intensively studied reptile groups in terms of karyology (*17*), only one report of triploidy has appeared (*14*). Diploid-triploid mosaicism has not been observed in other animals, although cells of certain tissues increase DNA content through endopolyploidy, gene amplification, or polyteny (*18*). Mosaicism in *P. platycephala* appears to be distinct from these phenomona because it is not limited to a specific tissue or cell type.

Our data demonstrate the existence of a diploid-triploid mosaic system in *P. platycephala*. Some individuals were predominantly triploid and others diploid (Table 1). Haploid gametes were produced by both reproductively active mosaic males examined (AK1227 and AK6630), thus indicating the fertility of these individuals. A female specimen (*14*) also was apparently fertile as she laid an egg while in captivity. Thus, triploidy in *P. platycephala* is not associated with parthenogenetic reproduction as is the case in all other normally triploid reptiles, nor is it associated with sterility as occurs in abnormal triploids (*8*).

We believe these mosaic individuals are autopolyploids and not the result of an interspecific hybridization event because (i) the individuals in question are morphologically typical *P. platycephala*, (ii) initial electrophoretic data do not indicate a higher level of heterozygosity in mosaics compared to diploids (*16*), and (iii) the karyotype does not suggest allopolyploidy. *Platemys platycephala* differs from all other members of the family Chelidae in diploid number and chromosome morphology. The other four species of the genus *Platemys* have karyotypes with diploid numbers of approximately 48 to 50 with several pairs of biarmed chromosomes (*19*).

The mechanism by which diploid-triploid mosaicism could be maintained in a sexually reproducing organism is unclear. A zygote must begin as either a diploid or triploid, with subsequent changes in chromosome number occurring in somatic or gonadal tissues during development. In either case, there may be an increase in ploidy to hexaploid and subsequent reduction to diploid or triploid. Furthermore, it is unknown whether the switch from diploid to triploid (or vice versa) occurs only once in development or independently in different tissues.

Mosaics would have the benefits of both sexual reproduction and polyploidy. Autopolyploids possess structural and biochemical properties not found in their diploid progenitors (*5*) and may have larger cell size, and slower development than diploids. They also show an increased ecological adaptation that allows them to exploit habitats that may not be available to diploids. Gene dosage effects might allow genes to be regulated differently in polyploids than in diploids (*5*). The presence of fertile male diploid-triploid mosaic specimens of *P. platycephala* and the presumably fertile female suggests that this cytogenetic system occurs naturally. Geographic variation is indicated as this population (or populations) is in Surinam but only diploids are known from Bolivia and Brazil.

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Isolation of the Gene for a Glycophorin-Binding Protein Implicated in Erythrocyte Invasion by a Malaria Parasite

Abstract. Plasmodium falciparum, *the most lethal of the malarial parasites that infect humans, undergoes three cycles of development in its vertebrate host and elicits stage-specific immune responses. This stage specificity of the immune response has made it difficult to isolate antigens that would be useful in developing a vaccine against malaria. A complementary DNA clone for a glycophorin-binding protein of* Plasmodium falciparum *merozoites has been isolated and characterized. The protein interacts with glycophorin, the erythrocyte receptor, during invasion of the host cell by the parasite. Antigenic determinants of this protein expressed in* Escherichia coli *have been used to produce antibodies to a glycophorin-binding protein. The antibodies show schizont-specific immunofluorescence and react with the merozoite protein. The primary sequence of these determinants reveals a 150-nucleotide tandem-repeating sequence coding for a 50-amino-acid repeat. The characterization of the* Plasmodium falciparum *glycophorin-binding protein represents one approach toward designing serologic agents to block the parasite's development in the vertebrate host.*

The malarial parasite has three cycles of development in its vertebrate host: the hepatic cycle; the asexual blood cycle, which includes the extracellular merozoite and the intraerythrocytic schizont; and the sexual gametocyte cycle. The erythrocyte cycle is responsible for the clinical symptoms of the disease and mortality. One of the obstacles in the development of a vaccine against the malarial parasite is that the immune response in the host is strictly stage specific. Thus, efforts have been directed toward identifying antigens of each stage which, when combined, could be used for vaccination.

The major surface antigen of sporozoites of *Plasmodium falciparum* has been

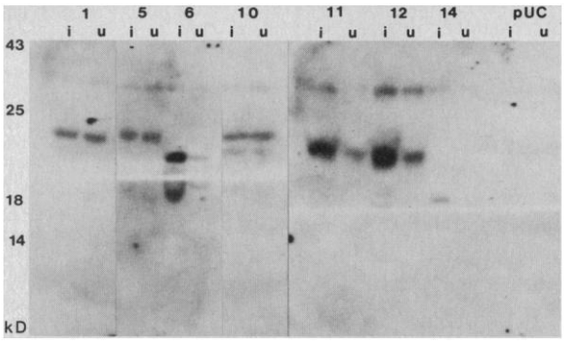

Fig. 1. Western blot analysis of fusion proteins expressed in *E. coli* between the *P. falciparum* GBP and β-galactosidase. Identical extracts (equivalent to 200 μl of *E. coli* or 8×10^7 bacteria) of induced (i) or uninduced (u) JM103 cultures containing cDNA clones expressing GBP determinants were fractionated by 12.5 percent SDS-PAGE and probed with rabbit antiserum to GBP155,130 (1:100 dilution) and then with ^{125}I-labeled protein A. Induction occurred for clones 6, 11, and 12 and for clone 14 at a lower level of expression. Constitutive expression occurred for clones 1, 5, and 10. No protein band is visible for the pUC-9 control. Clone 6 encodes a 28-kD fusion protein, yielding a major degradation product of 20 kD. Subsequent inductions for shorter times yielded greater proportions of the 28-kD band.

cloned in *Escherichia coli*, and its role in protection of humans is now being considered (*1, 2*). The surface of the merozoite appears to be more complex than that of the sporozoite and may be made up of many antigens. Several have been identified (*3*), and antibodies to some of these proteins are found in sera from infected subjects (*4*). A merozoite antigen suitable for use in immunization would be one that is nonvariant and is critical for survival of the parasite. With this in mind, we have identified two merozoite surface proteins of *P. falciparum* that appear to recognize the host erythrocyte. These proteins of 155 and 130 kilodaltons (kD) interact with high affinity and specificity with glycophorin, the erythrocyte receptor (*5*). Recent studies suggest that the 130-kD glycophorin-binding protein (GBP) is derived from the 155-kD GBP (*5*). Antibodies to these proteins effectively inhibit merozoite invasion of erythrocytes, which suggests that GBP's play a crucial role in the host-parasite interaction (*5*). The 155-kD GBP appears to be similar to the antigen identified on the surface of ring-infected erythrocytes, which also binds glycophorin (*6, 7*). Furthermore, there is a correlation between the presence in human serum of antibodies to the proteins and the acquisition of clinical immunity in humans (*6, 7*).

We now report the isolation of complementary DNA (cDNA) clones expressing a *P. falciparum* GBP selected with the antiserum that recognizes the 155- and 130-kD GBP (GPB155,130). Serum from mice immunized with *E. coli* extracts expressing GBP determinants was used to characterize the cloned protein. The immunofluorescence staining pattern with the mouse antiserum was specific for the schizont and merozoite surface and was identical to the pattern with rabbit antiserum to the schizont-derived GBP155,130. Immunoblot analysis of merozoites indicated that the mouse antiserum recognized a 130-kD protein that comigrated with a GBP. The primary sequence of these clones reveals a repeating nucleic acid sequence of 150 nucleotides coding for a 50-amino-acid tandem-repeat sequence. The gene for this protein is conserved in two strains of *P. falciparum* (Gambia and Honduras) and is expressed as a 6.6-kilobase (kb) messenger RNA (mRNA) that accumulates in late schizonts.

A cDNA library for late-stage schizonts (42 hours after invasion) of *P. falciparum* (FCR-3, Gambia) was constructed in the expression vector pUC-9

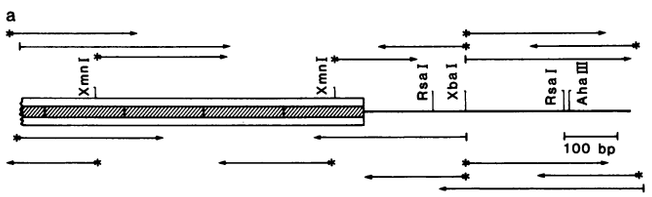

Fig. 2. (a) Restriction map and sequencing strategy for *P. falciparum* GBP clone pGBP-6. The coding sequence is indicated by the open rectangle, showing the tandemly repeating sequence (smaller rectangle); the 3′ untranslated sequence is indicated by the straight line. Arrows above the figure indicate DNA sequences from the coding strand; arrows below the figure represent sequences from the noncoding strand. Sequences determined by dideoxy chain termination (*21*) are indicated by arrows with blunt tails; sequences determined by chemical degradation (*22*) are denoted by arrows with asterisks as tails. Representative restriction sites are indicated. The map is oriented 5′ to 3′ (left to right); bp, base pairs. (b) Nucleotide sequence for pGBP-6 cDNA clone. The coding strand is shown with the predicted amino acid sequence above the nucleotide sequence. The sequence begins at the homopolymeric dG tails to demonstrate the continuous open reading frame with β-galactosidase. (c) Alignment of the predicted amino acid sequence of the repeat unit found in pGBP-6. A partial repeat begins the sequence.

b

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Gly Gly Gly Gly Gly Ala Asn Thr Asp Pro Asn Asp Asp Val Glu Arg Arg
GGG GGG GGG GGG GGG GCT AAC ACC GAT CCA AAT GAT GAC GTA GAA AGA AGA      51

Asn Ala Glu Asn Lys Glu Glu Leu Thr Ser Ser Asp Pro Glu Gly Gln Ile Met
AAT GCC GAG AAT AAA GAA GAA TTA ACC AGC TCA GAC CCA GAA GGT CAA ATA ATG  105

Arg Glu Tyr Ala Ala Asp Pro Glu Tyr Arg Lys His Leu Glu Ile Phe His Lys
AGA GAA TAT GCT GCT GAT CCA GAG TAT CGT AAA CAC TTA GAA ATA TTT CAT AAA  159

Ile Leu Thr Asn Thr Asp Pro Asn Asp Glu Val Glu Arg Arg Asn Ala Asp Asn
ATA TTA ACT AAC ACC GAT CCA AAT GAT GAA GTA GAA AGA AGA AAT GCC GAT AAT  213

Lys Glu Asp Leu Thr Ser Ala Asp Pro Glu Gly Gln Ile Met Arg Glu Tyr Ala
AAA GAA GAC TTA ACT AGT GCC GAT CCA GAA GGT CAA ATA ATG AGA GAA TAT GCC  267

Val Asp Pro Glu Tyr Arg Lys His Leu Glu Ile Phe Tyr Lys Ile Leu Thr Asn
GTT GAT CCA GAA TAC CGT AAA CAC TTA GAA ATA TTT TAT AAA ATA TTA ACT AAC  321

Thr Asp Pro Asn Asp Glu Val Glu Arg Arg Asn Ala Asp Asn Lys Glu Asp Leu
ACC GAT CCA AAT GAT GAA GTA GAA AGA AGA AAT GCC GAT AAT AAA GAA GAT TTA  375

Thr Ser Ala Asp Pro Glu Gly Gln Ile Met Arg Glu Tyr Ala Pro Asp Pro Glu
ACC AGT GCC GAT CCA GAA GGT CAA ATA ATG AGA GAA TAT GCT CCC GAT CCG GAA  429

Tyr Arg Lys His Leu Glu Ile Phe Tyr Lys Ile Leu Thr Asn Thr Asp Pro Asn
TAC CGT AAA CAC TTA GAA ATA TTT TAT AAA ATA TTA ACT AAC ACC GAT CCA AAT  483

Asp Asp Val Glu Arg Arg Asn Ala Asp Asn Lys Glu Asp Leu Thr Ser Ala Asp
GAT GAC GTA GAA AGA AGA AAT GCC GAT AAC AAA GAA GAT TTA ACT AGT GCC GAT  537

Pro Glu Gly Gln Ile Met Arg Glu Tyr Ala Ala Asp Pro Glu Tyr Arg Lys Asp
CCA GAA GGT CAA ATA ATG AGA GAA TAT GCT GCT GAT CCA GAA TAT CGT AAA GAC  591

Leu Glu Ile Phe His Lys Ile Leu Thr Asn Thr Asp Pro Asn Asp Glu Val Glu
TTA GAA ATA TTT CAT AAA ATA TTG ACT AAT ACC GAT CCA AAT GAT GAA GTA GAA  645

Arg Gln Asn Ala Asp Asn Asn Glu Ala
AGA CAA AAT GCT GAT AAT AAC GAA GCA TAAAATCAAAGAAAATTTACAAATGAATTAGAATG  707

TATTATTATGATAATTAAGAAATGTATGAATTTGAATGTAAAAATTCAATTGTTTATAATAATGTACAATG  778

TTTTAATAATTAATTATTAAAACTGAAGTATATAATACTATTTATGGGATTTTATAATATCTAGATGAGAA  849

AGAAAAAAAGAATTTTAACTTTGTTAATAGATATTATGAAATGTTATGTTTTGAAGAACATATAATGAAAT  920

CGCTTTGGTTACAGTATGTGAAATGCTATTAATAGAAACGGCATTTTTTTGTTCATTATTATTTGTTTCTT  991

AAATACTAAATAAATAAGTTTGAACCGTGCAAATGTTGTACACTTTAAAAATATTCTTTGAATAAAACTTA 1062

AAAATAGAAACTTCGTCCTAAAAATTAGATGACATGTTGAATTAATATGGAAAATGCATAAGATGTGTAAG 1133

AGAAAAAA                                                               1141
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c

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1         10        20        30        40        50

                                .......ANTDPNDDVERRNAENKEE
LTSSDPEGQIMREYAADPEYRKHLEIFHKILTNTDPNDEVERRNADNKED
LTSADPEGQIMREYAVDPEYRKHLEIFYKILTNTDPNDEVERRNADNKED
LTSADPEGQIMREYAPDPEYRKHLEIFYKILTNTDPNDDVERRNADNKED
LTSADPEGQIMREYAADPEYRKDLEIFHKILTNTDPNDEVERQNADNNEA
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by means of standard methods (*8, 9*). A library of 50,000 recombinants was screened for expression of antigenic determinants recognized by a rabbit antiserum to GBP155,130 (*6*) by an in situ colony immunoassay (*10*). Ten independent clones were obtained, colony-purified, and characterized by restriction analysis, cross-hybridization, and expression of immunoreactive proteins. For further characterization of these clones, the plasmid DNA's were transformed into *E. coli* JM103 (*11*), a strain inducible by isopropylthio-β-galactoside (IPTG), to identify fusion proteins between the β-galactosidase gene of pUC-9 and the cDNA inserts. Extracts (*12*) from induced and uninduced cultures were fractionated on 12.5 percent sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred to nitrocellulose filters, and probed with rabbit antiserum to GBP155,130 or with normal rabbit serum. Four isolates showed inducible proteins that reacted with the immune serum, and the other isolates showed constitutive expression (Fig. 1). Of the ten isolates, seven of which are shown in Fig. 1, the immunoreactive proteins varied in molecular size from 20 to 35 kD, which corresponds to the sizes of the inserts [400 to 1200 base pairs (bp)]. No immunoreactive band appeared with the vector alone (Fig. 1) or when preimmune serum was used. The inducible protein products indicate that fusion proteins between the ten amino acids of β-galactosidase and the cDNA inserts were constructed and were under the transcriptional control of the *lac* promoter. The inserts isolated from these clones all cross-hybridized, and identical genomic fragments were detected by Southern blot analysis, which showed their relatedness.

Clone 6, which exhibited inducible expression and contained the largest cDNA insert, was chosen for further analysis (Fig. 2, a and b). As expected, a continuous open reading frame was found between the ten amino acids of the pUC-9 vector and the cDNA insert that had had an oligo(dC) tail added at the 3′ end before cloning. A continuous open reading frame begins at position 1 and ends at position 672. Further analysis revealed that the sequence contains a tandemly repeating DNA sequence of 150 nucleotides encoding 50 amino acids (Fig. 2c). Four complete repeats occur in this clone, terminating with TAA (T, thymidine; A, adenine) at positions 673 to 675 (Fig. 2b) and an AT-rich sequence, which is characteristic of noncoding sequences for *Plasmodium* species (*1, 13, 14*). Because all the clones cross-hybridized and reacted with the rabbit antiserum to GBP155,130, we conclude that an epitope recognized by that serum is encoded within the 50-amino-acid repeat. Clone 6 revealed that the repeat occurs at the carboxyl end of the protein. No other repeats were evident by dot matrix analysis, nor was there an appreciable degree of identity to other proteins in the National Biomedical Research Foundation protein sequence library.

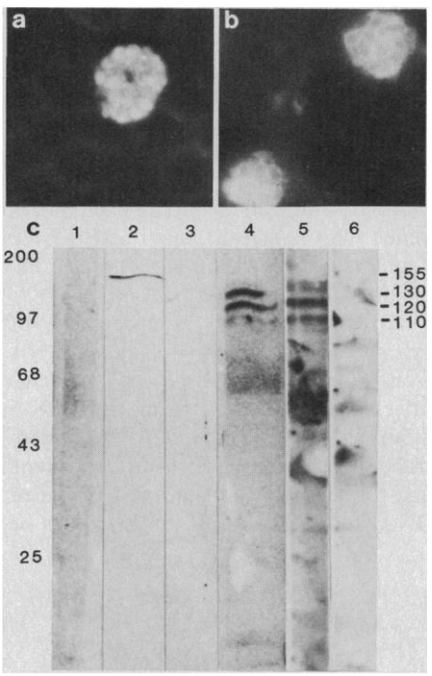

Fig. 3. (a and b) Indirect immunofluorescence of *P. falciparum* GBP antigen with mouse antibodies to pGBP-6. Thin smears of *P. falciparum* cultures were fixed in acetone for 10 minutes at 4°C. Dried culture smears were incubated at room temperature for 30 minutes with (a) mouse α-pGBP-6 expressed in *E. coli* and (b) rabbit antiserum to GBP155,130. Culture smears were washed extensively in phosphate-buffered saline and incubated with (a) FITC (fluorescein isothiocyanate) rabbit antiserum to mouse immunoglobulin G and (b) FITC goat antiserum to rabbit immunoglobulin G (Cappel). (c) Western blot of *P. falciparum* proteins stained with antiserum to GBP determinants. Late-stage *P. falciparum* proteins were fractionated by 10 percent SDS-PAGE and transferred to nitrocellulose at room temperature overnight in tris-glycine-methanol buffer. The filter was washed in tris-glycine buffer, blocked with 1 percent gelatin in tris-glycine buffer, and washed again in tris-glycine buffer. Lanes 1, 4, 5, and 6 are immunoblots of unlabeled merozoite proteins probed with the designated sera; lanes 2 and 3 are authentic ^{3}H-labeled GBP155 and GBP-130 subjected to electrophoresis on the same gel for comparison. (Lane 1) Normal mouse serum; (lane 2) ^{3}H-labeled GBP155; (lane 3) ^{3}H-labeled GBP130; (lane 4) culture supernatant immunoblotted with rabbit α-GBP155,130 (*5*) (diluted 1:50) and then with ^{125}I-labeled protein A; (lane 5) mouse α-pGBP-6 (diluted 1:10) treated with ^{125}I-labeled goat antiserum to mouse immunoglobulin G; (lane 6) mouse α-pUC-9 alone. Protein bands of varying intensity at 120 and 110 kD are characteristic degradation products. Staining was done in tris-buffered saline (TBS) and 20 percent fetal bovine serum. Filters were washed in TBS and 0.1 percent Nonidet P-40 after treatment with each antibody.

Complementary DNA clones expressing this repeat sequence were induced in *E. coli* strain JM103 with IPTG, and extracts were prepared (*15*). Mice were immunized with these extracts or with extracts prepared from *E. coli* transformed with vector sequences alone. The antisera were used in immunofluorescence studies and Western blot analysis. In the immunofluorescence pattern with the mouse antiserum (α-pGBP-6) to the repeating domain of the GBP (Fig. 3a), a pattern of schizont and merozoite immunofluorescence was seen, which is consistent with staining of a surface protein. A similar pattern occurred when the rabbit antiserum to GBP155,130 was used (Fig. 3b). Staining of rings was not seen with these antisera, which is in agreement with previous studies on the stage-specificity of GBP expression (*5*). The mouse α-pGBP-6 antiserum to the fusion protein also stained free merozoites.

Lysates of merozoite proteins (*5*) were fractionated on 10 percent SDS-polyacrylamide gels, transferred to nitrocellulose, and stained with the mouse antiserum to the repeating sequence expressed in *E. coli* (α-pGBP-6). Three protein bands were detected, corresponding to 130, 120, and 110 kD (Fig. 3c). An identical pattern was observed when rabbit antiserum to GBP155,130 was used. Normal mouse serum or mouse serum to vector alone did not stain any merozoite protein bands. Culture supernatant and heat-treated culture supernatant (boiled for 5 minutes) were also immunoblotted with mouse α-pGBP-6 antiserum. Identical protein bands of 130, 120, and 110 kD were detected in both cases, indicating that the proteins recognized by the mouse antiserum was released into the supernatant and was heat stable; these are properties characteristic of a GBP (*5*). Previous studies with this rabbit antiserum (*5*) identified two immunoprecipitable protein products with molecular sizes of 155 and 130 kD that bound to glycophorin-acrylamide. Additional experiments suggest that the 155- and 130-kD protein are related and share a common glycophorin-binding site. The 130-kD protein may represent a specific processing product of the 155-kD protein (*5*). The 130-kD protein band detected on Western blots (Fig. 3c) comigrated with the immunoprecipitable 130-kD protein from labeled schizonts, which shows that the repeating sequence encoded by the

cDNA clones corresponds to a GBP. In addition, the *E. coli* fusion proteins expressing GBP determinants (see Fig. 1) appeared to compete with the binding of the schizont-synthesized 155- and 130-kD GBP's to glycophorin-acrylamide (*16*). This suggests that the protein sequence encoded by the pGBP-6 cDNA clone (Fig. 2b) encodes the glycophorin-binding site of both GBP155 and GBP130. We have not been able to detect the 155-kD GBP by immunoblot analysis.

In view of the potential heterogeneity of the GBP's in *P. falciparum* merozoites, it was of interest to determine the genomic organization of the gene corresponding to a GBP. Isotopically labeled DNA of clone pGBP-6 was used to identify DNA fragments generated by restriction enzyme digestion of *P. falciparum* DNA from FCR-3 Gambia (the strain used to obtain the clone) and a variant strain isolated from Honduras. No differences were apparent with the six restriction enzymes used to analyze the DNA's of these parasites (Fig. 4a), which establishes that the gene for this protein is conserved in these two strains. This result is in agreement with the observation that the Honduran strain expresses a GBP that cross-reacts with the rabbit antiserum to the GBP of the Gambian strain by Western blot analysis (*17*). Further mapping of the genomic organization of this GBP gene with the 450-bp Xmn I fragment (see Fig. 2, a and b), which contains only the coding sequence, revealed a 2.0-kb Hind III–Xba I fragment. No sequences identical to the 150-bp repeat were found 5′ of this Hind III site, which establishes a maximum of 12 repeats of 150 bp each in this gene. This suggests that GBP155,130 contains no more than 65 kD of the 50-amino-acid repeat.

To determine the stage-specificity of this gene's expression, we isolated RNA from ring-, trophozoite-, and schizont-infected erythrocytes from the FCR-3 strain in culture. The RNA was size-fractionated under denaturing conditions and probed with the isotopically labeled DNA from clone pGBP-6. A species of RNA of 6.6 kb was observed that reached its highest level of steady-state expression during late schizogeny (Fig. 4b). Previous studies (*5*) have shown that the expression of the GBP's in metabolically labeled parasites is maximum during late schizogeny; this is consistent with these results.

The identification of the gene for the GBP of *P. falciparum* and its expression in prokaryotic hosts offers an opportunity to test the involvement of different protein domains in the invasion of erythrocytes by merozoites. The gene is not related to any of the erythrocyte-stage protein genes described earlier. Heat stability and relative richness in proline and glycine are properties of both GBP155,130 and the surface antigens of *P. falciparum* (*5*, *18*), which suggests that these proteins may be similar (*18*). However, several surface antigens have been cloned and partially sequenced (*19*) and are isolate-variant and composed of short repeating subunits of amino acids. In contrast, the partial sequence of GBP155,130 is conserved in all strains of *P. falciparum* tested so far (*16*) and is composed of a long (50 amino acids) repeating subunit. The gene for the RESA antigen, which is localized on ring-infected erythrocytes (*19*), encodes a different primary sequence and reacts with different DNA fragments on Southern blot analysis (*19*). Determination of the complete genomic organization of the GBP's should elucidate their relation to other merozoite surface antigens.

Studies on the characterization of genes for *P. falciparum* erythrocyte-stage antigens (*19*), the circumsporozoite antigen of *P. knowlesi* (*20*) and *P. falciparum* (*1*, *2*), and the histidine-rich protein of *P. lophurae* (*14*) reveal a common property of malarial antigens. Each contains tandemly repeating sequences of amino acids, with repeat units of between 4 to 12 residues. The gene for a GBP of *P. falciparum* similarly encodes a tandemly repeating sequence, but of 50 amino acids. Although the function of such an unusual protein structure in parasite survival is unknown, the GBP of *P. falciparum* is a useful protein in which to map ligand-binding sites and to determine the role of repeating protein sequences.

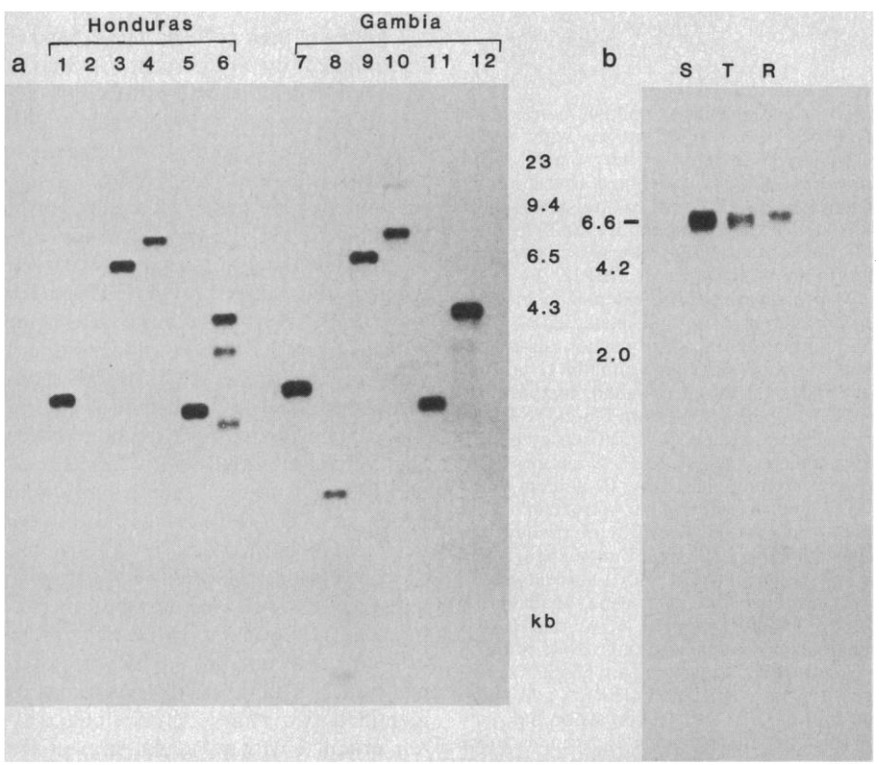

Fig. 4. (a) Genomic organization of a GBP in two *P. falciparum* strains. Total DNA from *P. falciparum* CDC-1 (Honduras, lanes 1 to 6) or FCR-3 (Gambia, lanes 7 to 12) was digested with Aha III (lanes 1 and 7), Xmn I (lanes 2 and 8); Xba I (lanes 3 and 9); Acc I (lanes 4 and 10), Hind III (lanes 5 and 11), and Eco RI (lanes 6 and 12). The digests were fractionated on 0.75 percent agarose, transferred to nitrocellulose, and probed with pGBP-6–labeled DNA under stringent conditions (50 percent formamide and 10 percent dextran sulfate at 40°C, then washed in 0.1× standard saline citrate at 52°C). Eco RI* activity is evident in lane 6. Lanes 2 and 8 reveal three hybridizing fragments of 2.0, 0.9, and 0.45 kb; lanes 3 and 9 reveal fragments of 6.0 and 1.35 kb. (b) Stage specificity of RNA expression for a GBP gene. Total RNA was isolated from FCR-3 (Gambia) parasites at ring (R), trophozoite (T), or schizont (S) stages as described (*23*). One microgram was fractionated on 1 percent agarose in 2.2*M* formaldehyde, transferred to nitrocellulose, and probed with pGBP-6–labeled DNA.

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12. *Escherichia coli* strain JM103, transformed with individual cDNA clones in pUC-9, were grown from single colonies in Luria-Bertani broth at 37°C to an optical density at 560 nm of 0.1, induced with IPTG to a final concentration of 1 m*M*, and grown for an additional 4 to 6 hours. Cultures were subjected to centrifugation, and the bacteria were resuspended in one-tenth volume of SDS loading buffer, boiled for 2 minutes, and subjected to SDS–polyacrylamide gel electrophoresis.
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15. Cultures were grown as described (*12*), and the centrifugate was resuspended in one-tenth volumes of 50 m*M* tris (*p*H 8.0), 25 percent sucrose, 1 percent Nonidet P-40, 0.5 percent deoxycholate, 2 m*M* dithiothreitol, and 5 m*M* EDTA and sonicated for 20 seconds. Mice of the BALB/c strain were inoculated intraperitoneally and subcutaneously with 0.1 ml of extract in complete Freund's adjuvant and then 2 weeks later with an equal dose in incomplete Freund's adjuvant. A final dose was administered intravenously 1 week later. The mice were bled 1 week later.
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Developmentally Controlled Expression of Immunoglobulin V_H Genes

Abstract. *Although antibody diversity arises mainly from apparently random combinatorial and somatic mutational mechanisms acting upon a limited number of germline antibody genes, the antibody repertoire develops in an ordered fashion during mammalian ontogeny. A series of early pre-B and B-lymphocyte cell lines were examined to determine whether an ordered rearrangement of gene families of the variable region of immunoglobulin heavy chains (V_H) may be the basis for the programmed development of the antibody response. The results indicated that the V_H repertoire of fetal B-lineage cells is largely restricted to the V_H 7183 gene family and that subsequent recruitment of additional V_H gene families occurs during neonatal development. These results have important implications in understanding the ontogeny of immune function.*

Analyses of genes encoding antibodies have resulted in the elucidation of the strategies that permit a limited number of sequences to direct the syntheses of millions of different antibody molecules (*1*). In particular, each antibody consists of heavy (H) and light (L) polypeptide chains that are encoded by multiple, discontinuous, germline gene segments that are juxtaposed in antibody-forming (B) cell precursors. Combinatorial joining of variable (V_H), diversity (D), and joining (J_H) gene segments (for heavy chains) and of V_L and J_L gene segments (for L chains), coupled with association of heavy and light polypeptides, can be calculated to yield more than 10^7 different antibody molecules from an estimated set of fewer than 10^3 gene segments (*2*). In addition, a process of localized somatic hypermutation introduces small deletions, insertions, and single base substitutions in and around rearranged antibody genes (*3*). In well-studied antibody populations, most of the observed sequence heterogeneity appears to result from the somatic hypermutation process acting upon rearrangements involving one or at most a few germline gene segments (*4*).

By analysis of available protein and nucleic acid sequence data it has been possible to group murine V_H gene segments into discrete families (*5*). Recombination studies suggest that the different murine V_H families are themselves grouped discretely and not interspersed and provide an order on murine chromosome 12 for the heavy chain locus: centromere–V_HJ558–V_HS107–V_HQ52–V_HM7183–D–J_H–C_μ, encompassing 2 to 3 map units (*6*). In most immune responses of restricted heterogeneity, antibodies of a given specificity use V_H gene segments derived from a single V_H family, although antibodies of many specificities may utilize members of the same V_H family (*7*).

Despite the randomness inherent in the mechanisms that propagate antibody variability, the development of the antibody repertoire follows a characteristic program (*8*). Antibody responses to some antigens, bacteriophage ϕX174 for example, appear during fetal life in both sheep (*9*) and mice (*10*), while responses to most protein antigens cannot be induced until some days after birth. Specific clones of antigen-responsive B cells appear at characteristic times during murine development (*11*). A comparable restriction in antibody diversity at early stages in development has also been described in the chicken (*12*), in the frog *Xenopus laevis* (*13*), and in humans (*14*).

Although complex mechanisms for immunoregulatory interactions between different cell types might explain the developmentally ordered acquisition of antibody specificities, there is considerable evidence that this is not the case; for example the early absence of B cells reactive with inulin (*15*) and α-(1 → 6) dextran (*8*) reflects an absolute lack of antigen-specific B cell precursors. In the mouse, B-lymphocyte precursors can be detected in the liver by day 12 of fetal life (*16*). Antibody gene rearrangement is developmentally ordered in these cells and begins with juxtaposition of D and J_H gene segments followed by V_H rearrangement to give a functional V_H–D–J_H–C_μ transcriptional unit (*17*). It continues with light-chain rearrangement (first κ and then, if necessary, λ) to produce a complete set of functional rearranged antibody genes, thus marking the differentiation of a B cell from a pre-B cell. Since the chronology of acquisition of antibody specificities reflects B cell development alone, and since B cell development is marked by an orderly process of gene rearrangements, we hypothesized that different V_H gene families might rearrange preferentially at different times during development.