Plasmodium falciparum Homologue of the Genes for *Plasmodium vivax* and *Plasmodium yoelii* Adhesive Proteins, Which Is Transcribed but Not Translated

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The 235-kDa family of rhoptry proteins in *Plasmodium yoelii* and the two reticulocyte binding proteins of *P. vivax* comprise a family of proteins involved in host cell selection and erythrocyte invasion. Here we described a member of the gene family found in *P. falciparum* (*PfRH3*) that is transcribed in its entirety, under stage-specific control, with correct splicing of the intron, but appears not to be translated, probably due to two reading frameshifts at the 5′ end of the gene.

Until we understand more fully the fundamental mechanisms that malaria parasites use to invade red blood cells, it will be almost impossible to design rational interventions to prevent this essential step in the parasite's life cycle. *Plasmodium falciparum* is the parasite responsible for almost all of the 2 million deaths that occur annually from malaria (69). With the *P. falciparum* genome sequencing project well under way (9, 24, 29) comes the opportunity to look for candidate genes for proteins that may be involved in erythrocyte invasion. *P. falciparum* is a member of the phylum Apicomplexa. These intracellular protozoan parasites are characterized by possession of a group of organelles, which include the rhoptries, micronemes, and dense granules (reviewed in references 55 and 59). The contents of these organelles are released at the time of invasion of host cells (59), some binding to host cell receptors and effecting cell penetration.

The sequence of events leading to erythrocyte invasion by *Plasmodium* parasites has been revealed by videomicroscopy and ultrastructural studies (1, 2, 16, 40). Malaria merozoites are released from infected cells at the end of schizogony. They invade fresh erythrocytes by first adhering to the uninfected cell. Merozoites can adhere in any orientation, and this initial attachment is reversible. Successful invasion occurs when the merozoite reorientates itself so that the apical end is in contact with the erythrocyte. At this stage, the attachments are irreversible and lead to the formation of a tight junction, the subsequent invagination of the erythrocyte membrane, and formation of a vacuole as the parasite moves into the cell.

The *P. falciparum* genome sequencing project provides the opportunity to look for homologues of proteins that are known to play a role in host cell invasion in other species. In *Toxoplasma*, sequential secretion occurs from the micronemes,

rhoptries, and dense granules mediating cell invasion (11). It is not known if such sequential secretion occurs in *Plasmodium* but several proteins involved in invasion are found in the micronemes and rhoptries. One well-characterized family is the erythrocyte binding protein family (EBP), whose members are located in the micronemes and include the Duffy binding protein (DBP) of *P. vivax* and the 175-kDa *P. falciparum* erythrocyte binding antigen (EBA-175). EBA-175 binds to sialic acid-dependent residues on glycophorin A during erythrocyte invasion (10, 48, 60, 61). EBA-175-independent pathways have also been identified (15); field isolates commonly use alternative invasion pathways (46), parasites with a targeted disruption of EBA-175 switch to a sialic acid-independent pathway (57), and at least one other erythrocyte binding protein (EBL-1) has been described (51).

Several rhoptry proteins are thought to play a role in host cell invasion and include MAEBL and apical membrane antigen 1 (AMA-1) (33, 52, 64). We were particularly interested in identifying homologues of a *P. yoelii* multigene family, encoding the 235-kDa rhoptry protein family (Py235) (20, 31, 34). There are several lines of evidence suggesting that these proteins play a central role in erythrocyte invasion. For example, in mice, passive immunization with monoclonal antibodies to these proteins (20) or active immunization with affinity-purified protein (31) attenuates the virulent parasite *P. yoelii* YM, limiting the infection to reticulocytes and mimicking the less-virulent strain, *P. yoelii* 17X. Furthermore, Py235 proteins bind to mature erythrocytes (44, 45).

The Py235 family has a small but significant similarity to two proteins in *P. vivax*, the reticulocyte binding proteins (RBPs) 1 and 2 (23, 35; the present study). *P. vivax* is much less flexible in its requirements for infection than either *P. yoelii* or *P. falciparum* (42), dependent as it is on the erythrocyte Duffy antigen for invasion (3, 41). Moreover, it exclusively invades reticulocytes (42, 43), and the interaction of the RBPs and the host cell is independent of the binding of the parasite DBPs to the erythrocyte Duffy antigen (22). It has been proposed that the RBPs act to select the appropriate cell for the parasite by

binding to a reticulocyte specific protein, triggering the release of the Duffy binding proteins and the formation of the tight junction (22).

We envisaged that there might be *P. falciparum* homologues of Py235 and PvRBPs, which play a role in interactions independent of those of the EBPs. Two such genes have recently been described (56, 67). The two genes, *Pfr2ha* and *Pfr2hb* (also called *PfRBP2-Ha* and *PfRBP2-Hb*) code for large type I membrane-bound proteins with some sequence similarity to both the PvRBPs and the Py235s. *Pfr2ha* and *Pfr2hb* are located centrally on chromosome 13 (67). Here we describe a *P. falciparum* putative pseudogene, *PfRH3*, located on chromosome 12, that is a member of the superfamily that includes these genes and those coding for Py235 and PvRBPs.

MATERIALS AND METHODS

Sequence analysis. Preliminary sequence data on The Institute for Genomic Research, The Sanger Centre, and Stanford University websites (http://www.tigr.org/, http://www.sanger.ac.uk/, and http://sequence-www.stanford.edu/), and sequence data submitted to EMBL (http://www.ncbi.nlm.nih.gov/Malaria/) were searched for sequence homology to Py235 and PvRBPs using tblastn. Alignment of predicted protein sequences was performed using Clustalx (65). For comparisons of predicted protein sequences, the Gap and Bestfit functions of GCG 10.1 were used (25). Prediction of transmembrane domains, hydrophobicity, and signal sequences in the protein sequences was performed using TmPred and PSort (http://www.expasy.ch/).

Plasmid construction. Genomic DNA from 3D7 parasites was used to PCR amplify a 710-bp DNA fragment (predicted amino acids 497 to 733). The primers were 5′-ctcgagCTGAAAAGTGTTTTTCGG-3′ and 5′-agatctAATTATTATGAATATAATGATG-3′ incorporating *Xho*I and *Bgl*II sites (shown in lowercase), respectively. The digested PCR product was cloned into the pHH1 transfection plasmid which contains a mutated human dihydrofolate reductase gene fragment encoding resistance to WR99210 (57). The resultant transfection plasmid was called pHH1-rh3.

Parasite culture and transfection. *P. falciparum* clones 3D7, E12, HB3, and FCB1 parasites were cultured in vitro in group O+ erythrocytes and RPMI 1640 (Life Technologies) supplemented with 2 mM glutamine and Albumax (5 g/liter), as described previously (6). Parasite development was synchronized by sorbitol treatment (37), Plasmagel flotation (50), or by centrifugation over 70% Percoll (30). Transfection of E12 and HB3 parasites with the pHH1-Δrh3 construct was carried out as described elsewhere (19, 66).

Parasite nucleic acid isolation and cDNA synthesis. Parasite DNA was isolated from cultures by incubating saponin-lysed pRBCs in 1% sodium dodecyl sulfate (SDS)–50 mM Tris–100 mM EDTA–200 mM NaCl (pH 9.0). Proteinase K was added to 1 mg/ml, and the reaction was incubated at 50°C overnight. DNA was obtained from the supernatant using phenol-chloroform extraction and isopropanol precipitation. DNA from wild isolates was extracted from filter paper blood samples from infected patients collected in 1992 in Igbo-Ora, Nigeria. Circles 5 mm in diameter were cut from the samples and incubated in TE (10 mM Tris, 1 mM EDTA; pH 8.0), 1% SDS, 1 mg of proteinase K per ml for 30 min at room temperature. DNA was extracted from the supernatants using phenol-chloroform and ethanol precipitation. RNA was extracted from parasite cultures (harvested at 5 to 15% parasitemia) using Trizol (Life Technologies) and stored in formamide at −80°C, as described previously (36, 63), or mRNA was purified directly using a MicroPolyA Pure Kit (Ambion, Inc.). RNA samples were treated with DNase (Life Technologies) to remove contaminating genomic DNA and reversed transcribed using Superscript reverse transcriptase (RT; Life Technologies) and random primers according to the manufacturer's instructions. cDNA was stored at −20°C or used immediately for PCR (0.5 µl/12.5-µl reaction). Control samples without the addition of RT were processed in parallel. 5′ RACE (rapid amplification of cDNA ends) was carried out using a Life Technologies 5′ RACE kit and the primers 5RHRa (5′-CACATAATGTGGGTATAAAG) and 5RHRb (5′-GGGGTCTAAATAAATTTGACG).

Southern and Northern blotting. Restriction digests were performed using a range of enzymes according to the manufacturer's instructions. Digested DNA was electrophoresed on agarose gels and transferred to Hybond N+ in 0.5 M NaOH. Pulsed-field gel electrophoresis (PFGE) was used to separate 3D7 chromosomes (8) which were transferred to Hybond N+. Gel electrophoresis of parasite RNA was carried out in 1% agarose gels as described elsewhere (36).

RNA was transferred to Hybond N+ in 7.5 mM NaOH for 12 h. The filter was neutralised in 2 × SSC (20× SSC is 3 M sodium chloride plus 0.3 M sodium citrate) and air dried prior to hybridization.

DNA fragments were labeled for hybridization reactions using the Prime-it II DNA labeling system (Stratagene). Gel-purified PCR products (25 ng) were labeled in a 50-µl reaction by using 1.11 MBq [α-^{32}P]dATP according to the manufacturer's instructions. Filters were prehybridized for 1 h in hybridization buffer (0.5 M sodium phosphate, 7% SDS). They were hybridized in the same buffer with the addition of the probe for up to 16 h and washed with buffers containing 0.1% SDS and between 0.1× and 2× SSC at between 55 and 65°C depending on the stringency required.

PCR. PCRs were carried out in 0.5-ml microfuge tubes with final concentrations as follows: 1× PCRII buffer (Perkin-Elmer), 1.5 to 3.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 1 µM forward primer, 1 µM reverse primer, 1 U of Amplitaq Gold DNA polymerase (Perkin-Elmer) per 50-µl reaction, and genomic DNA (~50 ng/50-µl reaction). For accurate amplification and sequence determination, the proofreading polymerases *Pfu* Turbo (Stratagene) or *Pfx* (Life Technologies) were used instead of Amplitaq Gold.

Production of recombinant GST fusion proteins and antibodies. Three *PfRH3* fragments were amplified and subcloned into pGEX vectors for expression as glutathione S-transferase (GST) fusion proteins (fragments RH3-1, RH3-2, and RH3-4; see Fig. 1). A 690-bp fragment, RH3-1, was amplified using the primers 5′-ggatccTGATGAAACCCATGTTGGATATGTCTCT and 5′-ggatccACCAGAAAACATTACTCTCTTAGCAC, cloned into TA vector, and subcloned into pGEX-3X (Amersham Pharmacia). Two other *PfRH3* fragments of 1,130 and 1,020 bp were amplified and subcloned into pGEX-4T to give the fusion proteins RH3-2 and RH3-4, respectively. The primers used for the RH3-2 protein had the following sequences: 5′-ggatccGTTTAAATCAAGTATTAGAAAAATATGAAGAA-3′ and 5′-gaattcAATATCATTTGTGTTAAATTTTGTTTGAATAAAATC-3′. The primers used for the RH3-4 protein had the following sequences: 5′-ggatccCAAGGAGTAATATCAAAACCAGCAGAAAGTGGA-3′ and 5′-gaattcATCTTCTTCTGCTTTTACAAGTTGTATTTC-3′. Plasmids with the expected sequence were used to produce recombinant GST fusion proteins in DH5α or BL21 cells, and the protein was purified using glutathione-agarose (Amersham Pharmacia). BALB/c mice were immunized intraperitoneally with 30 µg of the RH3-1 fusion protein in complete Freund adjuvant and boosted three times at 21-day intervals with 50 µg in incomplete Freund adjuvant. Serum was collected 14 days after the final boost. Similarly, the RH3-2 and RH3-4 fusion proteins were used to immunize rabbits. Antibodies were purified from the rabbit sera by the removal of GST specificities, followed by affinity chromatography on the appropriate fusion protein.

SDS-PAGE and Western blotting of parasite extracts. Either asynchronous 3D7 parasites or synchronised late-schizont stage parasites from E12, HB3, or the *PfRH3*-disrupted cultures were harvested. The cells were resuspended in phosphate-buffered saline (PBS) containing complete protease inhibitor cocktail. A 1% saponin-PBS solution was added dropwise until haemolysis was observed. The saponin was diluted out by flooding the reaction with PBS, and the released parasites were harvested by centrifugation. They were lysed in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer with or without the addition of 0.1 M dithiothreitol (DTT) and heated to 100°C for 5 min; the lysate was then resolved by electrophoresis on 12.5, 7.5, or 6% polyacrylamide gels. After transfer onto nitrocellulose, specific proteins were detected using polyclonal mouse or rabbit sera followed by horseradish peroxidase-linked secondary antibodies (ICN) and ECL (Pierce).

Metabolic radiolabeling of parasites. Parasites were radiolabeled by culturing them in the presence of [^{35}S]methionine and [^{35}S]cysteine (Promix; Amersham Pharmacia). Synchronized schizonts (estimated to be more than 33 h postinvasion) from a 70% Percoll layer were grown in methionine- and cysteine-free medium for 15 min, before the addition of 100 µCi of Promix per ml. Parasites were cultured in the presence of radiolabel for 2 h before being washed in PBS and harvested. Pelleted parasitized cells were stored at −70°C.

In vitro translation. Overlapping fragments of ca. 3 kb from *PfRH3* were cloned into the TA vector (Invitrogen) according to the manufacturer's instructions. Transcripts were made using T7 RNA polymerase and were translated using the rabbit reticulocyte lysate system (Promega).

Immunoprecipitation. Radiolabeled parasite pellets were thawed into denaturing buffer (1% SDS, 50 mM Tris-HCl, 5 mM EDTA; pH 8.0) and boiled for 5 min. Pellets were divided into the number of samples required, such that each sample was 100 µl. A total of 900 µl of DOC buffer (0.5% deoxycholate, 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA) was added to each sample and mixed. For in vitro-translated products, 5 µl of the 50-µl reaction was made up to 50 µl with PBS with complete protease inhibitor (Boerhinger Mannheim). Antibody was added, and the sample was left on ice overnight. Antigen-antibody complexes
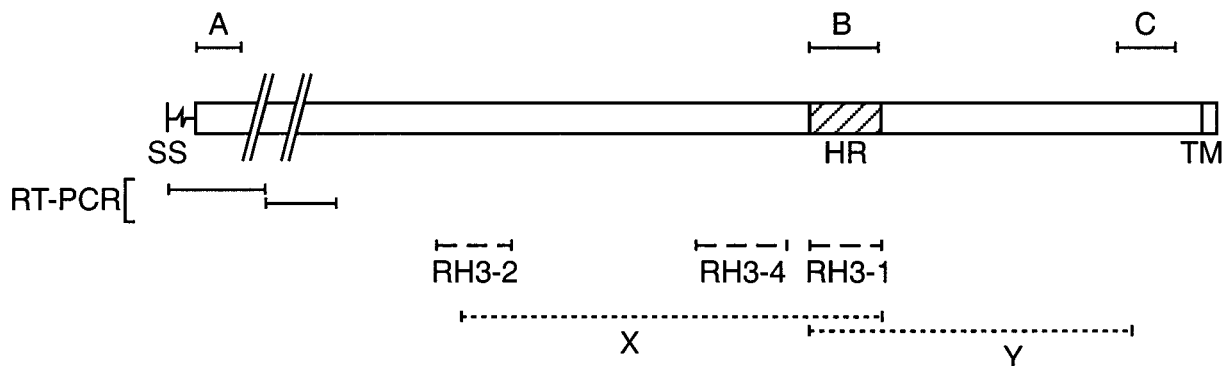
FIG. 1. Schematic of the predicted protein coded by *PfRH3*. In the diagram, 1 cm represents 200 amino acids. The cross bars represent shifts in the reading frame. SS, signal sequence; TM, transmembrane domain. The homology region (HR) represents an approximately 200-amino-acid fragment with the most similarity to the PvRBP1 and -2 and Py235 proteins. Two regions spanning the frameshifts that were amplified by RT-PCR are shown. Fragments A, B, and C represent regions of the gene used as probes. Dashed lines represent constructs used to make the GST fusion proteins RH3-1, RH3-2, and RH3-4. Dotted lines represent constructs X and Y used for in vitro translation.

were precipitated using protein G-Sepharose and resolved by SDS-PAGE, and the labeled proteins were detected by fluorography.

Tryptic peptide mapping. [^{35}S]methionine-labeled proteins, either derived from in vitro translation of *PfRH3* or immunoprecipitated from metabolically labeled schizonts with anti-RH3-1 antibodies, were resuspended in SDS-PAGE buffer containing 0.1 M DTT and heated to 100°C for 5 min. The samples were treated with 0.2 M iodoacetamide in 0.2 M Tris-HCl (pH 8.2) and incubated at 37°C for 30 min. Following SDS-PAGE, bands corresponding to the required proteins were visualized by autoradiography, cut out from the gel, and then digested with 0.1 mg of trypsin (Sigma; diphenylcarbamylchloride treated) per ml in 25 mM NH$_4$HCO$_3$ essentially as described previously (32). Tryptic peptides were analyzed by two-dimensional thin-layer chromatography as described earlier (7).

Nucleotide sequence. The nucleotide sequence reported here has been added to the GenBank database under accession number AF324831.

RESULTS

Database searching and sequence analysis revealed genes related to PvRBP and Py235. Screening the malaria genome sequencing project databases with the regions of the PvRBP1 and -2 and Py235 proteins that share the greatest degree of homology identified related sequences putatively on chromosomes 4, 12, and 13. Sequences on chromosome 13 have recently been described (*Pfr2ha* and *Pfr2hb*) (56, 67). Here we describe in detail the characterization of the sequence on chromosome 12.

Shotgun reads from chromosome 12 were used to construct a contig, containing a sequence expected to code for a protein with a similar overall structure to PvRBP and Py235. We designated this gene *PfRH3* (*P. falciparum* reticulocyte binding protein/rhoptry protein homologue 3). We chose this nomenclature because the predicted protein is as similar to the Py235 proteins as it is to the two PvRBPs. The 5′ end of the gene was confirmed by 5′ RACE, which revealed a 150-bp intron preceded by sequence coding for a hydrophobic signal sequence. Further sequencing of overlapping PCR amplified fragments of the whole gene verified the sequence and revealed that in two sites the reading frame shifts by +1 (Fig. 1). This was later confirmed by the assembled database sequence. A sequence analysis of RT-PCR products across these frameshifts revealed that these regions of the gene are transcribed, and the sequence of the cDNA is identical to that of the genomic DNA, except that the intron is removed. Amplification from contam-

inating genomic DNA was ruled out because amplification without RT did not produce any products. The proofreading polymerase *Pfu* Turbo was used to eliminate the possibility that errors were introduced by PCR. Furthermore, analysis of the products using primers spanning the intron and the frameshift proved that the template was cDNA (Fig. 1). No other introns were detected by RT-PCR.

The putative PfRH3 protein sequence (assuming translation through the frameshifts) contains one predicted transmembrane domain (amino acids 2830 to 2847), suggesting that the presumed protein contains a large N-terminal extracellular region and a short C-terminal cytoplasmic domain. This is similar to the predicted structures for PvRBP1 (22), PvRBP2 (23), and Py235 (27). PfRH3 was compared to the other members of the superfamily using the GAP and Bestfit functions of GCG 10.1 (25). The results are shown in Table 1. The different members of the family are around 34% similar to each other. However, there are areas where the similarity between the different members of the family increases. These include the transmembrane and intracytoplasmic regions and the area originally identified as the homologous part of the Py235 and RBP2 proteins. The alignment of the homology regions shows that all the proteins are related (Fig. 1 and 2).

PfRH3 is transcribed in schizonts. Given that frameshifts were present in the RNA transcripts made from the 5′ end of *PfRH3*, we wanted to know if the full-length transcript was made. RNA isolated from 3D7 trophozoites and schizonts was analyzed by Northern blotting. A PCR product corresponding to fragment B (Fig. 1) was used to probe the Northern blot. A large transcript (~10 kb) was present in schizont stage RNA but not in trophozoites (Fig. 3). There was no cross-hybridization with *P. yoelii* RNA (data not shown). To confirm that both the 5′ and 3′ ends of the putative gene *PfRH3* are part of the same transcript, PCR products corresponding to regions A and C (Fig. 1) were used to probe a duplicate filter. These probes hybridized to the same transcript. Control probes were used to confirm that the RNA was intact and loaded in equal quantities in all lanes (data not shown).

PfRH3 can be disrupted in two parasite lines. Since a large *PfRH3* transcript was made in schizont stages, it was possible

TABLE 1. Comparision of the predicted amino acid sequences of RBP1, RBP2, Py235 (E8), and PfRH3 made by using the Bestfit and Gap functions of GCG version 10.1 (25)[a]

Function and protein	RBP1		RBP2		Py235 E8	
	% Similar	% Identical	% Similar	% Identical	% Similar	% Identical
Bestfit						
PfRH3	33.7	21.3	35.7	24.5	36.3	24.9
RBP1			35.4	24.0	33.9	22.6
RBP2					34.4	23.4
Gap						
PfRH3	34.3	22.0	36.2	25.0	36.1	24.9
RBP1			35.2	23.9	33.7	22.5
RBP2					34.5	23.5

[a] The following parameters were used for both Bestfit and Gap: gap creation penalty, 12; gap extension penalty, 4; scoring matrix, blosum62 (25).

that some form of RNA editing or readthrough translation removed both frameshifts, resulting in a translated PfRH3 protein. To assess whether *PfRH3* is essential for parasite survival in vitro, two parasite lines were constructed in which the endogenous gene had been disrupted. The transfection vector pHH1-rh3 was used to introduce the truncated PfRH3 gene into both E12 (a clone derived from the Papua New Guinean isolate FC27) and HB3 parasite clones (Fig. 4A). Transfected parasites were selected with or without WR99210 for several cycles until homologous integration had occurred (14, 19). Hybridization of a *PfRH3* probe to an *Mfe*I/*Eco*RI-restricted genomic DNA from wild-type E12 and integrated clones (E12Δrh3 and HB3Δrh3) showed that *PfRH3* had been disrupted (Fig. 4B). The *PfRH3* probe hybridized to a 10.4-kb band in E12 but also to bands of 8.2 and 3.0 kb in both disrupted parasites and another band at 0.8 kb in the HB3Δrh3 parasite (Fig. 4A and B, left panel). The 0.8-kb band which was present at a copy number of two with respect to the other hybridizing bands, indicated that the HB3Δrh3 parasites contained two integrated copies of the pHH1-rh3 episome at the

PfRH3 locus. No extra copies of the episome were integrated into the E12Δrh3 parasite (Fig. 4A and B). Hybridization of the same probe to *Bsr*GI/*Xba*I-restricted DNAs showed once again that the endogenous gene had been disrupted and that only the HB3Δrh3 parasite had integrated two copies of the episome (the band at 4.3 kb) into the *PfRH3* locus (Fig. 4B, right panel).

Since disruption of *PfRH3* was readily obtained, we wanted to know if RNA was made from both truncated PfRH3 gene copies. Four primer pair combinations were used in RT-PCR experiments on cDNA produced from untransfected HB3 and disrupted HB3Δrh3 parasites. To ensure the cDNA was not contaminated by genomic DNA, PCR across the intron in *PfRH3* was carried out first. This showed that the cDNA was free of genomic DNA (data not shown). The P3-P4 primer combination showed this transcript was made in both wild-type and disrupted parasites (Fig. 4A and C). However, both the P2-P3 and the P2-P5 combinations only amplified a DNA fragment from wild-type parasites (Fig. 4C). Also, the P2-P5 combination showed that no truncated *PfRH3* transcript was
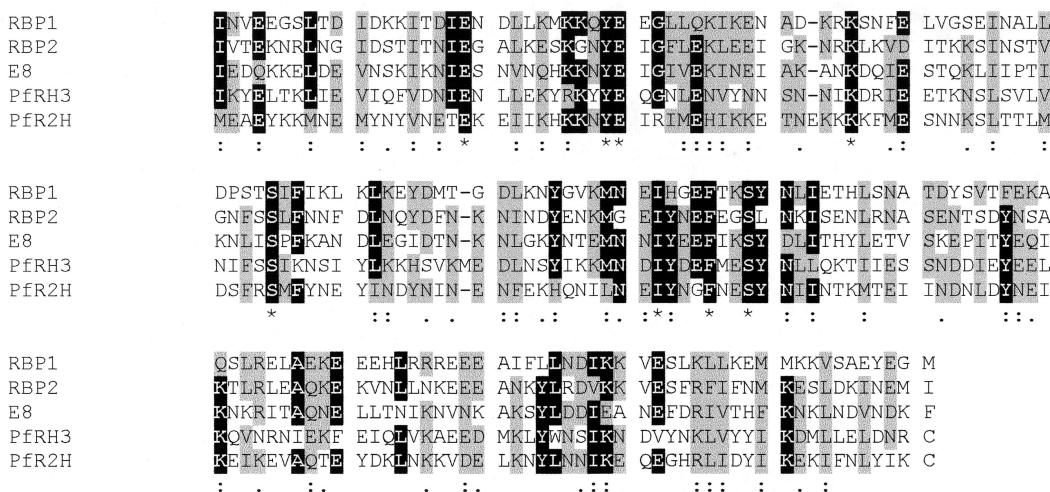

FIG. 2. Clustalx alignment of the homology regions in PfR2H, PfRH3, PvRBP1, PvRBP2, and Py235E8. The alignment uses the Gonnet Pam250 matrix, which defines groups of strongly conserved and weakly conserved amino acids (65). Conserved residues are marked with an asterisk; semiconserved residues are marked with double dots, and the weakly conserved residues are marked with a single dot. The shading represents the consensus sequence, for at least four out of five of the sequences, with conserved residues marked in black and semiconserved residues shaded.
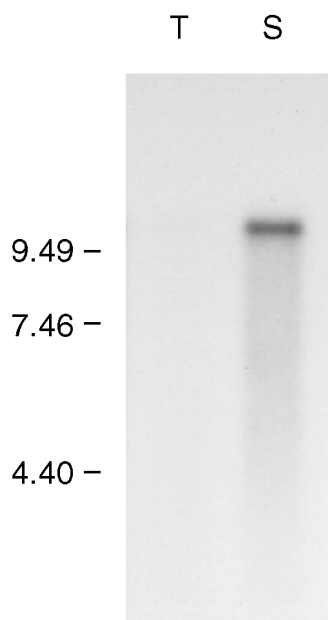
T   S

9.49 –

7.46 –

4.40 –

FIG. 3. A full-length transcript of *PfRH3* is made in schizonts. Northern blot analysis of 3D7 RNA hybridised with fragment B. Probes to fragments A and C hybridized to the same transcript. T, trophozoites; S, schizonts. Markers represent sizes in Kilobases.

made in disrupted parasites from the downstream PfRH3 gene lacking a promoter. This experiment also confirmed the absence of genomic DNA contamination in the cDNA from disrupted parasites. The P1-P3 combination only amplified a product from the disrupted parasites, confirming that a truncated transcript is made.

Neither disrupted parasite showed any demonstrable change in growth rate compared to untransfected controls (data not shown), suggesting that *PfRH3* is not essential for erythrocytic stage parasite growth in vitro.

PfRH3 is not part of a large family of closely related genes. Only a single band of the expected size was seen when a *PfRH3* probe was hybridized to *Mfe*I- and *Eco*RI-restricted genomic DNA in parasites where the gene has not been disrupted (Fig. 4B). Furthermore, hybridization at a high stringency of chromosomes separated by PFGE revealed that the gene is present only on chromosome 12 (data not shown). These results suggest that this gene is unique and not part of a large family of closely related genes.

Sequence analysis of different parasite strains reveals variation around the frameshifts. To address the possibility that the sequence may vary among different isolates, we amplified regions surrounding the frameshifts using the proofreading polymerase *Pfx*. While all isolates tested, including three field isolates from Nigeria, had identical sequences to 3D7 across the 5′ frameshift, differences were detected among isolates for the 3′ frameshift. Preceding the sequence where the reading frame changes is a run of adenosines (Fig. 5); there are 10 in 3D7, T996, and two field isolates but only 9 in FCB1 and A4. This means that the putative protein sequence stays in frame across this area for FCB1 and A4. Thus, there are minor differences in the gene sequence in different parasite isolates;

however, even in field isolates there are changes in the reading frame for this gene.

PfRH3 does not appear to be translated. Polyclonal sera to three GST fusion proteins of different fragments of *PfRH3* were raised (RH3-1, RH3-2, and RH3-4; Fig. 1). Serum against RH3-1 was raised in mice, while sera against RH3-2 and RH3-4 were raised in rabbits. All three sera gave a punctate pattern of staining in late schizonts by immunofluorescence assay (IFA), with anti-RH3-1 additionally staining late trophozoites (data not shown). To localize the staining of anti-RH3-1 more precisely, parasites were counterstained with antibodies to RAP1. In late schizonts and merozoites, anti-RAP1 and anti-RH3-1 antisera colocalize (data not shown).

To determine whether the three antisera recognized any parasite proteins of the predicted size, we tested them for their reactivity by Western blot or immunoprecipitation of parasite extract or labeled schizonts. First, we demonstrated the specificity of anti-RH3-1 antiserum to the immunizing protein by cloning overlapping fragments of ca. 3 kb of the gene in the TA vector and using in vitro transcription and translation to make in vitro-translated products (Fig. 6A). The predicted sizes for the proteins derived from construct X and construct Y are 130 and 103 kDa, respectively. A ladder of products is produced for each construct, presumably due to translation starting from internal methionines. For construct X, all of the products contain fragment RH3-1, but for construct Y only the longer products contain this fragment. Only products that contain fragment RH3-1 were immunoprecipitated by the serum to RH3-1, showing that it reacted specifically with proteins derived from the part of the gene to which it was raised. In vitro-translated products from unrelated proteins or different fragments of *PfRH3* were not immunoprecipitated by anti-RH3-1 antibodies (data not shown). Anti-RH3-1 serum immunoprecipitated a 200-kDa protein from schizont pellets (Fig. 6B) and reacted with a protein of identical size by Western blot of parasite extract (data not shown). This is smaller than the predicted 340-kDa for translation of *PfRH3* through the two frameshifts. Thus, to determine whether or not the 200-kDa immunoprecipitated protein was the product of *PfRH3*, we compared two-dimensional maps of tryptic peptides from both this immunoprecipitated protein and several of the products of in vitro translation from construct X as described above. The tryptic peptide maps were different (Fig. 6C). To ensure that the process was repeatable and that the ladder of products derived from construct X were related, the tryptic maps of several bands derived from translation of construct X were compared and were almost identical (complete identity was not expected as the bands were different sizes). Therefore, the 200-kDa protein is not the protein encoded by *PfRH3*.

The specificity of the rabbit anti RH3-2 and RH3-4 antibodies (depleted of anti-GST specificities) on the immunizing antigen is shown in Fig. 7A. While there was some proteolysis of both fusion proteins, the appropriate full-length fusion protein at ~72 kDa was recognized by both antibodies. The absence of a band at 26 kDa demonstrates that all of the anti-GST antibodies had been successfully removed (Fig. 7A). The antibodies were then used in a Western blot against late-schizont stage pellets from untransfected (E12 and HB3) and disrupted (E12Δrh3 and HB3Δrh3) parasites (Fig. 7B). Anti-RH3-4 antibodies reacted with neither high-molecular-weight proteins in
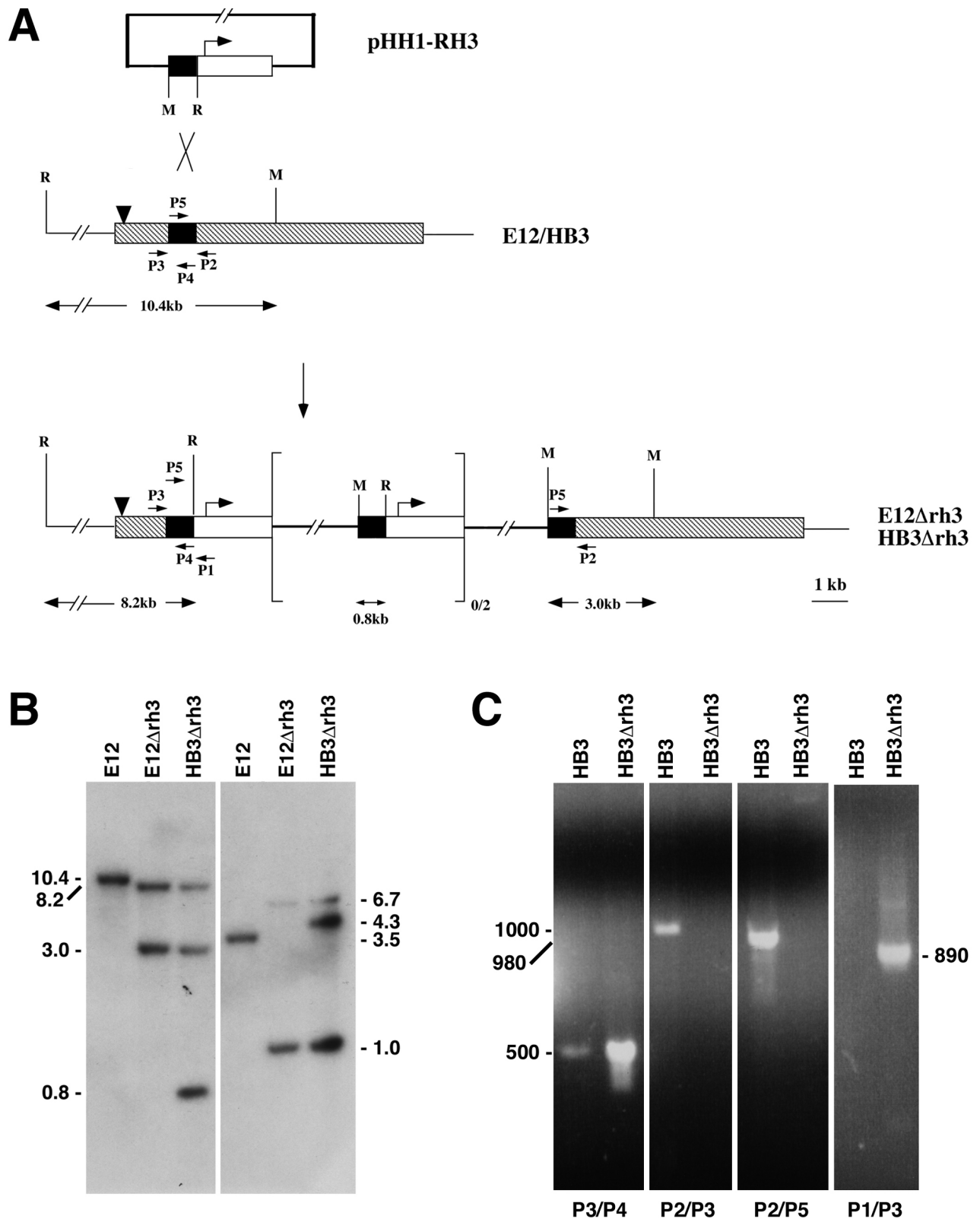
FIG. 4. *PfRH3* is transcribed and can be disrupted in 2 distinct parasite lines. (A) Schematic diagram of the *PfRH3* locus in the E12Δrh3 and HB3Δrh3 parasites. The plasmid construct, pHH1-RH3, contains a portion of *PfRH3* approximately one-quarter of the way into the gene (solid black shading), which has been cloned in the same direction as the WR99210 selection cassette (no shading). The remainder of *PfRH3* in the E12 and HB3 parasites is shown as diagonal hatching. The pGEM vector backbone is shown as the thick black line. The cross indicates the region where homologous integration has occurred. The downward-pointing arrow in the PfRH3 gene represents the single intron. The restriction sites shown are *Mfe*I (M) and *Eco*RI (R). The cloned parasite line E12Δrh3 has no copies, while the HB3Δrh3 parasite has two copies of the episome integrated

3D7/T996/field isolates

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TTAGTTCTAAATGATTATATAACAAAAAAAAAATAAGATATTAGATAATATAAATATTATTAATAAGGATTTTTT
 L   V   L   N   D   Y   I   T   K   K   K   *   D   I   R   *   Y   K   Y   Y   *   *   G   F   F
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A4/FCB1

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TTAGTTCTAAATGATTATATAACAAAAAAAAATAAGATATTAGATAATATAAATATTATTAATAAGGATTTTTT
 L   V   L   N   D   Y   I   T   K   K   N   K   I   L   D   N   I   N   I   I   N   K   D   F   F
```

FIG. 5. The sequence of the 3′ frameshift varies in different parasite isolates. In the laboratory isolates 3D7 and T996 and in two field isolates from Igbo-Ora, Nigeria, there is a run of 10 adenosines (highlighted) that introduce a stop codon. In the laboratory strains A4 and FCB1, there are 9 adenosines and no stop codon.

untransfected parasites nor any other proteins which were present in untransfected but absent from disrupted parasites. Similarly, while anti-RH3-2 antibodies reacted with a protein of >250 kDa in untransfected parasites, the same protein was also seen in disrupted parasites. Moreover, all three sera reacted in IFA with the parasites with the disrupted gene. Hence, *PfRH3* is transcribed but not translated.

DISCUSSION

The genome of *P. falciparum* contains several sequences with some homology to both Py235 and PvRBPs, including the putative pseudogene *PfRH3* described here, and two recently described genes, *Pfr2ha* and *Pfr2hb* (*PfRBP2-Ha* and *PfRBP2-Hb*) (56, 67). The genes from the different parasites form a superfamily of related genes, although it is not clear if the function of the proteins is the same.

The predicted proteins PfR2Ha and PfR2Hb, in *P. falciparum*, are identical to each other for most of their length, differing over the region coded for by the final 1 kb after a region of charged repeated sequence. They are expressed in late schizonts and merozoites and are located at the apical end of the merozoite. Although no erythrocyte binding activity has been demonstrated, antibodies to these proteins inhibit merozoite invasion of erythrocytes (67).

In *P. vivax*, the two RBPs are located at the apical end of the parasite. These proteins are proposed to select the appropriate cell for the parasite to invade, by attaching to specific receptors on reticulocytes, triggering the release of the apical complex organelles and the formation of the tight junction (22). RBP1 appears to form covalently linked dimers. Although there is no evidence for covalent association between RBP1 and RBP2, it is possible that they form a complex, since their distribution on the merozoite is closely associated. RBP1 and RBP2 are clearly related and ca. 35% similar to each other, which is the same order of similarity as between the hypothetical protein PfRH3 and the other family members. The exceptions are the individual members of Py235, which are much more closely related to one another than to the other members of the superfamily.

The Py235 proteins in *P. yoelii* appear to function as ligands for receptors on both mature and immature erythrocytes (44, 45). One possibility is that different members of the Py235 family have different specificities for host cell receptors, with some preferentially binding to reticulocytes. Individual merozoites from one schizont transcribe single but distinct transcripts (54). Such phenotypic variation may give the parasite an advantage by allowing it to adapt to changes in its erythropoietic environment (55).

PfRH3 potentially codes for a related family member in *P. falciparum*, though our work shows that it is possibly a pseudogene. While it is more common for pseudogenes to be partial sequences that are not transcribed (reviewed for vertebrate pseudogenes in reference 39), there are examples of full-length pseudogenes that transcribed (21, 38, 39, 47). In these cases, all of the elements involved in transcriptional control are present but the gene is not translated. In the case of *PfRH3*, translation of the whole transcript apparently does not occur. The most likely explanation is that mutations within the expected coding region lead to shifts in the reading frame. Nevertheless, the gene is transcribed both in its entirety (a process which is metabolically uneconomical) and under stage-specific control.

One possibility is that the mutations in *PfRH3* have arisen in parasites during in vitro culture. Distinct parasite isolates have different mutations within the gene. However, even in several field isolates, where the parasites are under selection pressures, *PfRH3* has mutated.

Pseudogenes often arise by duplication or partial duplication of existing sequences. Examples of this are the multiple copies

at the *PfRH3* locus. (B) Southern analysis of untransfected and *PfRH3*-disrupted parasites. The left panel shows genomic DNAs digested with *Eco*RI and *Mfe*I, while the right panel shows a *Bsr*GI/*Xba*I double digest. The Southern blot was probed with a 465-bp *PfRH3* fragment within the portion used for the gene disruption. The fragment was amplified using the primers 5′-CTGAAAAGTGTTTTTCGG-3′ and 5′-ATTTATATT ATCCATCTTTG-3′. (C) Transcriptional analysis of the *PfRH3* locus in HB3Δrh3 parasites. Synchronised late schizont stages from untransfected HB3 and HB3Δrh3 parasites were used for mRNA purification and conversion to cDNA. Primers used to ampify the cDNAs are labeled P1 to P5. Primer pair combinations used for each cDNA are shown below the panels. P1 is a primer within the calmodulin promoter with the sequence 5′-GGTTAACAAAGAAGAAGCTCAGAG-3′. P2 and P3 are from the PfRH3 gene outside the region used for disruption. The P2 sequence is 5′-CTTGATTTAAACTTTTTAGAGGATC-3′, and the P3 sequence is 5′-AAGAATATAACATCTAAAACT-3′. P4 and P5 primers are both within the PfRH3 gene fragment used for disruption. The P4 sequence is 5′-ATTTATATTATCCATCTTTG-3′, and the P5 sequence is 5′-CTGAAAAGTGTTTTTCGG-3′.
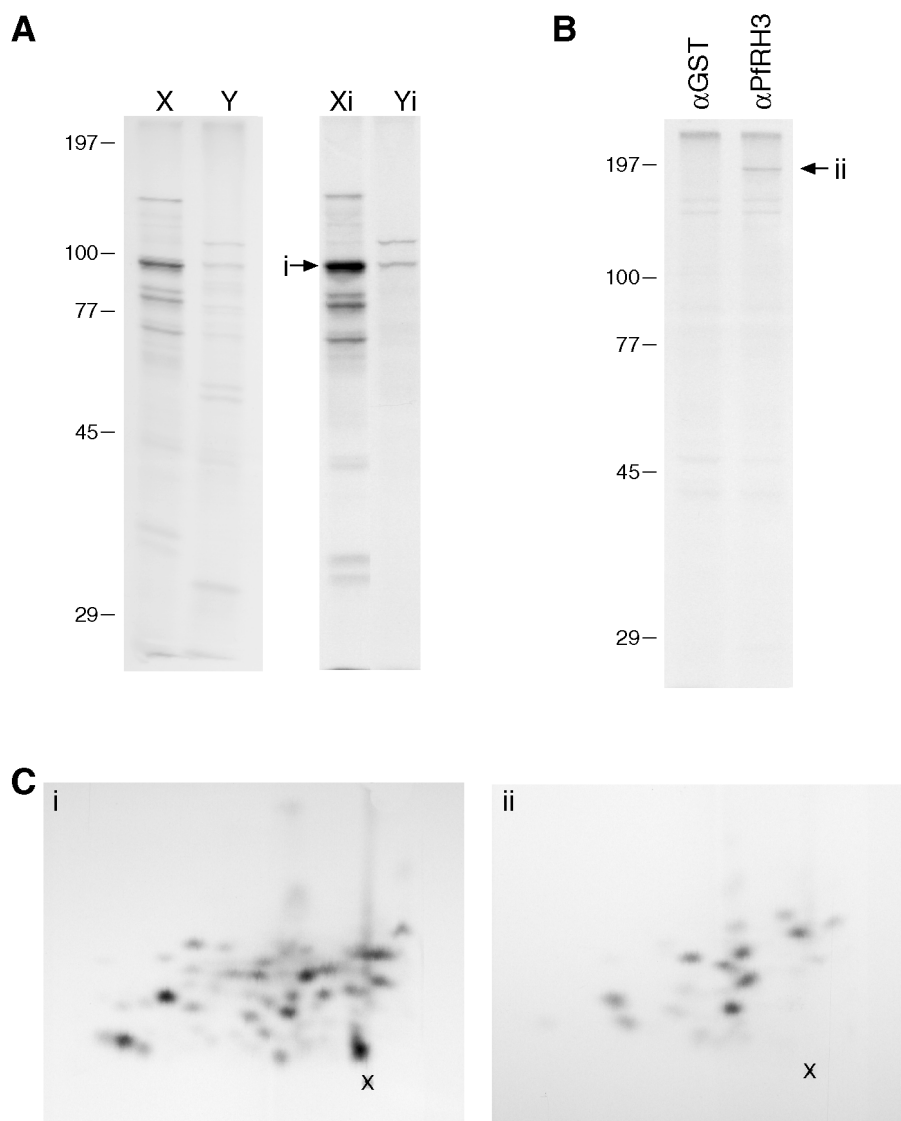
FIG. 6. Mouse anti-PfRH3 antibodies recognize proteins derived from *PfRH3* and immunoprecipitate a 200-kDa protein from metabolically labeled parasites, but this is not the protein coded by the gene. (A) Lanes X and Y, in vitro-translated products derived from *PfRH3* constructs X and Y, that overlap fragment RH3-1 (see Fig. 1); lanes Xi and Yi; in vitro-translated products from constructs X and Y that are immunoprecipitated by mouse anti-RH3-1 antibodies. In vitro-translated products from unrelated proteins or different fragments of *PfRH3* were not immunoprecipitated by anti-RH3-1 antibodies (data not shown). (B) Immunoprecipitated proteins from metabolically labeled 3D7 schizonts using a mouse polyclonal anti-GST serum (αGST) or anti-RH3-1 antibodies (αPfRH3-1). (C) Two-dimensional maps of tryptic peptides derived from (i) in vitro-translated product X (band i) or (ii) the 200-kDa parasite protein immunoprecipitated with anti-RH3-1 antibodies from a schizont pellet.

of *var* gene fragments present in the subtelomeric regions of at least chromosomes 2 and 3 in *P. falciparum* (9, 24). It is known that the subtelomeric regions of *P. falciparum* chromosomes are susceptible to rearrangements (including duplications, deletions, and translocations) (4, 13, 28, 49, 53, 68). Mapping the sequenced contigs onto the YAC map for chromosome 12 shows that *PfRH3* is subtelomeric (http://sequence-www .stanford.edu/ [58]), although, unusually for a pseudogene, it appears to be a single-copy gene (albeit with some distantly related sequences in the genome).

Not all genes that have stop codons disrupting the reading frame are pseudogenes. Programmed translational frameshift-ing and readthrough translation are well-recognized phenomena in both prokaryotes and eukaryotes (reviewed in references 17, 18, 26, and 70). Indeed, translation through an in-frame stop codon in *P. falciparum* has recently been described for a member of the Pf60 multigene family (5). Using luciferase fusion proteins and transient transfection of *P. falciparum*, readthrough of the internal stop codon in gene *Pf6.1* was demonstrated to be about 10% of that of an open reading frame without a stop codon. This translation efficiency correlates closely with that seen in other systems (5, 26). We were struck by the possibility that *PfRH3* may be translated through the frameshifts, particularly since one of the antisera raised
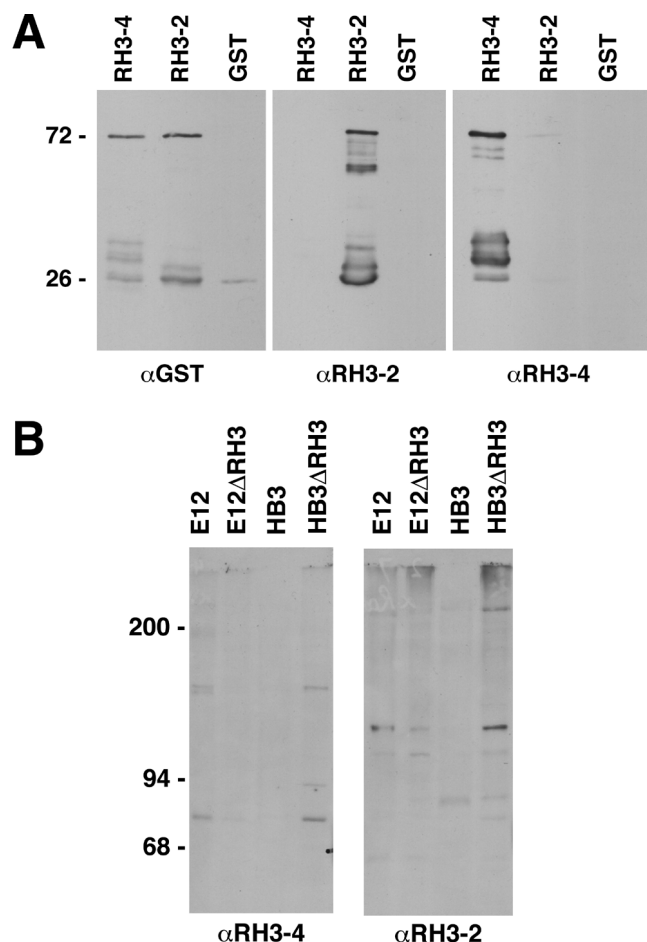
FIG. 7. Specificity and reactivity of rabbit anti-PfRH3 antibodies. (A) Western blot of RH3-4, RH3-2 fusion proteins and purified GST probed with anti-GST antibodies or rabbit antibodies to each fusion protein (anti-RH3-2 and anti-RH3-4). Sizes are shown in Kilodaltons. (B) Western blot of parasite protein samples from untransfected parasites (E12 and HB3) and *PfRH3*-disrupted parasites (E12Δrh3 and HB3Δrh3) probed with antibodies to RH3-2 and RH3-4.

be that the protein is not expressed because there are mutations within the coding sequence.

Since PfR2Ha and PfR2Hb are so closely related to each other, it seems likely that one of them has arisen by a gene duplication. One possibility is that the duplicated gene may have taken over the function of *PfRH3*, allowing this gene to mutate without detriment to the fitness of the parasite. However, we speculate that, although there is not a large family of closely related genes in *P. falciparum* as there is in *P. yoelii*, there are several less closely related proteins fulfilling a similar role. This redundancy is costly, so the genome of the parasite may be gradually evolving to get rid of the nonessential genes. It seems likely that the mutations in *PfRH3* demonstrate a relatively recent event in the evolution of the parasite.

Recent studies have demonstrated a correlation between the severity of the disease, the parasite multiplication rates, and cell selectivity (12, 62). In these studies, parasites causing severe malaria were less selective in erythrocyte invasion (measured by a cell selectivity index, which took into account multiply invaded erythrocytes and parasitemias). Furthermore, they multiplied at higher rates. Such parasites seem to have a greater invasion potential; one explanation could be that they express a greater repertoire of ligands involved in invasion. The members of both the PfRH and the EBP families may play a crucial role in this observation.

against it recognized a large apical protein. However, the weight of evidence suggests that the antiserum was cross-reacting with an unrelated protein, and there is no other evidence that the gene is translated along its whole length. It could be that either a truncated protein is made or the protein is translated at very low levels not detectable by Western blotting or immunoprecipitation, although this seems unlikely, since the antibodies reacted by IFA at a high titer. Alternatively, the protein may only be made for a very narrow time period. However, Western blots were carried out on a range of parasite stages, and immunoprecipitations were carried out on late schizonts (at the stage where the antibodies reacted with schizonts by IFA), so this seems improbable. In addition, disrupting the gene does not alter the phenotype of the parasite. We have not looked for the protein in parasite life cycle stages other than blood stages, so the possibility that some form of readthrough translation is occurring in these stages can not be absolutely ruled out. However, the simplest explanation would

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