Plasmodium falciparum Possesses a Cell Cycle-regulated Short Type Replication Protein A Large Subunit Encoded by an Unusual Transcript*

Till S. Voss‡§, Thierry Mini‡, Paul Jenoe‡, and Hans-Peter Beck¶

From the ‡Swiss Tropical Institute, Socinstrasse 59, 4051 Basel and §Biozentrum, Klingelbergstrasse 50-70, University of Basel, 4056 Basel, Switzerland

DNA replication in Plasmodium parasites takes place at multiple distinct points during their complex life cycle in the mosquito and vertebrate hosts. Although several parasite proteins involved in DNA replication have been described, the various mechanisms engaged in DNA metabolism of this major pathogen remain largely unexplored. As a step toward understanding this complex network, we describe the identification of Plasmodium falciparum replication protein A large subunit (pfRPA1) through affinity purification and mass spectral analysis of a purified 55-kDa factor. Gel retardation experiments revealed that pfRPA is the major single-stranded DNA binding activity in parasite protein extracts. The activity was expressed in a cell cycle-dependent manner with peak activities in late trophozoites and schizonts, thus correlating with the beginning of chromosomal DNA replication. Accordingly, the pfrep1 message was detected in parasites 20–24 h post-invasion which is in agreement with the expression of other P. falciparum DNA replication genes. Our results show that pfRPA1 is encoded by an unusual 6.5-kb transcript containing a single open reading frame of which only the C-terminal 42% of the deduced protein sequence shows homologies to other reported RPA1s. Like the orthologues of other protozoan parasites, pfRPA1 lacks the N-terminal protein interaction domain and is thus remarkably smaller than the RPA1s of higher eukaryotes.

Plasmodium falciparum causes one of the most life-threatening parasitic diseases in humans being responsible for up to 2 million deaths per year. Malaria pathogenesis is associated with the intracellular erythrocytic stage of the life cycle of the parasite involving repeated rounds of invasion, growth, and schizogony. Parasites that eventually differentiate into gametocytes are taken up by the female anopheline vector where the parasite (9, 10) and the fact that DNA replication occurs at five distinct developmental points, namely intrahepatocytic schizogony, intraerythrocytic schizogony, microgametogenesis, premeiotic DNA synthesis, and sporozoite development (11) indicate the operation of highly regulated mechanisms of DNA replication, recombination, and repair. In fact, dynamic processes of DNA metabolism may be one reason for the outstanding success of this parasite because this supports rapid adaptation to environmental challenges such as immune pressure and action of antimalarial drugs. In addition, the unusually high AT content of ~80% in the P. falciparum genome (12, 13) may also indicate peculiarities in the replication machinery of the parasite. Hence, investigation of the components involved in DNA metabolism of P. falciparum might reveal features unique to this parasite and may consequently lead to the identification of new potential drug targets for malaria therapy.

The eukaryotic single-stranded (ss) DNA-binding protein replication protein A (RPA) plays essential roles in various aspects of DNA metabolism, including replication, recombination, and repair (for review see Ref. 14). The protein has high affinity for ssDNA (15–17) and binds with much lower affinity to environmental challenges such as immune pressure and action of antimalarial drugs. In addition, the unusually high AT content of ~80% in the P. falciparum genome (12, 13)

Received for publication, January 4, 2002, and in revised form, February 27, 2002
Published, JBC Papers in Press, March 5, 2002, DOI 10.1074/jbc.M200100200

* This work was supported in part by the Swiss National Science Foundation Grant 351-059 064.99. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a scholarship of the Boehringer Ingelheim Fonds, Germany.
¶ To whom correspondence should be addressed: Dept. of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 59, 4051 Basel, Switzerland. Tel.: 41-61-284-81-16; Fax: 41-61-271-86-54; E-mail: Hans-Peter.Buck@unibas.ch.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: DNA pol α and δ, DNA polymerases α and δ; ssDNA, single-stranded DNA; RPA, replication protein A; RPA1, large subunit of RPA; EMSA, electromobility shift assay; hpi, h post-invasion; dsDNA, double-stranded DNA; PMSF, phenylmethylsulfonyl fluoride; ORF, open reading frame; DTT, dithiothreitol; aa, amino acid; oligo, oligonucleotides; MS, mass spectroscopy.

Printed in U.S.A.
Identification of P. falciparum Replication Protein A

enzyme in a fill-in reaction by incubating 1 pmol of DNA in 1× React2 buffer (Invitrogen) in the presence of 50 μM dATP/dCTP/dGTP/dTTP and 10 μCi (3000 Ci/mmol) of [α-32P]dCTP at 30 °C for 20 min. Probes were purified using Sephadex G-25 spin columns (Amersham Biosciences). The sequences of probes and competitors are shown in Table 1.

UV Cross-linking—To assess the molecular weight of the ssDNA-protein complex ~4 or 20 μg of crude nuclear or cytosolic protein extracts, respectively, were incubated with 60 fmol of labeled 5B1f oligo in EMSA buffer as described, followed by exposure to UV light for 10 min in a Stratalinker 1800 (Stratagene). Protein-DNA complexes were incubated at 95 °C in 1× SDS sample buffer for 5 min and separated on a 12% SDS-polyacrylamide gel. Gels were dried and analyzed by autoradiography. Pre-stained molecular weight markers were used to estimate the molecular mass of the ssDNA-protein complexes.

Limited Trypsin Digestion—6–10 or 20–40 μg of crude nuclear or cytosolic protein extracts, respectively, were incubated with 20 fmol of [γ-32P]dATP-labeled 5B1f oligonucleotide (in the presence of 1.25 mM PMSF) and UV cross-linked as described above. DNA-protein complexes were incubated with 2 μg of porcine pancreas trypsin at room temperature in the same buffer. Aliquots were removed at time points indicated in Fig. 4. Reactions were stopped by the addition of SDS-PAGE sample buffer followed by incubation at 95 °C for 5 min. Samples were analyzed by SDS-PAGE. Gels were dried and analyzed by autoradiography.

Affinity Purification of the Major ssDNA Binding Activity from Parasite Protein Extracts—As matrix for affinity purification, the biotinylated 90-base oligo 5B1AT (a trimer of 5B1) was tethered to magnetic streptavidin-coated Dynabeads (Dynal) according to the supplier’s instructions. Cytoplasmic extracts from a total of 7.5 liters of asynchronous parasite culture (5–8% parasitemia) were spun at 3000 × g for 10 min to pellet cellular debris. The supernatant was incubated with 2 mg of Dynabeads (80 pmol oligoing beads) in binding buffer BB (20 mM HEPES, pH 7.8, 120 mM KCl, 1 mM EDTA, pH 7.8, 1 mM PMSE, 0.1% Triton X-100, 0.65% Nonidet P-40) on a rotating wheel for 1 h at room temperature. Dynabeads were collected by use of a magnetic stand and washed twice with 1 ml of wash buffer W1 (20 mM HEPES, pH 7.8, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSE, 0.65% Nonidet P-40) and incubated in buffer W2 containing 0.1 M KCl. Bound proteins were eluted stepwise twice in 200 μl of elution buffer EB1 and twice in 200 μl of buffer EB2 (20 mM HEPES, pH 7.8, 2.5 mM EDTA, 200 mM KCl, 0.1% Triton X-100, 0.65% Nonidet P-40) on a rotating wheel for 1 h at room temperature. Purification from nuclear extracts was performed by incubation of 4.5 ml of crude nuclear extract (1–2 μg/μl) from a total of 2.8 liters of asynchronous parasite culture (5–8% parasitemia) with 1 mg of Dynabeads (10 pmol oligoing beads) in binding buffer BB (20 mM HEPES, pH 7.8, 2.5 mM EDTA, 200 mM KCl, 0.1% Triton X-100, 0.65% Nonidet P-40). The gel piece containing the Coomassie Blue-stained purified cytoplasmic ssDNA-binding factor was washed five times for 1 min each in 30 μl of 40% n-propanol alcohol followed by five 1-min washes each in 30 μl of 0.2 M NH4HCO3 (50% acetonitrile). The gel piece was dried in a SpeedVac concentrator and then digested with 0.5 μg of sequencing grade modified trypsin (Promega) in 10 μl of 0.1% NH4HCO3, for 2 h at 37 °C. The gel piece was extracted with 15 μl of 0.1% trifluoroacetic acid for 5 min followed by 15 μl of acetonitrile for 1 min. Extraction was repeated twice, and the pooled supernatant was dried in a SpeedVac concentrator. Peptides were redissolved in 10 μl of 0.1% trifluoroacetic acid, and 5 μl were used for mass spectral analysis. Separation of peptides was done on 100-μm inner diameter capillary columns packed with POROS R2 material. Mass spectral data were acquired on a TSQ7000 triple quadrupole instrument (Finnigan) with data-controlled switching between precursor ions and daughter ions (43). For precursor ion scan-
equal amounts of RNA extracted from synchronized parasite cultures formed at 42°C using random primers and Klenow polymerase. Hybridization was performed at www.sanger.ac.uk/Projects/P.falciparum/. Sequencing of accession number AL035475) was obtained from the Sanger Center Genome Project. Preliminary sequence data from the collaboration with the Naval Medical Research Center. website (www.tigr.org). This sequencing program is carried on in collaboration with the Institute for Genomic Research (Amersham Biosciences). Probes for Northern analysis of gene promoters, respectively, 5B1sub5 contains the sequence of oligonucleotide 5B1f. Yeast tRNA was present at 2000-fold weight excess, pCAM5/3 plasmid DNA (70), and sheared salmon sperm DNA at 100-fold weight excess. C, ssDNA-binding affinity analysis using a 20-fold molar excess of wild type oligo 5B1f and mutated oligos 5B1fmut1–5 (see Table I) as competitors. n.e., nuclear extract; pur, 30-base oligonucleotide of random pyrimidine sequence.

FIG. 1. Gel retardation analysis of the major ssDNA binding activity in P. falciparum nuclear extracts. A, incubation of radiolabeled oligonucleotide 5B1f with crude parasite nuclear extract led to the formation of a ssDNA-protein complex. B, affinity EMSAs. 5 fmol of radiolabeled 5B1f (0.05 ng) were incubated with 2 μg of nuclear extract. Double-stranded fragments 5B1sub5 (155 bp) and 4Asub3b (69 bp) are derived from chromosome central and subtelomeric P. falciparum var gene promoters, respectively. 5B1sub5 contains the sequence of oligonucleotide 5B1f. Yeast tRNA was present at 2000-fold weight excess, pCAM5/3 plasmid DNA (70), and sheared salmon sperm DNA at 100-fold weight excess. C, ssDNA-binding affinity analysis using a 20-fold molar excess of wild type oligo 5B1f and mutated oligos 5B1fmut1–5 (see Table I) as competitors. n.e., nuclear extract; pur, 30-base oligonucleotide of random purine sequence; pyr, 30-base oligonucleotide of random pyrimidine sequence.

Isolation of Parasite Total RNA and Northern Analysis—Parasite total RNA was isolated using Trizol (Invitrogen) as described (45), and RNA was stored in formamide at −80°C. For Northern blot analysis equal amounts of RNA from synchronized parasite cultures was electrophoresed on 1.2% agarose gels (5 mM guanidine isothiocyanate) (45) and vacuum-transferred to a Hybond-XL nylon membrane (Amersham Biosciences). Probes for Northern analysis of pfpr1p were gel-purified PCR products (see Fig. 6) radiolabeled with [α-32P]dCTP using random primers and Klenow polymerase. Hybridization was performed at 42°C in UltraHyb (Ambion).

Nucleotide Sequence Data—Sequence data for pfpr1p (GenBank™ accession number AL035475) was obtained from the Sanger Center website at www.sanger.ac.uk/Projects/P.falciparum/. Sequencing of P. falciparum chromosome 4 was accomplished as part of the Malaria Genome Project. Preliminary sequence data from the Plasmodium yoelii genome were obtained from the Institute for Genomic Research website (www.tigr.org). This sequencing program is carried on in collaboration with the Naval Medical Research Center.

RESULTS

Major DNA Binding Activity in P. falciparum Nuclear Extracts—In the course of investigations of P. falciparum promoters by gel retardation assays using dsDNA probes, we detected a dominant nonspecific DNA binding activity in parasite nuclear extracts derived from asynchronously growing cultures. This activity was only observed, however, when probes were end-labeled with T4 polynucleotide kinase which also labels ssDNA molecules. In contrast, when we used Klenow enzyme to fill in 4-base protrusions at the ends of double-stranded complementary oligonucleotides (ensuring that only double-stranded molecules are labeled), the DNA-protein complex was hardly detected (data not shown). EMSAs using end-labeled single-stranded oligonucleotides showed that this activity was due to the interaction of a nuclear factor with ssDNA. Fig. 1A shows the interaction between this factor and the radiolabeled 30-base single-stranded oligonucleotide probe 5B1f (the sequence of 5B1f corresponds to a conserved motif found in var gene promoters (46)). As shown in Fig. 1B the single-stranded oligonucleotides 5B1f and 5B1rc added at a 20-fold molar excess competed with binding to the labeled probe. However, a double-stranded 155-bp competitor restriction fragment containing the 5B1f sequence (5B1sub5) did not compete for binding. EMSA competition experiments further revealed that the affinity of the nuclear factor was higher for single-stranded polypurimidine than for polypurine oligomers (Fig. 1B). In contrast, heterogeneous dsDNA was a much weaker competitor, and yeast tRNA did not compete at all even if added at 2000-fold weight excess. To further investigate for sequence preferences, we used mutated forms of oligonucleotide 5B1f (5B1fmut1–5, see Table I) in gel retardation competition studies. In these oligos consecutive stretches of six nucleotides each were mutated, where A was replaced by G, G replaced by T, and T replaced by C. Whereas oligos 5B1fmut1, 5B1mut4, and 5B1fmut5 competed equally well or even better compared with the wild type sequence 5B1f, the ssDNA-binding factor had a clearly reduced affinity for oligonucleotides 5B1fmut2 and 5B1fmut3 (Fig. 1C), indicating a certain degree of sequence preference independent of the pyrimidine/purine content.

To investigate whether the ssDNA binding activity was present throughout the intra-erythrocytic life cycle, we performed gel retardation experiments using nuclear extracts prepared from synchronously growing cultures. The ssDNA binding activity was absent in mid-ring stage parasites (8–16 h post-invasion (hpi)) (Fig. 2). The activity faintly appeared in young trophozoites (16–24 hpi) and increased to maximal levels in parasites older than 34 hpi. Parasite nuclear extracts derived from the very early ring stage (0–8 hpi) also contained the ssDNA binding activity.

Comparison of Nuclear and Cytoplasmic ssDNA Binding Activities—A major ssDNA binding activity was also observed in cytoplasmic parasite extracts, but the complexed probe migrated at a slightly different position than the complex formed with the nuclear factor (Fig. 3A). However, when various protease inhibitors were used during protein isolation and EMSAs, and when using fresh cytoplasmic extracts in gel retardation
assays, an additional signal migrating at the same position as the nuclear complex was observed (Fig. 3B). This suggested identical activities in both subcellular compartments with protenolytic activities in cytosolic extracts acting on the ssDNA-binding factor during protein isolation and gel retardation experiments. In EMSA affinity assays using a variety of different competitor DNAs, the cytoplasmic and nuclear activities behaved identically (Fig. 3C). These observations supported the assumption that both activities were exerted by the same protein. To investigate this possibility in more detail, we compared limited trypptic digests of UV-cross-linked ssDNA reactions by SDS-PAGE (Fig. 4). Without trypsin digestion the major cross-linked ssDNA-protein complexes migrated on SDS-PAGE (Fig. 4). With trypsin, and the fragments were subjected to mass spectral analysis. Data base searches with the fragmentation spectra obtained by MS/MS analysis from four tryptic peptides (NVNLVNEALS, GTDSTDSIR, LNEFFFR, and YNNFISIDN) unambiguously identified this protein as putative P. falciparum replication protein A large subunit (pfRPA1) located on chromosome 4. The ssDNA-binding factor (55 kDa) was excised and digested with trypsin, and the fragments were subjected to mass spectral analysis. The characteristics of the purified proteins again indicated that the major ssDNA binding activities detected in nuclear and cytosolic extracts were exerted by the same protein.

**Mass Spectral Analysis of the Purified ssDNA Binding Factor**—To obtain enough protein for mass spectral analysis, the ssDNA binding activity was affinity-purified stepwise from crude cytosolic extracts obtained from a total of 7.5 liters of culture (8% parasitaemia) of asynchronously growing parasites yielding a total of 1–2 μg of purified protein. Eluates were pooled and separated on a 10% SDS-polyacrylamide gel. The ssDNA-binding factor (55 kDa) was excised and digested with trypsin, and the fragments were subjected to mass spectral analysis. Data base searches with the fragmentation spectra obtained by MS/MS analysis from four tryptic peptides (NVNLVNEALS, GTDSTDSIR, LNEFFFR, and YNNFISIDN) unambiguously identified this protein as putative P. falciparum replication protein A large subunit (pfRPA1) located on chromosome 4. The ssDNA-binding factor (55 kDa) was excised and digested with trypsin, and the fragments were subjected to mass spectral analysis. The characteristics of the purified proteins again indicated that the major ssDNA binding activities detected in nuclear and cytosolic extracts were exerted by the same protein.

**Affinity Purification of the ssDNA Binding Activity from Parasite Protein Extracts**—Both major ssDNA binding activities from nuclear and cytosolic extracts were purified by affinity purification. Straptavidin-coated magnetic beads with tethered biotinylated 90-base oligonucleotide 5B1Af (a 3-mer of 5B1f) were incubated with crude protein extracts in binding buffer. After washing, bound proteins were eluted with 0.5 and 1 M KCl, and all fractions were analyzed by SDS-PAGE followed by silver staining (data not shown). Testing for ssDNA binding activity using gel retardation revealed that most of the ssDNA binding activity was eluted at 0.5 M salt (data not shown). The electrophoretic mobilities of ssDNA-protein complexes formed with crude extracts and purified fractions were identical and are presented in Fig. 5A. Fig. 5B shows SDS-PAGE analysis of the 0.5 M cytosolic eluate revealing a dominant band at ~55 kDa and two additional enriched proteins at 30 and 25 kDa. Similar results were obtained for the nuclear 0.5 M eluate. However, the purified cytosolic 55-kDa protein had a slightly smaller size than the nuclear factor (data not shown) which is in agreement with the observed difference in mobility of the corresponding ssDNA-protein complexes in EMSA experiments. Furthermore, SDS-PAGE analysis of UV cross-linked ssDNA-protein complexes obtained with crude extracts and the purified factors revealed a size of ~65 kDa for each complex (Fig. 5C). These results strongly suggested that the 55-kDa factor was responsible for ssDNA binding because the size difference of 10 kDa observed between the purified protein alone and the UV cross-linked ssDNA-protein complexes was accounted for by the covalent attachment of the 30-base oligonucleotide 5B1f to the binding factor in the UV cross-linked samples. Gel retardation competition studies using the purified proteins from both the nuclear and cytosolic extracts again revealed an identical affinity pattern and the expected difference in complex mobility (Fig. 5D). However, the overall affinity pattern slightly diverged from the results obtained with the crude protein extracts, which might be due to the different binding conditions used in EMSAs (total amount of ssDNA-binding protein, presence of bovine serum albumin, and absence of poly(dI-dC) in EMSAs using the purified proteins). The characteristics of the purified proteins again indicated that the major ssDNA binding activities detected in nuclear and cytosolic extracts were exerted by the same protein.

**Identification of P. falciparum Replication Protein A**—Table I lists the oligonucleotide probes and competitors used in EMSA assays. Mutated hexanucleotide stretches in 5B1mut1–5 are highlighted in bold. The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) (accession numbers X65316, AF009179, AF035475). The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) located on chromosome 4 (GenBank™ accession number AL035475). Furthermore, peptide masses of 7 additional trypptic peptides—were observed indicating that the ssDNA binding activity was retained in tryptic fragments of equal size. Taken together, these findings clearly suggested that the activities present in nuclear and cytoplasmic extracts were identical.

**Effect of P. falciparum Replication Protein A on Cytokine Expression**—The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) located on chromosome 4 (GenBank™ accession number AL035475). Furthermore, peptide masses of 7 additional trypptic peptides—were observed indicating that the ssDNA binding activity was retained in tryptic fragments of equal size. Taken together, these findings clearly suggested that the activities present in nuclear and cytoplasmic extracts were identical.

**Effect of P. falciparum Replication Protein A on Cytokine Expression**—The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) located on chromosome 4 (GenBank™ accession number AL035475). Furthermore, peptide masses of 7 additional trypptic peptides—were observed indicating that the ssDNA binding activity was retained in tryptic fragments of equal size. Taken together, these findings clearly suggested that the activities present in nuclear and cytoplasmic extracts were identical.

**Effect of P. falciparum Replication Protein A on Cytokine Expression**—The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) located on chromosome 4 (GenBank™ accession number AL035475). Furthermore, peptide masses of 7 additional trypptic peptides—were observed indicating that the ssDNA binding activity was retained in tryptic fragments of equal size. Taken together, these findings clearly suggested that the activities present in nuclear and cytoplasmic extracts were identical.

**Effect of P. falciparum Replication Protein A on Cytokine Expression**—The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) located on chromosome 4 (GenBank™ accession number AL035475). Furthermore, peptide masses of 7 additional trypptic peptides—were observed indicating that the ssDNA binding activity was retained in tryptic fragments of equal size. Taken together, these findings clearly suggested that the activities present in nuclear and cytoplasmic extracts were identical.

**Effect of P. falciparum Replication Protein A on Cytokine Expression**—The nucleotide and deduced aa sequences of the putative P. falciparum replication protein A (pfRPA1) located on chromosome 4 (GenBank™ accession number AL035475). Furthermore, peptide masses of 7 additional trypptic peptides—were observed indicating that the ssDNA binding activity was retained in tryptic fragments of equal size. Taken together, these findings clearly suggested that the activities present in nuclear and cytoplasmic extracts were identical.
The binding activities and were of similar size are marked by tryptic fragments of the nuclear and cytoplasmic factors that retained protein complex migrated at an apparent mass of 65 kDa (arrow). The dried gel was analyzed by autoradiography. The undigested ssDNA-taken at the time points indicated were separated by SDS-PAGE, and fragments of partially digested cross-linked ssDNA-protein complexes binding activities were UV cross-linked to radiolabeled 5B1f. Fragments of partially digested cross-linked ssDNA-protein complexes taken at the time points indicated were separated by SDS-PAGE, and the dried gel was analyzed by autoradiography. The undigested ssDNA-protein complex migrated at an apparent mass of 65 kDa (arrow). Tryptic fragments of the nuclear and cytoplasmic factors that retained binding activity and were of similar size are marked by asterisks. Trypsin incubation times are indicated above each lane. Free probe is apparent as an intense signal at the bottom of the autoradiograph.

Identification of P. falciparum Replication Protein A

This paper describes for the first time the purification and identification of a DNA-binding protein from the malaria parasite P. falciparum. pfRPA binds ssDNA with high affinity, has a preference for single-stranded polypyrimidines over polypurines, a much lower affinity for dsDNA, and does not bind yeast tRNA. This affinity pattern is consistent for all eukaryotic RPAs investigated to date (14). In addition, we also detected preferences of pfRPA for oligonucleotides of mixed sequence that cannot solely be attributed to the ratio of pyrimidines and purines. This may reflect preferential binding of pfRPA to certain genomic regions like origins of replication or “hot spots” of recombination, and may therefore hold significance for pfRPA function. In fact, partial sequence dependence of RPA binding has also been reported in yeast and humans (18).

We would like to stress here that this ssDNA binding activity heavily interfered in gel retardation analysis of parasite promoters using dsDNA probes. We conclude that the presence of any labeled ssDNA molecules in binding reactions results in the formation of ssDNA-pfRPA complexes that may easily be

\* T. S. Voss, M. Kästli, D. Vogel, and H.-P. Beck, manuscript in preparation.
mistaken as dsDNA-protein interactions. These labeled ssDNA species might originate through labeling with T4 polynucleotide kinase or dissociation of double-stranded probes during preparation and purification. In addition, RPA preferentially binds to AT-rich regions of dsDNA under low salt conditions (26), and this finding merits special attention in light of the AT richness of *P. falciparum* DNA. We therefore propose to include a 50-fold molar excess of unlabeled oligonucleotides in EMSA reactions using *P. falciparum* extracts.

*pfRPA activity was present in both nuclear and cytosolic extracts. We exclude the possibility of cross-contamination of the cytosolic fraction with nuclear *pfRPA* during cell fractionation. By using the same protein preparations described in the present study, we recently identified sequence motifs in *P. falciparum var* gene promoters specifically interacting with activities present in nuclear fractions but completely absent in cytosolic fractions. Furthermore, in human cells the majority of RPA is present in the cytosolic fraction after gentle lysis of cells (15–17, 47), and purification of RPA from cytosolic fractions has been reported (18, 19). Compared with the nuclear activity, cytosolic *pfRPA* had a slightly decreased size, and the ssDNA-*pfRPA* complex showed a corresponding increase in electrophoretic mobility. This was probably due to proteolysis because the use of various protease inhibitors in fresh cytosolic preparations revealed a second complex similar to the one formed with crude nuclear extracts. Interestingly, Seroussi and Lavi (48) reported two closely comigrating complexes using mammalian whole cell extracts. By performing supershift EMSAs using antibodies against RPA subunits, they provided convincing evidence that the faster migrating complex was due to proteolysis of hsRPA1.

In other eukaryotes such as *Crithidia*, yeast, tobacco, rodents, and humans, purification schemes for RPA use prefractionation of cell lysates, ssDNA-cellulose columns for affinity purification, and gel retardation analysis comparing the ssDNA binding activities present in crude nuclear or cytosolic extracts and in the 0.5 M salt elutions obtained from affinity purification. B, SDS-PAGE separation of the cytosolic 0.5 M eluate with enriched bands at 55, 30, and 25 kDa. Crude nuclear and cytosolic extracts and molecular weight marker have been used as references. C, UV cross-linked ssDNA-binding reactions were analyzed by SDS-PAGE and autoradiography. The major ssDNA binding activities in crude nuclear extracts and in the 0.5 M eluate from nuclear affinity purification were of equal size. As size references molecular mass marker and an aliquot of the 0.5 M eluate were separated on the same gel and visualized by silver staining. D, competition EMSA comparing the ssDNA binding affinities of the purified nuclear and cytosolic activities. 5 fmol of radiolabeled 5B1f were incubated with ~0.1–0.5 ng of purified nuclear and cytosolic factors in the presence of various competitor DNAs. pur, 30-base oligonucleotide of random purine sequence; pyr, 30-base oligonucleotide of random pyrimidine sequence. Yeast tRNA was added at 2000-fold weight excess; the other competitors were added at 20-fold molar excess. n.e., nuclear extract; c.e., cytosolic extract.
chromatography, followed by liquid chromatography to obtain highly pure RPA. SDS-PAGE analysis of these preparations revealed three distinct protein bands corresponding to the dissociated subunits of RPA. Due to the *P. falciparum* in vitro culture conditions, it is almost inconceivable to obtain enough protein material for such purification procedures. We therefore purified pfRPA directly from crude nuclear and cytosolic extracts. This probably led to copurification of other proteins with affinity for ssDNA preventing a clear identification of the middle and small subunits (Fig. 5). Although we failed to identify malarial orthologues to these subunits using BLAST at PlasmoDB (www.plasmodb.org) and NCBI Malaria Genomics and Genomics section (www.ncbi.nlm.nih.gov/Malaria/), we clearly expect that *P. falciparum* is equipped with the middle and small subunits because the heterotrimeric structure of RPA is conserved from kinetoplastids to humans (14).

pfRPA1, with the apparent mass of 55 kDa, is remarkably smaller than its orthologues in most other eukaryotes. Sequence analysis revealed that this size difference is accounted for by the absence of the N-terminal protein-interaction domain. pfRPA1 is encoded by a continuous ORF on chromosome 4 (GenBank™ accession number AL035475) encoding a putative 1145-aa protein (134 kDa). Northern analysis using various *pfrpa1* probes detected a single large transcript of ~6.5 kb encoding the entire ORF. Furthermore, PCR analysis using genomic DNA from six different *P. falciparum* strains indicated that this locus is conserved (data not shown). Only the C-terminal 466 aa, however, share homology to other RPA large subunits, including a conserved zinc finger motif close to the C terminus. The predicted molecular mass of this 466-aa region (54.6 kDa) is in perfect accordance to the size predicted from SDS-PAGE analysis. The N-terminal part of the predicted protein shows no homologies to any other proteins. Several Asn-rich tracts are interspersed in this region, a feature that has also been reported for other *P. falciparum* proteins (2, 7, 53–56), and more examples emerge from the *P. falciparum* sequencing project (57, 58). These findings, together with the fact that all tryptic fragments from mass spectral analysis exclusively mapped to the C-terminal region, suggest post-translational proteolytic cleavage of a 1145-aa precursor protein generating the 55-kDa pfRPA1. In fact, such a process has been reported for the 51-kDa pfCDP-diacylglycerol synthase where the N-terminal third of a 78-kDa precursor protein generating the 55-kDa pfRPA1. Another explanation would be translation of *pfrpa1* from an ATG internal to the predicted 3435-bp ORF. However, the absence of frameshift mutations in the whole ORF argues against the latter possibility. Custom

**FIG. 6. Sequence of pfRPA1.** The nucleotide sequence (GenBank™ accession number AL035475) and deduced aa sequence of putative pfRPA1 are shown. Numbers indicate aa residues in the predicted protein sequence. Tryptic peptides identified by mass spectrometric analysis of the purified protein are boxed. The C-terminal part of the protein sharing sequence homology to other eukaryotic RPA large subunits is typed in *boldface* letters, and putative internal translation initiation methionines are indicated by black boxes. The conserved zinc finger motif found in all RPA large subunits is *shaded* in gray, and conserved residues are marked by *asterisks*. Asn-rich tracts found in the N-terminal half of the predicted protein sequences are indicated in *boldface italics*. Oligonucleotides used to generate PCR fragments rpaA-rpaE for Northern analysis are shown by *arrows.*
Identification of P. falciparum Replication Protein A

In summary, we identified the P. falciparum homologue of eukaryotic RPA1 encoded by an unusually long transcript which, like RPA1 of other protozoans, lacks the N-terminal protein-interaction domain. Expression of pfrPA is stage-specifically regulated, and the protein is present at the onset of S-phase in the erythrocytic parasite stages. Our findings support the assumption that major differences in DNA metabolism between P. falciparum and its host exist and may be an indication for peculiarities in the replication machinery of the parasite that could be exploited in novel antiparasite strategies.

REFERENCES

1. Chavalitshewinpoon, P., de Vries, E., Stam, J. G., Fransen, F. V., van der Vliet, P. C., and Overduin, J. P. (1993) Mol. Biochem. Parasitol. 61, 243–253.
2. White, J. H., Kilbey, B. J., de Vries, E., Goman, M., Alano, P., Cheesman, S., Kilbey, B. J., and Kelly, T. (1993) Nucleic Acids Res. 21, 3643–3646.
3. Fox, B. A., and Bzik, D. J. (1991) Mol. Biochem. Parasitol. 49, 289–296.
4. Ridley, R. G., White, J. H., McLaeese, S. M., Goman, M., Alano, P., de Vries, E., and Kelly, T. (1993) Nucleic Acids Res. 21, 3643–3646.
5. Kilbey, B. J., Fraser, I., McLaeese, S. M., and Kelly, T. R. G. (1993) Nucleic Acids Res. 22, 3, 249–253.
6. Tosh, K., and Kilbey, B. J. (1995) Gene (Amst.) 153, 151–154.
7. Cheesman, S. M., McLaeese, S. M., Johnson, D., Horrock, P., Ridley, R. G., and Kilbey, B. J. (1994) Nucleic Acids Res. 22, 2547–2551.
8. Inselburg, J., and Banyal, H. S. (1984) Mol. Biochem. Parasitol. 10, 79–87.
9. Geiges, J. G. (1988) Genet. Eng. 7, 57–90.
10. Weber, J. J. (1987) Gene (Amst.) 52, 103–109.
11. White, J. H., and Kilbey, B. J. (1996) Parasitol. Today 12, 151–155.
12. McCutchan, T. F., Dame, J. B., Miller, L. H., and Barnwell, J. W. (1984) Science 225, 808–811.
13. Pollack, Y., Katzen, A. L., Spira, D. T., and Golenser, J. (1982) Nucleic Acids Res. 10, 539–546.
14. Wold, M. S. (1997) Annu. Rev. Biochem. 66, 61–92.
15. Fairman, M. P., and Stillman, B. (1988) EMBO J. 7, 1211–1218.
16. Wobbe, C. R., Weissbach, L., Borowiec, J. A., and Kelly, T. J. (1991) J. Biol. Chem. 266, 2801–2809.
17. Brown, G. W., Melendy, T. E., and Ray, D. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10227–10231.
18. Dornreiter, I., Erdlie, L. P., Gilbert, I. U., von Winkler, K., Kelly, T. J., and Fanning, E. (1992) EMBO J. 11, 769–776.
19. Erdile, L. F., Heyer, W. D., Kolodner, R., and Kelly, T. J. (1991) J. Biol. Chem. 266, 3389–3395.
20. Brunn, R. A., Lao, Y., He, Z., Ingles, C. J., and Wold, M. S. (1997) Biochemistry 36, 8443–8454.
21. Erdile, L. F., Heyer, W. D., Kolodner, R., and Kelly, T. J. (1991) J. Biol. Chem. 266, 12900–12908.
22. Kenney, M. E., Lee, S. H., and Hurwitz, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9737–9741.
23. Tsuji, I., and Stillman, B. (1989) EMBO J. 8, 3883–3889.
24. Tsuji, I., and Stillman, B. (1991) J. Biol. Chem. 266, 1590–1960.
25. Tsuji, I., and Stillman, B. (1993) J. Biol. Chem. 268, 1961–1968.
26. Brown, G. W., Hines, J. C., Fisher, P., and Ray, D. S. (1994) Mol. Biochem. Parasitol. 63, 153–156.
27. Zhu, G., Marchewka, M. J., and Keithly, J. S. (1999) FEMS Microbiol. Lett. 176, 367–372.
28. Firnentin, A. A., Elisa-Arnaz, M., and Berg, P. (1995) Mol. Cell. Biol. 15, 1630–1632.
29. Gomes, X. V., and Wold, M. S. (1996) Biochemistry 35, 10558–10568.
30. Kim, D. K., Stigter, E., and Lee, S. H. (1996) J. Biol. Chem. 271, 15124–15129.
31. Lin, Y. L., Chen, C., Keshav, K. P., Winchester, E., and Dutta, A. (1996) J. Biol. Chem. 271, 15124–15129.
