gene (Fig. 5B and C). Cross-hybridization between individual PCR fragments, present in the complex var_{ex} probe, was not observed under stringent conditions (Fig. 4C). Assuming that the PCR generating the complex var_{ex} probe maintains the ratio between the different *var* gene transcripts, the different intensities of the hybridization signals would therefore give an indication of the relative level of *var* gene expression within the Dd2 parasite population. For example, *var* genes B and C appear to be expressed within the population to approximately the same level, whereas *var* genes A, D, and E appear to be expressed to much lower degrees (Fig. 5A).

To characterize the developmental stage- and population-dependent transcription of these *var* gene variants, we defined expressed sequence tags specific for three of the *var* gene variants (see Materials and Methods). This allows us to investigate the transcription of individual *var* gene variants within a given parasite population.

mRNA isolated from different developmental stages of Dd2 was subjected to RT-PCR using oligonucleotides specific for the *var* gene variants B, D, and E. It was found that the expression of *var* genes is developmentally controlled (Fig. 6). As demonstrated for the *var* gene variants B, D, and E, transcripts are detected only in rings (Fig. 6), suggesting that the *var* mRNAs are translated during early intraerythrocytic de-

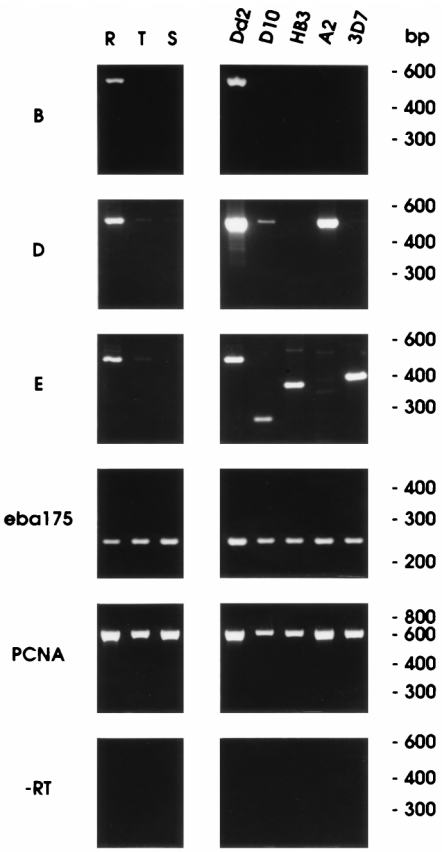

FIG. 6. Developmental stage- and population-dependent expression of three *var* gene variants. mRNA was isolated from different developmental stages of the *P. falciparum* line Dd2 (R, ring; T, trophozoite; S, schizont) or from asynchronous intraerythrocytic cultures of the other lines indicated. The mRNA was analyzed by RT-PCR with oligonucleotide primers defining expressed sequence tags for the *var* gene variants B, D, and E, *eba-175*, and *PCNA*. As a control, mRNA was subjected to PCR amplification in the absence of reverse transcriptase, using the oligonucleotide primers to *PCNA* (-RT). The geographic origins of the *P. falciparum* lines investigated are southeast Asia (Dd2), The Gambia (A2), Papua New Guinea (D10), Honduras (HB3), and unknown (3D7).

velopment. However, the exposure of the gene product, PfEMP1, on the surface of the host erythrocyte membrane appears to be delayed, as only trophozoites and schizonts sequester within the microvasculature (25, 26).

In comparison to *var* genes, *eba-175*, a member of the *var* gene superfamily (28, 36), appears to be constitutively transcribed during the asexual intraerythrocytic cycle, as is the housekeeping gene *PCNA* (17) (Fig. 6). No PCR products were obtained in the absence of reverse transcriptase in assays using the *PCNA*-specific oligonucleotide primers, indicating the absence of contaminating genomic DNA in the RNA preparation.

We next surveyed whether the *var* gene variants B, D, and E are also expressed within other parasite populations of different geographic origins. mRNA was isolated from four additional parasite lines from regions such as Honduras (HB3), The Gambia (A2), and Papua New Guinea (D10) to complement Dd2 from southeast Asia. Expression was examined by RT-PCR using oligonucleotides specific for the *var* gene variants B, D, and E. Transcripts from the *var* gene variants B and E are detected only in Dd2, although similar, but not identical, variations of *var* gene E may be expressed in D10, HB3, and 3D7, as indicated by PCR products of different sizes (Fig. 6). In contrast, *var* gene variant D appears to be expressed in several *P. falciparum* lines, including Dd2, D10, and A2 (Fig. 6). This was confirmed by the observation that internally located *var* genes expressed in geographically dispersed *P. falciparum* lines cross-hybridize with one another, unlike subtelomeric *var* genes, which are unique to a given parasite line (unpublished observation). Control RT-PCR experiments revealed that *eba-175* and *PCNA* are expressed in all parasite lines investigated.

DISCUSSION

Expression of *var* genes in the human malarial parasite *P. falciparum* is differentially controlled; that is, at any given time, a single infected erythrocyte expresses only one, or at most a few, *var* gene variants from a repertoire of an estimated 50 to 150 different *var* genes per genome (4, 31). However, within a parasite population, several different *var* gene variants are expressed (31), as also observed for a long-term culture of the *P. falciparum* line Dd2, in which at least seven different *var* gene variants are expressed (Fig. 4). The chromosomal locations of five of these expressed *var* gene variants were determined and found to map to chromosomes 1, 4/5, 7, 9/10, and 13. Interestingly, four of the five expressed *var* gene variants are located within 20 to 40 kb from telomeres. This places expressed *var* genes within the subtelomeric domains of *P. falciparum* chromosomes, which are approximately 80 kb in size and consist of arrays of repetitive sequence elements (11). Fine-scale restriction mapping showed that all of these expressed subtelomeric *var* genes are single copy and closely juxtaposed to the rep20 subtelomeric repeat sequence element, albeit in both orientations. A consistent organization was found for another single-copy *var* gene expressed from the subtelomeric domain of chromosome 11 in the *P. falciparum* line FCR 3 (15). The common genomic organization of subtelomerically expressed *var* genes suggests that this may play an important role in the variation of antigenic and adhesive phenotypes.

These data provide further evidence of transcriptional activity associated with subtelomeric domains of *P. falciparum* chromosomes. That subtelomeric domains of plasmodial chromosomes are not per se transcriptionally inactive is also supported by another report which showed that a selectable marker targeted by homologous recombination into a subtelomeric domain of a *Plasmodium berghei* chromosome is expressed (42). Thus, expression of subtelomerically located genes seems to be unaffected by the close proximity of telomere repeat sequences, which can exert a silencing effect on gene activity, as shown in other systems (14).

The *var* gene expression pattern observed raises the question as to why a majority of the expressed *var* gene variants lie within subtelomeric domains. The possibility that the methodology used to identify expressed *var* genes is biased toward subtelomeric *var* genes appears to be unlikely. The UNIEBP primers have been used extensively to characterize expressed *var* genes from a number of different *P. falciparum* isolates, including Dd2, and no bias toward subtelomerically located *var* gene variants has thus far been observed (28, 37, 38). In control experiments, UNIEBP primers were also able to amplify DBL domains from silent chromosome internal *var* genes present on artificial chromosomes in yeast (data not shown). This finding suggests that the *var* gene expression pattern observed reflects either the distribution and organization of *var* genes along *P. falciparum* chromosomes or the existence of specific expression sites within the subtelomeric domains, as is the case in African trypanosomes, where the antigenically variant *vsg* genes are exclusively expressed from telomeric expression sites (5).

The data available on the genomic organization of *var* genes

allow us to deliberate as to which model is applicable to *var* gene expression in *P. falciparum*. Using complete telomere-to-telomere YAC contigs, we have mapped the locations of *var* genes along chromosomes 2 and 8 and found that *var* genes are located at either end of both chromosomes. Similarly, other reports demonstrated the presence of *var* genes within the subtelomeric domains of other chromosomes (15, 34). Analysis of random telomeric YAC clones from three geographically dispersed *P. falciparum* lines confirmed that *var* genes are located at most, if not all, ends of the 14 *P. falciparum* chromosomes. These subtelomerically located *var* genes appear to be predominantly present in single copy, as shown here, although some subtelomeric domains contain two *var* genes (34). These data suggest that a substantial number of the *var* gene variants present in the *P. falciparum* genome are located at chromosome ends.

Chromosome internal *var* genes have been identified on chromosomes 7 (38), 8, and 12 (34). While there is a single copy of a *var* gene on chromosome 8, chromosomes 7 and 12 contain clusters of five and three *var* genes, respectively (34, 38). Screening of *var* gene-containing internally located YAC clones yielded no *var* gene clusters other than those already described (data not shown).

Thus, it appears that there are fewer *var* genes than initially thought, probably not more than 50 copies per haploid genome, which preferentially reside within subtelomeric domains, as also concluded by Rubio et al. (34). The data presented demonstrate that *var* genes can be expressed from both subtelomeric and chromosome internal domains. In addition to the five expressed *var* genes mapped in this study, two further expressed *var* genes which map to both chromosome internal and subtelomeric locations have been identified (15, 38). The fact that *var* gene expression is independent of the chromosomal location would argue against the existence of specific telomeric expression sites. The data are consistent with the model that all *var* genes can be expressed, independent of their chromosomal locations. Given that *var* genes appear to be preferentially located in subtelomeric domains, the *var* gene expression pattern observed in the long-term Dd2 culture seems to reflect the distribution and location of *var* genes along *P. falciparum* chromosomes.

It is intriguing that *var* genes are located within, and expressed from, subtelomeric locations, which places them in a highly recombinant background, as indicated by the extreme polymorphic nature of chromosome ends (8, 18, 20). The subtelomeric domains of *P. falciparum* chromosomes are characterized by arrays of repetitive sequence elements (11, 27), which are believed to facilitate ectopic recombination between heterologous chromosomes (9, 19). Such ectopic recombination events are known to occur in *P. falciparum*, are meiotically and mitotically stable, and render chromosome ends hot spots of recombination (16, 43).

Recent data have shown that within the same parasite population, subtelomeric *var* genes of different chromosomes are more closely related to each other than to internally located *var* genes (15, 34). This finding suggests that recombination occurs more frequently between subtelomeric, rather than between internally located, *var* genes. The two orientations of *var* genes within the subtelomeric domains may define subsets between which recombination can occur. Beyond a given parasite population, subtelomeric *var* genes are extremely polymorphic (38). It is therefore not surprising that expression sequence tags to the subtelomeric *var* genes investigated are unique to the *P. falciparum* line Dd2 (Fig. 6). In contrast, an expression sequence tag to an internally located *var* gene is conserved among several different *P. falciparum* lines, suggesting that there is little variation within chromosome internal *var* genes. Consistent with these data, it was shown that an internal *var* gene cluster on chromosome 7 is conserved among geographically dispersed parasite populations (38). This may be a reflection of the conserved nature of chromosome internal domains in *P. falciparum* (18).

Based on the data presented, we propose that the ability of chromosome ends to promote ectopic recombination has been recruited as a tool to introduce genetic variability into subtelomeric *var* genes and generate PfEMP1 variants with novel antigenic and adhesive phenotypes. In contrast, chromosome internal *var* genes may serve as a genetic pool of *var* genes which is stably maintained.

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