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Structural Diversity of the Major Surface Antigen of *Plasmodium falciparum* Merozoites

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The structures of the major merozoite surface antigen of *Plasmodium falciparum* and the gene encoding it were indistinguishable for the Wellcome strain and the Thai clone T9/94 but different for clones T9/96, T9/98, and T9/101. The central portion of the gene is subject to the greatest variation in structure. The protein from all five lines was found to be posttranslationally modified by covalent addition of both carbohydrate and fatty acid.

In one isolate of *Plasmodium falciparum*, the most dangerous of the four malaria-inducing species infecting humans, a 195,000-molecular-weight precursor to the major merozoite surface antigens (PMMSA) is synthesized and processed to three proteins, of molecular weights 83,000, 42,000, and 19,000, on the merozoite surface (5, 6). The gene for the 195,000-molecular-weight protein of this isolate (Wellcome strain) has been cloned, its nucleotide sequence has been determined, and selected peptides which may contain epitopes involved in the development of immunity have been expressed in *Escherichia coli* (7, 16). The isolation, sequencing, and expression of small parts of the gene coding for PMMSA of different lines of *P. falciparum* have also been described (1, 3), allowing first insights into the diversity of this gene.

A description of the polymorphism of the PMMSA from some 40 different isolates of *P. falciparum* in terms of reactivity with monoclonal antibodies has been reported (15). Here we describe the comparison of the PMMSA of the Wellcome strain and four cloned lines from a single isolate from Thailand at the level of the proteins and their corresponding genes.

RPMI 1640 tissue culture medium was obtained from Flow Laboratories Ltd., Ayrshire, Scotland, and methionine- or glucose-free RPMI 1640 was purchased from GIBCO Laboratories, Grand Island, N.Y. The radiolabeled compounds L-[^{35}S]methionine (specific activity, 1,315 Ci/mmol), D-[6-^{3}H]glucosamine hydrochloride (specific activity, 30 Ci/mmol), 5′-[^{32}P]dATP (specific activity, 3,000 Ci/mmol), and [9,10-*n*-^{3}H]palmitic acid (specific activity, 50 Ci/mmol) were obtained from Amersham International Ltd., Amersham, England. Restriction enzymes *Rsa*I and *Hin*fI were from Boehringer Mannheim Biochemicals, Indianapolis, Ind. DNA molecular weight standards were from Bethesda Research Laboratories, Gaithersburg, Md.

The Wellcome strain (a West African isolate) and four cloned lines (T9/94, T9/96, T9/98, and T9/101) (22; obtained from D. Walliker) of *P. falciparum* were cultivated in vitro and labeled with [^{35}S]methionine as described previously (5). Labeling of *P. falciparum* cultures with [6-^{3}H]glucosamine hydrochloride was done in 5 ml of RPMI 1640 in which glucose was replaced by 20 mM D-fructose at 60 μCi/ml at 37°C for 150 min and labeling with [9,10-*n*-^{3}H]palmitic acid was done at 75 μCi/ml. After incubation the cells were washed twice in cold phosphate-buffered saline (PBS) and stored at −70°C. Lysis of cells and immunoprecipitation were done as described previously (5). Immunoprecipitated complexes from labeled lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 20 mA in slab gels with 3% polyacrylamide in the stacking gel and 7.5% polyacrylamide in the separation gel (10). After electrophoresis the gels were stained with Coomassie blue, destained, impregnated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), and dried. Fluorography of radiolabeled material was done at −70°C with X-Omat S film (Eastman Kodak Co., Rochester, N.Y.). Molecular weight markers were human spectrin heterodimer (molecular weight, 240,000 and 220,000), β-galactosidase (molecular weight, 116,000), phosphorylase *b* (molecular weight, 93,000), bovine serum albumin (molecular weight, 68,000), and aldolase (molecular weight, 39,000).

Immunoprecipitates from extracts of [^{35}S]methionine-labeled parasites obtained by using rabbit anti-PMMSA serum were processed for peptide mapping (6).

P. falciparum genomic DNA was prepared from parasitized erythrocytes lysed by ammonium chloride (11, 13). Digestions of DNA with the restriction enzymes *Rsa*I and *Hin*fI were done for 3 h at 37°C in the buffers recommended by the manufacturer.

Nick translation was done as described by Rigby et al. (17) and Maniatis et al. (12) after the DNA probe was heated at 100°C for 3 min (23). Separation of the labeled probe from low-molecular-weight material was done by the spun-column method (11).

For the Southern blot analysis, 1.3 μg of restriction enzyme-digested genomic DNA was electrophoresed through a 1.25% agarose gel in 0.089 M Tris borate–2 mM EDTA–5 μg of ethidium bromide per ml for 14 h at 50 V. Standards (123-base-pair [bp] ladder and lambda bacteriophage *Hin*dIII digests [Bethesda Research Laboratories]) were run in parallel, and the relationship between molecular weight and migration was calculated for each electrophoretic run. The DNA was partially depurinated and transferred to a GeneScreen Plus (New England Nuclear) membrane by the procedure of Southern (21). Prehybridization was carried out in 50% formamide–1% sodium dodecyl sulfate–1 M NaCl–10% dextran sulfate–200 μg of single-stranded DNA

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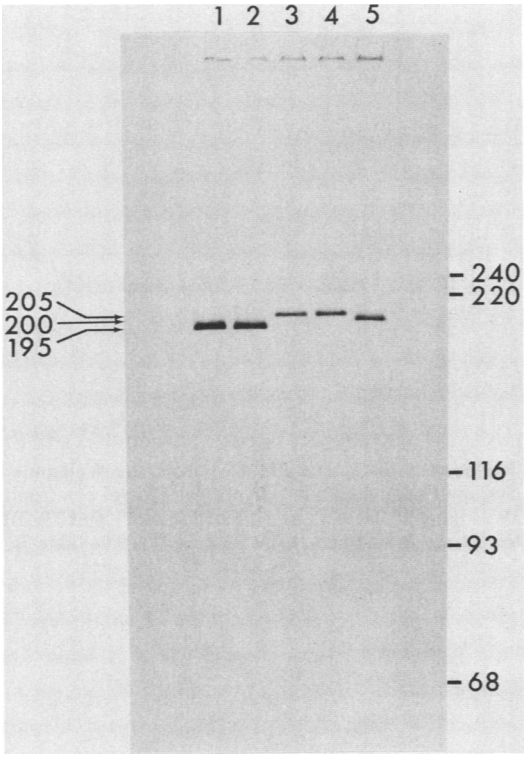

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated, [^{35}S]methionine-labeled PMMSA from the Wellcome strain (lane 1), and four cloned lines from a single Thai isolate, T9/94 (lane 2), T9/96 (lane 3), T9/98 (lane 4), and T9/101 (lane 5).

per ml at 37°C for at least 16 h. Hybridization with the ^{32}P-labeled nick-translated probe (3×10^6 to 6×10^6 cpm total) was done under the same conditions at 37°C overnight. The membrane was washed, as suggested by the manufacturer, with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min, 2× SSC–1.0% sodium dodecyl sulfate at 65°C for 30 min, and 0.1× SSC at room temperature for 30 min with constant agitation.

The electrophoretic mobilities of the PMMSA of the different strains are slightly but clearly different (Fig. 1), and the molecular weights fall into three groups: 195,000 (Wellcome and T9/94), 205,000 (T9/96 and T9/98), and 200,000 (T9/101). Maps of [^{35}S]methionine-labeled chymotryptic peptides from the PMMSA (Fig. 2) show that the pattern of peptides of the Wellcome strain and clone T9/94 (panel A and B) are virtually identical, while those of clones T9/96, T9/98, and T9/101 are distinct and similar but not identical to each other (e.g., there is an additional spot in the map of T9/101 [arrow in panel E]).

The posttranslational modification of the PMMSA of the different isolates was assessed by labeling either with [^{3}H]glucosamine (with fructose instead of glucose as an energy source [8, 20] to support the synthesis of malarial proteins) or with [^{3}H]palmitic acid. Figures 3A and B show that the PMMSA of each of the lines can be labeled with D-[^{3}H]glucosamine and [^{3}H]palmitic acid. The presence of fatty acids covalently bound to proteins has been demonstrated for a number of proteins (9, 18, 19).

To further substantiate the results obtained at the protein level, a comparative analysis was made of restriction enzyme-digested genomic DNA from the five *P. falciparum* lines by using the Southern blot technique and selected ^{32}P-labeled probes derived from the Wellcome strain (7).

Figure 4A shows a Southern blot analysis of *Rsa*I-digested genomic DNA by using probe A (bp 1065 to 1816 from clone pPfc1013). A fragment of 1,230 bp (Wellcome and T9/94) and 1,310 bp (T9/96, T9/98, and T9/101) hybridized, suggesting that the Wellcome strain PMMSA gene may be identical to that of T/94 within the region covered by this probe. Additional hybridization data obtained with two other probes (D, bp 209 to 519 from clone pPfc1013; E, bp 516 to 1460 from clone pPfc1013 [data not shown]) demonstrated the occurrence of hybridizing *Hin*fI restriction fragments of the same size and additional fragments extending outside the coding region of the gene for all stains. (For a summary of the results obtained with all probes, see Fig. 6.)

To investigate sequence homologies in the central area of the PMMSA gene, probe B (bp 1847 to 3559 from clone pPfg1) was used. This probe detected three fragments, with approximate sizes of 1,500, 1,230 and 369 bp, from the Wellcome and T9/94 DNA (Fig. 4B). Hybridizing fragments of these sizes are missing in *Rsa*I-restricted DNA from T9/96, T9/98, and T9/101. Instead, a band of 960 bp was detected. By using a probe derived from the region between probes B and C (F, bp 3556 to 4760 from pPfc1028) we found two fragments, of 1,500 and 800 bp, in the Wellcome strain and clone T9/94 and one, of 960 bp, in clones T9/96, T9/98, and T9/101 (see Fig. 6).

Figure 5A shows an analysis of the 3′-terminal region of

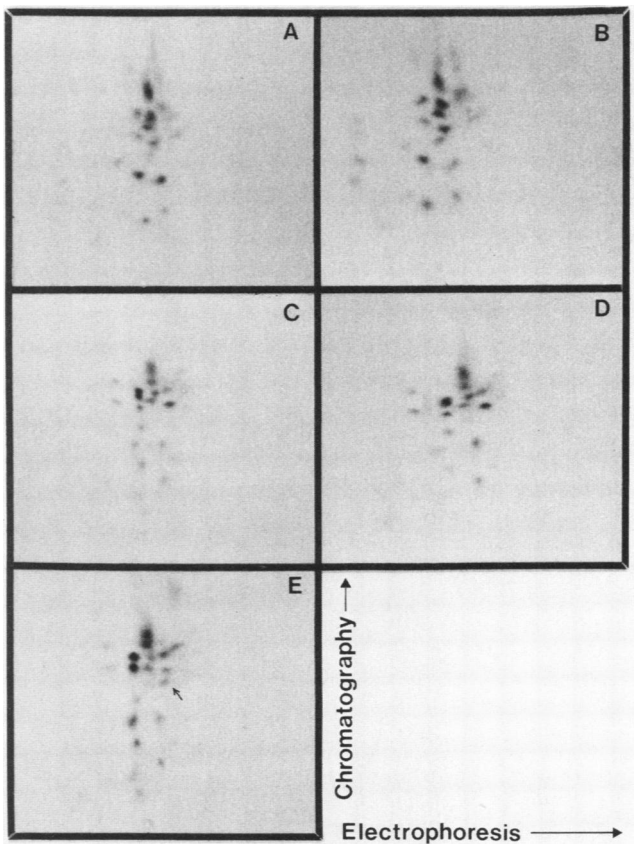

FIG. 2. Comparative chymotryptic peptide maps of [^{35}S] methionine-labeled PMMSA from the Wellcome strain (A), T9/94 (B), T9/96 (C), T9/98 (D), and T9/101 (E).

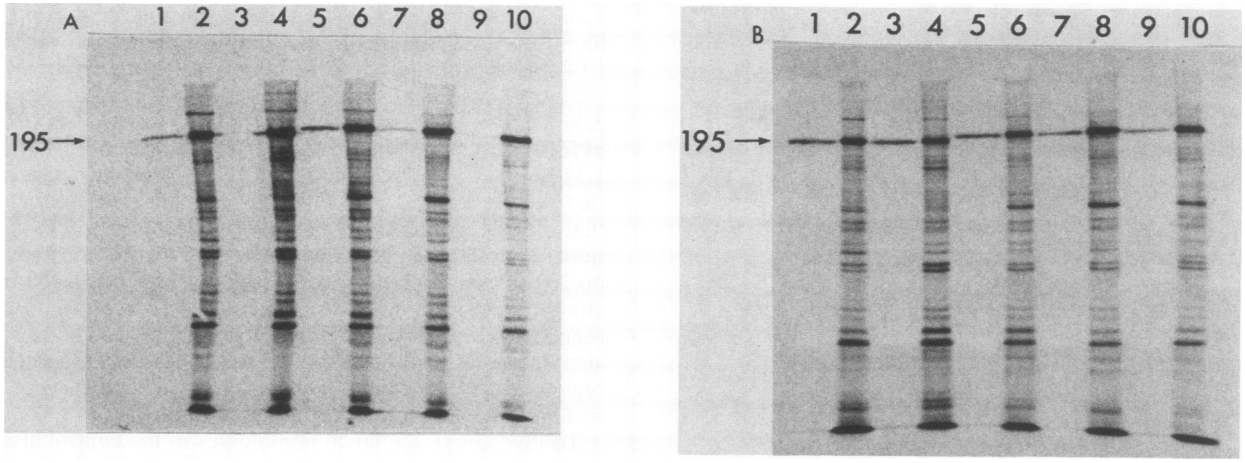

FIG. 3. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{3}H]glucosamine-labeled PMMSA from the Wellcome strain (lane 1), T9/94 (lane 3), T9/96 (lane 5), T9/98 (lane 7), and T9/101 (lane 9). (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of [^{3}H]palmitic acid-labeled PMMSA from the Wellcome strain (lane 1), T9/94 (lane 3), T9/96 (lane 5), T9/98 (lane 7), and T9/101 (lane 9). In parallel tracks in panels A and B, [^{35}S]methionine-labeled proteins from the Wellcome strain (lane 2), T9/94 (lane 4), T9/96 (lane 6), T9/98 (lane 8), and T9/101 (lane 10) are shown.

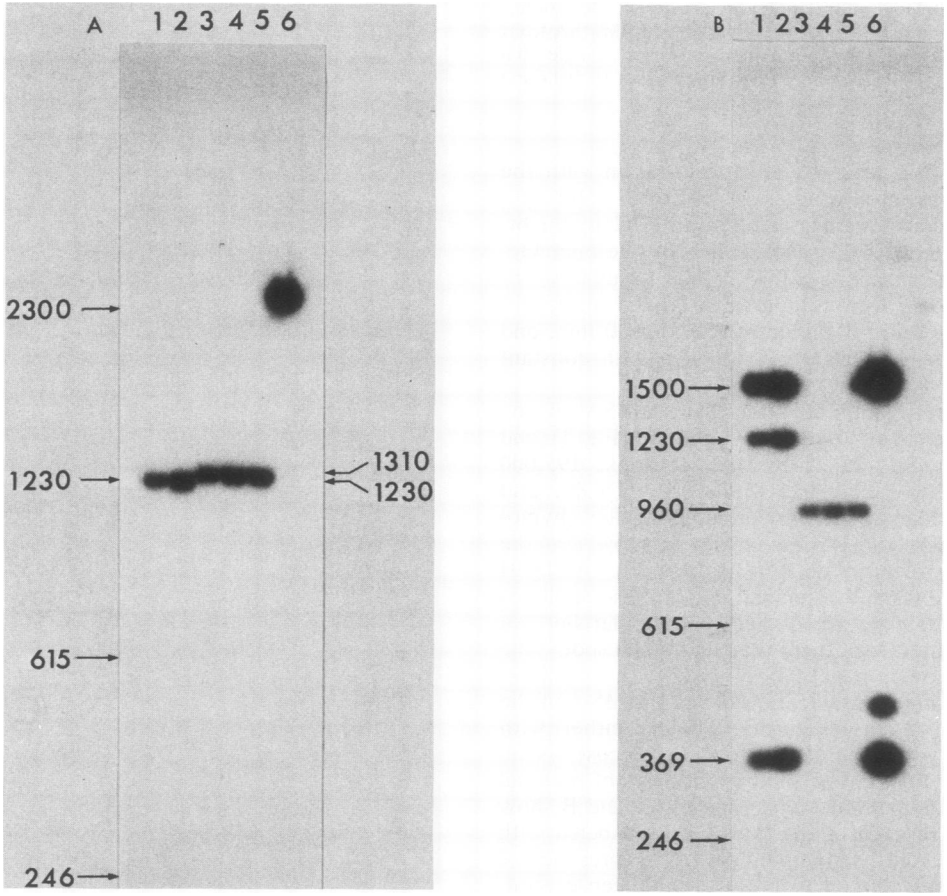

FIG. 4. Southern blot analysis of *P. falciparum* genomic DNA from the Wellcome strain (lane 1), T9/94 (lane 2), T9/96 (lane 3), T9/98 (lane 4), and T9/101 (lane 5). The genomic DNA was digested with *Rsa*I, and the blot was probed with (A) nick-translated probe A and (B) nick-translated probe B. *Rsa*I digests of pPfc1013 (panel A) and pPfc1028 (panel B) were run in parallel (lanes 6).

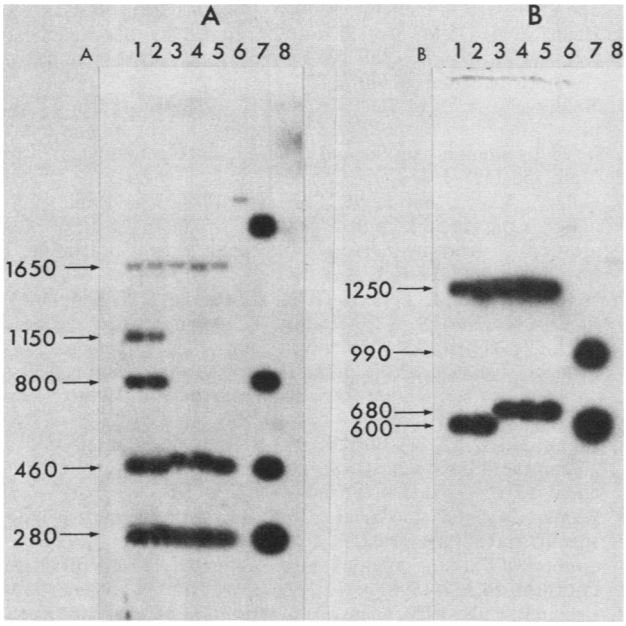

FIG. 5. Southern blot analysis of *P. falciparum* genomic DNA from the Wellcome strain (lane 1), T9/94 (lane 2), T9/96 (lane 3), T9/98 (lane 4), and T9/101 (lane 5). The genomic DNA was digested with *Rsa*I (panel A) or *Hin*fI (panel B) and probed after Southern transfer with nick-translated probe C. Digests with *Rsa*I or *Hin*fI, respectively, of pPfc1013 (lane 6), pPfc1028 (lane 7), and puc9 (lane 8) were run in parallel.

the gene by probe C (bp 4759 to 5920 from clone pPfc1028). The Wellcome and T9/94 DNA show an identical pattern of hybridizing *Rsa*I fragments with comparable signal strength, the sizes being 280, 460, 800, and 1,150 bp. The 280-bp fragment is common to all the lines, and the 460-bp fragment is common to the Wellcome, T9/94, and T9/101 DNA. A somewhat larger fragment, of approximately 490 bp, was found in the *Rsa*I digests from T9/96 and T9/98 DNA. The fragments of 800 and 1,150 bp appear to be unique to the Wellcome and T9/94 DNA. The faint signals, corresponding to sizes of 1,650 and 1,150 bp, may be a result of residual partially cleaved DNA. Alternatively, the 1,650-bp fragment may be due to hybridization of probe C to the region 3′ to the 3′-most *Rsa*I site shown in Fig. 6 extending 3′ to the map shown and common to all five lines.

The 3′ region and the area extending beyond the coding region of the PMMSA gene were also analyzed with probe C and by *Hin*fI digestion. Two bands, of 600 and 1,250 bp, were recognized in the Wellcome and T9/94 DNA, whereas in clones T9/96, T9/98, and T9/101 the restriction fragments had sizes of 680 and 1,250 bp, respectively (Fig. 5B). Thus, as for the 5′ end of the PMMSA gene, there is close homology in all lines at the 3′ end of this gene extending outside the coding sequence. The difference in size of the smaller *Hin*fI fragments, assumed to extend farther in the 5′ direction than the 280-bp *Rsa*I fragment, suggests a limited but distinct variability in the sequence coding for the middle region of the carboxy-terminal processed fragment of the PMMSA of the Wellcome strain and the T9/94 clone on one side and clones T9/96, T9/98, and T9/101 on the other. A schematic comparison of the structure of the gene for the PMMSA of all lines tested is shown in Fig. 6.

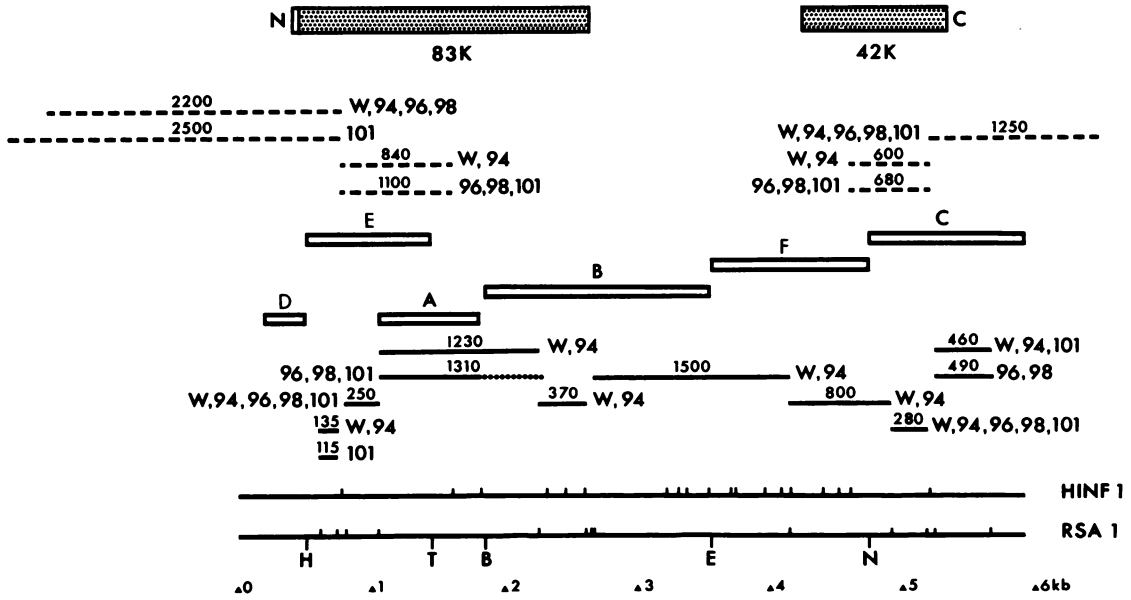

FIG. 6. Schematic representation of the gene for the PMMSA of the Wellcome strain (W), and four cloned lines of a single isolate from Thailand (T9/94 [94], T9/96 [96], T9/98 [98], and T9/101 [101]). Probes derived from parts of the PMMSA gene of the Wellcome strain of *P. falciparum* are shown as boxed bars and designated by capital letters. ——, Hybridizing fragments from *Rsa*I-digested genomic DNAs; ----, hybridizing fragments from *Hin*fI-digested genomic DNAs. The sizes of the fragments are indicated, but positions are tentative and have been chosen by analogy to those for the Wellcome strain. The restriction sites for *Hin*fI and *Rsa*I and other selected restriction enzymes (*Bgl*II [B], *Eco*RI [E], *Taq*I [T], *Hin*dIII [H], and *Nde*I [N]) are indicated. The positions of two of the processed protein fragments (83,000-molecular-weight [83K] including the putative signal sequence and 42,000-molecular-weight [42K]) are shown from the data of Holder et al. (7). The exact position of the 19,000-molecular-weight processed polypeptide fragment is not known. ⊢——···, Fragment hybridizes to probe A but not to probe B.

The data obtained show the extent of variability of the PMMSA of five different lines of *P. falciparum*, the Wellcome strain (a West African isolate) and four cloned lines (22) from a single isolate from Thailand, at the level of the protein and its modification and the structure of the gene. A notable finding is that the PMMSA of the Wellcome strain and of the cloned line T9/94 showed no detectable differences by any criterion. This result is remarkable, since the two lines stem from geographically remote areas, and more than 12 years elapsed between their isolation.

From the hybridization data it is apparent that there are conserved and variable regions in the gene for the PMMSA, in agreement with the results of a study of monoclonal antibody binding (14, 15).

The difference, as seen by hybridization, between the genes for PMMSA of the Wellcome strain and the clones T9/96, T9/98, and T9/101 appears to lie predominantly in the central part of the PMMSA gene. This is in addition to the region encoding the repeating peptide sequence described previously (1, 7).

According to the hybridization data, the 3′ end of the translated region, which codes for the C-terminal end of the 42,000-molecular-weight processing product, appears to be highly conserved in the lines investigated. This part of the gene shows little variation, and this conservation extends into the 3′ nontranslated region. The reason could be the functional pressures on the protein, as this area may be needed for attachment to the merozoite membrane and posttranslational modification. It is interesting to note that conservation which extends into noncoding regions was also detected at the 5′ end.

In all cases PMMSA was labeled with carbohydrate and palmitic acid. The sites of attachment were not investigated in this study, but available data (2) suggest that the 42,000-molecular-weight fragment is covalently acylated. A 180,000- to 185,000-molecular-weight glycoprotein in *P. falciparum* FCB1 has been described, and this may be a precursor to smaller merozoite glycoproteins in released merozoites (4). It is not known, however, whether these glycoproteins are related to the PMMSA.

Thus, from the results of this study it appears that although differences exist between the PMMSA of different clones, the repertoire for variation as detected by the methods used may be restricted. Also, it is important to note that all T9 clones were from one single isolate, so that the variations detected are within one population of parasites.

The full extent of sequence diversity between the PMMSA of different strains of *P. falciparum* must be determined, as this information may be fundamental for the development of a blood-stage malaria vaccine.

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