

Wheat Germ Cell-Free System-Based Production of Malaria Proteins for Discovery of Novel Vaccine Candidates^{∇†}

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One of the major bottlenecks in malaria research has been the difficulty in recombinant protein expression. Here, we report the application of the wheat germ cell-free system for the successful production of malaria proteins. For proof of principle, the Pfs25, PFCSP, and PfAMA1 proteins were chosen. These genes contain very high A/T sequences and are also difficult to express as recombinant proteins. In our wheat germ cell-free system, native and codon-optimized versions of the Pfs25 genes produced equal amounts of proteins. PFCSP and PfAMA1 genes without any codon optimization were also expressed. The products were soluble, with yields between 50 and 200 µg/ml of the translation mixture, indicating that the cell-free system can be used to produce malaria proteins without any prior optimization of their biased codon usage. Biochemical and immunocytochemical analyses of antibodies raised in mice against each protein revealed that every antibody retained its high specificity to the parasite protein in question. The development of parasites in mosquitoes fed patient blood carrying *Plasmodium falciparum* gametocytes and supplemented with our mouse anti-Pfs25 sera was strongly inhibited, indicating that both Pfs25-3D7/WG and Pfs25-TBV/WG retained their immunogenicity. Lastly, we carried out a parallel expression assay of proteins of blood-stage *P. falciparum*. The PCR products of 124 *P. falciparum* genes chosen from the available database were used directly in a small-scale format of transcription and translation reactions. Autoradiogram testing revealed the production of 93 proteins. The application of this new cell-free system-based protocol for the discovery of malaria vaccine candidates will be discussed.

Plasmodium falciparum is the protozoan responsible for the widespread return of malaria to tropical countries, particularly in Africa. This reemergence is generally credited to two causes: the development of multidrug-resistant parasites and the development of insecticide-resistant mosquitoes (10). Through decades of work, scientists have learned that vaccination could be a potent curative, but efforts to develop a successful vaccine have not yet succeeded (25). One of the bottlenecks in vaccine development is at the malaria protein production step and is mainly due to the lack of a methodology to enable preparation of quality proteins in an efficient manner. *P. falciparum* genes have a very high A/T content (average, 76% per gene), and a

number of them encode repeated stretches of amino acid sequences (8); these features have been proposed as the major factors limiting *P. falciparum* protein expression in cell-based systems. Moreover, the presence of glycosylation machinery in eukaryotic cell-based systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (9, 21, 26). In fact, the three pioneering genome-wide studies on the production of *P. falciparum* proteins in cell-based systems faced serious problems. For instance, Aguiar et al. (1) were able to obtain expression in *Escherichia coli* cells of only 39 of 292 malaria genes cloned into the glutathione S-transferase (GST) fusion vector. Mehlin et al. carried out an even more challenging trial in which 1,000 genes encoding relatively small (<450 amino acids) malaria cytosolic proteins were expressed in *E. coli* (24). In that study only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9 mg to 406 mg of protein per liter of culture medium. The other approach used an engineered *E. coli* strain with tRNAs genetically supplemented to allow reading of the high number of A/U codons in malaria mRNA (31). A significant improvement in protein solubility, up to 20.9%, was observed (38 out of 182 proteins tested were soluble). However, although the *E. coli* translation system is known to support folding of prokaryotic and small eukary-

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otic proteins, the multidomain proteins common in eukaryotes tend to fold incorrectly in the *E. coli* system, resulting in the formation of inclusion bodies.

Through decades of laborious work, scientists have identified three leading vaccine candidates from the pool of *P. falciparum* proteins: Pfs25 (19), PfCSP (5, 12, 34), and PfAMA1 (6, 11). Pfs25, a zygote/ookinete surface protein, is a promising candidate as a transmission-blocking vaccine. This protein is composed of four tandem epidermal growth factor-like domains, containing three putative N-linked glycosylation sites beside a signal peptide for the attachment of a glycosylphosphatidylinositol moiety (GPI anchor) at the C terminus. These characteristics render Pfs25 very difficult to express (18, 20). PfCSP, with its biased codon usage and lopsided amino acid composition, allows for only a minute amount of protein to be expressed in *E. coli* cells (34). The other antigen candidate is the PfAMA1 gene, which codes for a type 1 integral membrane protein of merozoites and is also difficult to express. Only a synthetic and codon-optimized gene has produced a fairly large amount of PfAMA1 protein in *E. coli* cells. Furthermore, a series of labor-intensive and technically complex refolding processes of the aggregates were required to use the protein as an antigen (6). The fact that only a few vaccine candidates are currently available (23) is most likely the result of difficulties in expressing malarial antigens in high quantity with their correct conformation.

We previously developed a wheat germ cell-free protein synthesis system for practical use in protein production. The system is especially powerful when used for the production of eukaryotic proteins because of its eukaryotic nature. We established two wheat germ cell-free protein protocols for practical use. The first can be used to produce a small amount of protein from a large number of cDNAs, in parallel, for the examination of product qualities and for the genome-wide biochemical annotation of gene products. In this approach, the templates for transcription are constructed using the split-PCR approach (29). The solution resulting from transcription is then directly used as the mRNA source in the small-scale bilayer translation system (28). The second protocol enables the production of large quantities of proteins. In this case, suitable gene products are first selected using the small-scale parallel production method and subsequent functional screening. Genes of interest are then cloned into the pEU plasmid (29), and the mRNA is transcribed. In the translational step, the protein production employs either the bilayer or the discontinuous batch translation method. The bilayer method has acceptable performance for the production of hundreds of micrograms of protein. Since 150 mg of a control protein in a reaction volume of 50 ml was produced in 5 h with the latter reaction method, the cell-free method can be scaled up (27). The system has been acknowledged in the fields of structural and functional genomics of eukaryotes (7, 32) and has proved advantageous due to its capacity to yield high-quality proteins. Taken together, the system seems to be powerful when used for the production of malaria parasite proteins, as no glycosylation takes place during the standard reaction. However, to date, there is no Good Manufacturing Practice facility for production of recombinant proteins for clinical studies using the wheat germ cell-free system in the world. In the present study, we first tested the versatility of the wheat germ cell-free

system using as control models the leading vaccine candidate genes from *P. falciparum*. In addition, a series of experiments was conducted to prove the value of the system for the parallel expression of malaria proteins. The results presented here suggest that the wheat germ cell-free system may be useful as an additional protein production method in the field of *P. falciparum* research.

MATERIALS AND METHODS

Genomic cloning and construction of genes encoding fragments of Pfs25, PfCSP, and PfAMA1. The nucleotide sequences for the signal peptide and the GPI anchor were excluded from the expression constructs for genes encoding the PfCSP and Pfs25 proteins. The truncated versions of the PfCSP and Pfs25-3D7 genes were amplified by PCR from the genomic DNA of the *P. falciparum* 3D7 strain and subcloned into pEU3 (a vector carrying the C-terminal His₆ tag) (29) at the EcoRV site. The gene encoding Pfs25-TBV was a generous gift from Anthony W. Stowers (NIAID, NIH, Rockville, MD) (35). Pfs25-TBV, a synthetic version of the Pfs25 gene, was codon optimized for expression in the yeast *Saccharomyces cerevisiae*, and the replacement of Asn with Gln at three N-glycosylation sites was performed (20). DNA encoding full-length PfAMA1 protein was amplified from the genomic DNA of *P. falciparum* 3D7 and cloned into pEU-E01-GST (a vector with an N-terminal GST tag followed by a tobacco etch virus protease cleavage site) between the XhoI and BamHI sites. These pEU plasmid vectors are the expression vectors designed specifically for the wheat germ cell-free system (16). The inserted nucleotide sequences were confirmed using the ABI PRISM 310 Genetic Analyzer and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

Parallel construction of the DNA template from the parasite RNA. We selected 124 genes annotated as dominantly expressed in the blood stages of *P. falciparum* based on the microarray data integrated in the PlasmoDB database (<http://www.plasmodb.org>) (see Table S1 in the supplemental material). Extracted total RNA from *P. falciparum* 3D7 asexual blood-stage parasites was reverse transcribed into cDNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and PCR amplification was performed using *LA Taq* DNA polymerase (Takara Bio, Otsu, Japan). The 5' primers were designed as 46-mers: 16-mer nucleotide sequences (5'-CCACCCACCACCACCA) as the S1 tag sequence followed by a 30-mer of unique sequence covering each 5' open reading frame containing the start codon. For the 3' primers, 30-mer nucleotide sequences covering each unique sequence upstream from the termination codon were prepared. The PCR products were then cloned into the pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen), and their sequences at both ends were confirmed. Translation templates were prepared by *in vitro* transcription from PCR products amplified by the split-primer PCR method described earlier (29).

Production and purification of the Pfs25-3D7/WG, Pfs25-TBV/WG, PfCSP/WG, and PfAMA1/WG proteins. We employed the wheat germ cell-free protein expression system for protein production using the bilayer translation reaction method described previously (28). Briefly, 250 μ l of transcription mixture containing 25 μ g of the plasmid DNA, 80 mM HEPES-KOH, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM dithiothreitol, 2.5 mM each of nucleoside triphosphates, 250 U of SP6 RNA polymerase (Promega, Madison, WI), and 250 U of RNasin (Promega) was incubated for 6 h at 37°C. After the incubation, the transcription solution containing transcribed mRNA was mixed with 250 μ l of wheat germ extract (60 *A*₂₆₀ units) supplemented with 2 μ l of 20-mg/ml creatine kinase in a single well of a six-well plate. The 5.5-ml substrate mix (30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM dithiothreitol, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate) from the ENDEXT Wheat Germ Expression S kit (CFS Co., Ltd., Matsuyama, Japan) was then added on top of the translation mix and incubated at 26°C for 12 h. After incubation, the reaction mixture was centrifuged at 21,900 \times g for 20 min. Recovered supernatants were passed through Amicon Ultra centrifugal filter units (10-kDa molecular mass cutoff) (Millipore, Billerica, MA) to replace the translation buffer with phosphate-buffered saline. The samples containing the synthesized Pfs25-3D7/WG, Pfs25-TBV/WG, and PfCSP/WG proteins were purified using the Ni-nitrilotriacetic acid agarose column (Qiagen, Valencia, CA). The PfAMA1/WG protein was purified by passing the supernatant through the glutathione-Sepharose 4B column (GE Healthcare Bio-Sciences, Piscataway, NJ), followed by tobacco etch virus protease (Invitrogen) cleavage to remove the

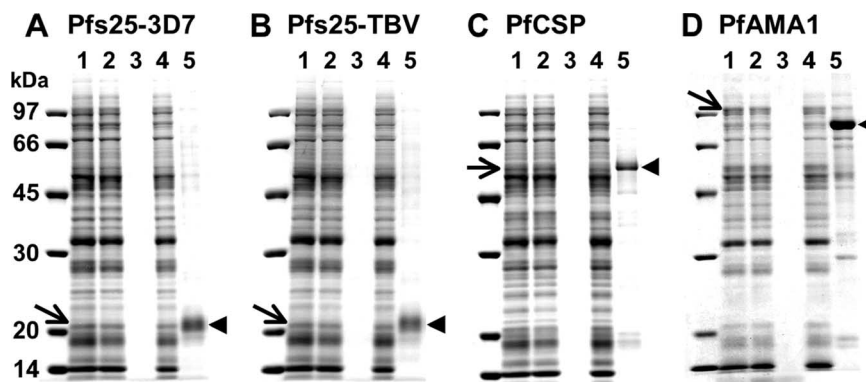


FIG. 1. SDS-PAGE analysis of the proteins expressed in the wheat germ cell-free system. Pfs25-3D7/WG (A), Pfs25-TBV/WG (B), PfcSP/WG (C), and PfAMA1/WG (D) were separated on SDS-12.5% polyacrylamide gels under reducing conditions and stained with Coomassie brilliant blue. The samples in each gel were as follows: total reaction mixture (lane 1), supernatant and precipitated fractions after brief centrifugation (lanes 2 and 3, respectively), and unbound and affinity-purified proteins (lanes 4 and 5, respectively). Products and purified proteins are indicated by arrows and arrowheads, respectively.

GST tag. Concentrations of affinity-purified proteins were determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (22), and the bands were visualized with Coomassie brilliant blue. Purified protein samples were stored in aliquots at -80°C until further use. For parallel protein synthesis from 124 malaria genes, the transcription and translation reactions were performed by a method similar to that described above. The 125- μl substrate mixture was overlaid on top of the 25- μl translation mixture containing transcribed mRNA in the presence of U- ^{14}C Leu (11.1 kBq; 15 GBq/mmol of Leu). The reaction was performed in 96-well plates. Proteins were separated by SDS-PAGE and identified by autoradiography using an imaging analyzer (BAS-2500; Fujifilm, Tokyo, Japan). The solubility of each product was expressed as the percentage of trichloroacetic acid-insoluble radioactivity (counted using a liquid scintillation counter [LSC-6100; Aloka, Mitaka, Japan]) in a supernatant fraction recovered from centrifugation at $21,900 \times g$ for 20 min compared to that of the total reaction mixture. The amount of target protein was estimated using the following formula where count is the radioactivity of the protein produced; Leu is the number of Leu residues in the protein, used to estimate the moles of Leu incorporated; MW is molecular weight; and ratio is the ratio of intensity of a specific protein band to the total intensity of bands on the autoradiogram: protein concentration = $\text{count/Leu} \times \text{MW} \times \text{ratio}$.

Preparation of antiserum. Groups of female BALB/c mice (five mice in each group) were subcutaneously immunized three times in the 1st, 3rd, and 5th weeks with 10 μg of affinity-purified proteins emulsified in Freund's adjuvant. As the control, a group of mice was administered GST in Freund's adjuvant, using the same protocol as described above. Antiserum preparation was as described elsewhere (2).

Preparation of *P. falciparum* asexual blood-stage parasites, ookinetes, and sporozoites. A mature schizont-rich fraction was obtained from cultured *P. falciparum* strain 3D7 (30). Parasite pellets were kept at -80°C until extract preparation.

To obtain ookinetes and sporozoites of *P. falciparum*, we used parasites derived from patient blood. The use of all human materials in this study was reviewed and approved by the Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects Research Review Board of the United States Army. Peripheral blood was collected with heparinized syringes under written informed consent from patients who came to the malaria clinics in the Mae Sod district, Thailand. Infection with *P. falciparum* was confirmed by the microscopic observation of Giemsa-stained thick and thin blood smears. The gametocytic patient blood was divided into two parts. One was used to grow zygotes/ookinetes in vitro for both Western blotting and immunocytochemical analyses, and the other half was subjected to propagation of sporozoites in mosquitoes for two further analyses, as described elsewhere (33). Western blot analysis and immunocytochemistry were performed as described previously (3, 17).

Transmission-blocking assays. We collected 20 ml of peripheral blood from a volunteer patient. Blood was divided into aliquots (300 μl /tube) and briefly centrifuged, and plasma was discarded. Mouse immune sera against both Pfs25-

3D7/WG and Pfs25-TBV/WG were serially diluted with heat-inactivated normal human serum prepared from malaria-naïve donors. Next, 180 μl of each diluted solution was added to the *P. falciparum*-infected blood cells and incubated for 15 min at room temperature. The mixture was placed in a membrane feeding apparatus kept at 37°C to allow *Anopheles dirus* A mosquitoes to feed on the blood in each apparatus for 30 min. Fully engorged mosquitoes were maintained for a week in the insectary. Oocysts that developed within the midgut were counted from 20 randomly selected mosquitoes. The Kruskal-Wallis test was applied to examine the differences in oocyst counts per mosquito between immunized groups and the control group fed on mouse serum raised against GST. Probability values (*P*) of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We were able to successfully express the Pfs25/WGs, PfcSP/WG, and PfAMA1/WG proteins using the wheat germ cell-free system. Expression of the Pfs25 (Pfs25-3D7/WG) protein from a gene with a native nucleotide composition was shown by subsequent SDS-PAGE analysis (Fig. 1A) to be comparable in amount to that of Pfs25-TBV/WG (Fig. 1B) expressed from the codon-optimized synthetic gene. On the SDS-polyacrylamide gels, two protein bands appeared at 20 kDa, the expected mobility of the Pfs25 truncated form, lacking the signal peptide and the GPI anchor. Almost all of the Pfs25-3D7/WG protein from the biased DNA was recovered in the supernatant fraction (Fig. 1A, lane 2) and was easily purified as a single dominant band along with other nonspecific faint bands by affinity chromatography (Fig. 1A, lane 5). The amount of purified Pfs25-3D7/WG was 35 μg per 6.0 ml of the reaction mixture, while that obtained from the codon-optimized gene was comparable, at 30 μg protein per reaction mixture. These results demonstrate that the wheat germ cell-free system that we employed produced equal amounts of proteins with and without prior optimization of their biased codon usage in the DNA. Similarly, the amounts of the other two proteins, PfcSP/WG (Fig. 1C), and PfAMA1/WG (Fig. 1D), produced from a gene with a native nucleotide composition were 26 and 102 μg per reaction, respectively.

Immunological characterization of the protein products. To determine the creation of conformation-dependent epitopes in Pfs25 and AMA1, we examined and confirmed the reactivity of

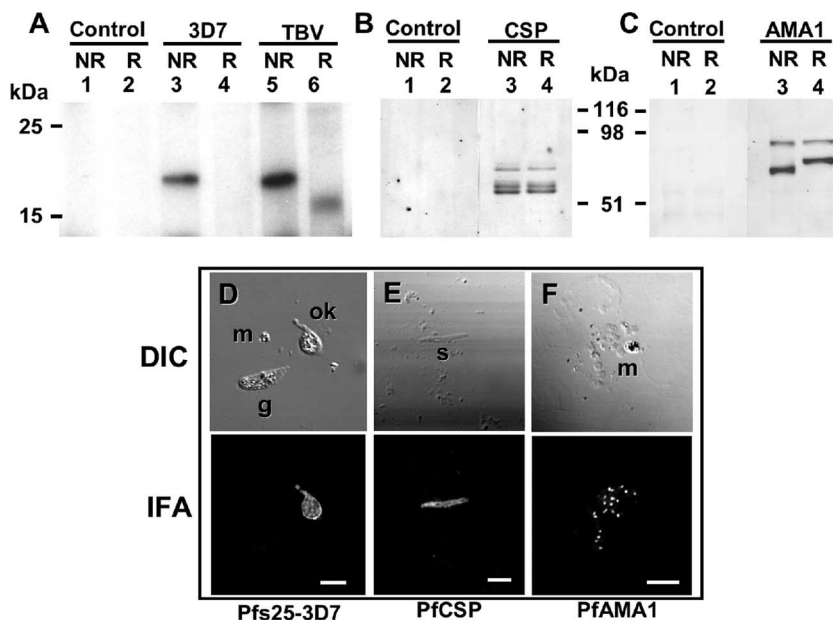


FIG. 2. Western blot and immunocytochemical analyses using antisera against Pfs25-3D7/WG, Pfs25-TBV/WG, PfCSP/WG, and PfAMA1/WG. Extracts prepared from *Plasmodium falciparum* zygotes/ookinetes (A), sporozoites (B), and schizonts (C) were separated on SDS-12.5% polyacrylamide gels under nonreducing (NR; lanes 1, 3, and 5) and reducing (R; lanes 2, 4, and 6) conditions. (A) Proteins on polyvinylidene fluoride membranes were immunostained either with mouse anti-Pfs25-3D7/WG serum (lanes 3 and 4) or mouse anti-Pfs25-TBV/WG serum (lanes 5 and 6) or with the negative-control serum (lanes 1 and 2). (B) The membrane was immunostained with either mouse anti-PfCSP/WG serum (lanes 3 and 4) or the control serum (lanes 1 and 2). (C) The membrane was immunostained with either mouse anti-PfAMA1/WG serum (lanes 3 and 4) or the control serum (lanes 1 and 2). (D to F) Samples prepared from *Plasmodium falciparum* immature ookinete (D), sporozoite (E), and schizont (F) were immunostained with the antiserum indicated at the bottom of the panel. Upper panels represent images obtained by differential interference contrast (DIC) microscopy, and lower panels represent immunostained images (immunofluorescence assay [IFA]) visualized with goat anti-mouse immunoglobulin G-fluorescein conjugate. These images have been taken by confocal scanning laser microscopy (LSM5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY). g, gametocyte; ok, immature ookinete; m, merozoite; s, sporozoite. Bars, 5 μ m.

anti-Pfs25 conformation-specific monoclonal antibody 4B7 (a generous gift from Carole A. Long [NIAID, NIH, Rockville, MD]) against Pfs25/WGs and the reactivity of anti-PfAMA1 3D7 conformation-specific monoclonal antibody 1E9 (a generous gift from Carole A. Long) against PfAMA1/WG by Western blotting under nonreducing conditions (data not shown). To evaluate the immunogenicity of each protein prepared, we then raised mouse antisera and determined their reactivity to the parasite-derived native proteins. Extract from approximately 5×10^5 zygotes/ookinetes per lane was separated by SDS-PAGE, and Western blot analysis was performed. Specific bands with the expected mobility of native Pfs25 protein were detected under nonreducing conditions using antisera against Pfs25-3D7/WG and Pfs25-TBV/WG. Anti-Pfs25-3D7/WG serum did not show any reactivity under reducing conditions (Fig. 2A). These results suggest that the Pfs25-3D7/WG protein prepared here retained a conformation similar to that of the native protein. The identity of the faint band detected at the lower position with anti-Pfs25-TBV/WG under reducing conditions is unclear at present (Fig. 2A). Similar experiments were performed using anti-PfCSP/WG and anti-PfAMA1/WG sera to study extracts from respective stages of the parasite. The analyses clearly showed specific reactivity of each antiserum to PfCSP and PfAMA1 proteins (Fig. 2B and C). Anti-PfCSP serum reacted to three protein bands in the sporozoite extract under both reducing and nonreducing

conditions (Fig. 2B). The upper and lower bands appeared to correspond to precursor and mature forms, respectively, as reported earlier by Coppi et al. (4). Anti-PfAMA1 serum gave two signals, with the upper and lower bands corresponding to mature and processed forms, respectively (15). The signal shift of the two bands upon introduction of a reducing reagent was most likely due to the high content of disulfide bonds within the protein (14). These results are consistent with previously reported findings (13).

Immunocytochemical staining was performed against immature ookinetes obtained in vitro short-term culture using anti-Pfs25-3D7/WG. As shown in Fig. 2D (differential interference contrast and immunofluorescence assay), the antiserum specifically stained the surface of the immature ookinete but not the gametocyte and the merozoite. Antiserum against Pfs25-TBV/WG yielded similar results (data not shown). These findings were consistent with our previous report in which Pfs25-TBV prepared from yeast cells was used to raise antiserum (2). These findings also verified that Pfs25 prepared using our protocols from a gene with an A/T-rich native nucleotide composition can yield a protein of sufficient quality to raise a specific antibody. Experiments using anti-PfCSP/WG and anti-PfAMA1/WG on the target stages of the parasite showed typical staining patterns. The entire surface of the slender sporozoite was stained by anti-PfCSP/WG serum (Fig. 2E), and the anti-PfAMA1/WG serum clearly visualized punc-

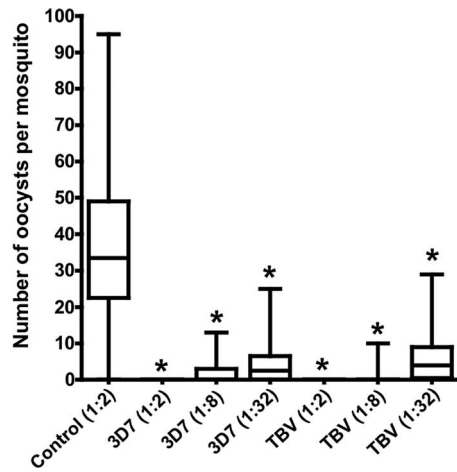


FIG. 3. Transmission-blocking efficacy of antibodies against *Plasmodium falciparum* parasites. The median numbers of oocysts per mosquito ($n = 20$) with quartiles (box plot) and ranges (lines on both top and bottom of the box) were compared among groups of mosquitoes fed on either anti-Pfs25 sera serially diluted or control mouse serum. Dilution of test immune serum is shown as 1:2 to 1:32. Statistical analysis was performed using the Kruskal-Wallis test for comparison of oocyst numbers between the test immune sera and control serum. Asterisks indicate statistically significant differences compared to the control group ($P < 0.05$).

tate localization of PfAMA1 at the apical end of merozoites (Fig. 2F).

Efficacy evaluation of the proteins as vaccine antigens. In view of a practical application of the system for discovery of malaria vaccine candidates, we evaluated the quality of antigens produced by performing a parasite growth inhibition assay using the antibodies raised against those antigens. We focused on Pfs25-3D7/WG and Pfs25-TBV/WG. Pfs25-TBV is currently the sole transmission-blocking vaccine candidate under clinical trial (23). A transmission-blocking assay was performed using both anti-Pf25-3D7/WG and anti-Pfs25-TBV/WG. A mixture containing *P. falciparum*-gametocyte infected erythrocytes and one of the antisera was fed to mosquitoes. The number of developed oocysts in the mosquitoes was then later counted. Both antisera at twofold dilution completely inhibited oocyst development, as we have seen no mosquito harboring oocysts (Fig. 3). The number of oocysts was inversely proportional to the concentration of antiserum added, findings consistent with previous experiments, in which Pfs25-TBV prepared from yeast was used to raise antiserum (2). It is important at this moment to stress the difference between this study and other studies: our proteins were produced from a non-codon-optimized gene in a cell-free system, while in other studies a codon-optimized engineered Pfs25-TBV gene was transformed into yeast cells (20). The results presented here strongly indicate the value of the wheat germ cell-free system for the production of malaria proteins that require complicated procedures in other systems.

Parallel syntheses of *P. falciparum* proteins. Although cell-based expression systems have been widely used in this field, they are limited mainly in their ability for efficient production of *P. falciparum* protein, primarily because of the complexity of

the genome. In order to evaluate the capability of our cell-free system for parallel expression from the parasite genes, we selected 124 genes (see Table S1 in the supplemental material) encoding asexual blood-stage parasite proteins, based on the PlasmoDB database. Autoradiography demonstrated that 93 of the 124 genes yielded protein products. The average yield of expressed protein estimated for each full-size product was 1.9 μg per 150 μl of reaction mixture, an amount sufficient for preliminary antigen discovery studies using hyperimmune serum. Average protein solubility was 65% (see Table S1 in the supplemental material). There was significant inverse correlation between yield and molecular size of the protein; the greater the size, the lower the protein yield. There was also weak but significant inverse correlation between the protein yield and the relative frequency of low-complexity regions. In addition, solubility was inversely correlated with the pI value (Table 1). These observations have already been documented in earlier studies (24, 31). Surprisingly, we did not see any correlation between yield and A/T content, pI value, or the existence of a transmembrane domain (data not shown). We then analyzed the statistical difference in molecular weights, pI values, A/T contents, and relative frequencies of low-complexity regions between the expressed and nonexpressed groups of molecules, using the Mann-Whitney U test. The molecular weights in the nonexpressed group were significantly higher than those of the expressed group ($P < 0.0001$). In contrast, pI values, A/T contents, and the relative frequencies of low-complexity regions did not differ significantly (see Table S1 in the supplemental material). We currently have no explanation for why 25% of the tested genes failed to produce proteins in our system. One possible explanation is the sequence errors most likely present in the PCR products that were used as templates for transcription and subsequent translation. Such templates would cause mistranslation of the protein by frameshift.

In summary, the ability of the wheat germ cell-free protein synthesis system to produce *P. falciparum* proteins was examined. We found that (i) without the need for codon optimization, the cell-free system is able to produce a sufficient amount of high-quality proteins of the leading malaria vaccine candidates, Pfs25, PfCSP, and PfAMA1; (ii) biochemical, immunocytochemical, and biological analyses demonstrated that the prepared proteins could be directly used for immunization after a simple affinity purification step; and (iii) the system proved suitable for use as a parallel

TABLE 1. Correlation of expression or solubility and characteristics^a

Parameter	Correlation coefficient (P value)	
	Protein concn	% Solubility
Mol wt	-0.3177 (0.0019) ^b	-0.1221 (0.2436)
pI	-0.1214 (0.2464)	-0.3519 (0.0005) ^b
% A/T	-0.1505 (0.1498)	
Low complexity ^c	-0.2276 (0.0283) ^b	
% Solubility	-0.0494 (0.6385)	

^a Spearman's correlation coefficients by rank were calculated among the 93 proteins expressed. The probability values of the statistical significance are shown in parentheses.

^b $P < 0.05$ was considered to indicate a statistically significant correlation.

^c Relative frequency of low-complexity regions per molecular weight.

way to produce parasite proteins. We believe that the wheat germ cell-free protein synthesis system may be a key tool for decoding genetic information above and beyond malaria vaccine research.

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REFERENCES

1. Aguiar, J. C., J. LaBaer, P. L. Blair, V. Y. Shamailova, M. Koundinya, J. A. Russell, F. Huang, W. Mar, R. M. Anthony, A. Witney, S. R. Caruana, L. Brizuela, J. B. Sacci, Jr., S. L. Hoffman, and D. J. Carucci. 2004. High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. *Genome Res.* **14**:2076–2082.
2. Arakawa, T., A. Komesu, H. Otsuki, J. Sattabongkot, R. Udomsangpetch, Y. Matsumoto, N. Tsuji, Y. Wu, M. Torii, and T. Tsuboi. 2005. Nasal immunization with a malaria transmission-blocking vaccine candidate, Pfs25, induces complete protective immunity in mice against field isolates of *Plasmodium falciparum*. *Infect. Immun.* **73**:7375–7380.
3. Arakawa, T., T. Tsuboi, A. Kishimoto, J. Sattabongkot, N. Suwanabun, T. Rungruang, Y. Matsumoto, N. Tsuji, H. Hisaeda, A. Stowers, I. Shimabukuro, Y. Sato, and M. Torii. 2003. Serum antibodies induced by intranasal immunization of mice with *Plasmodium vivax* Pvs25 co-administered with cholera toxin completely block parasite transmission to mosquitoes. *Vaccine* **21**:3143–3148.
4. Coppi, A., C. Pinzon-Ortiz, C. Hutter, and P. Sinnis. 2005. The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. *J. Exp. Med.* **201**:27–33.
5. Dame, J. B., J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, et al. 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science* **225**:593–599.
6. Dutta, S., P. V. Lalitha, L. A. Ware, A. Barbosa, J. K. Moch, M. A. Vassell, B. B. Fileta, S. Kitov, N. Kolodny, D. G. Heppner, J. D. Haynes, and D. E. Lanar. 2002. Purification, characterization, and immunogenicity of the refolded ectodomain of the *Plasmodium falciparum* apical membrane antigen 1 expressed in *Escherichia coli*. *Infect. Immun.* **70**:3101–3110.
7. Endo, Y., and T. Sawasaki. 2006. Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotechnol.* **17**:373–380.
8. Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, S. Bowman, I. T. Paulsen, K. James, J. A. Eisen, K. Rutherford, S. L. Salzberg, A. Craig, S. Kyes, M. S. Chan, V. Nene, S. J. Shallom, B. Suh, J. Peterson, S. Angiuoli, M. Pertea, J. Allen, J. Selengut, D. Haft, M. W. Mather, A. B. Vaidya, D. M. Martin, A. H. Fairlamb, M. J. Fraunholz, D. S. Roos, S. A. Ralph, G. I. McFadden, L. M. Cummings, G. M. Subramanian, C. Mungall, J. C. Venter, D. J. Carucci, S. L. Hoffman, C. Newbold, R. W. Davis, C. M. Fraser, and B. Barrell. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**:498–511.
9. Gowda, D. C., and E. A. Davidson. 1999. Protein glycosylation in the malaria parasite. *Parasitol. Today* **15**:147–152.
10. Greenwood, B., and T. Mutabingwa. 2002. Malaria in 2002. *Nature* **415**:670–672.
11. Gupta, A., T. Bai, V. Murphy, P. Strike, R. F. Anders, and A. H. Batchelor. 2005. Refolding, purification, and crystallization of apical membrane antigen 1 from *Plasmodium falciparum*. *Protein Expr. Purif.* **41**:186–198.
12. Heppner, D. G., Jr., K. E. Kester, C. F. Ockenhouse, N. Tornieporth, O. Ofori, J. A. Lyon, V. A. Stewart, P. Dubois, D. E. Lanar, U. Krzych, P. Moris, E. Angov, J. F. Cummings, A. Leach, B. T. Hall, S. Dutta, R. Schwenk, C. Hillier, A. Barbosa, L. A. Ware, L. Nair, C. A. Darko, M. R. Withers, B. Ogutu, M. E. Polhemus, M. Fukuda, S. Pichyangkul, M. Gettyacamin, C. Diggs, L. Soisson, J. Milman, M. C. Dubois, N. Garcon, K. Tucker, J. Wittes, C. V. Plowe, M. A. Thera, O. K. Duombo, M. G. Pau, J. Goudsmit, W. R. Ballou, and J. Cohen. 2005. Towards an RTS, S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. *Vaccine* **23**:2243–2250.
13. Hodder, A. N., P. E. Crewther, and R. F. Anders. 2001. Specificity of the protective antibody response to apical membrane antigen 1. *Infect. Immun.* **69**:3286–3294.
14. Hodder, A. N., P. E. Crewther, M. L. Matthew, G. E. Reid, R. L. Moritz, R. J. Simpson, and R. F. Anders. 1996. The disulfide bond structure of *Plasmodium* apical membrane antigen-1. *J. Biol. Chem.* **271**:29446–29452.
15. Howell, S. A., C. Withers-Martinez, C. H. Kocken, A. W. Thomas, and M. J. Blackman. 2001. Proteolytic processing and primary structure of *Plasmodium falciparum* apical membrane antigen-1. *J. Biol. Chem.* **276**:31311–31320.
16. Kamura, N., T. Sawasaki, Y. Kasahara, K. Takai, and Y. Endo. 2005. Selection of 5'-untranslated sequences that enhance initiation of translation in a cell-free protein synthesis system from wheat embryos. *Bioorg. Med. Chem. Lett.* **15**:5402–5406.
17. Kaneko, O., B. Y. Y. Lim, H. Iriko, I. T. Ling, H. Otsuki, M. Grainger, T. Tsuboi, J. H. Adams, D. Mattei, A. A. Holder, and M. Torii. 2005. Apical expression of three RhopH1/Clag proteins as components of the *Plasmodium falciparum* RhopH complex. *Mol. Biochem. Parasitol.* **143**:20–28.
18. Kaslow, D. C., I. C. Bathurst, T. Lensen, T. Ponnudurai, P. J. Barr, and D. B. Keister. 1994. *Saccharomyces cerevisiae* recombinant Pfs25 adsorbed to alum elicits antibodies that block transmission of *Plasmodium falciparum*. *Infect. Immun.* **62**:5576–5580.
19. Kaslow, D. C., I. A. Quakyi, C. Syin, M. G. Raum, D. B. Keister, J. E. Coligan, T. F. McCutchan, and L. H. Miller. 1988. A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. *Nature* **333**:74–76.
20. Kaslow, D. C., and J. Shiloach. 1994. Production, purification and immunogenicity of a malaria transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified using nickel-NTA agarose. *Biotechnology (New York)* **12**:494–499.
21. Kedees, M. H., N. Azzouz, P. Gerold, H. Shams-Eldin, J. Iqbal, V. Eckert, and R. T. Schwarz. 2002. *Plasmodium falciparum*: glycosylation status of *Plasmodium falciparum* circumsporozoite protein expressed in the baculovirus system. *Exp. Parasitol.* **101**:64–68.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
23. Malkin, E., F. Dubovsky, and M. Moree. 2006. Progress towards the development of malaria vaccines. *Trends Parasitol.* **22**:292–295.
24. Mehlin, C., E. Boni, F. S. Buckner, L. Engel, T. Feist, M. H. Gelb, L. Haji, D. Kim, C. Liu, N. Mueller, P. J. Myler, J. T. Reddy, J. N. Sampson, E. Subramanian, W. C. Van Voorhis, E. Worthey, F. Zucker, and W. G. Hol. 2006. Heterologous expression of proteins from *Plasmodium falciparum*: results from 1000 genes. *Mol. Biochem. Parasitol.* **148**:144–160.
25. Richie, T. L., and A. Saul. 2002. Progress and challenges for malaria vaccines. *Nature* **415**:694–701.
26. Samuelson, J., S. Banerjee, P. Magnelli, J. Cui, D. J. Kelleher, R. Gilmore, and P. W. Robbins. 2005. The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. *Proc. Natl. Acad. Sci. USA* **102**:1548–1553.
27. Sawasaki, T., and Y. Endo. 2008. The wheat germ cell-free protein synthesis system, p. 111–139. *In* A. S. Spirin and J. R. Swartz (ed.), *Cell-free protein synthesis, methods and protocols*. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim, Germany.
28. Sawasaki, T., Y. Hasegawa, M. Tsuchimochi, N. Kamura, T. Ogasawara, T. Kuroita, and Y. Endo. 2002. A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett.* **514**:102–105.
29. Sawasaki, T., T. Ogasawara, R. Morishita, and Y. Endo. 2002. A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA* **99**:14652–14657.
30. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science* **193**:673–675.
31. Vedadi, M., J. Lew, J. Artz, M. Amani, Y. Zhao, A. Dong, G. A. Wasney, M. Gao, T. Hills, S. Brox, W. Qiu, S. Sharma, A. Diassiti, Z. Alam, M. Melone, A. Mulichak, A. Wernimont, J. Bray, P. Loppnau, O. Plotnikova, K. Newberry, E. Sundararajan, S. Houston, J. Walker, W. Tempel, A. Bochkarev, I. Kozieradzki, A. Edwards, C. Arrowsmith, D. Roos, K. Kain, and R. Hui. 2007. Genome-scale protein expression and structural biology of *Plasmodium falciparum* and related Apicomplexan organisms. *Mol. Biochem. Parasitol.* **151**:100–110.
32. Vinarov, D. A., B. L. Lytle, F. C. Peterson, E. M. Tyler, B. F. Volkman, and J. L. Markley. 2004. Cell-free protein production and labeling protocol for NMR-based structural proteomics. *Nat. Methods* **1**:149–153.

33. **Wirtz, R. A., J. Sattabongkot, T. Hall, T. R. Burkot, and R. Rosenberg.** 1992. Development and evaluation of an enzyme-linked immunosorbent assay for *Plasmodium vivax*-VK247 sporozoites. *J. Med. Entomol.* **29**:854–857.
34. **Young, J. F., W. T. Hockmeyer, M. Gross, W. R. Ballou, R. A. Wirtz, J. H. Trospen, R. L. Beaudoin, M. R. Hollingdale, L. H. Miller, C. L. Diggs, and M. Rosenberg.** 1985. Expression of *Plasmodium falciparum* circumsporozoite proteins in *Escherichia coli* for potential use in a human malaria vaccine. *Science* **228**:958–962.
35. **Zou, L., A. P. Miles, J. Wang, and A. W. Stowers.** 2003. Expression of malaria transmission-blocking vaccine antigen Pfs25 in *Pichia pastoris* for use in human clinical trials. *Vaccine* **21**:1650–1657.

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