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Genetics

Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of *Plasmodium falciparum*

(malaria/gene duplication/gene evolution/introns)

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ABSTRACT **Two genes encoding distinct histidine-rich proteins in a *Plasmodium falciparum* clone exhibit high levels of homology, suggesting they have originated by duplication and divergence from a common ancestral sequence. Both genes have a similar interrupted structure and an exon that encodes closely related tandem repeats of very high histidine and alanine content. The most common repeat encoded by one gene, the hexapeptide Ala-His-His-Ala-Ala-Asp, differs in the sixth position from the most common repeat encoded by the other gene, the hexapeptide Ala-His-His-Ala-Ala-Asn. The divergence of the repeat domains is greater than that of the flanking regions, which exhibit 85–90% homology, including untranslated sequences. This suggests the tandem repeats are relatively labile elements within the genome that may provide the parasite with a means of rapid evolutionary change.**

The erythrocytic stages of two species of malaria parasite synthesize proteins with unusually high contents of histidine. *Plasmodium lophurae*, an avian malaria parasite, synthesizes an abundant protein of a histidine content of 73% (1–5). *Plasmodium falciparum*, which causes the most severe human malaria, synthesizes several proteins that are histidine-rich. One of these proteins (PfHRP-I) is associated with knob protrusions that mediate cytoadherence of infected erythrocytes to endothelium (6–11). Another histidine-rich protein produced by *P. falciparum*, PfHRP-II, is not associated with knob protrusions (7, 11). Studies using a monoclonal antibody specifically directed against this protein have shown that PfHRP-II is synthesized throughout the erythrocytic stage of the parasite life cycle and that it is released as a soluble protein from intact infected erythrocytes into the culture supernatant (33).

In this report we compare the genes of two histidine-rich proteins expressed in infected erythrocytes by a clone of the *P. falciparum*. One of these genes encodes PfHRP-II; the other encodes another histidine-rich protein, PfHRP-III. A very high level of homology is present between these genes. This homology implies the genes of PfHRP-II and PfHRP-III have originated by duplication and divergence from a common ancestral gene.

MATERIALS AND METHODS

DNA and RNA Isolation. Clone 7G8 of the Brazilian isolate IMTM22 of *P. falciparum* (12) was grown *in vitro* by established methods (13). Parasites were obtained from infected erythrocytes by saponin lysis (14). DNA was isolated by digestion of parasites with proteinase K in the presence of 0.5% $NaDodSO_4$/25 mM EDTA followed by ultracentrifugation in cesium chloride (15). RNA was isolated from parasites suspended in 100 mM NaCl/10 mM Tris, pH 8.0/2 mM $MgCl_2$/10 mM vanadyl-ribonucleoside complexes (Bethesda Research Laboratories)/1% $NaDodSO_4$ by sequential extraction with hot phenol and chloroform (16). RNA transfer and Southern hybridizations were performed as described (15, 17).

Genomic Libraries. Recombinant DNA experiments were performed under National Institutes of Health DNA research guidelines. Bacteriophage from the λgt11 expression library (18) were plated and screened with monoclonal antibody (19–21). Genomic libraries in pUC18 were constructed by digestion of 7G8 *P. falciparum* DNA with *Dra* I, isolation of fragments 0.75–1.2 kilobases (kb) in length by preparative agarose gel electrophoresis, and ligation into the *Sma* I site of pUC18 (22). *Escherichia coli* HB101 competent cells were obtained from Bethesda Research Laboratories and transformed as recommended. Clones from the plasmid library were identified by hybridization to the nick-translated insert from λMAB1 (23).

Nucleotide Sequence Analysis. Nucleotide sequences were obtained by the dideoxynucleotide method (24). mRNA sequence data were obtained by primer extension analysis (25) using 0.5 pmol of 5′ labeled synthetic oligonucleotide and 10 μg of *P. falciparum* RNA.

RESULTS

Immunological screening was performed on a genomic expression library of mung bean nuclease-digested *P. falciparum* 7G8 DNA in the vector λgt11 (18). Monoclonal antibody McAb87, which immunoprecipitates PfHRP-II (33), identified a clone (λMAB1) encoding an inducible fusion protein reacting with McAb87 and anti-β-galactosidase (data not shown). Characterization of λMAB1 revealed an insert containing tandemly repeated oligonucleotides. A 544-base-pair (bp) fragment containing these repeats was excised from the insert with *Fok* I + *Ssp* I, purified by preparative gel electrophoresis, and used to probe Southern blots of restricted genomic DNA (Fig. 1). Following moderately stringent washes (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 50°C, 1 hr), two bands of hybridization were identified in the restriction digests (Fig. 1*A*). Further washes of the same blots at higher stringency (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 65°C, 1 hr), yielded single bands of hybridization (Fig. 1*B*). Thus, two sequences were identified having different degrees of homology with the probe.

To explore the relationship of these two homologous genomic sequences, both *Dra* I fragments were cloned from restricted genomic DNA ligated into the *Sma* I site of the plasmid pUC18. Two clones, pDL4.1 and pDL11.3, each contained a 1.05-kb insert that hybridized to λMAB1 and was not removed by highly stringent washes. Another clone, pDS11.1, contained a 0.85-kb insert that remained hybridized

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Abbreviations: bp, base pair(s); kb, kilobase(s).

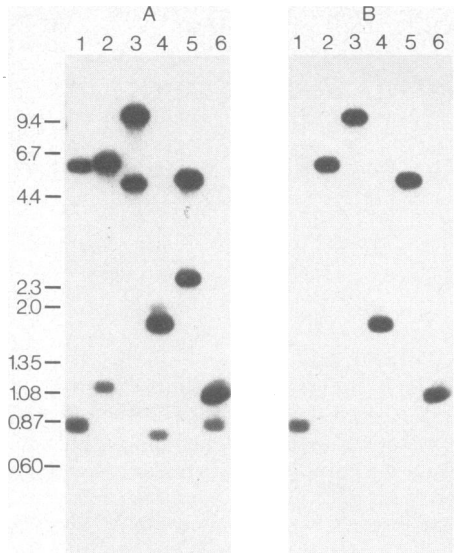

FIG. 1. Southern blot analysis of restricted 7G8 *P. falciparum* DNA probed with a nick-translated 544-bp *Fok* I–*Ssp* I fragment from λMAB1. Sizes are given in kilobases. (*A*) Following moderately stringent washes (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 50°C, 1 hr) each lane shows two bands of hybridization. (*B*) Autoradiogram of the same blot following highly stringent washes (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 65°C, 1 hr) shows only single bands. Lane 1, *Alu* I; lane 2, *Mbo* II; lane 3, *Nde* I; lane 4, *Ssp* I; lane 5, *Hinf*I; lane 6, *Dra* I.

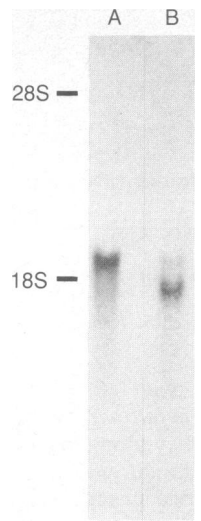

FIG. 2. RNA transfer blot analysis of RNA isolated from an asynchronous culture of 7G8 *P. falciparum*. Lanes were probed separately with nick-translated pDL11.3 (lane A) or pDS11.1 (lane B). The blots were washed at high stringency (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 65°C, 1 hr). The estimated size of the upper band is 2.1 kb and that of the lower band is 1.7 kb. Faint residual hybridization of pDS11.1 to the upper band is apparent. Positions of calf liver rRNA markers are shown.

to λMAB1 after moderately stringent washes but was removed by highly stringent washes.

RNA transcripts of both sequences in infected erythrocytes were demonstrated by RNA transfer blots of 7G8 RNA probed with nick-translated pDL11.3 and pDS11.1. Following moderately stringent washes (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 50°C, 1 hr), two bands of hybridization, approximately 2.1 kb and 1.7 kb in size, were identified with each probe. When the same blots were washed further at high stringency (15 mM NaCl/1.5 mM sodium citrate/0.5% $NaDodSO_4$, 65°C, 1 hr), only the 2.1-kb band remained hybridized to plasmid pDL11.3, whereas the 1.7-kb band remained hybridized to pDS11.1 (Fig. 2). The signal intensity from the 1.7-kb band was ≈5–10% of that from the 2.1-kb band, indicating a more abundant 2.1-kb transcript.

Sequence Analysis of PfHRP-II. Fig. 3 presents the genomic maps and sequencing strategy for the clones obtained in this work. The sequence of PfHRP-II (Fig. 4) contains an extended open reading frame including an extensive region of tandem repeats (nucleotides 141–941; R_{II}, Fig. 5) that encodes a polypeptide consisting almost entirely of histidine, alanine, and aspartic acid. Eighteen tripeptides and 33 hexapeptides, most commonly Ala-His-His and Ala-His-His-Ala-Ala-Asp, occur in this sequence. Near the 3′ end of the repeat region, hexapeptides alternate with three pentapeptides of the sequence Ala-His-His-Ala-Ala. The tripeptides and pentapeptides generally have the same amino acid sequence as the first three and five amino acids in the hexapeptide, indicating a close relationship to the hexapeptide.

Several observations indicate the oligopeptide repeats deduced for PfHRP-II are correct. (*i*) Monoclonal antibody McAb87, which immunoprecipitates PfHRP-II, recognizes the λMAB1 fusion protein incorporating these repeats. The RNA transfer blots show that mRNA transcripts corresponding to the λMAB1 insert are present in infected erythrocytes at the time PfHRP-II is expressed. (*ii*) Antisera directed against a synthetic peptide containing repeats from the deduced amino acid sequence immunoprecipitate PfHRP-II (11). (*iii*) The deduced amino acid sequence of the repeats agrees with data from biosynthetic labeling studies. PfHRP-II is labeled strongly with [^{3}H]alanine and [^{3}H]histidine but is not detected after labeling with [^{3}H]isoleucine or [^{3}H]methionine (11, 33).

The PfHRP-II nucleotide sequence contains an amber stop codon at nucleotides 39–41 (this codon is suppressed by the *supF* mutant tRNA of the *E. coli* strain used in these experiments, allowing translation of the λMAB1 fusion protein). Also, no initiator codon is in frame with the repeats, indicating the gene is interrupted. This has been confirmed by direct mRNA sequencing, using as primers synthetic oligonucleotides complementary to nucleotides 64–83 and 114–135 of the PfHRP-II genomic sequence (Fig. 4*B*). The overlapping sequence data obtained from each of these primers were unambiguous and in agreement with each other as well as with the coding region of the PfHRP-II genomic sequence. The specificity of this sequence for PfHRP-II is explained by the predominance of this transcript in the mRNA preparation. The genomic sequence diverges from the mRNA sequence at a 3′ intron splice site containing the polypyrimidine tract and A-G dinucleotide of the eukaryotic consensus sequence (28).

The mRNA sequence 5′ to the splice junction encodes a hydrophobic sequence of 13 amino acids, preceded by a lysine residue and followed by an aspartate residue. Although the structure of this region suggests it may act as a signal sequence, a consensus signal peptidase cleavage site (29) is not found. A single initiating methionine occurs upstream of the hydrophobic region (Fig. 4*B*).

Presumably the mRNA sequence 5′ to the splice junction is transcribed from a small exon 5′ to the exon encoding the tandem repeats. Support for linkage of the exons has been obtained from Southern blots of restricted genomic DNA probed with an oligonucleotide derived from the mRNA sequence encoding the hydrophobic region (data not shown). This sequence has been found within 600 bp of the exon encoding the tandem repeats, providing an upper limit on the size of the intron.

The nucleotide sequence of PfHRP-II potentially encodes a protein of M_r 35,138, a value much lower than the relative

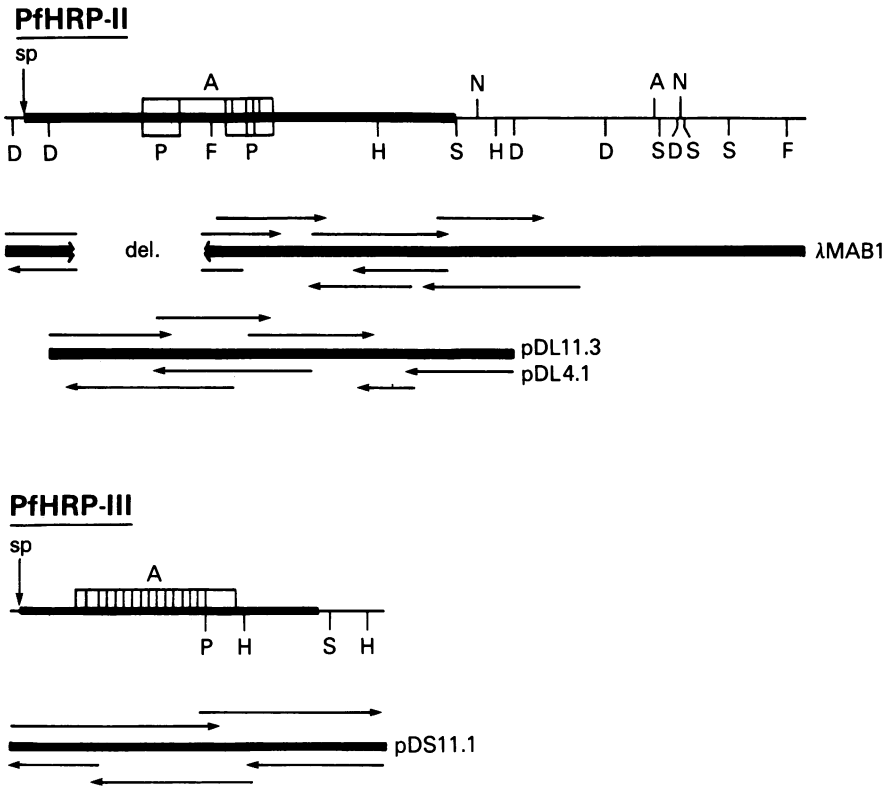

FIG. 3. Genomic maps and sequencing strategy for PfHRP-II and PfHRP-III. Coding regions are directed from left to right and are marked by heavy lines on the genomic maps. Comparison of the *Alu* I and *Dra* I restriction fragments from genomic DNA with those from λMAB1 revealed a 279-bp deletion (del.) had occurred within the region of tandem repeats but had preserved the reading frame. Restriction patterns of inserts from clones pDL4.1, pDL11.3, and pDS11.1 were compared with corresponding restriction digests of genomic DNA by Southern blotting and showed no evidence of deletion or rearrangement. Nucleotide sequence analysis was performed on subclones in M13 mp18 having targeted deletions generated by timed exonuclease III digestions (26). The coding strand of the pDS11.1 insert was determined from fragments digested with *Pvu* II and subcloned into M13 mp19. sp, Splice site at 3′ end of intron. A, *Alu* I; D, *Dra* I; F, *Fok* I; H, *Hin*fI; N, *Nde* I; P, *Pvu* II; S, *Ssp* I.

M_r of 60,000–80,000 obtained by NaDodSO$_4$/PAGE (11, 33). Possible explanations for this difference include posttranslational events and anomalous migration during NaDodSO$_4$/PAGE. Anomalously low mobilities occur with other malaria proteins containing tandem repeats (21, 30, 31), perhaps because of low NaDodSO$_4$ binding to the repeat domains (30). The deduced sequence contains 34% histidine, 37% alanine, and 10% aspartate.

Sequence Analysis of the PfHRP-III Genomic Fragment. The nucleotide sequence of clone PDS11.1 contains a single open reading frame encoding a histidine- and alanine-rich polypeptide (Fig. 4*A*). Two blocks of repeats occur in this sequence (Fig. 5). The first (R_{IIIA}) begins at nucleotide 122: three tripeptides (Ala-His-His) alternate with two hexapeptides (Ala-His-His-Val-Ala-Asp) before a stretch of 13 hexapeptides (Ala-His-His-Ala-Ala-Asn) and a 14th hexapeptide (Ala-His-His-Ala-Ala-Asp). Between the blocks of repeats is a 26 amino acid sequence without apparent repeat structure (nucleotides 437–514). The second block of repeats (R_{IIIB}) contains nine pentapeptides predominantly of the sequence Asp-Asp/Gly-Ala-His-His (nucleotides 515–648).

The encoded sequence for PfHRP-III is nearly identical to that of the cDNA clone corresponding to a small histidine- and alanine-rich protein (SHARP) reported by Stahl *et al.* (32). The sequences differ in that PfHRP-III contains an additional tripeptide and two hexapeptides in the first block of repeats and an additional pentapeptide in the second block of repeats. The nucleotide sequences 5′ to the first block of repeats are identical up to the intron splice site, where the genomic sequence diverges from the published cDNA sequence. As for PfHRP-II, the published cDNA sequence of SHARP encodes a hydrophobic region that may represent a signal region (32). Assuming these regions in SHARP and PfHRP-III are identical, we obtain a content of 28% histidine/28% alanine/12% asparagine and a calculated M_r of 26,739 for PfHRP-III. Antisera directed against a synthetic peptide containing repeats from region R_{IIIB} (Fig. 5) immunoprecipitate a parasite protein of M_r 35,000–40,000, which is labeled with [^{3}H]histidine and [^{3}H]alanine but not [^{3}H]isoleucine (unpublished data).

Homologies Between PfHRP-II and PfHRP-III. Comparison of the sequences for PfHRP-II and PfHRP-III shows the regions flanking the tandem repeat domains, including untranslated regions within the intron and following the stop codon, exhibit 85–90% homology in nucleotide sequence (Fig. 5). Although the sequence of the hydrophobic leader for PfHRP-III has not been obtained, the close relationships evident between PfHRP-III and SHARP indicate that a high level of homology can also be expected among the hydrophobic leader sequences. Greater differences occur between the tandem repeat domains of PfHRP-II and PfHRP-III. The arrangement and number of repeats differ in each protein, and the region between R_{IIIA} and R_{IIIB} in PfHRP-III is absent from PfHRP-II. Nevertheless, close relationships between the repeats are evident. Perhaps most remarkable is that the most common repeat in PfHRP-II, the hexapeptide Ala-His-His-Ala-Ala-Asp, differs in the sixth position from the most common repeat in PfHRP-III, the hexapeptide Ala-His-His-Ala-Ala-Asn. Of the 33 hexapeptide repeats in PfHRP-II, the sixth amino acid is aspartic acid in 30 and tyrosine in 3,

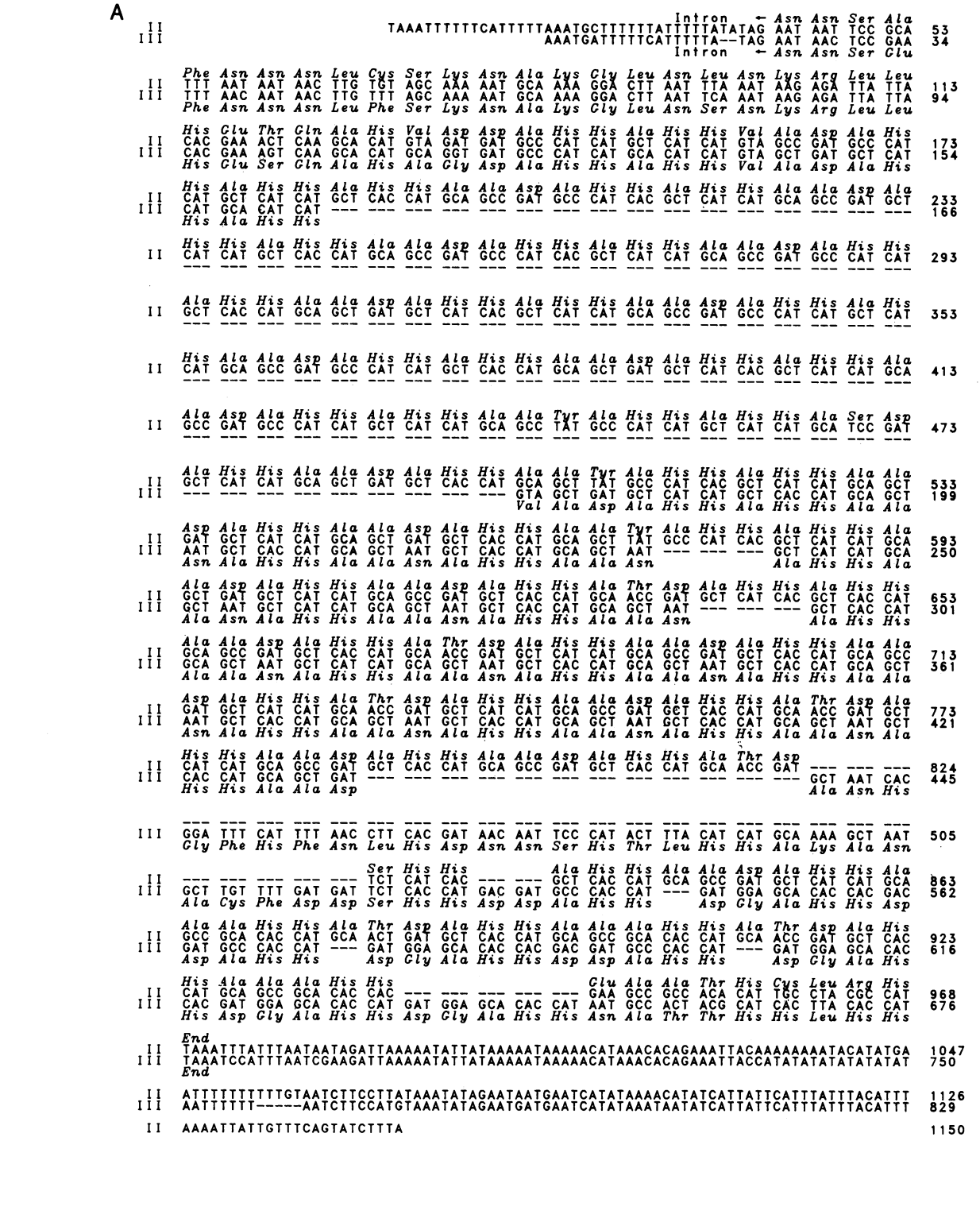

FIG. 4. Genomic sequences of PfHRP-II and PfHRP-III displayed by automated plotting (27). The register of the open reading frame of PfHRP-II is in phase with the gene for β-galactosidase in λMAB1, as expected since this clone produces a fusion protein reacting with McAb87 and anti-β-galactosidase. The sequence shown for PfHRP-II does not include the nucleotides added by ligation of *Eco*RI linkers (GGAATTCC) to the genomic fragment during library construction (18). (*B*) Sequence of PfHRP-II mRNA obtained by primer extension analysis. The splice junction is indicated by the arrow. The hydrophobic leader sequence is underlined.

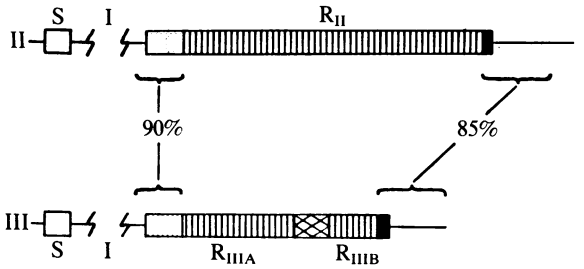

FIG. 5. Schematic representation of the genes for PfHRP-II and PfHRP-III. The lengths of the coding regions, represented by boxes, are drawn to scale. Introns (I) and repeat domains (R_{II}, R_{IIIA}, and R_{IIIB}) are indicated. S, exon encoding hydrophobic leader.

whereas in PfHRP-III the sixth amino acid is asparagine in 13 and aspartic acid in 3.

DISCUSSION

In this study we have used a clone of *P. falciparum* in the characterization of two closely related genes. Both genes are therefore found within a single parasite and are not variants of the same gene in different parasites. Both genes also occur in other clones of *P. falciparum* (unpublished data).

PfHRP-II and PfHRP-III contain unusually high levels of histidine and alanine within the tandem repeats of the proteins. In PfHRP-II the repeats comprise hexapeptides predominantly of the sequence Ala-His-His-Ala-Ala-Asp, interrupted at intervals by tripeptides and pentapeptides predominantly of the sequence Ala-His-His and Ala-His-His-Ala-Ala. Repeats varying from these sequences appear to have arisen by focal changes that have spread through the repeats. For example, two variants of the hexapeptide, Ala-His-His-Ala-Thr-Asp and Ala-His-His-Ala-Ala-Tyr, occur several times in the sequence of PfHRP-II. The tripeptides and pentapeptides in PfHRP-II generally have sequences that are the same as the first three and first five amino acids in the hexapeptide, suggesting deletions and/or insertions have occurred and also spread among the repeats.

The gene for PfHRP-III contains two repeat domains separated by a nonrepetitive stretch of 78 nucleotides. The first repeat domain, R_{IIIA}, encodes tandemly repeated hexapeptides that are homologous with the hexapeptide repeats in PfHRP-II. However, the predominant hexapeptide unit of PfHRP-III, Ala-His-His-Ala-Ala-Asn, differs from that of PfHRP-II by a change in the sixth amino acid from aspartic acid to asparagine. This striking change, from an acidic to a polar residue in the hexapeptide repeat unit, is the result of a change from adenosine to guanosine in the first nucleotide of the codon for this amino acid. It seems unlikely independent mutational events produced this change throughout the repeats; a more attractive hypothesis is that a single change in one of the repeat units was conserved and spread throughout the repeats.

The second repeat domain in PfHRP-III, R_{IIIB}, contains tandemly repeated pentapeptides that show less homology with PfHRP-II, though a clear relationship remains evident. When gaps are introduced to maximize alignment, approximately two-thirds of the aligned nucleotides and amino acids match. Tripeptides of the sequence Ala-His-His have been conserved in both repeat structures.

The homologies between the tandem repeat domains together with the homologies between the regions flanking the repeats imply the genes for PfHRP-II and PfHRP-III have originated in the duplication of an ancestral sequence. Divergence following duplication has evidently generated two genes having distinct primary structures. Divergence has been greater in the repeat domains than in the flanking regions, which include untranslated sequences that would be expected to be more susceptible to change than coding regions. This suggests the repeats constitute relatively labile elements within the genome that may provide the malaria parasite with a means of rapid evolutionary change. It remains to be determined whether the divergent structures of PfHRP-II and PfHRP-III reflect significant functional differences or whether both proteins have similar properties, perhaps deriving from their unusually high histidine content.

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