12. R. P. Hertzberg and P. B. Dervan, *Biochemistry* **23**, 3934 (1984).
13. G. B. Dreyer and P. B. Dervan, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 963 (1985).
14. S. S. Tavale and H. M. Sobell, *J. Mol. Biol.* **48**, 109 (1970); F. Jordan and H. Niv, *Biochim. Biophys. Acta* **476**, 265 (1977).
15. S. Arnott, P. J. Bond, E. Selsing, P. J. C. Smith, *Nucleic Acids Res.* **3**, 2459 (1976).
16. The *p*H values are not corrected for temperature or presence of ethanol and are given for the tenfold-concentrated buffer solutions at 25°C.
17. T. Maniatis *et al.*, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982); A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980).
18. We thank the National Institutes of Health (GM-35724), the Office of Naval Research, and the Parsons Foundation for generous support. We thank S. Singleton for valuable assistance in model building, A. Frankel for a gift of pHIV-CAT, and T. Povsic for large-scale plasmid preparation.

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Synthesis of Functional Human Hemoglobin in Transgenic Mice

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Human α- and β-globin genes were separately fused downstream of two erythroid-specific deoxyribonuclease (DNase) I super-hypersensitive sites that are normally located 50 kilobases upstream of the human β-globin gene. These two constructs were coinjected into fertilized mouse eggs, and expression was analyzed in transgenic animals that developed. Mice that had intact copies of the transgenes expressed high levels of correctly initiated human α- and β-globin messenger RNA specifically in erythroid tissue. An authentic human hemoglobin was formed in adult erythrocytes that when purified had an oxygen equilibrium curve identical to the curve of native human hemoglobin A (Hb A). Thus, functional human hemoglobin can be synthesized in transgenic mice. This provides a foundation for production of mouse models of human hemoglobinopathies such as sickle cell disease.

CORRECTLY REGULATED EXPRESSION of human β-globin genes in transgenic mice is well documented (*1, 2*). The human gene is expressed only in adult erythroid tissue and, in some animals with relatively high transgene copy numbers, the level of human β-globin mRNA is equivalent to endogenous mouse β-globin mRNA. Analysis of constructs with β-globin gene fragments inserted upstream of a reporter gene demonstrate that sequences located immediately upstream, within and downstream of the gene contribute to the correct temporal and tissue specific expression (*3*). Sequences located 50 kb upstream of the β-globin gene also have an effect on globin gene expression (*4–8*). When these sequences that contain erythroid-specific, DNase I super-hypersensitive (HS) sites are fused upstream of the human β-globin gene and injected into fertilized mouse eggs, large amounts of human β-globin mRNA are synthesized in virtually all transgenic mice that develop (*5, 7*). These experiments suggest that the super-hypersensitive sites define locus activation regions that "open" a large chromosomal domain for expression specifically in erythroid cells and dramatically enhance globin gene expression.

The human α1-globin gene is also expressed at high levels in erythroid tissue of transgenic mice when the injected gene is flanked by super-hypersensitive sites from the human β-globin locus (*8*). Thus a complete human hemoglobin could be synthesized in mice if human α- and β-globin gene constructs were coinjected into fertilized eggs. Previous studies demonstrated that two of the five HS sites in the β-globin locus were sufficient for high-level expression (*7, 8*). Therefore, we inserted HS I and II (a 12.9-kb Mlu I–Cla I fragment) upstream of the human α1- and β-globin genes (Fig. 1) and coinjected equimolar amounts of these constructs into fertilized mouse eggs (*9*). The eggs were transferred into the oviducts of pseudopregnant foster mothers, and seven transgenic mouse lines were established from founder animals that contained intact copies of the injected fragments. Total RNA from ten tissues of adult progeny were then analyzed for correctly initiated human α-, human β-, mouse α-, and mouse β-globin mRNA by primer extension (*10*) (Fig. 2A). Human α- and β-globin transgenes were expressed only in blood and spleen, which are both erythroid tissues in mice; detection in the lung is the result of blood contamination (*11*) because both human and mouse α- and β-globin mRNA are observed in this nonerythroid tissue. Human α- and β-globin mRNA levels in blood, as measured by solution hybridization, were 100% and 120% of endogenous mouse β-globin mRNA, respectively. Therefore, erythroid-specific, human α- and β-globin gene expression can be achieved in adult transgenic mice after coinjection of α- and β-globin constructs that contain HS I and II.

To determine whether complete human hemoglobins were formed, we separated hemolysates (*12*) of the blood of animals from two different transgenic lines by nondenaturing isoelectic focusing (IEF) (Fig. 2B). The first lane is a mouse control and the last lane is a normal human sample. The predominant band in each of the controls is the major adult hemoglobin; mouse $\alpha_2\beta_2$ or human $\alpha_2\beta_2$, respectively. In both transgenic mouse samples 5394 and 5393, bands that run at the same pI as human Hb A

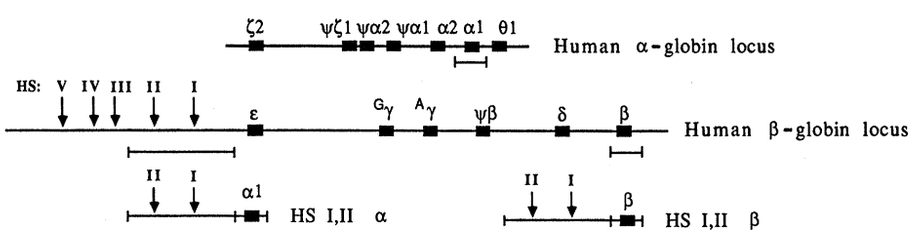

Fig. 1. HS I,II α-globin and HS I,II β-globin gene constructs. Eighty-five kilobases of the human β-globin locus and 35 kb of the human α-globin locus are drawn to scale. The brackets beneath the HS sites, α1-globin gene, and β-globin gene indicate fragments used for construction. A 12.9-kb Mlu I–Cla I fragment that contained erythroid-specific, DNase I super-hypersensitive (HS, arrow) sites I and II from the human β-globin locus was inserted into a modified pUC19 plasmid upstream of a 3.8-kb Bgl II–Eco RI fragment carrying the human α1-globin gene or a 4.1-kb Hpa I–Xba I fragment with the human β-globin gene. The 16.7- and 17.0-kb fragments with HS I,II α-globin and HS I,II β-globin were separated from plasmid sequences and coinjected into fertilized mouse eggs (*9*).

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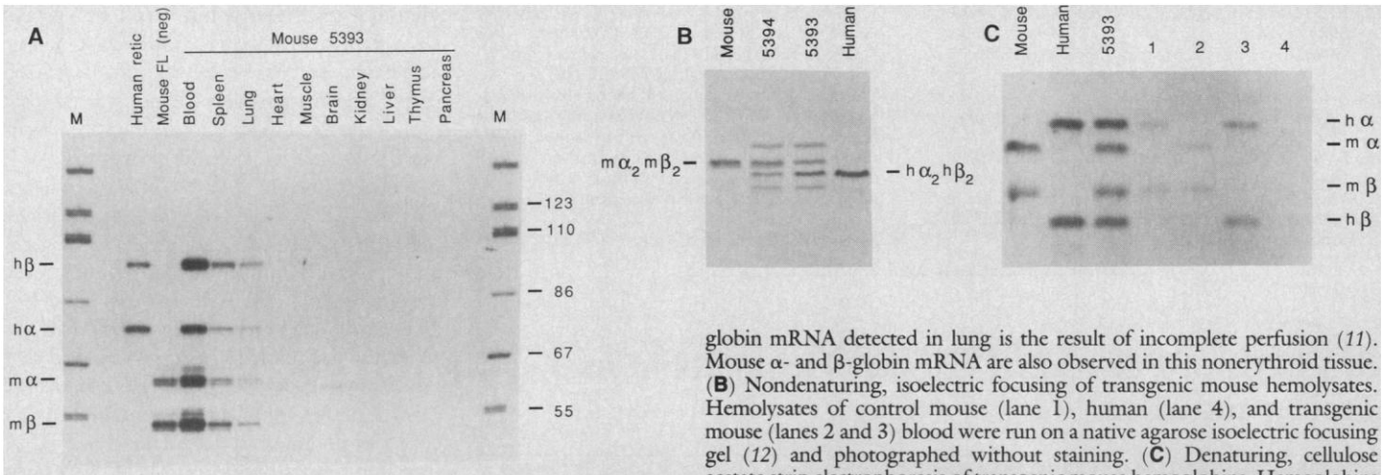

Fig. 2. Expression of human α- and β-globin genes in transgenic mice. (**A**) Primer extension analysis of total RNA from ten tissues of an HS I,II α-globin/HS I,II β-globin transgenic mouse. Human reticulocyte and mouse fetal liver RNAs are controls. Authentic human β- and α-globin primer extension products are 98 bp and 76 bp, respectively; correct mouse α- and β-globin products are 65 and 53 bp, respectively (*1, 8*). Human α- and β-globin mRNA detected in lung is the result of incomplete perfusion (*11*). Mouse α- and β-globin mRNA are also observed in this nonerythroid tissue. (**B**) Nondenaturing, isoelectric focusing of transgenic mouse hemolysates. Hemolysates of control mouse (lane 1), human (lane 4), and transgenic mouse (lanes 2 and 3) blood were run on a native agarose isoelectric focusing gel (*12*) and photographed without staining. (**C**) Denaturing, cellulose acetate strip electrophoresis of transgenic mouse hemoglobins. Hemoglobins were denatured in alkaline-urea buffer, electrophoresed on cellulose acetate strips, and stained with imido black (*13*). Lanes marked mouse, human, and 5393 are hemolysates of control mouse, human, and transgenic mouse (5393) blood, respectively. Lanes marked 1 to 4 are hemoglobins purified from individual bands (numbered 1 to 4 from top to bottom) of sample 5393 on the isoelectric focusing gel in (B).

($h\alpha_2h\beta_2$) and mouse hemoglobin ($m\alpha_2m\beta_2$) are observed. In addition to human and mouse hemoglobins, two other major bands were observed in both transgenic samples. To determine the composition of these bands and to confirm the human and mouse hemoglobins, the four bands in sample 5393 were excised from the gel and analyzed on a denaturing cellulose acetate strip (*13*) (Fig. 2C). Control lysates of mouse, human, and 5393 blood samples were separated in lanes on the left. Mouse α- and β-globin polypeptides, as well as human α- and β-globin polypeptides, were well separated on this strip. Sample 5393 contained all four polypeptides; the human α- and β-globin polypeptides were 110% and 106% of the amounts of mouse α- and β-globin, by densitometric analysis. The top band (band 1) of sample 5393 in Fig. 2B is composed of human α- and mouse β-globin chains. The second band is mouse α- and mouse β-globin and the third band is human α- and β-globin as expected. The polypeptides composing band 4 in Fig. 2B are barely visible in Fig. 2C but are clearly mouse α- and human β-globin. Therefore, normal amounts of human hemoglobin can be synthesized in adult mice, and multiple combinations of globin polypeptides are possible [see note (*14*)].

The functional properties of human, mouse, and hybrid hemoglobins synthesized by transgenic mice were assessed by determination of oxygen equilibrium curves (OEC) and by calculation of P_{50} values. The P_{50} is the partial pressure at which hemoglobin is half saturated with oxygen and is inversely related to hemoglobin oxygen affinity. All four hemoglobins described above were purified by preparative IEF (*15*) and the OEC for each was determined (*16*) (Fig. 3). The OEC were normal, sigmoid-shaped, and demonstrate that all four hemoglobins bind oxygen cooperatively. The P_{50} of human hemoglobin synthesized by transgenic mice is 8.0 mmHg, which is identical to the P_{50} of native human Hb A. Interestingly, the oxygen affinities of the two hybrid tetramers differ significantly from human and mouse hemoglobins. The $h\alpha_2m\beta_2$ hybrid has an extremely low O_2 affinity; the P_{50} is 15.7 mmHg. In contrast, the O_2 affinity for $m\alpha_2h\beta_2$ is extremely high; the P_{50} for this hemoglobin is 4.7 mmHg (*17*).

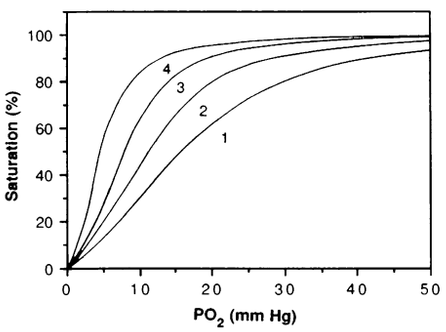

Fig. 3. Oxygen equilibrium curves (OEC) of hemoglobins purified from 5393 transgenic mouse progeny. Hemoglobins of 5393 progeny were separated on preparative isoelectric focusing gels (*15*). Bands 1 to 4 (top to bottom) illustrated in Fig. 2B were purified from gel slices and the OEC of each hemoglobin band was determined in 0.1*M* potassium phosphate, *p*H 7.0 at 20°C (*16*). The P_{50} of band 1 ($h\alpha_2m\beta_2$) is 15.7 mmHg, band 2 ($m\alpha_2m\beta_2$) is 11.1 mmHg, band 3 ($h\alpha_2h\beta_2$) is 8.0 mmHg, and band 4 ($m\alpha_2h\beta_2$) is 4.7 mmHg. The P_{50} of human hemoglobin in these transgenic mice is identical to the P_{50} of native Hb A.

Finally, the hematological values of six transgenic progeny were determined and compared to five normal animals. Red blood cell counts and hematocrits for transgenic animals were normal and, interestingly, the values for hemoglobin and mean corpuscular volume were in the normal range. Consequently, the calculated values of mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (MCHC) for transgenic animals were normal. Thus the total hemoglobin concentration in transgenic erythrocytes is not increased even though reticulocytes contain 100% more globin mRNA (*18*). Therefore, to maintain normal MCHC, all globin mRNAs are either translated at reduced rates or α- and β-globin polypeptides are less stable. Another possibility is that globin synthesis ceases when the maximum intracellular concentration of hemoglobin is attained. If the rate of globin synthesis is normal, then a full complement of hemoglobin could be synthesized in half the time leading to faster maturation of reticulocytes.

In summary, the results presented demonstrate that high levels of human α- and β-globin mRNA can be coexpressed in mice. The transgenes are expressed specifically in erythroid tissue and levels of human hemoglobin equivalent to mouse hemoglobin can be achieved. In addition, the human hemoglobin produced in these mice is fully functional and the transgenic animals are phenotypically normal. These results provide a solid foundaton for the production of transgenic mice that synthesize high levels of other human hemoglobins. We have initiated studies to synthesize high levels of human sickle hemoglobin in transgenic mice in an attempt to produce a mouse model of sickle

cell disease. Although sickle cell anemia was the first disease to be understood at the molecular level (*19*), there is still no cure or adequate treatment. If a transgenic mouse model can be developed, new drug therapies and even gene therapies could be tested. Once perfected in model systems, protocols that are safe and effective for humans could be developed.

REFERENCES AND NOTES

1. T. M. Townes *et al.*, *EMBO J.* **4**, 1715 (1985); T. M. Townes *et al.*, *Mol. Cell. Biol* **5**, 1977 (1985).
2. F. Costantini *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 361 (1985); G. Kollias *et al.*, *Cell* **46**, 89 (1986); P. Soriano *et al.*, *Science* **234**, 1409 (1986).
3. R. R. Behringer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7056 (1987); G. Kollias, J. Hurst, E. deBoer, F. Grosveld, *Nucleic Acids Res.* **15**, 5739 (1987); M. Trudel and F. Costantini, *Genes Dev.* **1**, 945 (1987).
4. D. Tuan, W. Soloman, Q. Li, I. London, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6384 (1985); W. Forrester, C. Thompson, J. T. Elder, M. Groudine, *ibid.* **83**, 1359 (1986).
5. F. Grosveld *et al.*, *Cell* **51**, 75 (1987); D. Talbot *et al.*, *Nature* **338**, 352 (1989).
6. W. Forrester *et al.*, *Nucleic Acids. Res.* **15**, 10159 (1987); A. K. Nandi, R. S. Roginski, R. G. Gregg, O. Smithies, A. I. Skoultchi, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3845 (1988); G. B. van Assendelft, O. Hanscombe, F. Grosveld, D. R. Greaves, *Cell* **56**, 969 (1989); W. Forrester, U. Novak, R. Gelinas, M. Groudine, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5439 (1989); M. C. Driscoll, C. S. Dobkin, B. P. Alter, *ibid.*, in press.
7. T. M. Ryan *et al.*, *Genes Dev.* **3**, 314 (1989).
8. T. M. Ryan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 37 (1989).
9. R. L. Brinster, H. Y. Chen, M. E. Trumbauer, M. K. Yagle, R. D. Palmiter, *ibid.* **82**, 4438 (1985).
10. Adult animals were made anemic with phenylhydrazine (*20*) to induce reticulocytosis, anesthetized, perfused (*11*), and tissues were removed. Total RNA was prepared from frozen tissue [*Anal. Biochem.* **162**, 156 (1987)] with the following modification. The final RNA pellets were resuspended in a solution containing 1.0% SDS, proteinase K (100 mg/ml), 25 m*M* NaCl, 1.0 m*M* EDTA, and 10 m*M* tris-HCl *p*H 7.5. After digestion for 3 hours at 50°C, the samples were extracted with phenol/chloroform, chloroform, and ethanol precipitated.
11. Animals were perfused by cutting the right atrium and injecting phosphate-buffered saline into the left ventricle. The lung is not perfused in this procedure and, therefore, is contaminated with blood.
12. Blood cells were washed twice with saline and lysed in a volume of water equal to the cell pellet. One-fourth volume of carbon tetrachloride was mixed with the hemolysate, and cell membranes were extracted by brief vortexing and mirocentrifugation. The aqueous phase was removed and frozen at 20°C. Samples were subsequently thawed, diluted with an equal volume of 0.05% KCN, and separated on an agarose isoelectric focusing gel (Resolve-Hb, Isolabs Inc., Akron, Ohio) according to the manufacturer's specifications. After focusing, proteins were fixed in the gel with 10% trichloroacetic acid for 10 min. The gel was then rinsed for 1 hour with water, dried, and hemoglobin bands were visualized without staining.
13. Hemoglobin bands were cut out of the agarose IEF gel and eluted in water for 1 hour at room temperature. After dialysing against water overnight at room temperature, the samples were lyophilized and resuspended in water. Equal volumes of sample (purified hemoglobin or whole hemolysate), alkaline-urea buffer (6.0*M* urea, 15 m*M* boric acid, 0.5 m*M* EDTA, 25 m*M* tris-HCl, *p*H 8.6), and β-mercaptoethanol were mixed and an aliquot was loaded onto a cellulose acetate strip (Gelman) that had been soaked overnight in alkaline-urea buffer. The samples were then electrophoresed for 1 hour at 190 V in alkaline-urea buffer. Proteins were subsequently stained with 0.5% imido black in methanol:acetic acid (45:10). The strips were destained in methanol: acetic acid (47.5:5), dried, and photographed.
14. Although only four hemoglobin bands are observed on the IEF gel in Fig. 2B, nine hemoglobins representing all possible combinations of mouse and human α- and β-globin polypeptides probably exist inside the cell. During electrophoresis oxy-hemoglobin tetramers ($\alpha_2\beta_2$) dissociate into dimers ($\alpha_1\beta_1$) that are separated by charge differences. Therefore, hemoglobin tetramers composed of dimers of unlike charge are not detected (*21*).
15. Mouse, human, and hybrid hemoglobins synthesized by transgenic mice were separated by preparative IEF on 4.0% acrylamide gels containing 2.0% Pharmalyte *p*H 5 to 8. Each of the four bands was sliced from the gel, homogenized, and the hemoglobin was eluted in 0.1*M* potassium phosphate buffer. The isolated fractions were concentrated with Amicon filters (YM 10).
16. Hemoglobins were maintained in the carbon monoxide (CO) form during separation and concentration procedures to avoid auto-oxidation. Prior to functional studies the hemoglobins were converted to the oxy-state by photolysis and vacuum removal of CO. The oxygen equilibrium curve of each hemoglobin fraction was determined using a Hemox Analyzer (TCS, Southhampton, PA) in 0.1*M* potassium phosphate buffer, *p*H 7.0 at 20°C (*22*). All samples were analyzed four times and the curves were drawn in continuous mode. The maximum error of measurement of the P_{50} values is ±1 mmHg [*Crit. Care Med.* 7, 391 (1979)].
17. OEC of whole blood and unfractionated hemolysates from transgenic mice were also determined and compared to mouse and human controls. The curve for whole blood of 5393 transgenic progeny is virtually identical to the mouse control, while the curve for an unfractionated transgenic hemolysate is shifted to the left of the mouse hemolysate control. The left shift of the transgenic hemolysate OEC can be attributed to the presence of high-affinity hybrid and human hemoglobin species. The similarity of the whole blood OEC for transgenic and control mice may be due to adaptive responses, such as an increase in allosteric effectors of oxygen affinity, in the transgenic mice.
18. Quantitative solution hybridizations of blood RNA from the seven transgenic lines indicate that mouse α- and β-globin mRNA levels (picograms of total RNA per microgram) are not decreased in mice expressing high levels of human α- and β-globin mRNA.
19. V. M. Ingram, *Nature* **178**, 792 (1956); *ibid.* **180**, 326 (1957)
20. T. Cheng, S. K. Polmar, H. H. Kazazian, Jr., *J. Biol. Chem.* **249**, 1781 (1974).
21. H. F. Bunn and B. G. Forget, *Hemoglobin: Molecular, Genetic and Clinical Aspect* (Saunders, Philadelphia, 1986), pp. 417–421.
22. T. Asakura and M. P. Reilly, in *Oxygen Transport in Red Blood Cells*, C. Nicolau, Ed. (Pergamon, New York, 1986), pp. 57–75.

23. We thank N. Martin, J. Askins, and M. Avarbock for excellent technical assistance, J. Prchal for providing human reticulocyte RNA, K. Hall for instructions on electrophoresis of hemoglobins on denaturing cellulose acetate strips, and J. Engler for synthesizing the human α-, human β-, mouse α-, and mouse β-globin oligonucleotides. Supported in part by grants HL-35559, HD-09172, HL-38632, and HD-23657 from the National Institutes of Health and predoctoral training grant T32 CA-09467 from NIH (to T.R.).

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Circumsporozoite Protein Heterogeneity in the Human Malaria Parasite *Plasmodium vivax*

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Phenotypic heterogeneity in the repetitive portion of a human malaria circumsporozoite (CS) protein, a major target of candidate vaccines, has been found. Over 14% of clinical cases of uncomplicated *Plasmodium vivax* malaria at two sites in western Thailand produced sporozoites immunologically distinct from previously characterized examples of the species. Monoclonal antibodies to the CS protein of other *P. vivax* isolates and to other species of human and simian malarias did not bind to these nonreactive sporozoites, nor did antibodies from monkeys immunized with a candidate vaccine made from the repeat portion of a New World CS protein. The section of the CS protein gene between the conserved regions I and II of a nonreactive isolate contained a nonapeptide repeat, Ala-Asn-Gly-Ala-Gly-Asn-Gln-Pro-Gly, identical at only three amino acid positions with published nonapeptide sequences. This heterogeneity implies that a *P. vivax* vaccine based on the CS protein repeat of one isolate will not be universally protective.

MALARIA, A DISEASE CAUSED BY A mosquito-borne protozoan parasite of red blood cells, is so widespread and causes disability so severe that many strategies to control it have been devised. Recently much effort has been focused on the construction of vaccines designed to elicit a host immune response to sporozoites, the parasite stage injected into humans by mosquitoes. The predominant surface, or circumsporozoite (CS), proteins of sporozoites are characterized by tandemly repeated peptide units that occupy about one-third of each molecule and are immunogenic (*1–3*). In *Plasmodium vivax*, one of four *Plasmodium* species naturally infecting humans, the unit has been found to be a nonapeptide repeated about 20 times (*2*, *3*).