

# A Reticulocyte-Binding Protein Complex of Plasmodium vivax Merozoites

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## Summary

**P**lasmodium vivax merozoites primarily invade reticulocytes. The basis of this restricted host cell preference has been debated. Here we introduce two novel P. vivax proteins that comigrate on reducing SDS-polyacrylamide gels, colocalize at the apical pole of merozoites, and adhere specifically to reticulocytes. The genes encoding these proteins, P. vivax reticulocyte-binding proteins 1 and 2 (PvRBP-1 and PvRBP-2), have been cloned and analyzed. Homologous genes are evident in the closely related simian malaria parasite, P. cynomolgi, which also prefers to invade reticulocytes, but are not evident in the genome of another related simian malaria parasite, P. knowlesi, which invades all red blood cell subpopulations. Native PvRBP-1 is likely a transmembrane-anchored disulfide-linked protein, and along with PvRBP-2 may function as an adhesive protein complex. We propose that the RBPs of P. vivax, and homologous proteins of P. cynomolgi, function to target the reticulocyte subpopulation of red blood cells for invasion.

## Introduction

A malaria blood infection becomes established when Plasmodium merozoites parasitize erythrocytes. Merozoites must invade susceptible host cells within minutes after being released into the circulation. Though merozoites can adhere to red blood cells in any orientation, prior to invading they must be oriented such that their apical prominence is juxtaposed to the erythrocyte surface. The initial surface contact of merozoites with erythrocytes is reversible. However, the apical attachment, which leads to the formation of a junction between parasite and host membranes, is not. After junction formation, the merozoite begins to move inward, causing a vacuole to form from the invaginating erythrocyte membrane. Reorientation of merozoites at the surface of erythrocytes and subsequent parasite-host interactions that make up the process of invasion have been observed by videomicroscopy (Dvorak et al., 1975) and ultrastructural studies (Bannister et al., 1975; Aikawa et al., 1978; Miller et al., 1979). These observations have led to the prediction that parasite molecules at the apical pole and at the surface of the merozoite perform specific and, perhaps, sequential adhesive functions that are requisite for invasion to occur.

Plasmodium vivax is one of the two principal species of Plasmodium causing most human malaria infections. However, because it cannot be cultured continuously in

vitro, this major pathogen remains poorly investigated. An important distinctive characteristic of P. vivax merozoites is that they invade primarily, if not exclusively, the reticulocyte subpopulation of red blood cells in vivo (Kitchen, 1938) and in vitro (Mons et al., 1988; Barnwell et al., 1989). Merozoites of P. cynomolgi, a simian malaria parasite closely related to P. vivax (McCUTCCHAN et al., 1984; DiGiovanni et al., 1990), also prefer to invade reticulocytes (Warren et al., 1966). Accordingly, the early intraerythrocytic stages of these species are rarely observed in normocytes. In contrast, merozoites of other primate malaria species, including the vivax-related simian malaria, P. knowlesi, and the other most prevalent human malaria, P. falciparum, invade all red blood cells.

P. falciparum merozoites utilize sialic acid on glycophorin as an erythrocyte receptor (reviewed in Hadley and Miller, 1988; and Perkins, 1989), whereas P. vivax merozoites require a Duffy blood group glycoprotein (Miller et al., 1976; Barnwell et al., 1989), and not glycophorin, for host cell entry. A 140 kd P. vivax merozoite protein, the Duffy adhesion protein (PvDAP-1), which binds specifically to the erythrocyte Duffy blood group glycoprotein, has recently been identified (Wertheimer and Barnwell, 1989). However, the Duffy glycoprotein is present on mature as well as immature red cells. Thus, though the interaction between the parasite Duffy-binding protein and the erythrocyte Duffy glycoprotein is essential for the successful invasion of human red blood cells by P. vivax merozoites, it does not account for their preferential invasion of reticulocytes.

In this article we describe two P. vivax reticulocyte-binding proteins, PvRBP-1 and PvRBP-2, that are concurrently expressed at the invasive, apical end of merozoites. These proteins are likely responsible for the selective invasion of P. vivax merozoites into reticulocytes. Together, the adhesive specificities of the PvRBPs and the Duffy-binding protein, PvDAP-1, begin to define the series of molecular interactions that are required for red blood cell invasion in vivax malaria.

## Results

### Identification of P. vivax Reticulocyte-Binding Proteins

An in vitro erythrocyte-binding assay (EBA) (see Experimental Procedures) was used to identify merozoite proteins that adhere to erythrocytes. To perform the EBA, purified mature trophozoite-stage intraerythrocytic parasites are cultured through schizogony in the presence of [<sup>35</sup>S]methionine and allowed to rupture, releasing merozoites in the absence of fresh target red blood cells. Under these conditions, free merozoites are not very stable and rapidly deteriorate, producing a culture supernatant that contains numerous merozoite proteins. Two P. vivax proteins frequently bind strongly to Duffy-positive human erythrocytes: a 210 kd protein and most consistently a 140 kd protein, the Duffy-binding protein, PvDAP-1 (Figure 1A).

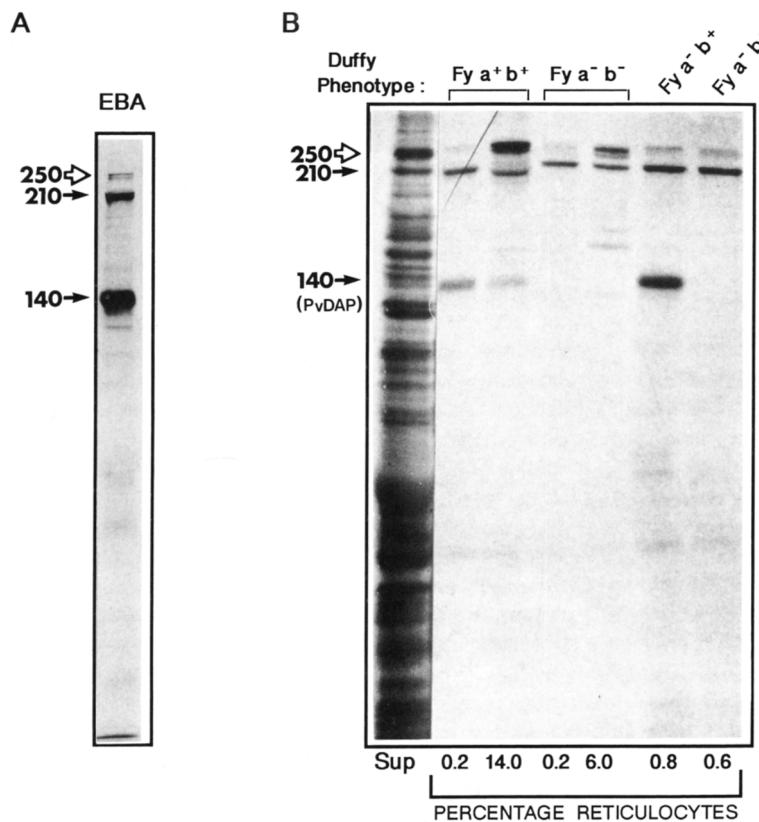


Figure 1. Demonstration of PvRBPs: EBA Analysis by Reducing SDS-PAGE and Fluorography

(A) EBA Profile of *P. vivax* erythrocyte-binding proteins. A weak doublet is noted at approximately 250 kd and intense bands at 210 kd and 140 kd.

(B) The degree of binding of the 250 kd proteins correlates directly with the percentage of reticulocytes used in the EBA. First lane, supernatant obtained from cultures of [<sup>35</sup>S]methionine biosynthetically labeled *P. vivax*, after schizont rupture and merozoite release in the absence of fresh target red blood cells. Remaining six lanes, EBA analyses. Percentages of reticulocytes for each experiment are noted; 0.2% indicates depletion; 14.0% and 6.0% indicate enrichment; 0.8% and 0.6% indicate normal percentages in whole blood. Duffy glycoprotein phenotypes are indicated for each erythrocyte preparation; Duffy positive = Fy a<sup>+</sup>b<sup>+</sup> and Fy a<sup>+</sup>b<sup>+</sup>; Duffy negative = Fy a<sup>-</sup>b<sup>-</sup>. PvDAP-1 refers to the 140 kd Duffy-binding protein (Wertheimer and Barnwell, 1989; Fang et al., 1991).

In addition to the *P. vivax* 210 kd and PvDAP-1 erythrocyte-binding proteins, in some experiments we observed the binding of faint, high molecular weight (HMW) protein bands (as shown in Figure 1A). These protein bands migrated at approximately 250 kd and 280 kd on 7.5% and 5.0% reducing SDS-polyacrylamide gel electrophoresis (PAGE) gels, respectively.

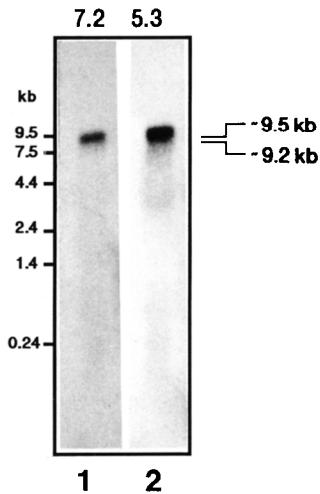
We questioned whether these HMW protein bands signified low affinity binding to all erythrocytes or binding restricted to a minor subpopulation. Because *P. vivax* preferentially invades reticulocytes, we tested the hypothesis that the faint bands represented protein binding specifically to the reticulocyte subpopulation of red blood cells. EBAs were repeated using fresh whole-cell populations of erythrocytes and cell populations enriched for, or depleted of, reticulocytes. There was a dramatic increase in the intensity of the HMW protein bands when the EBA was performed using the reticulocyte-enriched erythrocytes, and a decrease in intensity when reticulocyte-depleted preparations were used (Figure 1B). Thus, the intensity of the HMW protein bands correlated directly with the percentage of reticulocytes present in the erythrocyte preparation used for each EBA. The reticulocyte-binding protein bands sometimes appeared as one band, but often as two closely spaced HMW bands, indicating the possibility that there is more than one *P. vivax* reticulocyte-binding protein (PvRBP) or that the intact binding protein may tend to degrade. The PvRBP(s) bind reticulocytes regardless of their Duffy phenotype. In contrast, PvDAP-1 only binds

Duffy-positive erythrocytes, regardless of maturity, and the 210 kd protein binds erythrocytes independent of their Duffy phenotype or stage of maturation. Thus, the PvRBP, Pv210, and PvDAP-1 proteins bind to different red blood cell receptor determinants.

*P. vivax* also infects a number of other primates, in addition to humans. We therefore investigated whether the PvRBP(s) bound specifically to the reticulocytes of other susceptible primate species. Chimpanzees and *Aotus* or squirrel monkeys are susceptible to *P. vivax* blood stage infections and the PvRBP(s) bound specifically to reticulocytes from these primates (see below and data not shown). Rhesus monkeys (*Macaca mulatta*) are not susceptible to *P. vivax* blood infections, and the PvRBP(s) did not bind to the mature red blood cells or reticulocytes of this primate species. The PvRBP(s) also bound to reticulocyte-rich red blood cells from rabbits (see below), but not to the mature or immature erythrocytes of rats, mice, or hamsters.

#### Identification of Gene Clones Encoding Portions of Two Distinct PvRBPs

Serum from one squirrel monkey (*Saimiri boliviensis*) immunized with *P. vivax* merozoites (see Experimental Procedures) specifically recognized a subset of merozoite proteins including HMW protein(s) with a  $M_r$  of  $\geq 250$  kd. A *P. vivax* genomic DNA λgt11 expression library was screened with this immune serum. Two of the recombinants identified, clones 7.2 and 5.3, contain 2.0 kb and 3.8 kb EcoRI DNA inserts, which hybridize to closely migrating

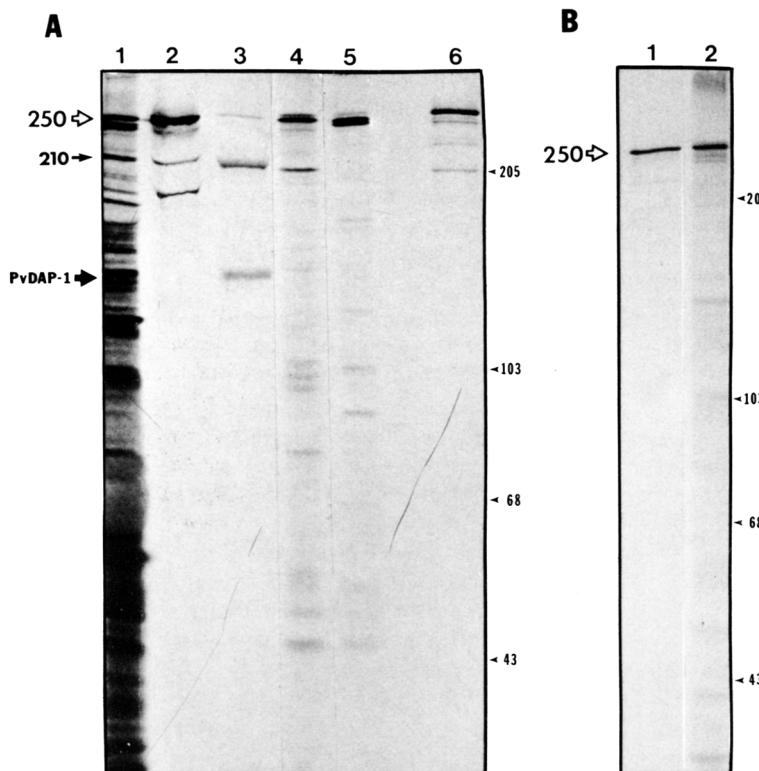


**Figure 2.** The 7.2 and 5.3 Clones Hybridize to Distinct RNA Transcripts

Sequential hybridization of the 7.2 (lane 1) and 5.3 (lane 2)  $\lambda$ gt11 DNA inserts to a Northern blot containing 30  $\mu$ g of total RNA isolated from mature *P. vivax* schizonts detected closely migrating transcripts of approximately 9.2 kb and 9.5 kb, respectively. Locations of the RNA markers (Bethesda Research Laboratories) are indicated.

RNA transcripts of approximately 9.2 kb and 9.5 kb, respectively (Figure 2). The two cloned DNA fragments did not hybridize to one another, and Southern analyses of restricted genomic DNA also indicated that they represented portions of two genes (see below).

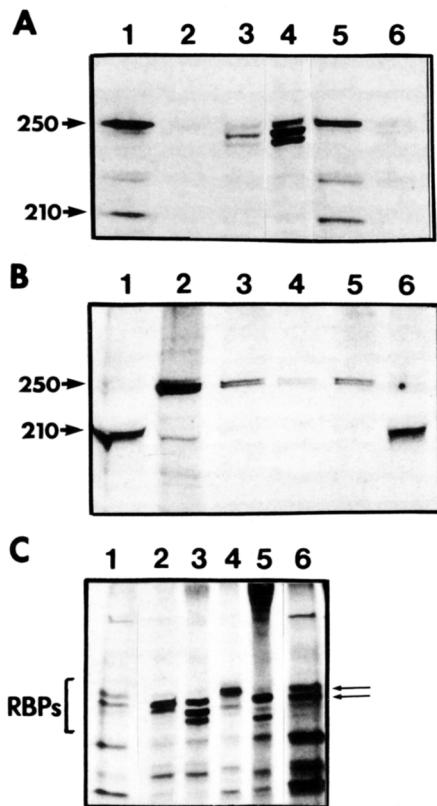
To characterize the proteins partially encoded by clones 7.2 and 5.3, rabbit antisera were prepared against each  $\beta$ -galactosidase fusion protein and, in addition, the squirrel monkey antibodies from the immune serum that recognized these recombinant proteins were plaque purified. The 7.2 and 5.3 rabbit antisera and plaque-selected antibodies recognized the corresponding 7.2 and 5.3 fusion proteins, and no cross-reactivity was observed. These antibodies specifically immunoprecipitated [ $^{35}$ S]methionine metabolically labeled HMW merozoite proteins from the culture supernatants used for EBAs (Figure 3, lanes 4 and 5) and from detergent extracts of mature *P. vivax* schizonts (Figure 3B, lane 2 and data not shown). In each instance, the immunoprecipitated protein bands comigrated with the HMW protein(s) that bound to human or rabbit red blood cells (Figure 3A, lanes 2, 3, and 6). Because the SDS-PAGE mobility of the immunoprecipitated proteins corresponded closely to the mobility of the HMW protein bands that bound to reticulocytes, we sought to determine directly whether either, or both, the 7.2 and 5.3 gene fragments encoded portions of the HMW PvRBP(s). The 7.2 and 5.3 antibodies each immunoprecipitated HMW proteins that had been bound to, and eluted from, reticulocytes (Figures 4A and 4B, and data not shown). In some experiments, the 7.2 and 5.3 antibodies immunoprecipitated several bands (Figure 4A, lanes 3, 4, and 6); the top bands correspond to the reticulocyte-bound protein band (Figure 4A, lane 1) and to the 250 kd band immunoprecipitated with the immune Saimiri monkey antiserum (Figure 4A, lane 5); lane 6 shows a weak though significant signal. In other experiments, both antibody specificities, 7.2 and



**Figure 3.** The 7.2 and 5.3  $\lambda$ gt11 Fusion Protein Antibodies Immunoprecipitate Proteins of Approximately 250 kd

(A) Lane 1, supernatant from cultures of [ $^{35}$ S]methionine biosynthetically labeled *P. vivax*, after schizont rupture in the absence of fresh target red blood cells. Lanes 2 and 6, EBAs performed using rabbit erythrocytes. Lane 3, EBA performed using human Fy a<sup>b+</sup> erythrocytes. Lane 4, immunoprecipitation of a protein of approximately 250 kd from the culture supernatant using plaque-purified squirrel monkey 7.2 antibodies. Lane 5, immunoprecipitation of a protein of approximately 250 kd from the culture supernatant using plaque-purified squirrel monkey 5.3 antibodies. The SDS-polyacrylamide gel contained 7.5% acrylamide and 0.2% bisacrylamide. Positions of prestained protein MW standards (Bethesda Research Laboratories) are indicated.

(B) Lane 1, EBA performed on rabbit erythrocytes. Lane 2, immunoprecipitation of a protein of approximately 250 kd from NP-40 detergent-extracted [ $^{35}$ S]methionine biosynthetically labeled *P. vivax* schizonts using rabbit fusion protein 7.2 antiserum. The SDS-polyacrylamide gel contained 7.5% acrylamide and 0.2% piperazine diacrylamide cross-linker. Positions of prestained protein MW standards (Bethesda Research Laboratories) are indicated.



**Figure 4.** PvRBP-1 and PvRBP-2: Reducing SDS-PAGE and Fluorographic Analysis

(A) Lane 1, *P. vivax* EBA on human Fy  $a^+b^+$  erythrocytes enriched for reticulocytes (8%). Proteins were eluted from these erythrocytes with 0.5 M salts (3  $\times$  Hank's buffer salt solution) and immunoprecipitated with: lane 2, normal rabbit serum; lane 3, plaque-purified squirrel monkey 5.3 antibodies; lane 4, rabbit fusion protein 5.3 antiserum; lane 5, squirrel monkey *P. vivax* antiserum; and lane 6, plaque-purified squirrel monkey 7.2 antibodies.

(B) Lanes 1 and 2, EBAs using Saimiri monkey reticulocyte-depleted (0.2%) and reticulocyte-enriched (15%) erythrocyte preparations, respectively. Proteins were eluted from the reticulocyte-enriched erythrocytes with 0.5 M salts (3  $\times$  Hank's buffer salt solution) and immunoprecipitated with: lane 3, rabbit fusion protein 7.2 antiserum; lane 4, plaque-purified squirrel monkey 7.2 antibodies; lane 5, rabbit fusion protein 5.3 antiserum; and lane 6, a control rabbit antiserum against the major merozoite surface protein (MSP-1) of *P. cynomolgi*.

(C) Lanes 1 and 6, total [ $^{35}$ S]methionine biosynthetically labeled *P. vivax* culture supernatants and merozoite 1% NP-40 detergent extracts, respectively; lanes 2 and 3, culture supernatant immunoprecipitates using 7.2 and 5.3 antiserum, respectively. Lanes 4 and 5, merozoite extract immunoprecipitates using 7.2 and 5.3 antiserum, respectively.

All SDS-polyacrylamide gels were 7.5% acrylamide and 0.2% bisacrylamide or piperazine diacrylamide cross-linker, except in (C), where piperazine diacrylamide cross-linker with mixed lauryl SDS (Pierce) was used in preparation of a 5.0% acrylamide gel. In this experiment electrophoresis was continued until the 97 kd phosphorylase b prestained marker (Bethesda Research Laboratories) had migrated through the gel slab.

5.3, immunoprecipitated very closely spaced bands (Figure 4B, lanes 3, 4, and 5), corresponding to a doublet evident in the fluorograph of the reticulocyte eluate prior to immunoprecipitation. These HMW bands were not im-

munoprecipitated with normal rabbit serum (Figure 4A, lane 2) or a rabbit antiserum that recognizes the Pv210 kd protein (Figure 4B, lane 6). Additional analyses of the 7.2 and 5.3 immunoprecipitates from both culture supernatants and merozoite extracts (Figure 4C and data not shown) indicate that the culture supernatant PvRBP precipitates are more prone to degradation and highlight the fact that the 7.2 and 5.3 immunoprecipitates have distinctive degradation patterns (see also Figures 3A and 4A). Immunoprecipitation of merozoite detergent extracts (Figure 4C, lanes 4 and 5) indicate that the 7.2 immunoprecipitated protein corresponds to the higher of the two proteins when recognizable as a doublet. These proteins can also be distinguished by their different mobilities in nonreducing SDS-polyacrylamide gels (see below). We have designated the HMW proteins recognized by the 7.2 and 5.3 antibodies as PvRBP-1 and PvRBP-2, respectively.

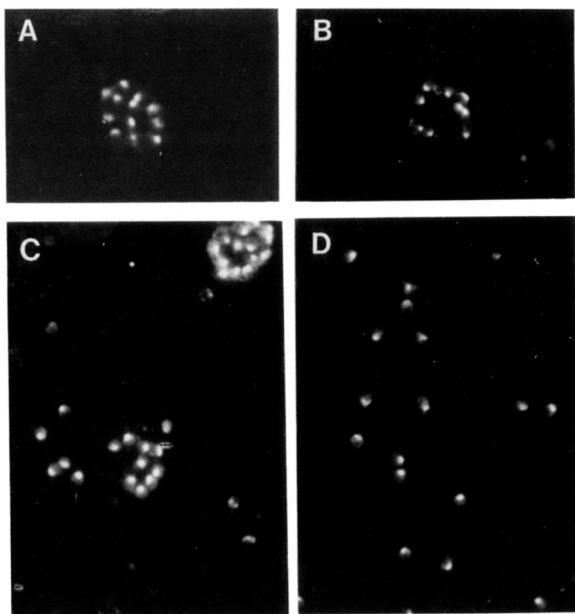
#### Localization of PvRBP-1 and PvRBP-2

The plaque-purified 7.2 and 5.3 squirrel monkey antibodies and rabbit anti- $\beta$ -galactosidase fusion protein antisera were used as the primary antibody reagents in indirect immunofluorescence analyses (IFAs) on air-dried *P. vivax*, *P. cynomolgi*, *P. knowlesi*, and *P. falciparum* mature schizonts. Both the 7.2 and 5.3 antibodies reacted with the apical (anterior) pole of *P. vivax* merozoites, and identical cap-like fluorescence patterns extending beyond the merozoite conoid prominence were observed. The apical fluorescence patterns were evident on both free merozoites and merozoites contained within mature, segmented schizonts (Figure 5). These antibodies reacted similarly with *P. cynomolgi* merozoites, but did not react with *P. knowlesi* or *P. falciparum* merozoites (data not shown). Fluorescence reactivity was observed only on mature, well differentiated merozoites; no reactivity occurred with multinucleated, nonsegmented, schizont-stage parasites.

#### Cloning the PvRBP Genes

Using mung bean nuclease in the presence of formamide, we established digestion conditions that potentially would isolate both PvRBP genes and their immediate flanking sequences on large pieces of DNA (see Experimental Procedures). Large fragments of *P. vivax* genomic DNA were thus generated and cloned into  $\lambda$  Dash II. A portion of this library was screened with a cocktail solution containing the  $\lambda$ gt11 7.2 and 5.3 DNA inserts as probes.

The 7.2 (PvRBP-1) DNA probe hybridized to six  $\lambda$  Dash recombinant clones. A schematic representation of the physical maps of the DNA inserts of four of these clones is presented in Figure 6. Each contains either two or three internal XbaI sites, which were utilized to generate XbaI fragments (labeled A to D). The 7.2 DNA insert hybridized to the XbaI fragment B of each clone. DNA sequence analysis (data shown below) showed that the complete gene encoding PvRBP-1 lies within the XbaI A, B, and C fragments and is contained in its entirety in  $\lambda$  Dash clones 11 and 16. Accordingly, the 16-A, 16-B, and 16-C XbaI fragments hybridized to the PvRBP-1 mRNA band identified previously (Figure 2, lane 1), and the 16-D XbaI fragment did not (data not shown).



**Figure 5.** The 7.2 and 5.3 Antibodies Recognize Proteins That Colocalize at the Merozoite Apical Pole

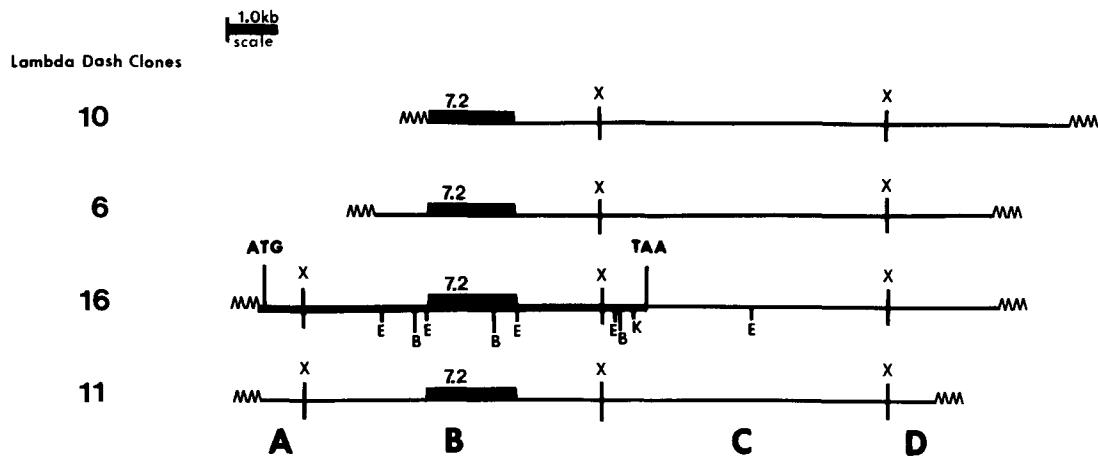
IFAs were performed on air-dried blood smears containing mature *P. vivax* schizonts and merozoites using plaque-selected squirrel monkey 7.2 antibodies (A); plaque-selected squirrel monkey 5.3 antibodies (B); rabbit fusion protein 7.2 antiserum (C); and rabbit fusion protein 5.3 antiserum (D) as primary antibody reagents. FITC-conjugated rabbit anti-Saimiri IgG or goat anti-rabbit IgG was employed as the second fluorochrome probe. Apical staining was initially determined with the use of 4,6-diamidino-2-phenylindole to identify the nucleus, which is known to be at the posterior pole of the merozoite (data not shown). Double fluorochrome staining with FITC and rhodamine-conjugated, species-specific, anti-IgG antibodies indicated overlapping anti-5.3 and anti-7.2 immunofluorescence (see Experimental Procedures).

The 5.3 (PvRBP-2) DNA probe hybridized to one recombinant  $\lambda$  Dash clone, which contains a 16 kb DNA insert (data not shown). We have confirmed by limited mapping

and DNA sequencing that the sequence corresponding to the 5.3 DNA probe is situated 3 kb downstream of the 5' end of this insert. There is no hybridization between the DNA insert of the  $\lambda$  Dash PvRBP-2 clone and the inserts of the  $\lambda$  Dash PvRBP-1 clones.

#### Sequence Analysis of PvRBP-1

The PvRBP-1 gene sequence was determined from the original  $\lambda$ gt11 7.2 DNA insert and from the  $\lambda$  Dash 16-A, 16-B, 16-C, and 6-B XbaI fragments (Figure 6) subcloned into pBluescript. The coding region has been determined to be 8.49 kb contained on two exons (Figure 7). To date, no entries have been detected in either GenBank/EMBL or the Swiss Protein Data Bank that have significant sequence similarities with any portions of the PvRBP-1 gene or deduced protein. Exon I of the PvRBP-1 gene encodes a putative signal peptide sequence; the initial methionine residue is followed by two charged amino acids and a short region of hydrophobic amino acids (Perlman and Halvorson, 1983; von Heijne, 1986). Consistent with the premise that this sequence encodes the true N-terminal portion of PvRBP-1 is the fact that it is preceded by three in-frame stop codons and an adenine at position -3 (Kozak, 1986; Saul and Battistutta, 1990). This short sequence is separated from the remaining 8.4 kb of continuous coding sequence, designated as exon II, by a short intron. Introns following a short signal peptide coding sequence have been found in a number of malaria genes (Weber, 1988; Brown and Coppel, 1991). The PvRBP-1 intragenic region, proposed to be 200 bp, is demarcated by consensus intron junctions cataloged for malarial (Weber, 1988) and other eukaryotic (Darnell et al., 1986) intron sequences. Immediately upstream of the predicted 3' intron splice site is a long pyrimidine tract, which is typical for the 3' region of an intron (Weber, 1988) and not characteristic of the remaining 8.4 kb of continuous coding sequence. Direct analysis of the PvRBP-1 cDNA will verify the boundaries of this intron; no other introns are evident

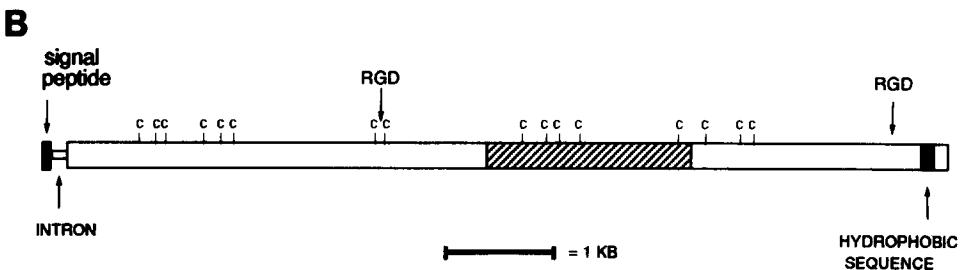


**Figure 6.** Schematic Representing Four PvRBP-1  $\lambda$  Dash Clones

The deduced start (ATG) and stop (TAA) codons and internal restriction sites are indicated for clone #16. Restriction enzyme cut sites are indicated by: X, XbaI; E, EcoRI; B, BamHI; K, KpnI. The letters A-D designate fragments generated with XbaI, the black box corresponds to the 2.0 kb insert of  $\lambda$ gt11 clone 7.2, and the zigzag lines represent the  $\lambda$  Dash II polylinker sequence containing XbaI, SalI, and EcoRI restriction sites. The restriction fragment sizes were calculated using standard DNA MW markers;  $\lambda$  DNA digested with HindIII or EcoRI, and  $\Phi$ X174 RF DNA digested with HaeIII.

A

GAAAAAAAGGGGAGTCAGTGTCTTTAACATGAAAAGGGGATTGCGCTAGCGCTCTTGTGCTTCAACTTAAAGGATGGTCTATTTAACATGAGTCAGTGATGTGAC  
 M K R G I C L A A L L C F N Y I  
 ↓  
 TTATGCGTGTGGTTAATTTGTATTTGGTATCTCCACTGGGCTTCTCATGTAACCATTCTATGACCATAGCAC  
 ↓  
 CTCTCTATGAGTCGCTTTAGTACTCCCCATTAGTGAGCAGAGACATAAGGAATGCCAAGGGAAAGTAGTACCTTCTCG  
 G A G H G E N A E D I R N S E G K V N F F S L  
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 K D A E G S R P S H D S F V N G L H V D G G S L S Y S V H V K E S T P H S T  
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 V D M S E K L K E N D K Y N L V F D H I E F D V D L O F F N L I L E P I K D S  
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 V N S L K K L L G E E I S E V S H L Y V I N S T L I D D A A K K L E S I N E E D E  
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 L E N D T S K V N L F H O I R K I N T E K T K I D E S I L O T V E F K Y K E I L  
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 K M S A E V T N A E G I K K E I A O K O F E N V H K K M K K E F S D A F S T K F E  
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 A L O N S H O O Y N O E G D A I E K H K O N R S E K E E E Y F N E S V E E D L  
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 S R E E T E E O Y T K H K N N F S R K R G E I S A E I T V N K I E S  
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 N Y Y G V I E F Y F S L I G D O N V E S T A K C A L K E K I V S D S S L R D K I  
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 D O Y E T E F K E K T S A V E N T V S T I O S L S K I A D S L K P R L N G S C I N N  
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 I L K S L R M G K I N E L N D G R L N S L D T K K E D L L E P Y S E S K S  
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 L E T T L D E M V V O U V N M N L O S A I O G N A G I S K E L N E L K V G V I E  
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 G D G G K K A N I T D S C H E E V G N Y V S K A E H A F T H V E A O D V K T K A F  
 TCGGAATTCATGGTCACTGAGGAGAACAGGAGAACAGGAGAACAGGAGAACAGGAGAACAGGAGAACAGGAGAACAGGAGAACAGGAGAACAGGAGAACAG  
 S V P L I S E L G A E K S I D L E V Y E A K F E S Y E K N I F T V S M S R I H V  
 5005



**Figure 7.** The PvRBP-1 Gene and Deduced Protein Sequences

(A) A putative TATA box, signal peptide sequence, GT...AG splicing sites, transmembrane domain (*italicized*), and cleavage/polyadenylation signals (AATAAC, AATAAT, and AATAAA) are underlined. The boundaries of the 200 bp intervening sequence are demarcated with downward arrows. The two RGD motifs are in bold print and are underlined. Nucleotide +1 corresponds to the adenine of the initial methionine codon.

(B) Schematic presentation of PvRBP-1. Positions of the signal peptide, putative intron, RGD motifs, and C-terminal hydrophobic sequence are indicated by arrows. The hatched box represents the region encoded by the 2.0 kb DNA insert of λgt11 clone 7.2. The "C" notations represent positions of cysteine residues.

in the genomic DNA sequence of the remainder of the gene. Tyrosine is the final amino acid encoded by this open reading frame, which is followed by stop codons in all three reading frames. In the 3' noncoding sequence

there are two stretches of thymidine residues and three putative cleavage/polyadenylation signal sequences (reviewed in Birnstiel et al., 1985). Thymidine stretches are also in the 3' noncoding region of the *P. vivax* merozoite

surface protein-1 gene (del Portillo et al., 1991) and other merozoite genes of *P. vivax* (M. R. G. and J. W. B., unpublished data).

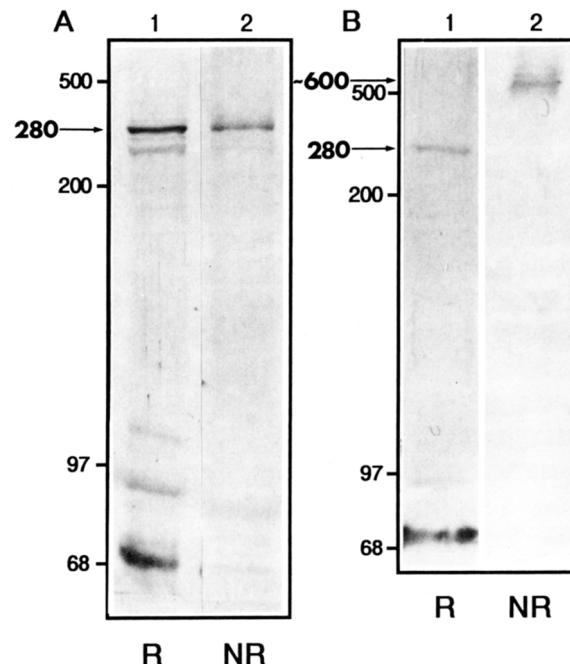
PvRBP-1, as deduced from the gene sequence, is a very hydrophilic polypeptide composed of 2829 aa, predicted to form mostly  $\alpha$ -helical secondary conformations. Its calculated molecular weight (MW) is 325.8 kd; PvRBP-1 is thus slightly (16%) larger than predicted from its mobility in a 5% reducing SDS-polyacrylamide gel. In addition to the presumptive signal peptide sequence, the only other region of PvRBP-1 that is significantly hydrophobic is an 18 aa segment located 43 aa from the carboxyl terminus. This hydrophobic region has the features of a transmembrane domain. Like many known membrane-spanning protein domains, this region is immediately bound by positively charged (lysine) residues. PvRBP-1 also has two Arg-Gly-Asp (RGD) sequence motifs, which are integrin-binding sites for a number of proteins (reviewed in Ruoslahti and Pierschbacher, 1986; and Hynes, 1987). Whether these RGD sequences function as adhesive domains of PvRBP-1 remains to be ascertained. Lastly, in contrast with the sequences of numerous Plasmodium proteins (reviewed in Kemp et al., 1987), there are no motifs of tandemly repeated amino acids in PvRBP-1.

#### Comparison of PvRBP-1 and PvRBP-2

The  $\lambda$ gt11 5.3 DNA insert (3.8 kb) was subcloned into pBluescript and sequenced (GenBank accession #M88098). There are 1250 aa encoded in this insert in a complete open reading frame. We estimate that this polypeptide sequence corresponds to roughly 45% of PvRBP-2. As for PvRBP-1, there were no significant homologies detected in data bank searches for any portion of the PvRBP-2 (5.3) gene fragment or deduced protein sequence. The 5.3 polypeptide also has no repetitive amino acid motifs, is predicted to be largely  $\alpha$ -helical, is very hydrophilic, and is similar to PvRBP-1 in overall amino acid composition. Nonetheless, the best-fit alignment of the PvRBP-1 and -2 sequences (amino acids encoded by nucleotides 3764–7508 of PvRBP-1) indicates an amino acid identity of only 22%, and no cysteine residues or other amino acids that might indicate similar tertiary conformations align. If conservative amino acid substitutions are allowed in analyses, the extent of similarity between the PvRBP sequences only increases to 34%. Additional sequence information deduced for PvRBP-2, N-terminal to the 5.3 sequence, also shows no significant similarity to PvRBP-1 (M. R. G., unpublished data). Completion of the PvRBP-2 gene sequence will allow a more thorough comparison of these proteins.

#### Native PvRBP-1 Is a Disulfide-Linked Protein

PvRBP-1 contains 19 cysteine residues (0.6%). Sixteen of these residues appear to be clustered, thereby leaving stretches of the protein sequence void of cysteines (Figure 7B). The remaining 3 cysteines are located in the hydrophobic regions, 2 in the putative signal sequence and 1 in the putative transmembrane domain. The carboxy-terminal 660 aa contain no other cysteines. To determine whether any of the cysteines in the large, presumably ex-



**Figure 8. Native PvRBP-1 Is a Disulfide-Linked Protein Complex**  
*P. vivax* merozoite proteins were extracted in SDS-PAGE sample buffer with (R, reduced) or without (NR, nonreduced) 2-mercaptoethanol, separated on a 5% SDS-polyacrylamide gel, and transferred electrophoretically to nitrocellulose. The transferred proteins were probed with rabbit 5.3  $\beta$ -galactosidase fusion protein antiserum (A) or rabbit antiserum to the glutathione S-transferase PvRBP-1D fusion protein (see Experimental Procedures) (B). Protein MW markers are apolipoprotein B (500 kd) (Sigma), myosin (200 kd), phosphorylase b (97.5 kd), and albumin (68 kd) (Bio-Rad).

tracellular domain of PvRBP-1 contribute to the conformation of the native protein, we performed Western immunoblot analyses of reduced and nonreduced PvRBP-1. As previously indicated, PvRBP-1 migrates in 5% reducing SDS-polyacrylamide gels at an approximate  $M_r$  of 280 kd (Figure 8B, lane 1). In the absence of a reducing agent, however, it migrates slower than apolipoprotein B (500 kd) and is estimated to be about 600 kd (Figure 8B, lane 2). This result suggests that PvRBP-1 is part of a protein complex composed of at least two large peptide chains linked via one or more interchain disulfide bonds. The actual quaternary composition of native PvRBP-1 remains to be clarified. In contrast with PvRBP-1, the results of Western immunoblot indicate that interchain disulfide bonds do not contribute to the conformational structure of PvRBP-2 (Figure 8A).

#### The PvRBP-1 and PvRBP-2 Genes Are Not Members of a Gene Family

The PvRBP-1 XbaI subclones 16-A, 16-B, and 16-C (Figure 6), which together contain the entire PvRBP-1 gene and flanking sequences, and the PvRBP-2 gene fragment (5.3) were used sequentially as probes under stringent conditions on Southern blots containing *P. vivax*, *P. cynomolgi*, and *P. knowlesi* restricted genomic DNAs (Figure 9A and data not shown). The PvRBP-1 probes were first applied

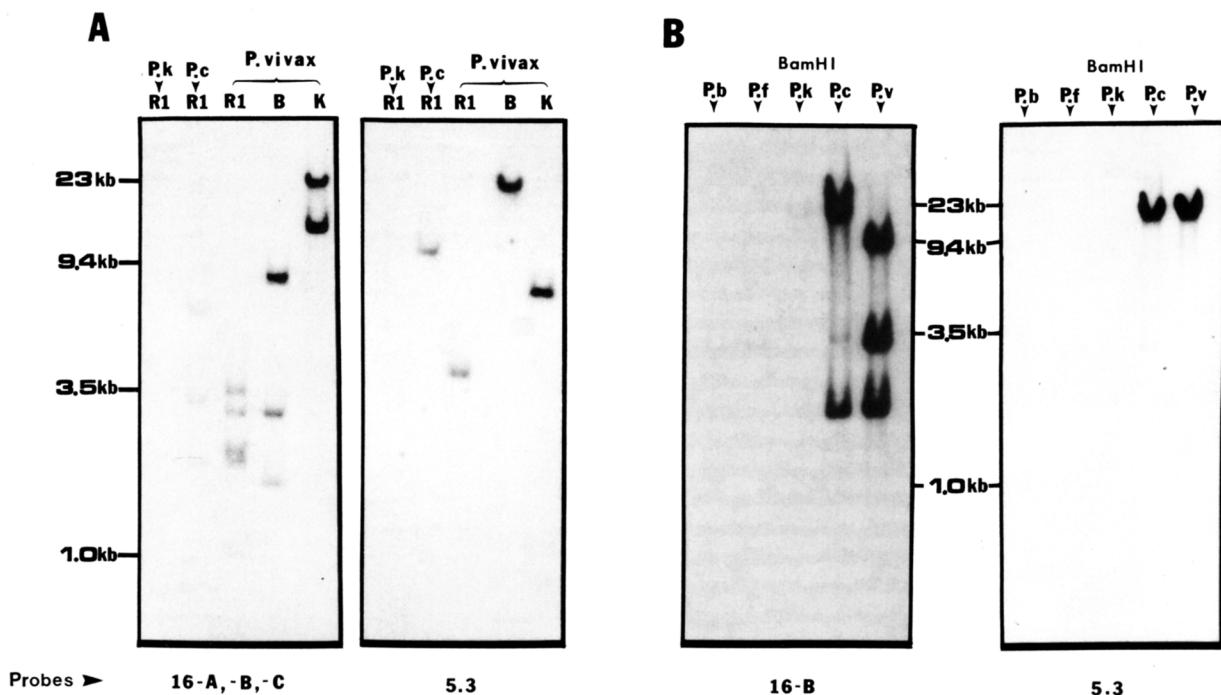


Figure 9. Genomic DNA Analysis of the PvRBP-1 and PvRBP-2 Genes

(A) Sequential hybridization of the PvRBP-1 16-A, -B, and -C subclones and the PvRBP-2 (5.3) subclone under stringent conditions (see Experimental Procedures) to restricted DNA of *P. knowlesi*, *P. cynomolgi*, and *P. vivax*. Plasmodium species, restriction enzymes, and DNA markers are indicated. RI, EcoRI; B, BamHI; K, KpnI. Exposure time shown, 24 hr.

(B) Sequential hybridization of the PvRBP-1 (16-B) and the PvRBP-2 (5.3) subclones under low stringency conditions (see Experimental Procedures), to BamHI-restricted DNA of *P. berghei*, *P. falciparum*, *P. knowlesi*, *P. cynomolgi*, and *P. vivax*. Plasmodium species and DNA markers are indicated.

individually and then as a cocktail solution. Figure 9A shows the hybridization patterns of the PvRBP-1 16-A, -B, and -C probe cocktail and the PvRBP-2 (5.3) probe, on DNA restricted with EcoRI, BamHI, and KpnI. The hybridization patterns are in agreement with the restriction enzyme sites determined for the cloned *P. vivax* DNAs: the PvRBP-1 probes detected the internal PvRBP-1 EcoRI (1.0 kb, 2.0 kb, and 2.1 kb) and BamHI (2.8 kb and 1.8 kb) gene fragments and hybridized to two KpnI fragments, consistent with the fact that there is one KpnI site within the PvRBP-1 gene (Figure 6). The PvRBP-2 (5.3) DNA insert detected the corresponding 3.8 kb EcoRI fragment in the genome and hybridized to single BamHI and KpnI fragments, consistent with the fact that it does not contain these enzyme sites. For these and other restriction enzyme digests, there was no hybridization of the PvRBP-1 probes with the PvRBP-2 gene-containing region of the genome under high or low stringency, supporting the finding deduced from analysis of the cloned genes that the PvRBP-1 and PvRBP-2 genes do not share any regions of significant identity. Thus, these data support the notion that the PvRBP genes are not members of a gene family. The PvRBP-1 and -2 probes hybridized to different size restriction fragments in *P. cynomolgi*, indicating, as would be expected, that the RBP homologs in *P. cynomolgi* are not identical to their *P. vivax* counterparts. The PvRBP gene probes did not hybridize to the DNA of *P. knowlesi* nor to the DNA of *P. berghei* and *P. falciparum* (data not shown).

#### The PvRBP Genes Are Not Conserved in *P. knowlesi*

Because *P. knowlesi* is closely related to *P. cynomolgi* and *P. vivax*, we tested whether *P. knowlesi* genes homologous to the PvRBP genes could be detected under non-stringent conditions. Freshly prepared Southern blots containing restricted genomic DNA of *P. vivax*, *P. cynomolgi*, and *P. knowlesi*, as well as the more distantly related species *P. falciparum* and *P. berghei*, were probed sequentially with the PvRBP-1 16-A, -B, and -C probes and the PvRBP-2 (5.3) probe (Figure 9B and data not shown). No hybridization was detected with the DNA of *P. knowlesi*, *P. falciparum*, or *P. berghei*, even after excessive autoradiographic exposures. The faint signal observed for these species represents background hybridization of labeled pBluescript that occurs at 59°C with low stringency washes.

#### Discussion

Entry of a malarial merozoite into a susceptible erythrocyte is a multistep process involving a cascade of specific adhesive interactions, which are just beginning to be unraveled at the molecular level. These interactions are mediated by the parasite, are precise, and have evolved to differ among species of Plasmodium. We have determined that two *P. vivax* proteins, PvRBP-1 and PvRBP-2, adhere specifically to the reticulocyte subpopulation of human and other primate erythrocytes. Antisera to recombinantly ex-

pressed polypeptides encoded by each PvRBP gene immunoprecipitate the respective PvRBP adhesins. Because reticulocytes are the primary cells invaded by *P. vivax* merozoites, it is predicted that these reticulocyte-binding proteins play a major role in determining the specificity of *P. vivax* merozoite host cell selection.

The apical pole of *Plasmodium* merozoites, as in other members of the phylum Apicomplexa, is a specialized region containing distinct organelles and proteins that function in processes of both host cell recognition and entry (reviewed in Perkins, 1992). The apical RBP fluorescence pattern observed by IFA is distinctly different from the small double-dot pattern typical for malarial antigens associated with the apically oriented rhoptry organelles. The micronemes are the only other apically situated organelles of *Plasmodium* merozoites. The apical distribution of the RBPs appears to be more extensive than that of the *P. knowlesi* Duffy-binding proteins (unpublished observations), which are sequestered prior to invasion within the micronemes (Adams et al., 1990). Furthermore, whereas the internal *P. knowlesi* Duffy-binding proteins could only be identified by IFA after fixation of the parasites and permeabilization with mild detergent (Adams et al., 1990), the RBP proteins are evident in IFA analyses on unfixed and nonpermeabilized merozoite preparations. In fact, formaldehyde fixation and detergent permeabilization of merozoites abrogate RBP recognition by antibodies. The precise localization of PvRBP-1 and PvRBP-2 will require immunoelectron microscopic and other analyses. The present evidence, however, suggests that PvRBP-1 and PvRBP-2 are located at the apical surface of the merozoite. Perhaps following translocation through the microneme organelles, a process known to occur for the circumsporozoite protein of malaria sporozoites (Fine et al., 1984; Stewart and Vanderberg, 1991), the PvRBPs become organized externally at the apical surface.

PvRBP-1 exhibits structural features of an integral membrane protein with a large hydrophilic extracellular N-terminal domain, a transmembrane domain, and a short charged cytoplasmic tail. Furthermore, this polypeptide is apparently part of a much larger native protein that is formed by interchain disulfide bonds. It is not likely that the decreased mobility of RBP-1 under nonreducing conditions is a result of aggregation; the same result occurs whether or not the samples are heat denatured (Kumar and Davidson, 1992). Although other eukaryotic membrane and cell matrix proteins such as the transferrin receptor, fibronectin, and thrombospondin are known to be sulfhydryl-linked dimeric or trimeric proteins, PvRBP-1 is the first such example indicated for malaria parasites. Whether the native protein consists of two disulfide-linked PvRBP-1 polypeptide chains or PvRBP-1 complexed with a heterologous polypeptide requires further study. However, at this time we favor a homologous dimeric-chain protein model, because reducing SDS-PAGE analysis of undegraded biosynthetically labeled immunoprecipitates of PvRBP-1 does not indicate the presence of a heterologous protein coprecipitate, and because the estimated size of the complex (600 kd) is approximately twice that of the PvRBP-1

polypeptide. In contrast, our analyses indicate that native PvRBP-2 does not contain interchain disulfide bonds.

Although the PvRBP genes and their encoded proteins do not appear to be related, it remains possible that these proteins have a functional relationship. The fact that both proteins bind reticulocytes and colocalize precisely to the same region of the apical portion of the merozoite supports the possibility that they may function in concert. Interestingly, both are predicted to be largely  $\alpha$ -helical, and it is not improbable that PvRBP-1 and PvRBP-2 could be physically but noncovalently associated and function together as a complex. As such, these ligands might form a higher affinity interaction with reticulocytes than possible with either alone. Completion of the PvRBP-2 gene sequence will likely shed more light on the possible structural and functional relationships of these molecules.

That both the PvRBP-1 and PvRBP-2 gene probes hybridize to the DNA of *P. cynomolgi*, and not to the DNA of *P. knowlesi*, is in agreement with the functional role experimentally deduced for the RBPs. Generally speaking, the lack of significant hybridization of a *P. vivax* gene probe to the DNA of *P. knowlesi*, or vice versa, would at first appear inconsistent with the phylogenetic relationship of these three species (McCutchan et al., 1984; Galinski et al., 1987; DiGiovanni et al., 1990). In keeping with the fact that these species are closely related, a portion of the *P. knowlesi* Duffy-binding protein gene (Adams et al., 1990) was used successfully as a probe to isolate the *P. vivax* homolog (Fang et al., 1991). Also, genes encoding other proteins that are essential for the invasive parasite stages of *Plasmodium* have been maintained and conserved as speciation occurred (Peterson et al., 1990; Walters et al., 1990). Because *P. knowlesi* is not restricted to reticulocytes, it is possible that extreme divergence or perhaps even extinction of the RBP ancestral genes has occurred in this species. The converse, that these large genes evolved only and specifically in *P. vivax* and *P. cynomolgi*, intuitively is less likely. The rodent parasite, *P. berghei*, also preferentially invades reticulocytes. However, the lack of any hybridization of the RBP gene probes to the DNA of this species is not surprising, considering the extent of evolutionary divergence between *P. berghei* and *P. vivax*.

While it has been known for decades that *P. vivax* tends to exclusively invade reticulocytes instead of normocytes, the basis for this host cell exclusivity, until now, has remained uninvestigated. One hypothesis previously set forth to explain this "preferential" invasion takes the position that *P. vivax* merozoites invade reticulocytes primarily because the spectrin-based cytoskeletal network is incompletely packed in the membrane of young immature red blood cells, and therefore the membrane is more "pliable" and permissive to invasion (Mons, 1990). Although this explanation may have some validity, it is implicit that the selective parasitization of reticulocytes, which represent less than 1% of the total red blood cell population, would occur by essentially nonspecific trial and error probing. However, for *P. vivax*, such selection of a host cell by default could result in predominantly futile and abortive

invasion attempts. Alternatively, reticulocyte-specific binding molecules would provide the merozoites with an efficient means to discriminate between reticulocytes and normocytes. Our experimental data support this hypothesis. The selective interaction of a merozoite ligand to a reticulocyte receptor would signify the commitment of a merozoite to that reticulocyte.

Formation of the irreversible junction between parasite and host membranes, which precedes invasion, is known to be dependent specifically upon the binding of the merozoite to the erythrocyte Duffy glycoprotein receptor (Miller et al., 1979). Also of significance, these investigators and others (Mitchell and Bannister, 1988) have shown that prior to, or in the absence of, the formation of a junction, malaria merozoites attach apically to erythrocytes via long (40–120 nm), fine filaments. Since the merozoite Duffy-binding proteins are sequestered in the microneme organelles prior to invasion, some signal presumably must initiate their release. Interaction of the RBPs with receptors on the reticulocyte membrane could provide that signal. Particularly since the merozoite is likely to be transiently in contact with mature circulating erythrocytes, this reticulocyte-specific signal would eliminate the possibility of premature release and irreversible binding of PvDAP-1, and thus merozoites, to normocytes. This model of reversible contact, committed contact, signaling, and irreversible adhesion for merozoite invasion has similarities to models proposed for leukocyte–endothelial cell interactions (Butcher, 1991).

The reticulocyte membrane is not nearly as well characterized as the membrane of the mature red blood cell. However, it is known that during the maturation process of erythrocytes, a number of constituent proteins are lost from the membranes of reticulocytes (Patel et al., 1985; Rapoport, 1986; Lazarides, 1987). Any of these proteins, or others as yet unidentified, could potentially constitute or be a component of the receptor site(s) of the PvRBPs. That PvRBP-1 contains two RGD motifs in its sequence may provide one clue to the nature of its host cell receptor. RGD and other integrin-binding sites have been implicated in cellular interactions of a number of bacterial (Isberg and Leong, 1990) and fungal (Gabius et al., 1985) adhesins, as well as cell adhesion proteins of higher animals (reviewed in Hynes, 1992).

In essence, we propose that the immature red blood cells are actively selected for invasion by *P. vivax* merozoites through the specific interaction of their PvRBP adhesins with a receptor(s) present on the surface of reticulocytes that is absent from mature erythrocytes, and that this contact may initiate subsequent molecular contacts, including the binding of PvDAP-1. Furthermore, it is not unlikely that analogous proteins (not specific for reticulocytes) will be identified that serve a similar function in the invasion process for other malaria parasite species that are not restricted to reticulocytes.

#### Experimental Procedures

##### Parasites and In Vitro Culture of *P. vivax*

*P. vivax* (Belem strain) late-stage trophozoite-infected erythrocytes were acquired from splenectomized Saimiri boliviensis (squirrel) mon-

keys. *P. knowlesi* (H strain) and *P. cynomolgi* (Mulligan strain) schizont-infected erythrocytes were acquired from Rhesus monkeys (*Macaca mulatta*). *P. berghei* was isolated from infected hamsters. All parasite infections were initiated by intravenous injection of blood stage parasites. *P. falciparum* was grown in continuous in vitro blood cultures (Trager and Jensen, 1976). *P. vivax* trophozoite-infected erythrocytes were purified from whole blood and cultured in vitro to the mature schizont stage in RPMI 1640 tissue culture medium supplemented with 15% human AB serum, 50 mg/liter hypoxanthine, 2 g/liter D-glucose, and 35 mM HEPES, as described (Barnwell et al., 1989). Metabolically labeled parasites for immunoprecipitation and EBA analyses were obtained by culturing the infected erythrocytes ( $2.5 \times 10^7$  per ml) in methionine-deficient medium supplemented with 100  $\mu$ Ci/ml of [<sup>35</sup>S]methionine (New England Nuclear) to the desired stage of maturity.

#### Preparation of *P. vivax* Extracts and Culture Supernatants

[<sup>35</sup>S]methionine metabolically labeled mature schizonts and merozoites were extracted in 1% NP-40 in 10 mM HEPES or phosphate-buffered saline (PBS) with 25  $\mu$ g/ml each leupeptin, chymostatin, E-64, and pepstatin protease inhibitors. Extracts were clarified by centrifugation at 16,000  $\times$  g for 30 min at 4°C. Culture supernatants were produced essentially as described (Wertheimer and Barnwell, 1989). *P. vivax*-infected erythrocytes (over 90% purity) were allowed to mature, rupture, and release merozoites in the absence of fresh target erythrocytes. Six to 8 hr after initial merozoite release, culture supernatants were collected, centrifuged at 18,000  $\times$  g for 30 min at 4°C, and either used immediately or stored in liquid nitrogen. Culture supernatants were adsorbed with protein A-conjugated Sepharose CL-4B (Pharmacia) at a volumetric ratio of 2:1 to remove human immunoglobulin G (IgG).

#### Immunoprecipitation of Parasite Proteins

Immunoprecipitation of [<sup>35</sup>S]methionine metabolically labeled parasite proteins was accomplished by incubating 150  $\mu$ l of plaque-purified antibody preparations or 25  $\mu$ l of the IgG fraction of rabbit antiserum with 400  $\mu$ l of *P. vivax* culture supernatant or 100  $\mu$ l of detergent extract for 2 hr at 4°C, followed by 60  $\mu$ l of a 50% suspension of protein A-Sepharose 4B beads for 1 hr. The Sepharose beads were washed twice in NETT buffer (0.5% Triton X-100, 5 mM EDTA, 50 mM Tris, 150 mM NaCl), once in NETT with 0.5 M NaCl, and again twice in NETT, with the exception of the experiment in Figure 4B, in which the immunoprecipitates were washed five times, solely with NETT. Immunoprecipitates were analyzed by standard methods of SDS-PAGE and fluorography.

#### EBA

[<sup>35</sup>S]methionine-labeled culture supernatants (400  $\mu$ l) were mixed with PBS-washed erythrocytes ( $1 \times 10^9$ ) for 20 min, washed by microcentrifugation through 0.75 ml of silicone oil (Dow Corning 550), resuspended in RPMI 1640 basal medium with 5% fetal calf serum, and again washed through silicone oil. Adsorbed protein was eluted in 75  $\mu$ l of 0.75 M saline or 3  $\times$  Hank's buffer salt solution and analyzed by SDS-PAGE and fluorography. For subsequent immunoprecipitation analyses, eluates were diluted to 0.15 mM salts with 0.1% NP-40 in 10 mM HEPES.

Rabbit erythrocytes were obtained from reticulocytic animals used routinely for normal mosquito feedings. Human erythrocytes had a normal reticulocyte count of 0.5%. Reticulocyte-enriched, or depleted, fractions of blood were separated as described (Barnwell et al., 1989) using Percoll (Pharmacia) density cushions. The percentage of reticulocytes present was determined by light microscopy. Erythrocyte samples were incubated in PBS containing 0.5% brilliant Cresyl blue stain for 30 min, smeared on a glass slide, fixed briefly in methanol, counterstained with Giemsa, and counted.

#### Squirrel Monkey Immune Serum Production

A squirrel monkey (*Saimiri boliviensis*, #7251) was immunized with  $2 \times 10^6$  mature *P. vivax* schizonts, twice by intravenous inoculation, and twice by intramuscular injection within a period of 9 months, using Freund's incomplete adjuvant. The antiserum tested positively by IFA only with schizonts and merozoites, and immunoprecipitated 12 to 15 *P. vivax* proteins ranging in size from approximately 50 kd to 300 kd

as determined by reducing SDS-PAGE analysis. The antiserum was adsorbed sequentially with  $\lambda$ gt11 plaques that had been transferred to nitrocellulose filters and *E. coli* protein extracts coupled to Affigel 10 and 15 beads (Bio-Rad).

#### **$\lambda$ gt11 Library Construction and Immunoassay**

*P. vivax* genomic DNA was extracted and purified from mature blood stage parasites essentially as described (Ozaki et al., 1983). A  $\lambda$ gt11 expression library was constructed (contributed by D. E. Arnot) by ligating *P. vivax* genomic DNA digested with EcoRI into  $\lambda$ gt11/EcoRI calf intestinal alkaline phosphatase-treated vector arms (Stratagene). The recombinant phage were packaged using Packagene extracts (Promega) and immunoscreened (Young and Davis, 1983) using the immune serum described above from Saimiri monkey #7251, diluted 1:500. Plaques transferred to nitrocellulose filters were first incubated with this antiserum for 4 hr, washed three times for 20 min with 1 x Tris-buffered saline (TBS), 0.1% Tween 20, and then incubated with rabbit anti-Saimiri monkey IgG (2  $\mu$ g/ml) for 1 hr. The filters were washed as above and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG (diluted 1:7500) for 90 min. After being washed again in the same manner, immunoreactivity was detected using the NBT-BCIP chromogenic substrate as recommended by the manufacturer (Promega). Positive phage were purified through three cycles of immunoscreening, grown as liquid lysates (Sambrook et al., 1989), and purified using  $\lambda$  Sorb (Promega).

#### **Saimiri Monkey Plaque-Selected Antibody Purification**

Saimiri monkey antibodies specific for the fusion proteins expressed by  $\lambda$ gt11 phage clones 7.2 and 5.3 were purified by plaque selection. Purified positive phage were plated at a density of  $1 \times 10^5$  pfu/100 mm plate. When plaques appeared, two nitrocellulose filters, impregnated with 10 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), were applied consecutively for 2 hr incubation periods each, at 37°C. The filters were blocked overnight at 4°C in 3% Carnation nonfat dry milk in TBS and then incubated for 4 to 6 hr with the Saimiri monkey *P. vivax* antiserum. After three 20 min washes in TBS, the bound antibodies were eluted with 0.2 M glycine (pH 2.5), neutralized with Tris (1.6 M, 0.1 vol), dialyzed overnight, and concentrated on an Amicon filter.

#### **Rabbit Fusion Protein Antiserum Production**

Y1089 *E. coli* cells were infected with purified  $\lambda$ gt11 recombinant phage as described (Huynh et al., 1985). Cultures of the resulting lysogens were grown to a density of 0.5–0.8 OD<sub>600</sub>, heat shocked at 42°C for 20 min, and then grown for 2 hr at 37°C with the addition of 10 mM IPTG. The cells were centrifuged, resuspended in 100 mM Tris, 10 mM EDTA containing 1 mM phenylmethylsulfonyl fluoride and 1.2 mM each of TLCK and TPCK protease inhibitors, and sonicated. The soluble fusion proteins from clones 7.2 and 5.3 were purified on LacZ HA anti- $\beta$ -galactosidase beads (Promega). The  $\beta$ -galactosidase fusion proteins were analyzed by SDS-PAGE, silver staining (Pierce), and Western immunoblot using the 7.2 and 5.3 plaque-selected antibodies and an anti- $\beta$ -galactosidase monoclonal antibody (Promega). Clones 7.2 and 5.3 produced fusion proteins of 190 kd and 260 kd, respectively. A glutathione S-transferase fusion protein was also produced by expressing a 2.1 kb EcoRI fragment of the PvRBP-1 gene in the plasmid pGEX-2T (Pharmacia). This 2.1 kb fragment (RBP1-D) is encoded by PvRBP-1 nucleotides 5786–7848 immediately 3' to the position of the 2.0 kb 7.2 coding region. SURE cells (Stratagene) were transformed with the RBP1-D pGEX plasmid, cultured, and the cell pellet sonicated. The soluble glutathione S-transferase (RBP1-D) fusion protein (110 kd) was purified by glutathione-Sepharose CL-4B affinity chromatography (Pharmacia). The purified 7.2, 5.3, and RBP1-D fusion proteins were resuspended in PBS and emulsified with an equal volume of Freund's complete adjuvant for the first immunization and with Freund's incomplete adjuvant for two subsequent immunizations in rabbits.

In addition to analyses presented, the rabbit antisera against the 5.3 and 7.2 fusion proteins were also tested for inhibitory activity in *P. vivax* and *P. knowlesi* in vitro invasion assays (Barnwell et al., 1989). The 5.3 antiserum inhibited *P. vivax* invasion 46% and 31% at 1:10 and 1:20 dilutions, respectively. The 7.2 antiserum inhibited *P. vivax* invasion 23% at a 1:10 dilution. *P. knowlesi* merozoite invasion was not significantly affected by either antiserum.

#### **Microscopic Immunofluorescence Assays**

Thin film smears of mature schizonts and merozoites in 50% fetal bovine serum were made on glass slides and allowed to air dry. The slides were placed in a humidified chamber, and sections of the smears were incubated at room temperature for 90 min with antiserum diluted 50- to 500-fold in PBS or plaque-purified antibody diluted 2- to 5-fold. The slides were washed in PBS, 0.1% bovine serum albumin and subsequently incubated with either FITC-conjugated goat anti-rabbit IgG or rabbit anti-Saimiri monkey IgG for 1 hr, washed, and mounted in glycerol (pH 8.0) containing antifade (phenylenediamine) for viewing by epifluorescent microscopy. Apical localization was confirmed by coincubation with 4,6-diamidino-2-phenylindole for nuclear staining. Colocalization studies utilized two-color fluorescence microscopy with sequential applications of rabbit antiserum, rhodamine-conjugated goat anti-rabbit IgG, Saimiri monkey plaque-purified antibodies, and FITC-conjugated rabbit anti-Saimiri IgG.

#### ***P. vivax* RNA Purification and Northern Blot Analysis**

Late-stage schizonts were extracted with 4 M guanidinium thiocyanate, 25 mM sodium acetate (pH 7.0), 0.5% sarcosyl, and 100 mM  $\beta$ -mercaptoethanol, and total RNA was purified, using a kit from Stratagene based upon the method of Chomczynski and Sacchi (1987). For Northern blot analyses, 30  $\mu$ g of total *P. vivax* RNA and 3  $\mu$ g of RNA markers (Bethesda Research Laboratories) were dissolved in 20 mM MOPS (pH 7.0), 50% formamide, and 6% formaldehyde, separated by agarose gel electrophoresis (1% agarose, 6% formaldehyde, 20 mM MOPS [pH 7.0]), and transferred overnight in 20 x SSPE (0.2 M phosphate buffer [pH 7.4], 2.88 M NaCl, 0.02 M EDTA) onto a Duralon nylon filter (Stratagene). The filter was rinsed briefly in 0.2 x SSPE, and the RNA was cross-linked using ultraviolet light (UV) (Stratagene). Prehybridization was carried out for 4 hr at 42°C in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.5% SDS, and 200  $\mu$ g/ml denatured herring sperm DNA. The prehybridization solution was removed and replaced with new solution containing 5  $\times$  10<sup>6</sup> cpm/ml of probe labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random priming with 9-mer primers and T7 DNA polymerase (Stratagene). Hybridization was carried out for about 20 hr at 42°C. Washes (two times 5 min in 5 x SSPE; one time 15 min in 2 x SSPE, 0.1% SDS; one time 30 min in 0.3 x SSPE, 0.1% SDS; and one time 30 min in 0.1 x SSPE, 0.1% SDS) were performed in a shaking waterbath at 42°C. The filters were exposed at -70°C with an intensifying screen, first overnight, and then again for 48 hr. The radiolabeled 7.2 probe was removed by washing the filters three times with boiling 0.1% SSPE, 0.1% SDS. Prior to hybridization of the 5.3 probe, the filter was checked by autoradiography to ensure that the 7.2 probe had been completely removed. The distinct 7.2 and 5.3 transcript signals were evident when the two autoradiographs were superimposed.

#### ***P. vivax* $\lambda$ Dash II Genomic DNA Library Construction and Screening**

Vernick et al. (1988) reported that mung nuclease in the presence of 30% to 35% formamide digested *P. falciparum* DNA immediately 5' and 3' of a number of genes. This methodology was adapted to isolate the PvRBP-1 and PvRBP-2 genes. Pilot experiments were performed with *P. vivax* DNA (M. R. G., unpublished data), which indicated that 40% formamide was optimal for the isolation of large *P. vivax* gene-containing fragments, ranging in size from 9 to 23 kb, suitable for cloning in a  $\lambda$  replacement vector. *P. vivax* genomic DNA (20  $\mu$ g) was digested with 2 U/ $\mu$ g mung bean nuclease (Pharmacia) at 50°C in 0.2 M NaCl, 30 mM sodium acetate (pH 4.6), 1 mM ZnSO<sub>4</sub>, and 40% formamide for 15 min. The digested DNA was blunt ended with T4 DNA polymerase (Bethesda Research Laboratories), methylated with EcoRI methylase (Bethesda Research Laboratories), and ligated overnight with EcoRI linkers (Bio-Labs) that had been phosphorylated with [ $\gamma$ -<sup>32</sup>P]dATP using T4 polynucleotide kinase (Bethesda Research Laboratories). Excess linkers were removed by digestion with 300 U of EcoRI for 4 hr followed by two ethanol precipitations with 2 M ammonium acetate. The resulting *P. vivax* DNA fragments were treated with calf intestinal alkaline phosphatase (Stratagene), and an aliquot (900 ng) was ligated to 1  $\mu$ g of  $\lambda$  Dash II/EcoRI arms (Stratagene) overnight at 4°C, using T4 DNA ligase (U.S. Biochemical). The recombinant DNAs were packaged with Gigapack Gold extracts (Stratagene). Non-amplified recombinant  $\lambda$  Dash II phage ( $2 \times 10^5$ ) were plated on P2392

cells. The phage plaques were transferred in duplicate to nitrocellulose (Schleicher and Schuell), denatured as described in Sambrook et al. (1989), and their DNAs cross-linked with UV (Stratagene). Prehybridizations were carried out at 50°C for 3 hr in 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate), 50 mM sodium phosphate, 5 × Denhardt's solution, and 100 µg/ml sheared and denatured salmon sperm DNA. Hybridization was carried out overnight at 65°C in a fresh aliquot of the same solution, with the addition of a probe cocktail containing the DNA inserts of λgt11 clones 7.2 and 5.3 ( $5 \times 10^5$  cpm of each probe per ml). Each probe was radiolabeled with [ $\alpha^{32}$ P]dCTP by random priming with 9-mers and T7 DNA polymerase (Stratagene). The filters were washed three times for 20 min at 50°C in 0.2 × SSC, 0.1% SDS, and exposed to Kodak XAR-5 film overnight at -70°C with an intensifying screen. Positive clones were replated and screened separately with each radio-labeled DNA insert. Six recombinants hybridized to the 7.2 (PvRBP-1) DNA insert and one to the 5.3 (PvRBP-2) DNA insert.

#### Physical Characterization of the λ Dash PvRBP-1 Clones

The basic physical maps of four of the PvRBP-1 gene clones were determined and compared (Figure 6). Because we have observed cloning artifacts in a few instances in other analyses of such large cloned inserts, care was taken to be confident in the integrity of these clones. Toward this end, each XbaI fragment released from λ Dash clones 10, 6, 16, and 11 was subcloned into pBluescript, and the sequences of about 300 bp were determined from the ends of each. The sequences of regions in common between each clone proved to be identical. The length of the A and D XbaI fragments varied either because of the extent of mung bean nuclease activity on the ends of each individual genomic DNA fragment or because all EcoRI sites were not methylated and were subsequently digested with EcoRI in the cloning process. DNA sequencing of the complete PvRBP-1 gene confirmed that the B fragments of clones 10 and 6 contain natural P. vivax EcoRI sites ligated to λ Dash and are thus the result of incomplete EcoRI methylation.

#### DNA Sequencing

DNAs to be sequenced were subcloned into pBluescript SK(+) (Stratagene). Nested deletions of 100–300 bp intervals were created using exonuclease III and mung bean nuclease (U.S. Biochemical) and standard methodology. DNA sequences were generated using the dideoxy termination sequencing methodology (Sanger et al., 1977), [ $^{35}$ S]dATP, and Sequenase reagents and protocols (U.S. Biochemical). In a few reactions PvRBP-specific oligonucleotides were used as primers. Over 90% (about 8.5 kb) of the PvRBP-1 gene and the entire PvRBP-2 gene fragment (3.8 kb) were sequenced on both DNA strands. Each gene and deduced protein sequence was analyzed using Pustell and MacVector software programs (IBI). GenBank (release 70) and the Swiss Protein Data Bank (release 20) were screened for DNA and protein sequence homologies using the GCG Sequence Analysis Software Package, Version 7.0 (Genetics Computer Group, Inc.).

#### Genomic DNA Southern Blot Analysis

Aliquots (1 µg) of genomic DNA, digested for 3 hr with designated restriction enzymes, were separated on 1% vertical agarose gels by standard electrophoresis, visualized by ethidium bromide staining, transferred by vacuum blotting for 1 hr to Duralon nylon filters, and UV cross-linked (Stratagene). Prehybridizations (3 hr at 50°C) and hybridizations (about 20 hr) were carried out in 6 × SSC, 50 mM sodium phosphate, 5 × Denhardt's solution, and 100 µg/ml sheared, denatured salmon sperm DNA. Probes were labeled with [ $\alpha^{32}$ P]dCTP by random priming with 9-mers and T7 DNA polymerase (Stratagene) and were added to hybridization reactions at  $1 \times 10^6$  cpm/ml. Stringent hybridizations were performed at 65°C with filter washes carried out in 0.2 × SSC, 0.1% SDS. Low stringency hybridizations were performed at 59°C with filter washes in 2 × SSC, 0.1% SDS. All filters were washed, three times for 20 min with rocking, and exposed for 24 and 72 hr to Kodak XAR-5 film at -70°C with an intensifying screen.

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#### GenBank Accession Numbers

The accession numbers for the sequences reported in this article are M88097 (PvRBP-1 gene) and M88098 (PvRBP-2 gene fragment 5.3).