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Identification and expression in *Escherichia coli* of merozoite stage-specific genes of the human malarial parasite *Plasmodium falciparum*

(developmental regulation/cDNA cloning/differential hybridization/blood stage antigens/malaria vaccine)

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ABSTRACT **The key steps in the development of a malaria vaccine through gene cloning are the identification of the proteins involved in host protective immunity and the cloning, identification, and expression of the genes coding for these proteins. Recent data have indicated that certain proteins synthesized at the late schizont-merozoite stage of *Plasmodium falciparum* play a major role in malaria immunity. This paper reports the identification, in a cDNA library, of recombinant clones corresponding to genes expressed specifically during the late schizont-merozoite stage of *P. falciparum* development. The 132 cDNA clones thus identified out of 10,000 were found to correspond to only 12 different genes, probably representing most of the major schizont-merozoite specific genes. The stage-specific cDNAs can be efficiently expressed in *Escherichia coli* cells. The protein products of some of these clones are recognized by monoclonal antibodies specific for late schizont-merozoite proteins. We conclude that only a small set of genes is specifically induced in the schizont-merozoite stage and that the stage-specific cDNA clones we have isolated are very likely to include the genes coding for the immunologically relevant proteins of *P. falciparum*.**

Malaria remains a major cause of morbidity and mortality in many parts of the world (1). Increased resistance of the insect vectors (mosquitos) to insecticides and of the malarial parasites to chemotherapeutic agents has made the long-standing goal of a vaccine against malaria more urgent than ever.

Immunity in a vertebrate host can operate against two distinct developmental stages of the parasite: the sporozoite form, which is introduced by the bite of the mosquito, and the asexual blood stages, which are largely responsible for the clinical symptoms of malaria (2). The latter involves the maturation through a number of steps called ring, trophozoite, and schizont. At the end of the asexual cycle, mature schizonts rupture the erythrocytes and merozoites are released into the circulation. Antigens of the asexual blood stage, exposed on the surface of schizonts and merozoites and expressed late in the asexual cycle, play a major role in protective immunity against malaria (2–11). A major step in the study of *Plasmodium falciparum* biology has been the development of procedures for the *in vitro* culture of the human parasite *P. falciparum* in infected human erythrocytes (12).

Cloning the genes for parasite antigens and expressing them in bacteria represents a strategy for the preparation of *P. falciparum* proteins to be used as a vaccine. Progress has been made in the cloning of genes expressed in both the sporozoite (13) and the asexual blood stages (14–16). One of the major difficulties remains the identification of recombinant DNA clones corresponding to the schizont-merozoite specific polypeptides that are involved in immunity against malaria. We have studied this problem with an approach based on the developmental regulation of stage-specific genes at the transcriptional level (17) and have identified cDNA clones corresponding to a small set of genes expressed specifically at the schizont-merozoite stage of *P. falciparum* development, which represent a small fraction of a cDNA library.

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MATERIAL AND METHODS

Preparation of Ring and Schizont-Merozoite Forms of *P. falciparum*. Enrichment for these two developmental forms of *P. falciparum* from *in vitro* cultures was performed by using a modification of a procedure previously described (18). Parasitized erythrocytes from the *P. falciparum* isolate SGE2 originating from Zaire were cultivated in 10-cm (diameter) Falcon Petri dishes at a hematocrit of 6% using 11 ml of culture medium (12, 18). The cells from 25 Petri dishes at 22% parasitemia were synchronized by two treatments of 5% mannitol at 7-hr intervals. Twenty-two hr later, these cultures had a parasitemia of 9% and contained >80% schizonts. The schizonts were purified by flotation on Physiogel and after washing in culture medium were mixed at a ratio of 5:1 with uninfected erythrocytes. The re-invasion period was limited to 6 hr, after which the remaining mature forms were lysed by mannitol treatment. The cells were then dispersed into 10 Petri dishes. Cells from 5 Petri dishes were collected after an additional culture period of 6 hr (ring form preparation) and the cells from the 5 remaining plates were collected after an additional culture period of 33 hr (mature segmented schizonts). These cultures had a parasitemia of 12% and contained >97% ring forms and >95% segmented schizonts, respectively.

Biosynthetic Labeling of *P. falciparum* and Electrophoretic Analysis. Synchronized cultures were sequentially labeled *in vitro* at various intervals with 5-hr pulses of [^{35}S]methionine (50 μCi/ml; 1 Ci = 37 GBq) in methionine-free minimal essential medium supplemented with 10% normal human serum.

Following lysis with 1% Nonidet P-40 and centrifugation, aliquots containing 30,000 trichloroacetic acid-precipitable cpm were analyzed on NaDodSO$_4$/8% acrylamide gels under reducing conditions (19). The different extracts (Fig. 1) represent proteins synthesized in rings, trophozoites, early schizonts, and mature segmented schizonts, the latter consisting of erythrocytes filled with merozoites.

Preparation of *P. falciparum* RNA. Human erythrocytes were infected with *P. falciparum* and asynchronous cultures were grown to a parasitemia of 15%. Total RNA was prepared by homogenization of infected cells in a solution containing 6 M guanidine isothiocyanate, 0.1 M 2-mercapto-

ethanol, 25 mM sodium citrate (pH 7.0), and 0.5% *N*-lauroylsarcosine, followed by centrifugation of the RNA through 5.7 M cesium chloride (20). Poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose and used for the construction of a cDNA library.

RNA was isolated from the enriched preparations of rings and schizont-merozoites (see above) by the same procedure and used as total RNA [without oligo(dT)-cellulose chromatography] for the synthesis of stage-specific [^{32}P]cDNA probes.

Construction of a *P. falciparum* cDNA Library. Complementary DNA was synthesized, tailed with oligo(dC), and cloned in plasmid pBR322, which had been linearized with *Pst* I and tailed with oligo(dG). All procedures were as described elsewhere (21). Following transformation of *Escherichia coli* HB 101 cells, a library of ≈10,000 cDNA colonies was prepared and replicated on nitrocellulose filters (22). Another cDNA library (15,000 clones) was constructed in the expression vector pPL31A (kindly provided by H. Küpper), in which the cDNA insert is under the control of an inducible PL promoter.

Analysis of Recombinant Clones by Differential Hybridization. The cDNA library constructed in plasmid pBR322 was screened by colony-hybridization on nitrocellulose filters (23) with single-stranded [^{32}P]cDNA probes made from either total RNA from the ring stage or total RNA from the mature schizont-merozoite stage (see above). The filters were prehybridized for 2 hr at 65°C in 0.3 M NaCl/0.03 M sodium citrate, pH 7/1× concentrated Denhardt's solution with 50 μg of denatured salmon sperm DNA per ml and 1 μg of (poly)A per ml, and hybridization was performed for 16 hr. Filters were washed for 30 min five times in 0.3 M NaCl/0.03 M sodium citrate/ 0.1% $NaDodSO_4$ at 65°C and autoradiographed for ≈16 hr.

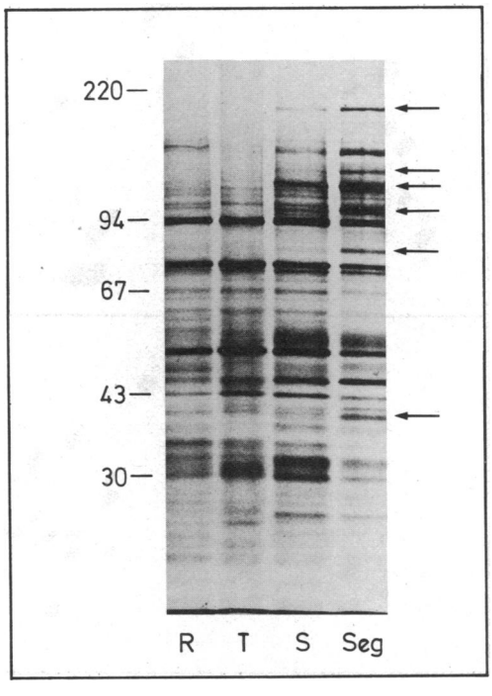

FIG. 1. Autoradiography of $NaDodSO_4$/PAGE of [^{35}S]methionine-labeled proteins from the asexual erythrocytic stages of *P. falciparum*. Proteins of *P. falciparum* were pulse-labeled *in vitro* with [^{35}S]methionine (5 hr): rings (R), trophozoites (T), early schizonts (S), and mature segmented schizonts (Seg), which contain merozoites. The arrows (top to bottom) indicate major schizont-merozoite specific polypeptide bands with apparent M_rs of 200,000, 160,000, 140,000, 105,000, 82,000, and 41,000. Reference molecular weights are indicated as $M_r \times 10^{-3}$.

RESULTS

Proteins Synthesized Specifically in Mature Schizonts. When synchronized cultures of *P. falciparum* are grown in human erythrocytes *in vitro* (12), the different stages of the asexual blood cycle, rings, trophozoites, and finally mature schizonts (corresponding to erythrocytes filled with merozoites) can be successively observed. When such cultures are pulse-labeled with [^{35}S]methionine at different times and the labeled *P. falciparum* proteins are analyzed on $NaDodSO_4$/polyacrylamide gels, it can be seen that the majority of the proteins synthesized are common to the different developmental stages (Fig. 1). However, as we and others have reported earlier (2, 3, 5, 24, 25), a small number of proteins are specifically synthesized late in the asexual cycle, in mature or segmented schizonts that contain merozoites (see arrows in Fig. 1). The relative intensity of the bands on autoradiography and Coomassie-stained gels (8) indicates that these proteins are major constituents of late schizonts and merozoites. Several groups have reported similar findings, although there are minor differences in the molecular weights assigned to these polypeptides (2–5, 10, 24–26). The possibility exists that some of the merozoite-specific bands in Fig. 1 represent processing or breakdown products of larger proteins (25, 26).

Identification of Stage-Specific cDNA Clones by Differential Hybridization. Poly(A)$^+$ RNA prepared from unsynchronized infections was shown to be active in cell-free protein synthesis (unpublished observations). This RNA was used to synthesize cDNA and to construct a cDNA library in the plasmid pBR322. In parallel, RNA was prepared from preparations enriched for the ring stage and for mature schizont-

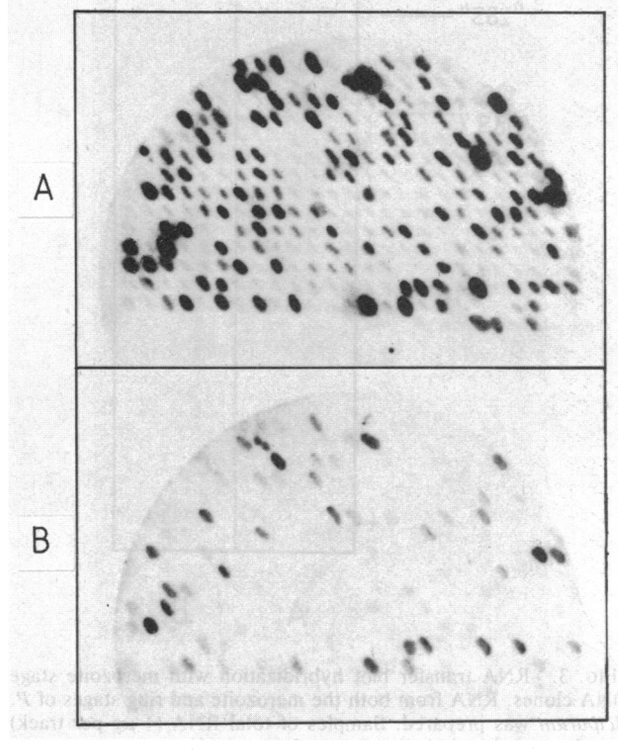

FIG. 2. Identification of schizont-merozoite stage-specific cDNA clones. A cDNA library prepared from *P. falciparum*-infected erythrocytes in plasmid pBR322 was screened by colony-hybridization with [^{32}P]cDNA probes specific for the ring and the schizont-merozoite stages. About 10,000 individual clones were examined on duplicate filters (not shown) and those showing a stronger signal with the merozoite stage probe were picked onto a gridded filter that was replicated (see text). These selected cDNA clones were hybridized with the same probes as before. (*A*) Schizont-merozoite stage probe; (*B*) ring stage probe.

merozoites, from which [^{32}P]cDNA probes were synthesized and used to screen the cDNA library by colony-hybridization (23). Although most colonies hybridized with both cDNA probes, about 150 colonies out of about 10,000 tested hybridized preferentially or exclusively with the schizont-merozoite cDNA probe. This was confirmed by a second round of differential hybridization with the same cDNA probes (Fig. 2).

To confirm that the selected colonies corresponded to genes that are activated in a stage-specific manner in the course of *P. falciparum* development, RNA blots from both the ring and late schizont-merozoite stages were hybridized with plasmid [^{32}P]DNA prepared from some of the selected clones. Fig. 3 shows the result of two such hybridizations demonstrating mRNAs of different size and abundance. These RNA transfer blots suggest that activation of new genes at the transcriptional level takes place during parasite development.

Only a Small Set of Genes Is Turned on Specifically at the Schizont-Merozoite Stage. To find out how many different genes were represented in the selected clones, we used the inserts of some clones as probes to identify identical sequences in the other clones by hybridization. These "relatedness" experiments allowed the grouping of most of the schizont-merozoite specific clones into families of various sizes. Two such families of cross-hybridizing clones are shown in Fig. 4, with 19 and 32 members, respectively. This analysis (Table 1) allowed us to assign 132 stage-specific clones to 12 nonoverlapping families corresponding to 12 schizont-merozoite specific genes. On the basis of the number of clones in each family (Table 1), it can be concluded that these 12 families probably include the genes coding for the abundant and moderately abundant proteins expressed specifically at the schizont-merozoite stage shown in Fig. 1.

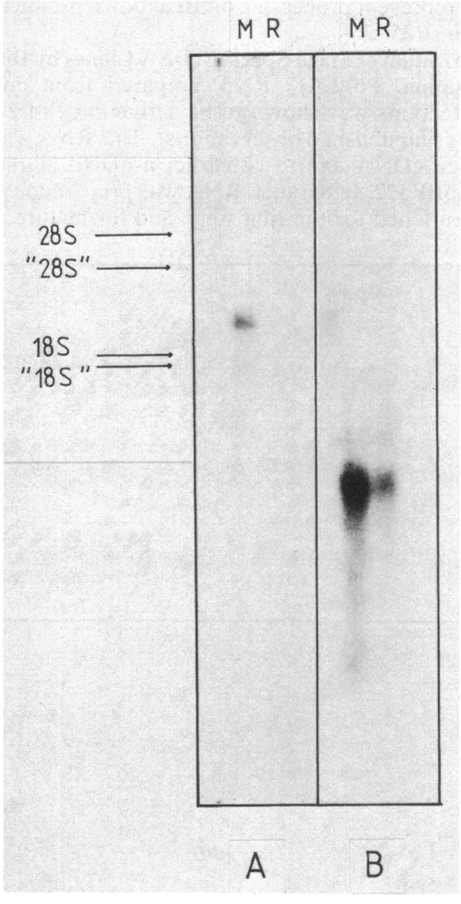

FIG. 3. RNA transfer blot hybridization with merozoite stage cDNA clones. RNA from both the merozoite and ring stages of *P. falciparum* was prepared. Samples of total RNA (4 μg per track) were denatured and electrophoresed on a 0.8% agarose gel as described by McMaster and Carmichael (27). Ethidium bromide staining showed that there were equal amounts of RNA in both the merozoite (M) and ring (R) tracks. The RNA was transferred to diazobenzyloxymethyl-paper, hybridized, and washed according to Wahl *et al.* (28). Plasmid DNAs of individual merozoite specific cDNA clones were nick-translated (29) and used in the hybridizations at a concentration of 5×10^5 cpm/ml. (*A*) cDNA clone D4; (*B*) cDNA clone X11. Arrows indicate the positions of 28S and 18S human liver ribosomal RNAs and "28S" and "18S" *P. falciparum* ribosomal RNAs.

Expression of Schizont-Merozoite Specific Genes in Bacteria. A second cDNA library made in the inducible expression vector pPL31A was screened with inserts from the 12 schizont-merozoite stage-specific genes. Clones corresponding to each of these genes were obtained and, as shown in Fig. 5, certain clones produce, upon induction, large amounts of an additional polypeptide. In certain cases, the cDNA-coded fusion protein represents >5% of the total bacterial proteins. The protein product of some stage-specific cDNA clones was found to react with monoclonal antibodies specific for the M_r 200,000 and the M_r 41,000 schizont-merozoite polypeptides by using ELISAs and electrophoretic transfer blot assays (unpublished data).

DISCUSSION

The data presented here imply that during the developmental cycle of *P. falciparum*, the maturation of the parasite from the ring stage to the merozoite stage is associated with the selective activation of a relatively small set of genes. These genes exhibit various degrees of differential activation. The

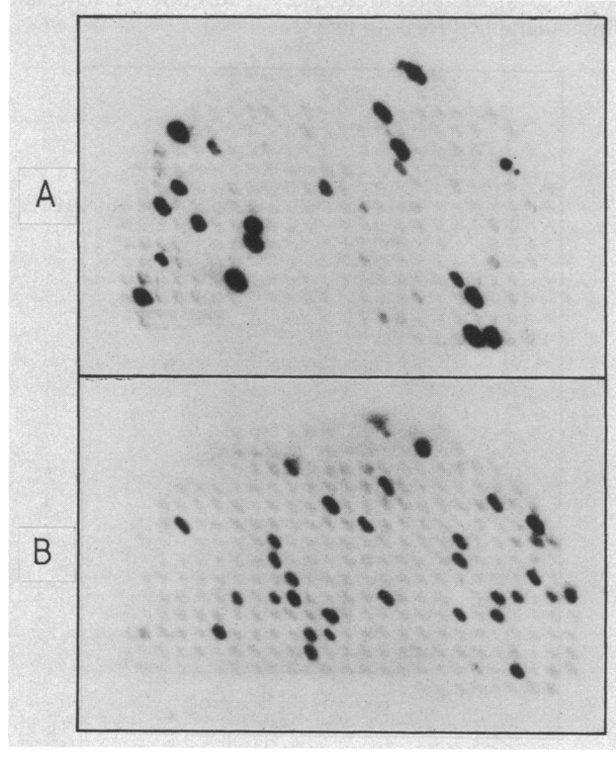

FIG. 4. Relatedness of schizont-merozoite stage-specific cDNA clones. Replicas of the selected schizont-merozoite cDNA clones shown in Fig. 2 were prepared on nitrocellulose filters (22). Inserts of some of these stage-specific recombinant plasmids were cut out with *Pst* I, purified on a 1% agarose gel, eluted with DEAE-cellulose paper (30), and nick-translated (29). Hybridization conditions are described in the text. (*A*) Insert from clone D4; (*B*) insert from clone V14. Identical results were obtained under high stringency conditions (last washing in 15 mM NaCl/1.5 mM sodium citrate at 65°C).

Table 1. Cross-hybridization of schizont-merozoite specific cDNA clones

cDNA clone (used as probe)	Cross-hybridizing clones, no.
V14	31
G2	23
R14	20
D4	19
X11	18
M2	7
M7	3
N2	3
V4	3
D8	2
I4	2
E8	1

Merozoite specific clones as described in the legend to Fig. 2 were hybridized with *Pst* I inserts from individual stage-specific recombinant plasmids clones (see examples in Fig. 4). The number of clones that cross-hybridize with each insert probe is shown.

identification of cDNA clones corresponding to these stage-specific clones represents a considerable enrichment compared to unselected cDNA libraries.

Parasite-specific components are exposed to immune effector mechanisms of the host at the schizont and at the merozoite stages. Immunization experiments have been conducted in monkeys susceptible to *P. falciparum* using whole schizonts or merozoites (or both) as immunogens (32–34). Recent data indicate that certain specific antigens of the schizont and merozoite form of the asexual blood stage play a major role in protective immunity against malaria. Immunoglobulins from immune donors recognize schizont-merozoite specific protein antigens (2–4). Studies *in vitro* with immune serum and monoclonal antibodies reacting with schizont-merozoite specific antigens have demonstrated an inhibitory activity on parasite growth (3, 4, 6, 7). Finally, it has been shown recently that monkeys immunized with defined schizont-merozoite specific polypeptides of *P. falciparum*, the most lethal species to man, were protected from a lethal intravenous challenge infection with parasites (8, 9). These protective polypeptides, of M_rs 200,000, 140,000, 82,000, and 41,000, are the same as those recognized by monoclonal antibodies that have demonstrated an inhibition of the infectivity and the growth of *P. falciparum in vitro* (6, 7). The variety of protective polypeptides present in mature schizonts and merozoites indicates that there is not just a single immunodominant protective antigen in the asexual blood form of the parasite, in contrast to the single surface antigen found in sporozoites (13).

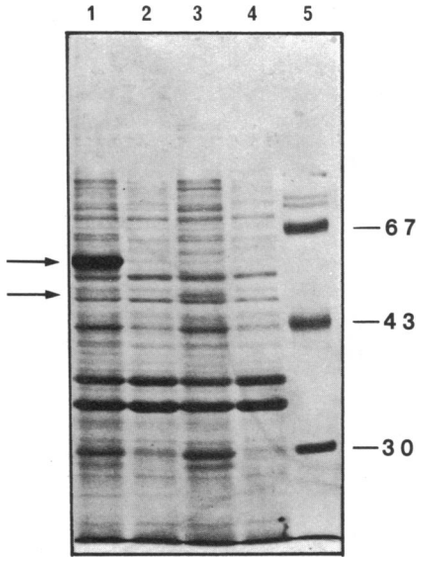

FIG. 5. Electrophoretic analysis of proteins from bacterial extracts. Clones corresponding to the 12 stage-specific genes (Table 1) were identified in the cDNA library constructed in the inducible expression vector pPL31A (unpublished data). In this vector, the product of the cloned gene is expressed as a fusion protein containing 99 amino acids of the phage MS2 polymerase (31). Cultures from such clones were grown overnight at 28°C and induced at 42°C for 2 hr, and the bacterial pellet was sonicated, washed with phosphate-buffered saline (to remove soluble proteins), and finally extracted with 4 M urea and analyzed by $NaDodSO_4$ gel electrophoresis (19). Four examples of protein extracts from different clones are shown. Lanes 1 and 3, clones with a strong and a weak additional polypeptide, respectively (arrows). No additional polypeptides are present in lanes 2 and 4. Lane 5, molecular weight markers (indicated as $M_r \times 10^{-3}$).

Developmental regulation of gene expression is known to occur in other systems. For example, selective gene activation has been described in the development of *Drosophila* (35), *Bombyx mori* (36), and *Dictyostelium* (37). It will be of interest to see whether this coordinate expression of stage-specific genes involves genes that are physically linked in the genome of *P. falciparum*.

Perhaps the most important practical implications of the work described here are the identification and cloning of the genes coding for late schizont-merozoite specific proteins (in a cDNA library in which these stage-specific clones represent only a small percent) and the fact that these genes number only about 12. It is very likely that the cDNA clones identified here do correspond to the genes encoding the merozoite polypeptides that are important in the development of host immunity to *P. falciparum*. As expected, the protein product of certain stage-specific cDNA clones was recognized by monoclonal antibodies specific for some of the relevant merozoite proteins (unpublished data). However, it is of interest that the procedure used here for the identification of the merozoite specific clones does not rely solely on reactivity with antibodies (and therefore on the immunogenicity of parasite proteins). Indeed, some of the most immunogenic plasmodium antigen proteins may turn out not to be protective and, conversely, some truly protective antigens may elicit primarily a T-cell protective response with little or no antibody production. The preparation in bacteria of the protein product corresponding to each of the merozoite specific genes identified will now allow us to test for their capacity to protect against malaria *in vivo*.

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