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Novel Target Antigens of the Variant-Specific Immune Response to *Plasmodium falciparum* Identified by Differential Screening of an Expression Library

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A primary infection by the *Plasmodium falciparum* Palo Alto O and R antigenic variants induces a variant-specific immunity in the *Saimiri sciureus* monkey. We have shown that these variants express distinct PfEMP1 antigens and differ in their levels of expression of additional antigens, including two conserved erythrocyte membrane-associated proteins, HRP1 and PfEMP3. To identify the antigens eliciting a variant-specific response, we conducted a differential screening of a λgt11 library with variant-specific sera. We report here the analysis of the 46 anti-R-specific clones. Two specific targets of the anti-R response were identified: (i) PfEMP3, suggesting that immunogenicity of this antigen is modulated by its relative abundance in different variants, and (ii) Asn-rich motifs. Most anti-R-specific clones, derived from so-far-undescribed genes, were detected by a cross-reaction on poly(Asn) stretches, as indicated by elimination of the signal after absorption on Asn-rich sequences. Reverse transcription-PCR (RT-PCR) showed that expression of the gene defined by clone 13 was R specific. Pepscan analysis of clone 13 identified three Asn-rich polypeptides and one unique peptide reacting specifically with antibodies eluted from the R-infected erythrocyte surface. Antisera raised to the unique peptide reacted with an R-specific protein. Attempts to demonstrate that clone 13 was derived from a *var* gene by using PCRs combining clone 13 and *var*-derived primers were unsuccessful. The *var* genes expressed by O and R parasites were identified not by this strategy but by RT-PCR with *var*-specific primers. This work has provided novel insights into immunity to antigenic variants and has identified a novel gene switched on during antigenic variation.

Experimental *Plasmodium falciparum* inoculations in humans indicated that the immunity acquired after an infection by one strain protected against a second inoculation with the same strain but not with a different one (7). Molecular typing of strains causing clinical episodes experienced by children living in an area of endemicity showed that the successive clinical episodes were caused by genetically different *P. falciparum* parasites and, furthermore, that the children restrained parasite multiplication of some strains while being apparently incapable of preventing other ones from reaching a high density and causing a clinical episode (8). This indicated that, at least in its early phase of acquisition, immunity to *P. falciparum* has a strain-specific component. Individuals living in areas of endemicity are exposed to numerous serologically diverse isolates, which differ both in their merozoite surface antigen serotypes and in the serotype of the variant antigen exposed onto the infected erythrocyte membrane. Longitudinal surveys showed that protection against clinical malaria was positively correlated with serum reactivity to serologically diverse antigens exposed on the infected erythrocyte surfaces of a broad range of isolates (5, 19). This reactivity was shown by the agglutination reaction, targeting the PfEMP1 variant antigen (3, 19, 24). However, these data do not prove that the variant antigen is the target of protective immune effectors contributing to parasite clearance or that recognition of this single antigen is involved in the elimination process. There is evidence for a role of merozoite-targeted effector mechanisms involved in protection acquired by adults living in areas of endemicity (4, 18). Thus, to date, it has been difficult to determine the respective roles of antimerozoite and antierythrocyte surface recognition in protection, and as a consequence, the strain-specific target antigens remain to be identified.

Experimental infection in the *Saimiri sciureus* monkey allows an investigation of both variant-specific and strain-specific immunity, as in this model infection-challenge experiments with different antigenic variants of the same strain or with two distinct strains can be carried out. We have used this experimental host to address the issue of variant-specific immunity and its target antigens. It has been previously shown that the protection afforded after a primary infection was variant specific (9) and that parasites expressing a particular serotype at the surface of the infected erythrocyte are negatively selected under serotype-specific immune pressure (9, 14). Immunological analysis of O and R parasites, two antigenic variants of the FUP/SP Palo Alto line (9), indicated that the R parasites presented a different PfEMP1 molecule and had increased amounts of PfEMP3 and decreased levels of HRP1 as compared to O parasites (17). Thus, these antigenic variants exhibited differences in the expression of several antigens associated with the erythrocyte membrane.

In order to identify the targets (antigens or epitopes) of the variant-specific immune response, we have used here an expression cloning approach, which has proved to be quite useful for molecular characterization of numerous important malaria antigens in the last decade. We have capitalized on the specificities of the sera raised after a primary infection in monkeys

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and have carried out differential expression cloning with anti-O- and anti-R-specific antisera in order to detect clones expressing antigens specifically recognized by one or the other reagent. A differential screening of a genomic expression library rather than of a cDNA library was chosen, as it had the advantage of permitting the isolation of clones reacting specifically with either one of the variant-specific sera, whether or not the relevant genes were expressed in the variant used to construct the library. A large number of clones were isolated, among which a fraction reacted specifically with one variant-specific serum. In the work reported here, we have concentrated our analysis on the clones reacting exclusively or with a much stronger signal with the anti-R antiserum and thus representing targets of the R variant-specific immune response potentially involved in protection.

MATERIALS AND METHODS

Parasites. The parasites used here were two antigenic variants of the *Saimiri*-adapted Palo Alto FUP/SP strain of *P. falciparum*, as described in detail elsewhere (9). Briefly, we refer to the standard *Saimiri*-adapted Palo Alto FUP/SP strain, more precisely the 93rd blood passage in the *Saimiri* monkey, as O parasites. The R variant was isolated during the passive transfer of fractionated hyperimmune immunoglobulin (Ig) into monkey S1413, which was experiencing a primary infection by the O parasites. The O and R parasites are two distinct antigenic variants of the FUP/SP line, as indicated by their identical genetic backgrounds and distinct erythrocyte surface phenotypes and serotypes. O-parasitized cells form rosettes and have knobs with a normal morphology, and the trophozoites spontaneously autoagglutinate in the absence of immune serum. The R parasites do not show any of these properties (i.e., they do not form rosettes, have abnormal knobs, and do not autoagglutinate). Furthermore, O and R parasites express distinct serotypes on the surface of the infected erythrocyte and form large agglutinates in the presence of homologous but not heterologous serum (9, 17). Challenge experiments indicated that a primary infection with O or R parasites induced a variant-specific protection (9). These phenotypic differences were stable upon serial blood passages into naive splenectomized *Saimiri* monkeys.

Serum and antibody preparations. For the differential screening of the library and purification of positive clones, the anti-O-specific reagent used was a pool of sera collected at various time points between days 30 and 60 after a primary infection with O parasites from monkeys S1410, S1417, and S1393, which did not subsequently resist a heterologous challenge by R parasites. The anti-R reagent was a pool of serum samples collected at various time points (between days 30 and 66) from monkey S1413, in which the R parasites were selected (9). This monkey had developed a protective immunity to R parasites, as it subsequently resisted challenge with R parasites (9).

A polyspecific pool of immune *Saimiri* sera (SIS) was prepared by pooling sera from 12 animals rendered resistant to challenge by O or R variants as a result of several successive infections with O and R parasites and cures by drug treatment. Antibodies reacting with erythrocyte surface-exposed antigens were affinity purified from this pool as described previously (17). A pool of *P. falciparum*-immune human sera (HIS) was prepared from five sera collected from hyperimmune adults living in Dielmo, a Senegalese village where *P. falciparum* is holoendemic (31). Purified human hyperimmune gamma globulins prepared from West African hyperimmune adults and protective in passive transfer assays in humans (HIG_1) (25) were kindly provided by P. Druilhe. These Igs were also efficient at clearing infections of *Saimiri* monkeys with the FUP/SP-O parasites (12). The activity on R parasites has not been tested in passive transfer assays. Purified human hyperimmune gamma globulins prepared from hyperimmune Malawian adults (HIG_2) were a kind gift of M. Hommel.

The rabbit antiserum raised to infected erythrocyte membranes (RIS) was kindly provided by M. Schreiber. It was prepared by immunizing rabbits with the membranes of schizont-enriched preparations of FUP/CP parasites cultivated in human A+ erythrocytes (15). The FUP/CP parasites have an FCR3-type genetic background, distinct from that of the monkey-adapted FUP/SP parasites (10), and in particular do not have knobs and have deletions of the HRP1 (10) and PfEMP3 (20a) genes.

Electrophoresis and immunoblots. The procedures for protein separation on a sodium dodecyl sulfate (SDS)-polyacrylamide gel have been described in detail elsewhere (17). Fractionated proteins were transferred onto nitrocellulose sheets (BA85; Schleicher & Schuell) (17). Blots reacted with the HIS were incubated with alkaline phosphatase-conjugated anti-human IgG (Cappel), while monkey antibodies were detected with a laboratory-made rabbit anti-*Saimiri* Ig immune serum, followed by alkaline phosphatase-conjugated anti-rabbit IgG (Promega). Blots reacted with the rabbit antiserum were incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). Staining of immune complexes was done in phosphatase buffer with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate solutions (Promega) according to the manufacturer's instructions.

Construction and screening of the genomic DNA library. The library was prepared as described by Mattei et al. (20), with minor modifications. Briefly, 20 μg of FUP/SP genomic DNA (prepared from the second blood passage of R parasites in the *Saimiri* monkey) was digested with DNase I, and the endogenous *Eco*RI sites were methylated with *Eco*RI methylase in the presence of *S*-adenosylmethionine (Promega). DNA was blunt ended with T4 DNA polymerase (Pharmacia), treated with *Escherichia coli* DNA ligase, and ligated with phosphorylated *Eco*RI linkers (New England Biolabs). After *Eco*RI digestion, the DNA fragments were introduced into *Eco*RI-cut phosphatase-treated λgt11 arms and encapsidated by using Gigapack extracts (Promega). About 4×10^5 recombinant bacteriophage, corresponding to approximately 10 genome equivalents (the average insert size was 500 bp), were plated on Y1090 bacteria and transferred onto nitrocellulose filters as described previously (21). The primary differential screening was performed by incubating duplicate filters of each plate with either the anti-O (dilution, 1/50) or the anti-R (dilution, 1/700) reagent. Procedures for immunological screening were as described previously (21), except that bound antibodies were detected with alkaline phosphatase-labeled conjugates. The sera were depleted of anti-*E. coli* antibodies before use by serial incubations with filters blotted onto λgt11 plaques (about 30 filters per ml of serum). This depletion did not modify the anti-*P. falciparum* immunoblot titer. For subsequent analysis with specific sera or antibody preparations, about 10^3 purified recombinant bacteriophage were spotted onto a freshly plated lawn of Y1090 bacteria.

PCR and RT-PCR analysis. Two-microliter aliquots of a recombinant bacteriophage suspension or of parasite genomic DNA were used as templates for PCR amplification (26). Amplification reactions were performed in a 50-μl final volume of a solution containing 0.2 mM deoxynucleoside triphosphates, 2 μM (each) sense and antisense oligonucleotide primers (see below), and 2 U of *Taq* DNA polymerase (Promega) in the buffer supplied by the manufacturer. After 30 rounds of amplification, PCR products were examined by agarose gel electrophoresis. For reverse transcription-PCR (RT-PCR) (32), total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method (6) and reversed transcribed as follows. RNA samples were heat treated at 90°C for 5 min, immediately chilled on ice, and incubated at 37°C for 1 h in a 20-μl final volume of a mixture containing 1 μM deoxynucleoside triphosphates (Pharmacia), 25 μM $MgCl_2$, 1 U of RNase inhibitor (RNasin; Promega) per μl, 100 pmol of random hexamer (Pharmacia), and 100 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia). The reaction was stopped by heating at 90°C for 5 min. Two microliters of each preparation was subsequently used for PCR. The following primers were used: λgt11-specific primers λFBH1 (5′-AAA GGA TCC TCC TGG AGC CCG TCA GTA) and λRH3 (5′-AAA AAG CTT AGC GAC CGG CGC TCA GCT); HRP1-specific primers HRP1 P5′ (5′-CCG GGA TCC ATG AAA AGT TTT AAG AAC AA) and HRP1 P3′ (5′-TGA ATT CCC TGC ACC ATG GGG TGG G); clone 13-specific primers I-13A (5′-GGA GTA ATA TGA GTT TCA GCA AAG G) and I-13B (5′-CGA TTC CAT TTT TCT TTT GAA GTG G); clone 15-specific primers I-15C (5′ ATT ATT AAC TTA ATA ATA TTA GTG ATC) and I-15D (5′-ATT TTG TTG CAC GTT ATT ATT AAT G); clone 16-specific primers I-16A (5′-ACC ATA TGA AAA CCT TTA AAT CCT GG) and I-16C (5′-ATA AGA AAA AAT ATA ATA TGT ATG ATG); clone 17-specific primers I-17A (5′-AAA TCA TAT AAT AAT AGT GAT ATA) and I-17B (5′-GGT ATT TAT TTT TAT AAT ACT TTG); *var* gene-specific primers UNI-EBP 5′ [5′-CC(A/G) AG(A/G) AG(A/G) CAA (G/A)AA (C/T)TA TG] and UNI-EBP 3′ [5′-CCA (A/T)C(T/G) (T/G)A(A/G) (A/G)AA TTG (A/T)GG] (which recognize all Duffy binding-like [DBL] domains [23]), varA5.2 [5′-GCC TG(T/C) GC(T/G) CC(A/G) T(T/A)(T/C) AG(A/G) CG] (which is specific for DBL-1 [13]), DBL4anti-S [5′-TCT TCA A(A/G)A AAA (T/G)AT TCT A(C/G)C CAT C(T/G)T TT] (which is specific for DBL-4 [8a] and was designed from alignments of the complete *var* gene sequences as reported elsewhere [2, 30]), and reverse primer XCRI [5′-GGT ATA TCA TAA (A/T)CA CTT TTG G], mapping to a conserved region of exon 2 (28).

Classification of the clones by cross-hybridization. Two microliters of each bacteriophage preparation was spotted onto a Y1090 lawn on a tryptone agar plate and incubated at 37°C for 4 to 5 h, and DNA was transferred onto a nylon filter (Hybond-N; Amersham) according to the manufacturer's instructions. The filters were prehybridized at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na citrate, pH 7.0) containing 2.5% nonfat powdered milk and hybridized with the radiolabeled probes overnight in 6× SSC–2.5% nonfat powdered milk in the presence of 100 μg of herring sperm DNA per ml. After hybridization the filters were washed at 65°C twice with 6× SSC, 2× SSC, and 0.5× SSC and autoradiographed on KODAK XOMAT films. Probes were prepared by nick translation of the various bacteriophage-derived inserts in the presence of [α-^{32}P]dATP (nick translation kit; Boehringer). Inserts were prepared either by *Eco*RI digestion of the purified phage DNA (for the >1-kb inserts) or by PCR amplification (for inserts of <1 kb) with the λgt11-derived primers λFBH1 and λRH3, located on both sides of the cloning site. Inserts were purified from agarose gels with Geneclean (Bio101) according to the manufacturer's instructions.

Southern blots. O or R genomic DNA was prepared by phenol-chloroform extraction of infected *Saimiri* blood cells (9). For Southern blot analysis, 2 to 5

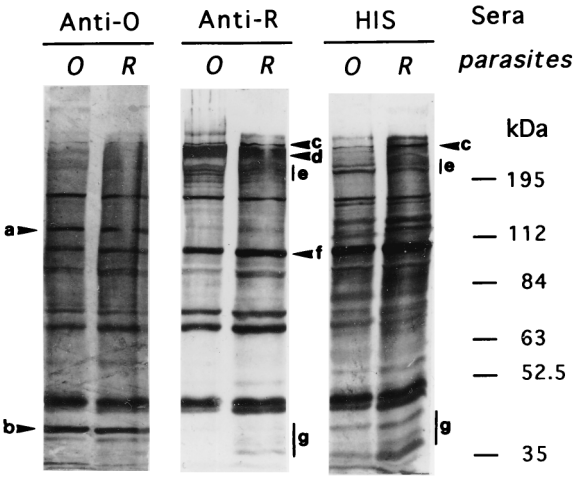

FIG. 1. Immunoblot analysis of O and R SDS extracts. Extracts prepared from O- or R-infected monkeys and adjusted so as to contain similar ratios of the various developmental stages were loaded onto an SDS–7.5% polyacrylamide gel, blotted onto a nitrocellulose filter, and reacted with a 1/50, 1/700, and 1/100 dilutions of anti-O, anti-R, or HIS antisera. Anti-O and anti-R reactions were visualized by using anti-Saimiri IgG rabbit antibodies (1:1,000 dilution) and a commercial anti-rabbit IgG alkaline phosphatase conjugate (1:15,000 dilution) (Sigma). Human antibodies were detected by using an anti-human IgG alkaline phosphatase conjugate adjusted to a 1:5,000 dilution (Cappel). The migrations of prestained molecular mass markers (Sigma) are indicated on the right. a to g indicate differences in reactivity of the anti-O and anti-R antisera on parasite extracts.

μg of DNA was digested with an excess of *Eco*RI, *Rsa*I, *Eco*RV, *Taq*I, *Pst*I, or *Alu*I restriction enzyme (Promega) according to the manufacturer's recommendations. After digestion, DNA fragments were size fractionated by agarose gel electrophoresis, transferred onto nylon membranes (Hybond-N; Amersham), and probed with α-^{32}P-radiolabeled inserts (nick translation kit; Boehringer) (10, 16).

Sequencing. The various inserts, prepared as described above, were subcloned into M13mp18 and M13mp19 sequencing vectors and sequenced by the dideoxy chain termination method (27) with the Sequenase version 2.0 kit (U.S. Biochemicals).

Pepscan analysis of clone 13 and preparation of antisera. A set of 83 15-residue-long peptides overlapping by 9 amino acids scanning the entire deduced amino acid sequence of clone 13 was purchased from Chiron Mimotopes, Lyon, France. They were provided bound to a solid polyethylene pin support in a standard enzyme-linked immunosorbent assay plate layout. Antibodies affinity purified on the surface of O- or R-infected erythrocytes from hyperimmune *Saimiri* monkeys were used at a 1:200 dilution. Binding was visualized by using homemade anti-*Saimiri* IgG rabbit antibodies, at a 1:5,000 dilution, followed by anti-rabbit IgG conjugate (Biosys) diluted 1:4,000. All incubation procedures were carried out in enzyme-linked immunosorbent assay plates according to the manufacturer's recommendations. The same set of pins was used sequentially. Removal of antibodies was done as recommended by the manufacturer and was controlled by using the appropriate anti-Ig conjugate. The positive standard control was the SIS hyperimmune serum, which was used to check the pin reactivity after each assay with antibodies eluted from the surfaces of infected erythrocytes.

Rabbit antisera raised to peptide 13 conjugated to keyhole limpet hemocyanin were prepared by Genosys (Cambridge, United Kingdom). Control antibodies were laboratory-raised sera to irrelevant keyhole limpet hemocyanin conjugates.

Nucleotide sequence accession numbers. The nucleotide sequence of clone 13 reported in this paper was submitted to GenBank and assigned accession no. AF091239 (insert 13 complete sequence obtained from the Dd2 genomic DNA) and AF091850 and AF091851 (insert 13 partial 5′ and 3′ sequences, respectively, obtained from the Palo Alto FUP/SP genomic DNA).

RESULTS

Immunoblot analysis of sera used for screening. The anti-O and anti-R antisera were titrated on immunoblots of SDS extracts prepared from samples of *Saimiri* erythrocytes infected with O or R parasites containing similar proportions of various blood stages (17) in order to adjust the dilution to be used to screen the library according to their immunoblot titer. Figure 1 shows the reactivity profiles produced by each reagent at the dilution used to screen the library (1/50 and 1/700 for anti-O and anti-R, respectively). As previously observed for individual variant-specific *Saimiri* sera, the reagents used here generated very similar patterns on both parasite types, but a few differences in reactivity were identified (labeled a to g in Fig. 1). These differences in reactivity of variant-specific sera on antigens present on both extracts have previously been detected by using several individual anti-O or anti-R reagents and antibodies eluted from the surfaces of O- or R-infected erythrocytes (17).

Differential screening. The anti-O- and anti-R-specific sera were used for a differential screening of a λgt11 genomic expression library. Clones were classified according to their reactivities into three distinct groups: anti-R^+/anti-O^+ clones, producing signals of similar intensities with the anti-R and the anti-O reagents (165 clones); anti-R^-/anti-O^+ clones, reacting specifically or with significantly stronger signals with the anti-O reagent (25 clones); and anti-R^+/anti-O^- clones, reacting specifically or with markedly stronger signals with the anti-R reagent (46 clones). In the work reported here, we have analyzed the 46 clones which gave a strong positive reaction with the anti-R antiserum and a weak to no signal with the anti-O antiserum and which are thus predicted to express antigenic determinants either not expressed by O parasites or not immunogenic in this context.

Classification of the clones by hybridization. Of the 46 recombinant clones retained, 6 were unstable and were not further studied. The clones containing identical or overlapping sequences were identified by hybridization with individual inserts recovered from each recombinant bacteriophage and used to probe the panel of clones. As indicated in Table 1, such a strategy allowed the identification of four distinct cross-hybridization clusters, with 14, 4, 2, and 2 clones in clusters A, B, C, and D, respectively. Eighteen clones were unique (did not hybridize to any other one); for convenience, they were all placed in a separate group, group U. The size of the insert carried by each recombinant bacteriophage is indicated in Table 1. Twelve of 14 clones in cluster A carried a 1,350-bp insert; the other 2, namely, clones 3 and 33, carried 980- and 1,380-bp inserts, respectively. The various 1,350-bp inserts could not be distinguished by restriction analysis with *Rsa*I, *Eco*RI, *Bcl*I, or *Taq*I or by partial sequencing. Likewise, in cluster B the four clones had an identical 2,350-bp insert, and in cluster D both clones had a 1,400-bp insert. We interpret this as indicating that each cluster contains several redundant clones carrying the same sequence. The size of the inserts in cluster C or of the unique clones was variable, ranging from 280 to 3,600 bp.

Immunological reactivity. One randomly selected clone from each cross-hybridizing cluster and each of the 18 unique clones were further tested for reactivity with a panel of immunological reagents. The reactivities with the anti-O- and anti-R-specific antisera used to screen the library are indicated in Fig. 2, rows a and b, respectively. The subset of clones shown in Fig. 2 was analyzed with the HIS pool from hyperimmune Senegalese adults (row c). As shown in Fig. 1, this pool produced the same differential labeling as the anti-R antiserum, specifically reacting with the antigens designated c, e, and g. However, it reacted with very few clones. The restricted reactivity of human sera with this panel of clones was confirmed by using a pool of human hyperimmune IgG (HIG_1) protective in passive transfer assays both in humans (25) and in *Saimiri* (12) (Fig. 2, row d).

In order to identify clones coding for antigenic determinants putatively associated with the membranes of the infected

TABLE 1. Molecular sorting and characterization of the anti-R^+/anti-O^- clones

Cluster[a]	Clone no.	Insert size (bp)[b]	Southern blot[c]
A	1, 3, 7, 9, 16, 19, 20, 23, 28, 29, 33, 38, 39, 40	1,350,980 (3),1,380 (33)	Multiple
B	2, 15, 31, 35	2,350	Multiple
C	5, 25	1,450 (5),3,000 (25)	2
D	36, 37	1,400	1
U	10	1,300	Multiple
	11	580	1
	13	1,450	1
	17	280	Multiple
	18	1,350	Multiple
	21	1,700	1
	22	1,500	1
	24	1,600	ND[d]
	26	1,750	1
	27	1,250	ND
	30	780	1
	32	3,600	1
	41	3,300	1
	42	3,100	1
	43	480	ND
	44	2,300	ND
	45	1,330	ND
	46	2,300	ND

[a] Clones were classified according to the results of hybridization of each nick-translated insert on the whole panel of clones (clusters A, B, C, and D). Unique clones, which failed to hybridize to any other one tested here, were grouped together in group U.

[b] The size of the insert excised or amplified from each clone is indicated for each unique clone. For the clones grouped in clusters, the size of the insert carried by the majority of the clones is the one indicated; the number of the clone showing an insert with a different size is indicated in parentheses.

[c] Number of *Eco*RI fragments hybridizing under 2× SSC stringency is indicated.

[d] ND, not determined.

erythrocytes, additional reagents were used: (i) a serum pool (RIS) from rabbits immunized with membranes prepared from FUP/CP-infected human erythrocytes (Fig. 2, row e) and (ii) *Saimiri* antibodies eluted from the surfaces of O-infected (row f) or R-infected (row g) erythrocytes. The reactivity profiles with the various reagents permitted a classification into five distinct groups of reactivity, as shown in Fig. 2. Clones 5 and 36, which reacted with both human reagents, were placed together in reactivity group 1. The other clones which were not recognized by human antibodies but reacted with reagents raised to or eluted from infected erythrocyte membranes were classified in reactivity groups 2 to 4, according to their specificities. Clones 16, 32, 22, 24, 17, 41, and 45, which reacted with the rabbit serum and with the antibodies eluted from the surfaces of R-infected erythrocytes and reacted faintly or not at all with antibodies eluted from the surfaces of O-infected erythrocytes, formed reactivity group 2. Clones 10 and 13, which reacted with the antibodies eluted from the surfaces of R-infected erythrocytes but failed to react with any of the other reagents tested were classified in group 3. Eight clones which reacted with the rabbit serum but did not react with surface-eluted antibodies formed reactivity group 4. Finally, clones 21, 30, and 46, which did not react with the rabbit serum but still were strongly positive with the anti-R antiserum, were grouped separately in reactivity group 5.

Hybridization patterns on O and R genomic DNAs. The nick-translated inserts from a large number of clones were used to probe Southern blots of O and R genomic DNAs digested with a variety of restriction enzymes. The restriction profiles of O and R DNAs were superimposable, further confirming their genetic relatedness. Figure 3 illustrates some typical examples, obtained with *Eco*RI-restricted DNAs. Probes from clusters A and B, as well as several probes derived from unique clones (Table 1), generated a multiple-band hybridization pattern. Insert 16 (cluster A) hybridized strongly with some fragments and more weakly with numerous others under nonstringent conditions (2× SSC). Under conditions of increased specificity (0.5× SSC), it hybridized to the subset of the three most strongly labeled fragments and in particular with an approximately 1.35-kb *Eco*RI fragment (Fig. 3). Probing with additional inserts from cluster A generated the same multiple-band hybridization pattern (data not shown). Insert 15 (cluster B) produced a distinct, multiple-band pattern under nonstringent conditions, which resolved under stringent conditions into a single, strongly hybridizing, approximately 2.35-kb *Eco*RI fragment. Here again, identical results were observed with insert 2, also belonging to cluster B (data not shown). These results show that the size of the insert carried by the sibling clones grouped in cluster A or in cluster B was identical to that of the *Eco*RI genomic fragment strongly hybridizing with the respective probe. This indicates that the sibling clones in each cluster contain an *Eco*RI genomic fragment, generated by the *Eco*RI digestion performed during construction of the library, indicating incomplete protection of the endogenous *Eco*RI sites by the *Eco*RI methylase treatment.

Some inserts from group U, such as inserts 10 and 18, also produced a multiple-band pattern and, for each digestion tested, reacted with the same bands as those detected with probes derived from cluster A. Interestingly, however, the relative intensities of the signal observed on the various bands differed, and under stringent conditions, inserts 10 and 18 strongly hybridized with a specific, unique fragment different from that detected with cluster A inserts (data not shown). This suggests that the inserts from cluster A, clone 10, and clone 18 are derived from the same group of homologous sequences or genes and that each defines a specific member of that group.

The other inserts hybridized to a more limited number of fragments. As shown in Fig. 3, insert 5 (cluster C) hybridized in 2× SSC to only two (15- and 3.3-kb) *Eco*RI restriction fragments, while inserts 13 and 32 (unique clones from group U) hybridized to single 1.6- and 4-kb *Eco*RI fragments, respectively. Likewise, the other unique members examined so far (clones 11, 17, 21, 22, 26, and 30 to 42) also hybridized to a specific single *Eco*RI fragment (data not shown). In each case, the size of the genomic *Eco*RI fragment differed from that of the insert carried by the recombinant bacteriophages, as predicted for randomly DNase-generated fragments.

Analysis of gene products. We concentrated further investigation on representative members of the various immunological and cross-hybridization groups. We first attempted to affinity purify specific antibodies on recombinant bacteriophage plaques (21) so as to identify the corresponding parasite antigen. Affinity purification unfortunately resulted in elution of denatured Igs which failed to react with replica plates of the bacteriophage itself and with native or SDS-denatured parasite proteins, precluding identification of the corresponding parasite antigen. With the major aim of rapidly obtaining information on the nature of the various clones and in order to design primers to be used in an RT-PCR analysis of expression of the

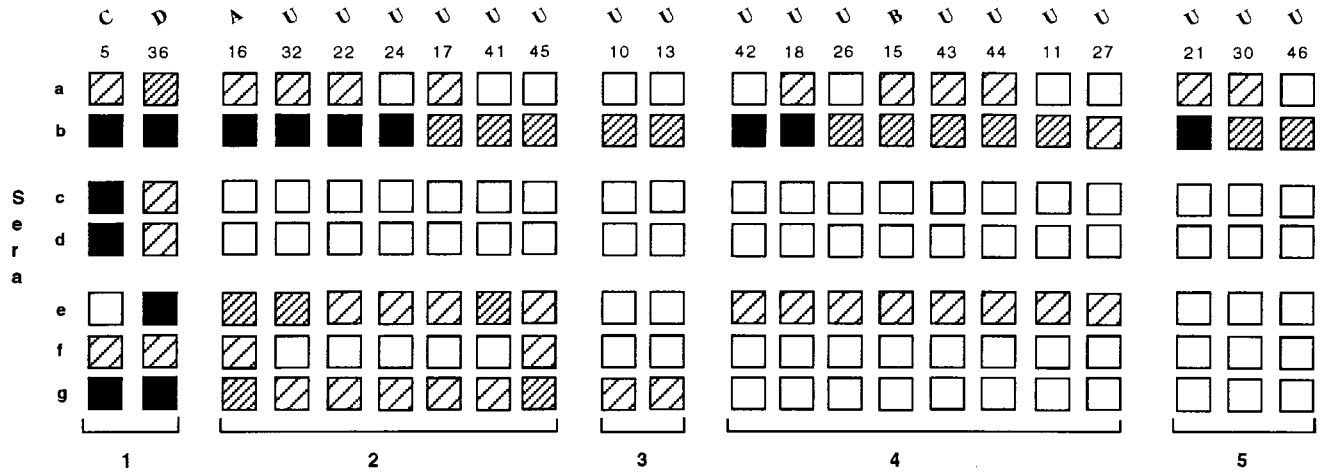

FIG. 2. Summary of the immunological reactivities of 22 representative clones (one from each hybridization cluster and the 18 single clones), reacted by using the plaque immunoassay with various sera: *Saimiri* anti-O and anti-R reagents used to screen the library (a and b, respectively), the HIS pool of human hyperimmune antisera from Senegalese adults (c), the HIG_1 pool of immune human IgG (d), RIS, a rabbit serum raised to the membrane fraction of FUP/CP-infected human erythrocytes (e), and antibodies eluted from the surfaces of O- or R-infected *Saimiri* erythrocytes (f and g, respectively). A reactivity score was established for each plaque immunoassay; the scores used ranged from 4 (very strong) to 1 (marginal or no reactivity) (■, ▨, ▨, and □, respectively). Clones were classified in five groups, depending on their reaction with the human hyperimmune reagents (HIS and HIG_1), the RIS sera, and the surface-eluted antibodies. The hybridization cluster or group for each clone is indicated above the clone number.

various genes in the O and R contexts (see below), the inserts from most clones were partially sequenced. A homology search of the databases indicated that clone 5 was derived from the PfEMP3 gene described by Pasloske et al. (22). Clone 5 encoded 24 copies of the 13-amino-acid repeat described by Pasloske et al. and an additional 135 amino acids upstream from the N terminus of the published PfEMP3 sequence (17a). All other recombinant phages carried novel *P. falciparum* sequences. However, the partial or full sequences of these clones revealed a remarkable common property, namely, the presence of stretches of variable length encoding Asn-rich sequences. There were poly(Asn) stretches ranging from 4 to 14 contiguous Asn residues encoded by most of the clones. Several examples are shown in Fig. 4. Each clone had otherwise a specific

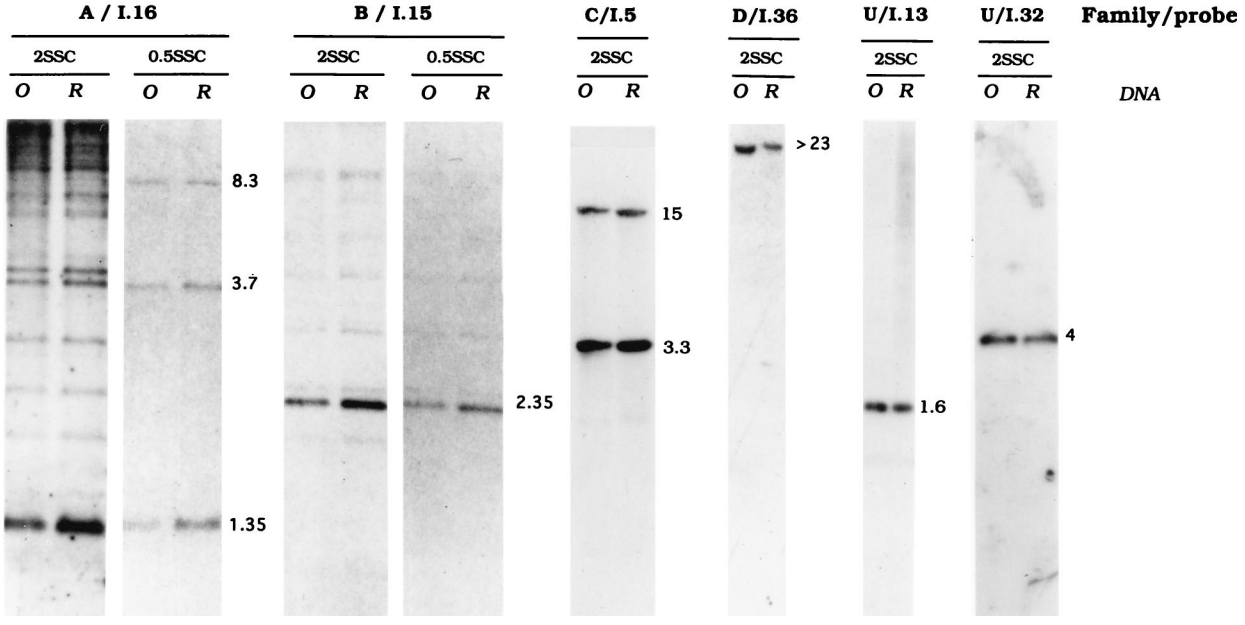

FIG. 3. Southern analysis of *Eco*RI-restricted genomic DNAs from O FUP/SP (lanes *O*) or R FUP/SP (lanes *R*). The restricted DNAs were run in parallel lanes on the same agarose gels before transfer to Hybond-N nylon membranes, as indicated in Materials and Methods. The representative example shown here shows the autoradiographs of filters probed with nick-translated inserts I-16, I-15, I-5, I-36, I-13, and I-32. The probe used and its respective hybridization cluster or group are indicated above the lanes. Blots were autoradiographed first after being washed in 2× SSC at 65°C and later after being washed in 0.5× SSC at 65°C. Results observed under both conditions of stringency are shown for the probes which produced multiple banding patterns (inserts 16 and 15). For the blots probed with insert 5, 36, 13, or 32, the profile with 0.5× SSC (not shown) was identical to the one observed with 2× SSC. The sizes (in kilobases) of the restriction fragments hybridizing with the probes are indicated on the right of each blot.

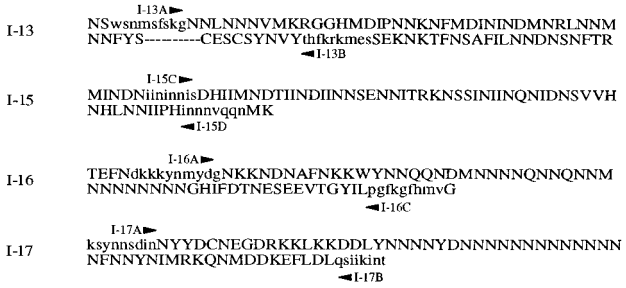

FIG. 4. Partial deduced amino acid sequences of inserts I-13, I-15, and I-16 and complete deduced amino acid sequence of insert I-17. The positions of the primers used for PCR and RT-PCR analyses are indicated in lowercase letters. The sequences of the primers are indicated in Materials and Methods.

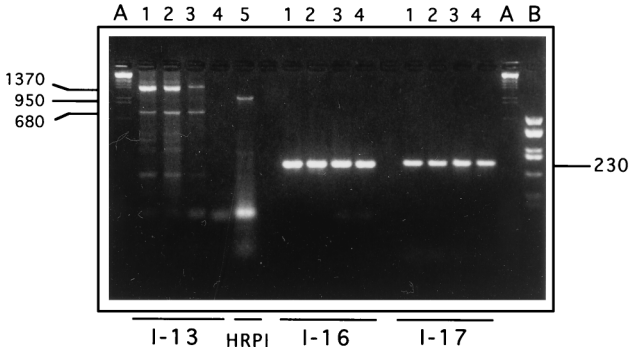

FIG. 5. Analysis of the PCR and RT-PCR products generated with insert I-13-, I-16-, or I-17-specific primers. Templates used were R (lanes 1) or O (lanes 2) genomic DNA or reverse-transcribed R (lanes 3) or O (lanes 4) RNA from FUP/SP parasites. The primers used are indicated below each group of reactions. The RT RNA sample from O parasites which did not generate a product after amplification with the I-13-specific primers (lane 4) was subsequently subjected to a second amplification reaction driven by HRP1 primers, located 5′ and 3′ of the intron of the gene (lane 5) The markers on the right and left are *Hin*dIII-digested λ DNA (lanes A) or *Hae*III-digested pUC18 (lane B) (Sigma). The sizes of the amplified fragments are indicated.

unique sequence, and in addition, the arrangement, number, and primary sequence of the Asn-rich motifs were specific for each insert. This indicated that most anti-R^+/anti-O^- clones coded for antigens containing poly(Asn) clusters, suggesting that the reaction of the anti-R antiserum on this series of clones was due to binding to these Asn-rich clusters. This interpretation was confirmed by the marked reduction of the signal on the various Asn-rich clones after absorption of the anti-R serum onto replica filters of clone λgt11-13 (one such Asn-rich clone), while the reaction on clones which did not possess such Asn-rich motifs (such as clones 5 and 25, as well as a panel of control bacteriophages expressing irrelevant *P. falciparum* or *Toxoplasma gondii* antigens) was unaffected (data not shown). The reaction with the Asn-rich clones remained unchanged after absorption on plaques of an irrelevant recombinant bacteriophage (data not shown). These data indicated that PfEMP3 on one hand and a series of Asn-rich antigens on the other hand were the main targets of the R-specific immune response identified by this strategy.

Previous immunological analysis of O and R parasites showed that PfEMP3 was much more abundant in R than in O parasites (17), an observation consistent with the higher titer of anti-PfEMP3 antibodies in the anti-R antiserum than in the anti-O antiserum. In order to study expression of the various genes coding for Asn-rich motifs identified by this strategy in O and R parasites, we developed RT-PCR assays, using specific primers derived from the 5′ and 3′ sequences of the clones. The locations of the primers used for the amplification reaction are shown in Fig. 4, and their sequences are indicated in Materials and Methods. PCRs were first done with genomic DNA. As shown in Fig. 5, priming of R and O DNAs with clone 16- or clone 17-specific oligonucleotides amplified a single PCR fragment of the predicted size (230 bp in both cases), which was identical for both parasite types (lanes 1 and 2, respectively). Clone 13-derived primers generated three fragments, one of the predicted 1,370 bp and two additional ones of 680 and 200 bp, the significance of which is uncertain. RT-PCR analysis was then carried out with the same cDNAs preparations for all reactions. R and O cDNAs were prepared by randomly primed reverse transcription of RNAs prepared from asynchronous parasites collected from R- or O-infected *Saimiri* monkeys. No transcript was detected in the cDNA preparations with clone 15-specific primers (not shown). RT-PCR products were detected in both R and O cDNA preparations (Fig. 5, lanes 3 and 4, respectively) with clone 16- and clone 17-specific primers, indicating that the corresponding mRNA was present in R as well as in O parasites. In contrast, the clone 13-specific assay showed that while both the 680- and 1,350-bp products were generated from the R cDNA (lane 3), no PCR product was detected in the reaction where the O cDNA preparation was used as the template (lane 4). To verify that this was not due to degradation of the O cDNA introduced in this tube, primers specific for the HRP1 gene, which is known to be expressed in both O and R parasites (7, 15), were added together with fresh *Taq* polymerase, and a second PCR was carried out. This resulted in amplification of a 1,000-bp fragment (Fig. 5, lane 5), corresponding to the size predicted for an adequately spliced HRP1 cDNA. This indicated that the quality of the cDNA preparation introduced in the tube was satisfactory and, moreover, that contamination by genomic DNA was negligible. Therefore, we concluded that the clone 13-specific mRNA was present in the R but not in the O parasites.

Characterization of clone 13. The deduced amino acid sequence of clone 13 (Fig. 6) was characterized by a high content of Asn residues, which represented 29% of the amino acids and were frequently clustered. The Asn content reached 50% in the region between amino acids 130 and 260. As indicated above, searches of various databases indicated that clone 13 carries a novel *P. falciparum* sequence.

As this clone reacted exclusively with anti-R reagents (reactivity group 3 in Fig. 2), including with antibodies eluted from the surfaces of R-infected erythrocytes and with several other individual anti-R antisera (data not shown), we decided to map the binding sites of the surface-eluted antibodies. We carried out a Pepscan analysis of the entire deduced amino acid sequence of clone 13 with a set of 83 synthetic 15-mer peptides overlapping by 9 residues. The reactivity of the antibodies eluted from the surfaces of O-infected erythrocytes was marginal, while significant reactions were observed with antibodies eluted from the surfaces of R-infected erythrocytes. Figure 7 shows that the reaction concerned mainly four specific peptides, three of which contained Asn-rich stretches: INNNNNNIN NNNNIN, NINNNNNINNNNNSV, and NNNNNNFFQNN NN. Interestingly, the strongest reaction was obtained with a specific unique polypeptide which is also Asn rich: VTNNLG STNFNVNNQ, which was called peptide 13. Other Asn-rich peptides did not produce any signal, indicating that the reactions observed with the subset of four peptides were specific.

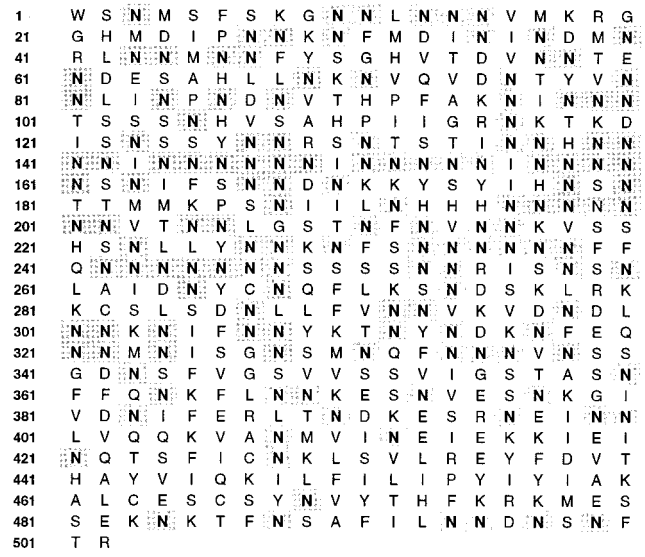

FIG. 6. Deduced amino acid sequence of clone 13. Asn residues are in boldface and shaded.

These data indicated that surface affinity-purified antibodies reacted with specific peptides derived from clone 13 and suggested that Asn-rich determinants were exposed on the surfaces of R-infected erythrocytes. Antibodies to peptide 13 were raised in rabbits and used to probe O and R immunoblots. As shown in Fig. 8, these antibodies reacted with a high-molecular-mass antigen of approximately 250 kDa present in R and absent from O parasites. Whether the additional, lower-molecular-mass bands correspond to proteolysis fragments or cross-reacting antigens is still unclear. This exclusive pattern confirms the RT-PCR data and furthermore indicates that there was no cross-reaction with other potentially cross-reacting proteins containing Asn-rich residues. Unfortunately, the antibodies raised to this linear sequence failed to react by immunofluorescence on fixed or live parasites, probably because the peptide does not mimic the conformation of the antigen in situ and elicited antibodies reacting with the denatured parasite antigen only.

Analysis of *var* gene expression. In order to determine whether clone 13 was part of a *var* gene, PCRs combining clone 13-derived primers with *var*-specific primers were carried out. No product was generated (data not shown). A nested PCR primed in a reaction with a DBL-1-specific forward primer (varA5.2) and a DBL-4-specific reverse primer (DBL4anti-S) was then carried out. The first reaction generated an abundant, approximately 4-kb band, which was then used as template in a second reaction primed with clone 13-specific primers. Two pairs of clone 13-specific primers were used, but no product was generated in either case (data not shown).

Other PCRs with an exon I-derived forward primer coupled to XCRI, an exon II-derived reverse primer, did amplify large genomic fragments of *var* genes. However, none of the nested PCRs carried out with these fragments and insert 13-derived primers was able to amplify any product, strongly suggesting that clone 13 is not present within the region of the *var* genes analyzed.

In order to identify the *var* genes expressed specifically by O and R variants, RT-PCR was carried out with the UNI-EBP primers reported to amplify DBL domains from *var* genes and other DBL domain-containing genes (23). As shown in Fig. 9, RT-PCR carried out with O or R cDNA (lanes 3 and 4, respectively) generated different patterns, contrasting with the very similar profiles observed for the UNI-EBP-driven PCR with O and R genomic DNAs (lanes 1 and 2, respectively). The predicted *eba-175*-derived 350-bp band (23) was present in O and R cDNAs. A 390-bp band was observed in the O cDNA (Fig. 9, lane 3) and was faintly detected in the R cDNA (lane 4). Conversely, a 460-bp band was observed in the R cDNA (lane 4) and was more faintly detected in the O cDNA. Sequencing indicated that the 390- and 460-bp bands contained distinct DBL-3 domains (data not shown).

DISCUSSION

Since a differential screening of an expression library relies on the comparison of the intensities of the signals generated by two reagents with distinct specificities, we have adjusted the working dilutions of the anti-O and anti-R reagents according to their immunoblot titers on *P. falciparum* extracts, assuming that presentation of *P. falciparum* antigens on immunoblots would be the closest approximation for nitrocellulose filters containing replicas of bacteriophage plaques. This assumption was validated by the fact that the majority of the positive clones (165 of 236) produced similar signals with both reagents. A subset of 46 clones showed an anti-R^+/anti-O^- reactivity, indicating that the antibody titers to the corresponding antigenic determinants were higher in the anti-R reagent than in the anti-O sera and hence that the immune response against these antigens could be interpreted as variant specific. Molecular analysis indicated that many of these were redundant. A total of 25 different anti-R^+/anti-O^- clones have been isolated (3 distinct clones in cluster A, 1 clone type each in clusters B and D, 2 distinct clones in cluster C, and 18 single clones in group U). Eleven clones reacted with the antibodies eluted from the surfaces of the R-infected erythrocytes and faintly or not at all with those eluted from the surfaces of the O-infected erythrocytes. The reaction of the surface-eluted antibodies with a subset of clones is consistent with their reaction with a limited number of antigens on immunoblots (17). This suggests that these clones encode determinants either exposed on the surfaces of the R-infected erythrocytes or mimicking surface-exposed ones. Sequencing revealed that a large proportion of clones encoded Asn-rich determinants and indicated that two major antibody specificities could be considered specific for the anti-R response: antibodies reacting with PfEMP3 and antibodies reacting with Asn-rich motifs.

The strongest reaction with surface-eluted antibodies was detected with clone 5, which is derived from the PfEMP3 gene. PfEMP3 is a conserved protein associated with the erythrocyte membrane (22). The failure of clone 5 to react with the rabbit antiserum raised to membranes of human erythrocytes infected with FUP/CP parasites was due to the fact that this serum does not contain any anti-PfEMP3 antibodies, as the PfEMP3 gene is deleted in the FUP/CP strain. Clone 5 reacted strongly with antibodies eluted from the surfaces of R-infected parasites. Data published by Pasloske et al. (22) indicate that PfEMP3 is not surface exposed in human infected erythrocytes from in vitro cultures. The situation might be different here. In *Saimiri*-infected erythrocytes collected from splenectomized animals, surface accessibility of parasite-derived molecules might differ from that in human cells cultivated in vitro. In addition, the recombinant protein expressed by clone 5 is not strictly identical to the protein used by Pasloske et al. (22) to raise anti-PfEMP3 antibodies, as it contains an additional N-terminal 135-amino-acid sequence. We cannot exclude the possibility that this extra sequence is the region where binding

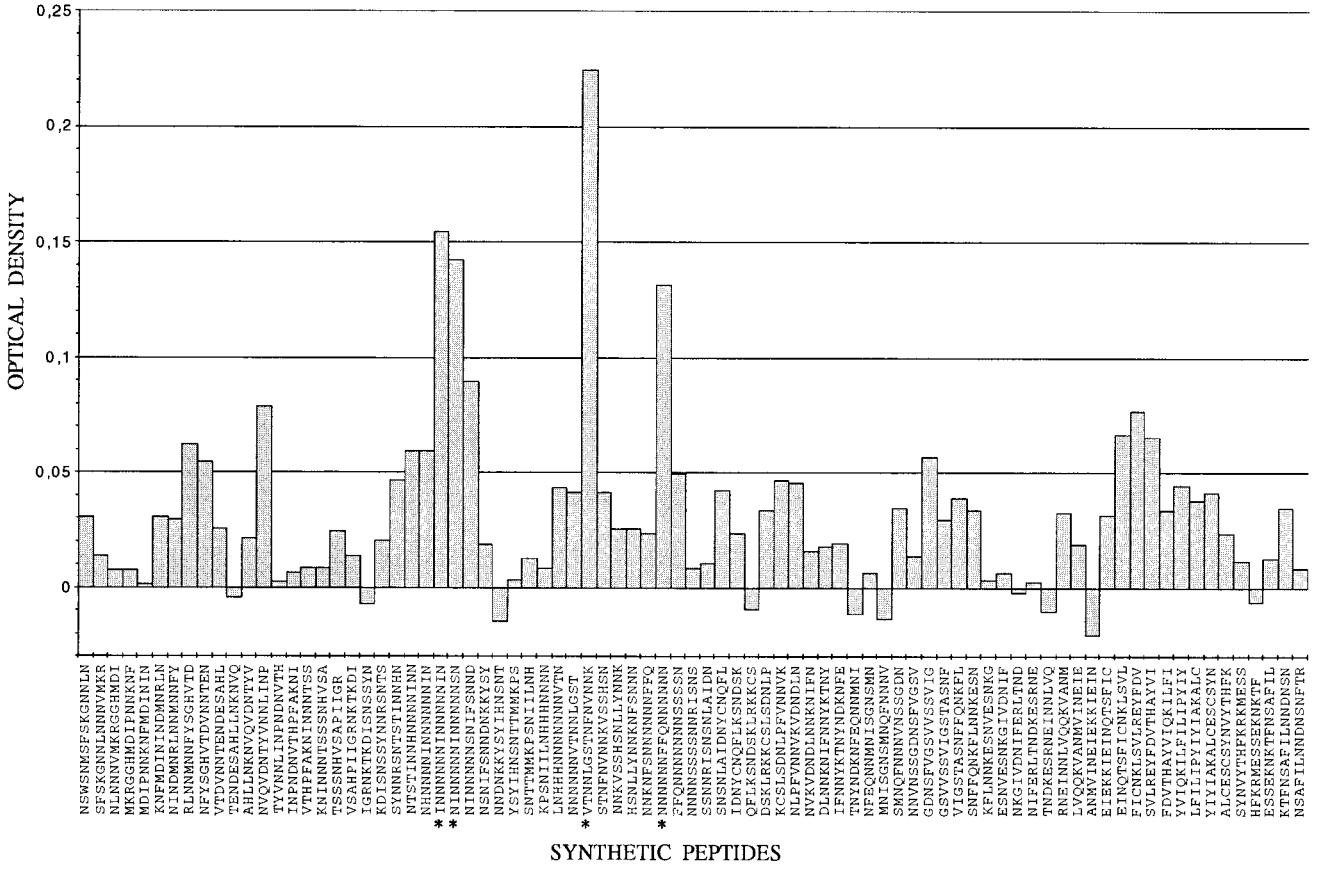

FIG. 7. Pepscan analysis of the deduced amino acid sequence of clone 13, using antibodies eluted from the surfaces of infected erythrocytes at a 1:200 dilution. The peptides were produced as noncleavable peptides on pins. The reactivity of a pool of sera collected from nonimmune *Saimiri* monkeys and the faint reactivity of antibodies eluted from the surfaces of O-infected erythrocytes were deduced from signals obtained with the antibodies eluted from the surfaces of R-infected erythrocytes. The first peptide (on the left) contains two extra amino acids (SF) encoded by the *Eco*RI linker used to construct the library. These residues are omitted in the sequence shown in Fig. 6.

of the surface-eluted antibodies occurs. While the surface exposure of PfEMP3 in R-infected erythrocytes is still to be confirmed, it must be stressed that the present results convincingly show that the titer of antibodies reacting with PfEMP3 was significantly higher in the anti-R than in the anti-O antisera. This variant-dependent immune response to PfEMP3 is interesting in view of our previous data which showed larger amounts of PfEMP3 in R than in O parasites (17). The work reported here indicates that the increased expression of PfEMP3 by R parasites and/or its surface exposure results in an increased immunogenicity of this protein in the R context.

The second major specificity identified for the anti-R response was the reaction with Asn-rich motifs. Indeed, many of the anti-R^+/anti-O^- clones sequenced so far contain Asn-rich motifs. There was no association between the presence of Asn-rich motifs and assignment to any specific hybridization cluster or immunological reactivity group. The reaction on the various Asn-rich clones was markedly reduced by prior absorption of the antiserum on plaques of a single clone which expresses an Asn-rich protein (clone 13). This suggests that the reaction of the anti-R reagent involved essentially binding to the Asn-rich motifs and that the large number of Asn-rich clones isolated here have been detected as a result of a cross-reaction of the anti-R reagent on the various Asn-rich motifs expressed by individual clones. Such cross-reactions of Asn-rich motifs have already been observed by other groups (1, 29). The isolation of numerous Asn-rich clones from a genomic *P. falciparum* library is not surprising, as the *P. falciparum* repertoire of Asn-rich proteins is quite large. Indeed, poly(Asn) stretches are usually encoded by $(AAT)_n$, which occurs frequently in such an A/T-rich genome (34). An illustration of the richness of the *P. falciparum* genome in such sequences was the multiple-band hybridization patterns observed on Southern blots under nonstringent conditions. Several groups have reported the cloning of genes coding for Asn-rich sequences or Asn clusters, and interestingly, some of these genes have been isolated by using antisera raised to or eluted from infected erythrocyte membranes (11, 15, 33). Other Asn-rich sequences, scattered in numerous genes, are found in databases. However all of the clones isolated here present novel Asn-rich sequences. The difficulty caused by the presence of such a cross-reacting specificity was in identification of the antigen expressed by R parasites which elicited such anti-Asn-rich antibodies. A likely hypothesis is that the antigen in question is specifically expressed by R parasites or, alternatively, that it is surface exposed on R but not O parasites, eliciting antibodies in the R but not in the O context. We therefore attempted to analyze expression of the genes defined by various clones coding for Asn-rich motifs in O and R parasites. As anticipated for clones isolated from a genomic DNA library, which permits the isolation of expressed as well as silent sequences, we did not find any evidence for transcription in either R or O parasites

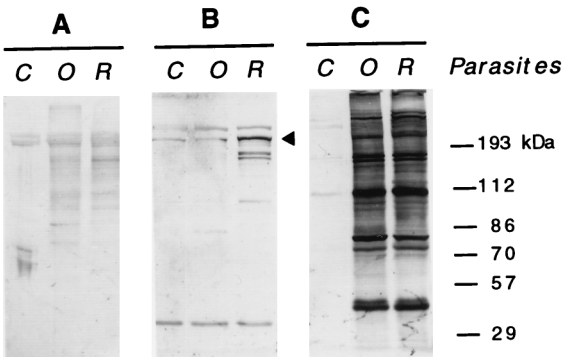

FIG. 8. Immunoblot analysis of O and R SDS extracts. Extracts were loaded onto an SDS–7.5% polyacrylamide gel, blotted onto a nitrocellulose filter, and reacted with a 1:200 dilution of a rabbit preimmune serum (A), with a 1:200 dilution of a rabbit antiserum raised to peptide 13 (B), and with a 1:400 dilution of SIS antiserum (C). Rabbit antibodies were detected with anti-rabbit IgG alkaline phosphatase conjugate adjusted to a 1:15,000 dilution (Sigma). Monkey antibodies were visualized with anti-*Saimiri* IgG rabbit antibodies (1:1,000 dilution) and a commercial anti-rabbit IgG alkaline phosphatase conjugate adjusted to a 1:15,000 dilution (Sigma). *C*, noninfected erythrocyte extract analyzed in parallel. The migrations of prestained molecular mass markers (Sigma) are indicated on the right.

for some genes (e.g., clone 15). Other clones, such as clones 16 and 17, are derived from genes expressed in both parasite types. On the other hand, there was convincing evidence that expression of the gene defined by clone 13 was switched on in R parasites and silent in O parasites. Importantly, the plaques of λgt11 clone 13 reacted with the anti-R-specific antisera and also with the antibodies eluted from the surfaces of R-infected erythrocytes, but they failed to react with any anti-O reagent. The localization of this protein in R parasites is still unclear, as specific antibodies reacting with the parasites by immunofluorescence are not yet available. Affinity purification of antibodies on replica plates of clone 13 was unsuccessful, and attempts to express the protein encoded by clone 13 as a recombinant antigen have so far failed. The Pepscan analysis with surface-eluted antibodies outlined a few peptides containing Asn-rich motifs and allowed identification of peptide 13, which had a unique sequence. Antibodies raised to peptide 13 reacted with a high-molecular-weight antigen present in R but not in O parasites. Our data are compatible with the interpretation that gene 13 codes for a protein exported to the surface of the R-infected erythrocyte, but this awaits definitive demonstration. We can nevertheless conclude that Asn motifs are exposed on the surfaces of the R-infected erythrocytes and not on the surfaces of O-infected erythrocytes, as antibodies eluted from the surfaces of the R-infected erythrocytes reacted with Asn-rich motifs in the Pepscan analysis. Preliminary evidence indicates that treatment with *E. coli* L-asparaginase results in profound modifications in the surface reactivity of R-infected but not O-infected erythrocytes, further supporting this conclusion.

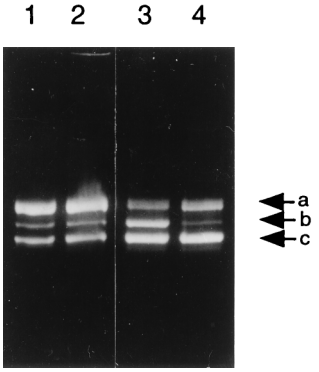

FIG. 9. Analysis of the PCR and RT-PCR *var* gene products generated by using UNI-EBP primers. The templates used were genomic DNA from O (lane 1) or R (lane 2) parasites or reverse-transcribed O (lane 3) or R (lane 4) RNA. The arrows indicate the R-specific 460-bp (a), O-specific 390-bp (b), and *eba-175*-specific 350-bp (c) products.

Immunological analysis of O and R parasites showed that the switch from the O to the R phenotype was accompanied by the expression of a distinct PfEMP1 molecule, with the presence of a larger amount of PfEMP3 concomitant with a reduction in the HRP1 content (17). The work reported here, which was undertaken to identify the potential targets of the variant-specific immune response, confirms and expands on these findings. By RT-PCR analysis we have demonstrated that distinct *var* genes were expressed in O and R parasites. We have not yet identified which among the clones isolated here (if any) corresponds to a *var* product. One obvious candidate was clone 13, which could encode a variable domain of a *var* gene. Attempts to carry out RT-PCR assays combining insert 13-derived and *var*-derived primers have been unsuccessful. Further investigations by nested PCR with amplified large *var* fragments and clone 13 primers were also unsuccessful. The present data therefore do not support the hypothesis that clone 13 is a variable region of an R-specific *var* gene. Cloning of the full-length gene from which clone 13 is derived and of the R-specific *var* gene identified by the UNI-EBP-primed RT PCR should help resolve this issue.

In summary, the results reported here show that we have identified two antibody specificities which characterize the anti-R immune response, namely, high levels of antibodies reacting with PfEMP3 and with Asn-rich motifs. This has been observed with several individual monkey sera. These data provide evidence that the variant-specific immune response concerns several distinct target antigens, two of which have been identified in the work reported here, and depends on the level of expression and possibly surface exposure of these antigens in different variants. The involvement of these antigens in variant-specific protection is under study.

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