A Family of Cation ATPase-like Molecules from *Plasmodium falciparum*

Sanjeev Krishna, Gill Cowan, John C. Meade,* Richard A. Wells, James R. Stringer,‡ and Kathryn J. Robson

Medical Research Council Molecular Haematology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom OX3 9DU; *Department of Preventive Medicine, Division of Parasitology, University of Mississippi Medical Center, Jackson, Mississippi 39216-4505; and ‡Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, Cincinnati, Ohio 45267-0524

Abstract. We report the nucleotide and derived amino acid sequence of the ATPase 1 gene from *Plasmodium falciparum*. The amino acid sequence shares homology with the family of "P"-type cation translocating ATPases in conserved regions important for nucleotide binding, conformational change, or phosphorylation. The gene, which is present on chromosome 5, has a product longer than any other reported for a P-type ATPase. Interstrain analysis from 12 parasite isolates by the polymerase chain reaction reveals that a 330-bp nucleotide sequence encoding three cytoplasmic regions conserved in cation ATPases (regions a–c) is of constant length. By contrast, another 360-bp sequence which is one of four regions we refer to as "inserts" contains arrays of tandem repeats which show length variation between different parasite isolates. Polymorphism results from differences in the number and types of repeat motif contained in this insert. Inserts are divergent in sequence from other P-type ATPases and share features in common with many malarial antigens. Studies using RNA from the erythrocytic stages of the malarial life cycle suggest that ATPase 1 (including the sequence which encodes tandem repeats) is expressed at the large ring stage of development. Immunolocalization has identified ATPase 1 to be in the region of the parasite plasma membrane and pigment body. These findings suggest a possible model for the genesis of malarial antigens.

INFECTION with *Plasmodium falciparum*, the most virulent human malaria parasite, kills an estimated 0.5–2 million children in Africa alone every year (20, 68). The organism multiplies within erythrocytes during the clinically evident phase of infection (77). To achieve this, it subverts both the homeostatic mechanisms of erythrocytes and host defense systems. As it develops, novel permeation pathways are induced which result in increased uptake by the parasite of synthetic precursors and energy substrates, the best studied of which are nucleosides (15), amino acids (12), and glucose (70). Some of these processes depend upon the establishment of electrochemical gradients at the host-parasite interface (secondarily active transporters). These gradients must be maintained in spite of changes in the ionic composition of the infected erythrocyte as the parasite feeds and matures (31).

It has been shown that calcium uptake by infected erythrocytes increases with parasite development, although the distribution of calcium within the infected erythrocyte has not been adequately defined (for reviews see references 30, 33). In *Plasmodium chabaudi* infection the uptake of Ca^{2+} may depend upon a H^+-ion gradient generated by a parasite plasma membrane ATPase (71). Intraerythrocytic parasites also maintain a membrane potential susceptible to protonophores probably through the same mechanism (42). This H^+-ion pump may regulate parasite pH, possibly in conjunction with a K^+/H^+ exchanger (31). Erythrocytes infected with *P. falciparum* gain Na^+ and lose K^+ ions because of inhibition of erythrocyte Na^+/K^+-ATPase activity (17). These alterations in cation status do not interfere with parasite development which is also insusceptible to artificial elevations in Na^+ and depletion of K^+ ions within erythrocytes (72). These observations imply that the parasite is capable of internal regulation of the concentrations of these cations. Recently, a malarial vacuolar membrane ATPase ("V" type) has been partially characterized (6), but the mechanisms by which parasites regulate ionic homeostasis in infected cells are poorly understood. Cation ATPases from *Leishmania spps* have been more extensively studied both physiologically and by sequence analysis (16, 41, 78). No plasma membrane cation-motive (P-type) ATPases have been isolated from *P. falciparum*.

The P-type ATPases are a ubiquitously distributed class of multi-pass membrane proteins which contribute to electrochemical gradients by pumping ions using energy derived from the hydrolysis of ATP. Although they share many structural features, similar hydropathy profiles and conserved structural motifs such as a nucleotide binding domain and phosphorylation site (18), they are phylogenetically more diverse than many "housekeeping" gene families such as the tubulins or calmodulin (for review see reference 32). This diversity suggests interventions to inhibit the potentially crit-

ical ATPases in *P. falciparum* may not affect homologues in the host, particularly as selective inhibition is achievable for this class of enzyme, and isoform-specific ATPase inhibitors are already in clinical use (1, 58).

Murakami et al. (43) have recently sequenced a P-type ATPase from *P. yoelii* similar to a sarcoplasmic reticulum Ca^{2+}-ATPase (>60% identity in conserved cytoplasmic domains). By contrast, parasite-encoded plasma membrane cation ATPases may be expected to differ from host pumps because they face an environment of unusual (intracellular) composition for most of the erythrocytic phase of the life cycle. To allow more detailed studies on the structure, expression, and possible functions of these molecules, we have isolated a family of cation ATPase-like molecules (called ATPases 1-3) from *P. falciparum*. We report here the complete nucleotide sequence of one member of this family (ATPase 1).

ATPase 1, the largest putative cation-motive ATPase (1,956 amino acids) isolated thus far, has unusual structural features including arrays of tandemly repeated amino acid sequence. Interstrain analysis demonstrates polymorphism in a region of the ATPase 1 sequence which is unique for this class of enzyme. This report describes the isolation of this parasite cation ATPase, analyzes the nature of the polymorphism in ATPase 1, and compares the structure of malarial with published ATPase sequences. It extends significantly the diversity within the family of cation-motive ATPases and has important implications for understanding the cell biology of host-parasite interactions. Furthermore, it provides a potentially novel chemotherapeutic target.

Materials and Methods

Parasites

The parasite isolates used in this paper (47, 56) were cultured as described before (56). For experiments on stage specificity, synchronization of parasite cultures to yield a population restricted to a developmental time span of <4 h was carried out by standard methods (11, 34, 46).

Oligonucleotide Synthesis and Hybridization

Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and eluted at room temperature with NH_4OH (Aldrich Chem. Co., Milwaukee, WI; HPLC grade) before deprotection overnight at 55°C. Oligonucleotides (10 pmol) were radiolabeled by incubation with 50 pmol of [γ^{32}P]ATP and T4 polynucleotide kinase in 6.6 mM Tris-HCl, pH 8, 1 mM magnesium acetate, and 1 mM DTT for 30 min at 37°C, followed by removal of unincorporated label by centrifugation (800 *g*) through a G25/50 column equilibrated with 3 × SSC.

Hybridization of labeled oligonucleotides to Southern blots and filter lifts from libraries was carried out under identical conditions: 37°C in hybridization solution (6 mM EDTA, 5 × Denhardt's solution, 100 mg/ml sheared salmon sperm DNA, 0.5% NP-40, 0.9 M NaCl, 90 mM Tris-HCl, pH 8). To remove unbound probe, filters were washed twice in 6 × SSC/0.1% SDS at room temperature for 5 min each time, followed by a final 5-min wash at 54°C in the same solution.

Southern and Northern Blot Analyses

For Southern blot transfer, purified genomic (10 μg) or plasmid DNA (0.1 μg) was digested with restriction enzymes (2-3 U/μg), resolved by agarose gel electrophoresis, and visualized by staining with ethidium bromide (59). The DNA in agarose gels was denatured in 0.5 M NaOH, 1.5 M NaCl and neutralized with 0.5 M Tris-HCl, pH 7, 1.5 M NaCl before transfer to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) in 10 × SSC. DNA was fixed to the filters by baking for 2 h at 80°C. Hybridized probe was removed from blots by rinsing in ddH_2O at 100°C before reuse.

RNA was extracted from parasites by a single step method (7) or, in some experiments, used after purification through a gradient of caesium chloride (55). Total RNA (10 μg) was resolved on standard formaldehyde agarose denaturing gels (5) and transferred to nylon membrane (Hybond-N, Amersham International, Amersham, UK; or Schleicher & Schuell, Inc.) by capillary blotting in 20 × SSC. Hybridization conditions for Northern blots were identical to those used for Southern blots. Prehybridization and hybridization temperatures were the same (65°C) when carried out in Church buffer with albumin (8) or were carried out as described previously (54). All probes (activity of 2-5 × 10^6 cpm/ml) were incubated overnight with filters and unbound probe was removed by washing filters at a final stringency indicated in the results or figure legends.

Libraries and Screening

For the initial isolation of *P. falciparum* ATPase-like sequences, an EcoRI-digested genomic library in λgt11 (54) was screened by hybridization of duplicate nitrocellulose filter lifts with a degenerate antisense oligonucleotide (Mal2 ATPase). Mal2 ATPase was designed to detect the phosphorylation site of cation ATPases and consists of the following sequence: NGT TAA NGT TCC NGT TTT ATC (N indicates any nucleotide). A clone (λ7.6.2; Fig. 1 *a*) containing a 500-bp insert was isolated from the EcoRI library. To obtain additional ATPase gene sequence, two "partial RsaI" libraries were constructed. A time course for RsaI digestion of *P. falciparum* DNA was analyzed by agarose gel electrophoresis. A DNA sample from the time point giving an average fragment size of 3-5 kb was attached to HindIII linkers and ligated into λCharon 28 (53), or attached to EcoRI linkers and ligated into λgt11. The 500-bp EcoRI fragment, isolated from λ7.6.2, was used as a radiolabeled probe to screen the Charon 28 RsaI library. The λgt11 (26) RsaI library was screened with ATPase 1 sequence obtained from the Charon 28 RsaI library.

The sequence encoding the terminal transmembrane hairpins of ATPase 1 was not available from the λ libraries, even after repeated screening. This may have been a result of instability of this region in conventional cloning vectors and hosts. Consequently, a recently devised strategy to isolate new DNA sequences using polymerase chain reaction (PCR)[1] technology was applied. A "vectorette" library (52) was made from HincII-digested genomic T9/96 DNA by ligating the DNA to a partially mismatched double-stranded oligonucleotide (vectorette). The enzyme HincII was chosen to construct this library because Southern blot analysis (Fig. 1 *b*) had revealed a HincII restriction site ~500 bp downstream of the available *P. falciparum* ATPase sequence. Directional amplification of further ATPase 1 sequence was achieved using one primer (Seq 61, Table I) to initiate the reaction in a 5′ to 3′ direction from known ATPase 1 sequence, and a second primer (the universal vectorette primer; see Table I) which hybridizes specifically to the mismatched region of the vectorette. The resulting PCR product was cloned in pUC and sequenced. Subsequent screening or blot hybridizations utilized *P. falciparum* ATPase sequences from plasmids (20-40 ng) which had been labeled by random hexanucleotide priming with a kit (Amersham International) in the presence of [^{32}P]αdCTP.

DNA Sequencing Analysis and PCR

Sequencing was by the dideoxy chain termination method using a modified T7 polymerase (Sequenase™; United States Biochemical Corp., Cleveland, OH) with both single-stranded (M13) and double-stranded templates (pUC or pBS-Bluescript™) (59). Sequencing of inserts derived from the λgt11 and Charon 28 libraries was carried out on subclones prepared after digestion of DNA with one or more of the following enzymes: DraI, RsaI, HincII, ClaI, and HinfI. Complete sequence was obtained from both DNA strands.

Sequence compilation was aided by the DBUTIL program of Staden (66). Nucleotide and derived amino acid sequence analyses were carried out with the following programs: ANALYSEQ, PSQ, PIP, NAQ, and DBSEARCH (67). Additional database searches (PIR 26) were carried out using a Distributed Array Processor with contouring of the protein sequence (in view of its length), at low stringency (150 PAM, noise reduction 1.5, indels −17) or higher stringency (100 PAM) (9).

PCR conditions for all experiments are given in Table I, with the primer positions indicated. The reagents were as supplied in the manufacturer's kits (Amplitaq kit™, Cetus Corp., Berkeley, CA). Cloning of PCR products into plasmid and M13 vectors was as described previously (56). For expres-

1. *Abbreviations used in this paper*: ORF, open reading frame; PCR, polymerase chain reaction.

sion studies, cDNA from *P. falciparum* was obtained from the large ring/early trophozoite stage of erythrocytic development.

Immunofluorescence Studies

To raise polyclonal antibodies, groups of 3 BALB/c mice were injected intraperitoneally with the peptides (Pep 1 and Pep 2) shown below. The sequences of the peptides are compared with equivalent sequences in the other ATPases (ATPase 2, ATP2 and ATPase 3, ATP3) that we have identified when available. Purity and sequence of peptides was confirmed by microsequencing and HPLC, and identified an inadvertent substitution in Pep 1 at residue 495 (Ser for Phe).

PEP1	M A G Q I N T M V S D K T G T L	(position 486-501)
ATP2	E L G Q I E Y I F S D K T G T L	
ATP3	I C G K I R V F F F D K T G T L	
PEP2	M D I I T D A I P P A L P T T L T	(position 444-460)
ATP2	V V I T G N F V P I S L I V T M S	

Primary immunizations were with peptide conjugated to Keyhole-Limpet Haemocyanin (50 μg) given with Freund's complete adjuvant. Boosting was with an equivalent amount of peptide and Freund's incomplete adjuvant, after 2 wk. A final boost was given 10 d later with no adjuvant. Responses were assayed by an ELISA (data not shown) and the polyclonal antibodies applied to slides of *P. falciparum* to identify and localize ATPase 1. Slides used in immunofluorescence studies were prepared by washing infected or uninfected erythrocytes (1% haematocrit) in PBS, making smears and fixing at −20°C in acetone. Antiserum was applied to the slides for 30 min at room temperature in a humid atmosphere, and after removing excess serum and three washes in PBS/0.1% BSA, the detecting antibody (FITC goat anti-mouse, Becton-Dickinson, Immunocytometry Sys., Mountain View, CA) was incubated at a concentration recommended by the manufacturers. After washing as before, coverslips were mounted with glycerol and viewed under a model IIIRS (Carl Zeiss, Inc., Thornwood, NY).

Results

Isolation and Mapping of Clones Containing Sequence for ATPase 1

An antisense oligonucleotide (Mal2 ATPase), with 64-fold degeneracy that incorporated the malarial bias for A+T residues, was designed to detect the conserved phosphorylation site (DKTGTLT) which is the signature of the P-type class of cation ATPases. Hybridization to a Southern blot of restriction-digested DNA from *P. falciparum* clone T9/96 revealed four bands in the track digested with the enzyme EcoRI, after washing at high stringency (6 × SSC/0.1% SDS, 54°C, data not shown). Screening of 40 genome equivalents of a λgt11 library containing EcoRI-digested *P. falciparum* DNA (clone T9/96) under conditions identical to Southern blot analysis yielded 129 positive clones. These were classified by crosshybridization experiments, using the EcoRI fragments as probes, into three subclasses (ATPase 1, 35% of clones, 500-bp-sized inserts, subclone designated p7.6.2, Fig. 1, *a* and *b*; ATPase 2, 42% of clones, 1.53-kb-sized inserts; and ATPase 3, 23% of clones, 1.48-kb-sized inserts, data not shown). The size of the fourth EcoRI band (11 kb) detected by Mal2 ATPase oligonucleotide probe precluded cloning in λgt11 (maximum insert size 7 kb). Sequence analysis identified the conserved phosphorylation site in representatives from all three classes of clones (Fig. 3 *a*). The degeneracy in the sequence for Mal2 ATPase allowed almost perfect matches with genomic sequences obtained from this region (one nucleotide mismatch in ATPase 1). Genes for all three ATPases were seen to be present in the genome as single copy (Fig. 1 *b* and not shown).

To identify further ATPase 1 sequence contiguous with the 500-bp EcoRI fragment, 20 genome equivalents of the partial RsaI library in Charon 28 (average insert size 3 kb) were screened at high stringency of washing (0.1 × SSC/0.1% SDS, 65°C 45 min) with this fragment (from p7.6.2) as probe. Two overlapping clones which hybridized to p7.6.2 (λ3.3 and λ7.8; insert sizes 3.5 and 2.7 kb, respectively) were purified (Fig. 1). Subcloning and sequence analysis confirmed contiguity of sequence with p7.6.2. An open reading frame (ORF) extended from a putative initiating methionine residue in clone p3.3 to the end of insert in the distal overlapping clone p7.8 (Fig. 1 *a*). An M13 subclone containing ~400 bp of sequence from the 3′ end of p7.8 was radiolabeled (25), and the second (partial RsaI-digested) genomic library from clone T9/96 (average insert size 3 kb) ligated into the vector λgt11 via EcoRI linkers, was screened.

One positive clone from this library (λ7CA, Fig. 1 *a*) which contained a 4-kb insert was subcloned into plasmid (p7CA) and M13 vectors and its sequence determined. An ORF that was contiguous with the ORFs from p3.3, p7.6.2, and p7.8 extended to the end of p7CA insert, but did not contain the terminal transmembrane segments essential for ion transport in other ATPases. The HincII vectorette library enabled amplification of the remaining portion of ATPase 1 by PCR initiated by the oligonucleotide Seq 61 (for conditions see Table I). Three independent clones were analyzed. A coarse and fine restriction map of ATPase 1 is shown in Fig. 1 *a*.

ATPase 1 Is a Member of the P-Type Family of Cation Pumps

The nucleotide sequence and predicted amino acid sequence for ATPase 1 is shown in Fig. 2. The initiating methionine was identified as the first in-frame ATG codon (nucleotides 927–929, Fig. 2) of the ORF. As with other malarial sequences, there is poor agreement with the Kozak eukaryotic consensus for the context of initiation (54). The gene has no introns and ends at a termination codon (UGA; 6,795 bp; Fig. 2) which forms part of a strong tetranucleotide consensus for eukaryotic termination signals (UGA [A/G]) (4), and is soon followed by another termination signal (UAAG). After the second termination signal, there is a polypyrimidine tract (25 "T" residues), a feature which is also present in other malarial genes (73).

The calculated molecular mass of the unmodified protein is 230 kD; it contains 1,956 amino acid residues and has 27 sequons (potential N-linked glycosylation sites) (50). The predicted topography of the derived amino acid sequence for ATPase 1 conforms to that of other P-type ATPases with 8–10 membrane-spanning domains organized into three regions, and conserved cytoplasmic domains including the phosphorylation site DKTGTLT (amino acids 496–502, region f, Figs. 2 and 3 *a*). The longest of the membrane-spanning regions corresponds to the terminal transmembrane hairpins which contribute to ion transport specificity. The last two membrane-spanning regions (M3, M4, and M5–M9) are separated by an unusually long cytoplasmic region (containing conserved regions f–j) of 1,353 amino acids).

A comparison of the conserved cytoplasmic domains in ATPase 1 with representatives from other ion transporting ATPases (with the ATPase 1 sequence as template) is shown in Fig. 3 *a*. There is preservation of functionally critical

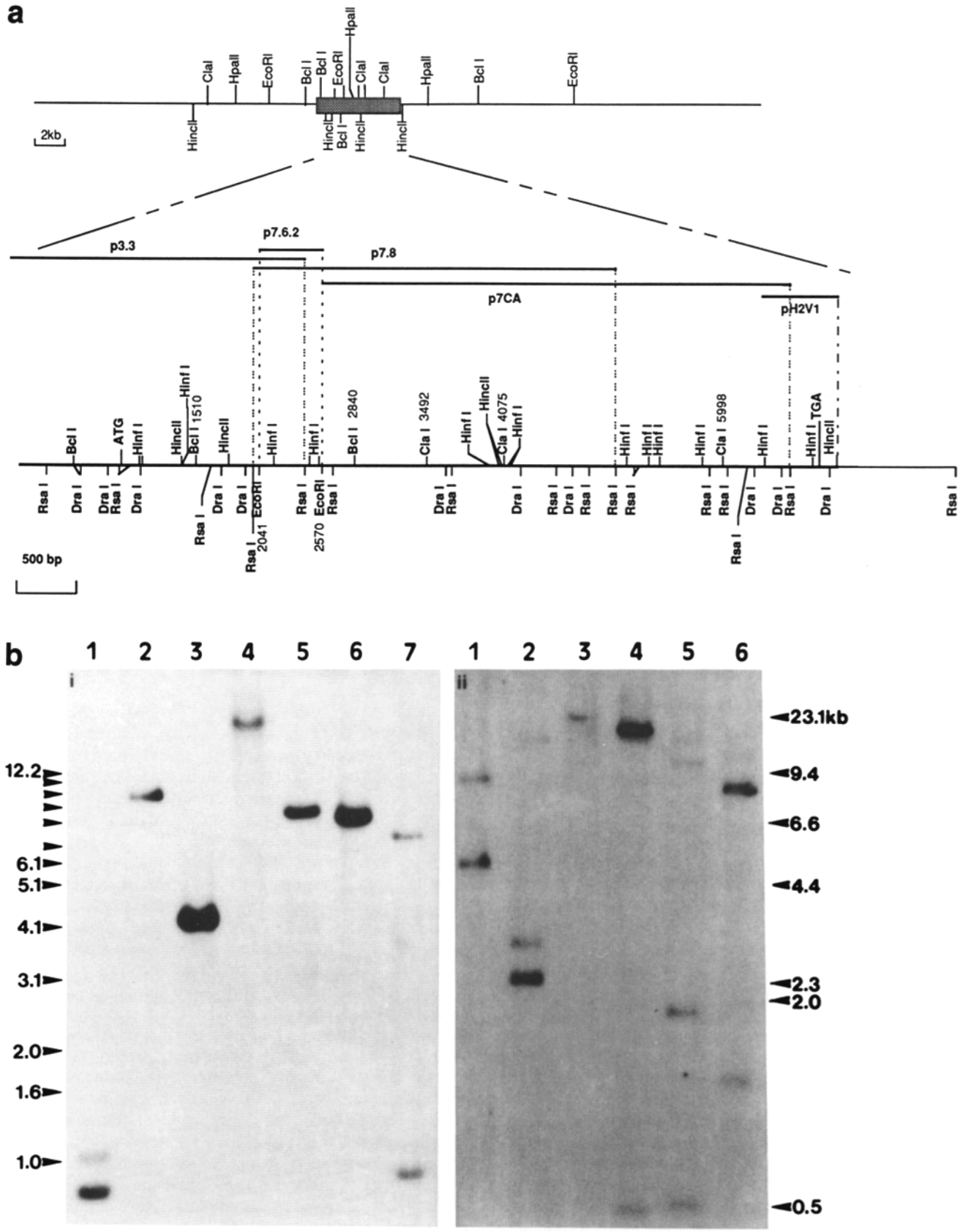

Figure 1. (*a*) Restriction map of ATPase 1. The restriction map for ATPase 1 was derived from Southern blot and sequence analysis. The ORF is indicated by the shaded box, and the overlapping clones from which the sequence was obtained are shown below it. The order of isolation of clones is from top to bottom. The positions of some of the enzyme sites in the nucleotide sequence are shown to allow identification in Fig. 2. Clone p7.6.2 was isolated by screening with an oligonucleotide (Mal2 ATPase) and was used to screen for clones p3.3 and p7.8. Clone p7CA was obtained by screening with the 3′ end of p7.8 and pH2V1 by the vectorette technique (see Materials and

Table I. PCR Conditions

Name	Sequence (5′ to 3′)	Position (nucleotides)	Tm (°C)	Annealing Temp(°C)/Time(s)	Extension Temp(°C)/Time(s)
VAR 1	GGG AGA TGT CTT AGA AAT ATT GAT G	3377 S	68	60/90	72/120
VAR 2	CCC CAA AAA ATG CAA ATT AGA TTC	3965 A	64		
VAR 3	GGA GAT GTA ATA CCT GTT GTT GGT	4134 S	68	59/90	72/60
VAR 4	GGT ATT CTG TGG GAC AAT AAT TCC	4500 A	68		
VAR 5	GTA TGG AGC CTA GAT AAC TAC	1089 S	60	55/90	72/60
VAR 6	CCT TCA AAA GGT AAC CGC TC	1417 A	60		
BIVS5′	CTG ATG AGC ATG GAA TAG ATC CAG	284 S	70	60/120	72/60
BIVS3′	CTG TCC CCA CAA TAG GTA CTA CTC	674 A	72		
BIVS5′	CTG ATG AGC ATG GAA TAG ATC CAG	284 S	70	60/120	72/60
BIVSe	CCA AAG GGG CCA GCA CGA ACA CTA TCC	799 A	86		
Seq 61	GAA CGT GTA CTG TAT ATG CTA GAA TG	6103 S	72	56/90	72/240
UVP	CGA ATC GTA ACC GTT CGT ACG AGA ATC GCT		90		

S, sense; *A*, antisense; *UVP*, universal vectorette primer. For all these reactions 1.5 mM $MgCl_2$ was used. The primers BIVS5′, BIVS3′, and BIVSe were used in PCRs across one of the introns in the β-tubulin gene to establish that cDNA was free of contamination by genomic DNA. Genomic DNA subjected to PCR across this intron yielded a product sized at ~340 bp. Complementary DNA subjected to PCR with the same primers (BIVS5′ and BIVS3′) did not give any product. When the PCR was applied to cDNA with primers (BIVS5′ and BIVSe) which included 150 bp of coding sequence (exon), a product of 150 bp was visualized. Genomic DNA subjected to PCR with these primers (BIVS5′ and BIVSe) yielded a product 500 bp in size, as predicted (data not shown).

amino acid motifs (18) in all these domains including the transduction domain (region c, containing the motif TGES, amino acids 149–152), the ATP phosphorylation site (region f, containing the phosphorylated aspartic acid residue, Asp 496), the FITC binding site (region g, containing an invariant lysine residue, Lys 949), and the ATP binding domain (region i). The linear organization of the conserved domains is also interesting. Some are spaced in a similar fashion in ATPase 1 as in other ATPases (e.g., regions a–c) while others are much further apart (e.g., regions f and g: in the putative proton pump from *Leishmania donovani* the intervening length is 85 amino acid residues, in the Ca^{2+} pump from *P. yoelii* it is 246 residues, and in ATPase 1 it is 442 residues). Presumably, folding patterns still bring together functionally related regions which may be separated by some distance in the primary sequence, with the membrane-spanning domains acting as "anchors" (Fig. 3 *a*).

Partial sequences from the other two ATPases (508 amino acids for ATPase 2 and 492 amino acids for ATPase 3) from *P. falciparum* also contain the highly conserved phosphorylation site (DKTGTLT, region f in Fig. 3 *a*, and Materials and Methods). In ATPase 1 there is a Phe residue preceding the phosphorylated Asp residue, unlike the eukaryotic consensus (which is ICSD). The only other published sequence of an ATP phosphorylation site containing Phe residues in this position comes from the cadmium pump of *Staphylococcus aureus* (44).

ATPase 1 Resembles the α Subunit of Na^+/K^+ ATPases

ATPase 1 shares greatest similarity to a mammalian Na^+/K^+ ATPase with 32% amino acid identity (39.7% similarity) in a comparison of conserved regions a–j containing a total of 282 amino acids (Table II). Comparison with ATPases of different ion transport specificities reveals similar though somewhat lower values (identity range 18–32.3%). The two malarial cation ATPase sequences available (*P. falciparum* ATPase 1 and the Ca^{2+} ATPase from *P. yoelii*) share 30% identity in a similar comparison with each other, and calmodulin and phospholamban binding sites (features of Ca^{2+} ATPases) are absent from ATPase 1.

Ouabain binding to mammalian Na^+/K^+ ATPases depends on two regions which were first identified in the sheep α_1 subunit, an ouabain-sensitive enzyme. The first lies between M1 and M2 (the first and second membrane-spanning segments), and requires a Gln residue (position 111) and an Asn residue (position 122). Mutagenesis at these positions increases similarity to the rodent form of enzyme (^{111}Gln→Arg or ^{122}Asn→Asp) and confers the property of ouabain resistance (23). ATPase 1 possesses both an Asn (amino acid 60, preceded by a conserved Asp residue) and a Gln residue (amino acid 49) separated by 10 amino acids in the appropriate region between M1 and M2 (Fig. 2 *a*), a feature shared with H^+/K^+ ATPases (69). The second region contains the motif EYTWLE which binds the steroid ring in ouabain and is found in the extracellular loop between M3 and M4 in the mammalian sequence. The malarial sequence has the motif EYTNHI (amino acids 434–439) in the corresponding position, a motif not found in ATPases with other ion transport specificities. Ouabain does not inhibit the multiplication of cultured *P. falciparum* in concentrations which inhibit the host enzyme (72).

Methods). The initiating codon (*ATG*), the termination signal (*TGA*), and nucleotide positions at key enzyme sites (Fig. 2 *a*) are indicated on the fine restriction map. (*b*) Southern blot analysis of ATPase 1. DNA from strain T9/96 was digested with the following enzymes and hybridized with inserts from plasmid clone p3.3 (*i*) or p7.8 (*ii*). Washing was with 0.2 × SSC/0.1% SDS at 65°C for 45 min followed by 0.1 × SSC/1%SDS at 65°C for 30 min. For *i* enzymes were (lane *1*) BclI; (lane *2*) ClaI; (lane *3*) EcoRI; (lane *4*) BglII; (lane *5*) HincII; (lane *6*) HpaII; (lane *7*) BstNI. Molecular weight markers were a 1-kb ladder (Bethesda Research Laboratories, Gaithersburg, MD). For *ii* enzymes were (lane *1*) HpaII; (lane *2*) HincII; (lane *3*) BglII; (lane *4*) EcoRI; (lane *5*) ClaI; (lane *6*) BclI. Molecular weight markers were HindIII-digested λ DNA.

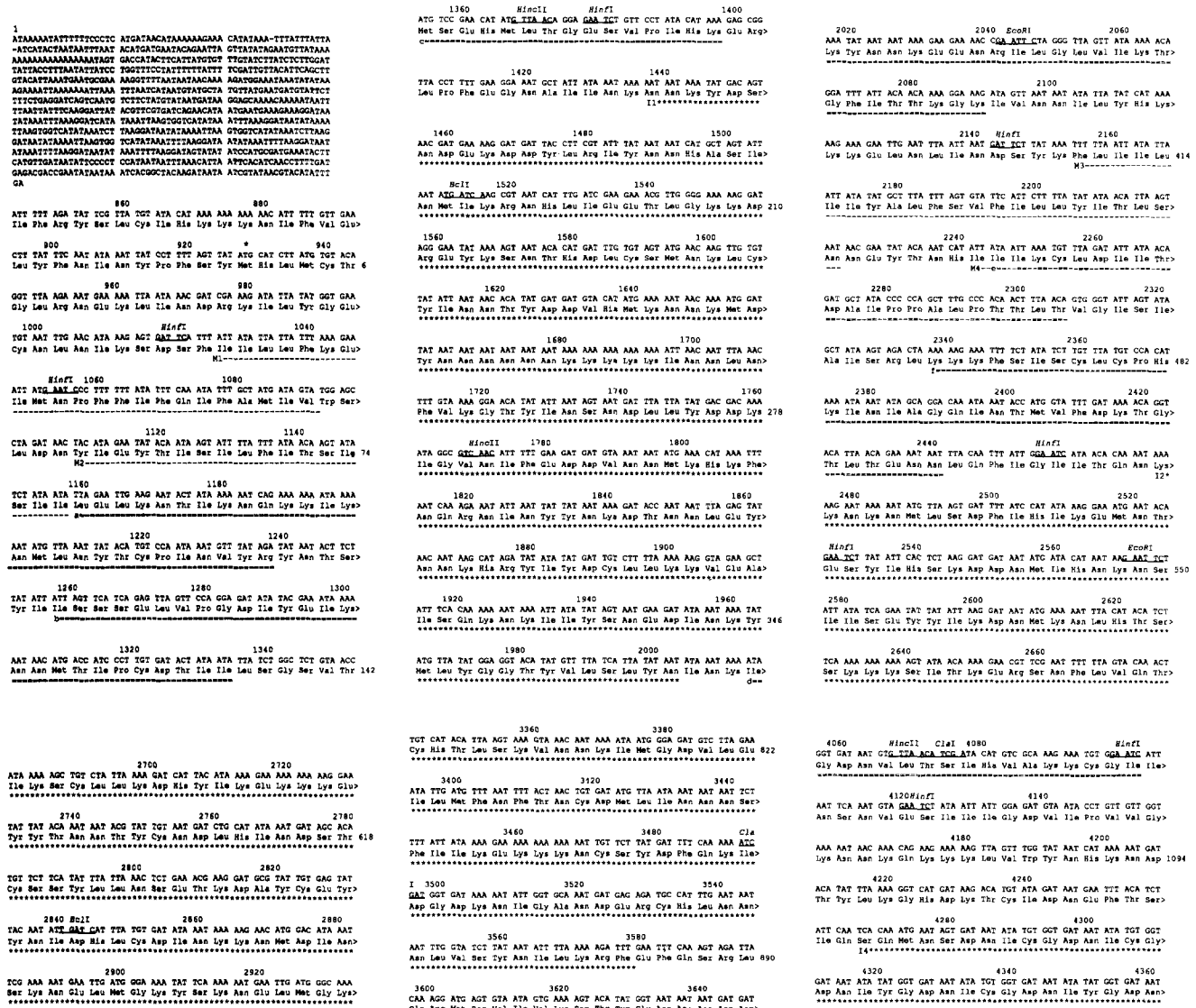

Figure 2. Nucleotide and derived amino acid sequence of ATPase 1. Nucleotide residues are numbered consecutively from the most 5′ nucleotide. Dashes in the 5′ untranslated portion of the sequence indicate nucleotides that could not be assigned unambiguously. Restriction enzyme sites (HinfI, HincII, BclI, EcoRI, ClaI) are shown with the nucleotides representing the respective recognition sites underlined. Amino acid sequences (three-letter code) are numbered consecutively from the initiating methionine which is marked by an asterisk above it. Transmembrane regions (*M1–M9*) are highlighted by underlining with a dashed line, and are indicated below the amino acid sequence. Conserved regions common to other P-type ATPases conventionally labeled a–j are underlined with a double-dashed line and are also indicated by lower case letters below the amino acid sequence. Inserts (I1–I4) peculiar to the malarial ATPase 1 sequence are shown by labels at the start of each insert sequence, followed by asterisks below the amino acid sequence. The boundaries for each insert have been determined after alignments with other ATPase sequences carried out by eye. These sequence data are available from EMBL/GenBank/DDBJ under accession number X65738.

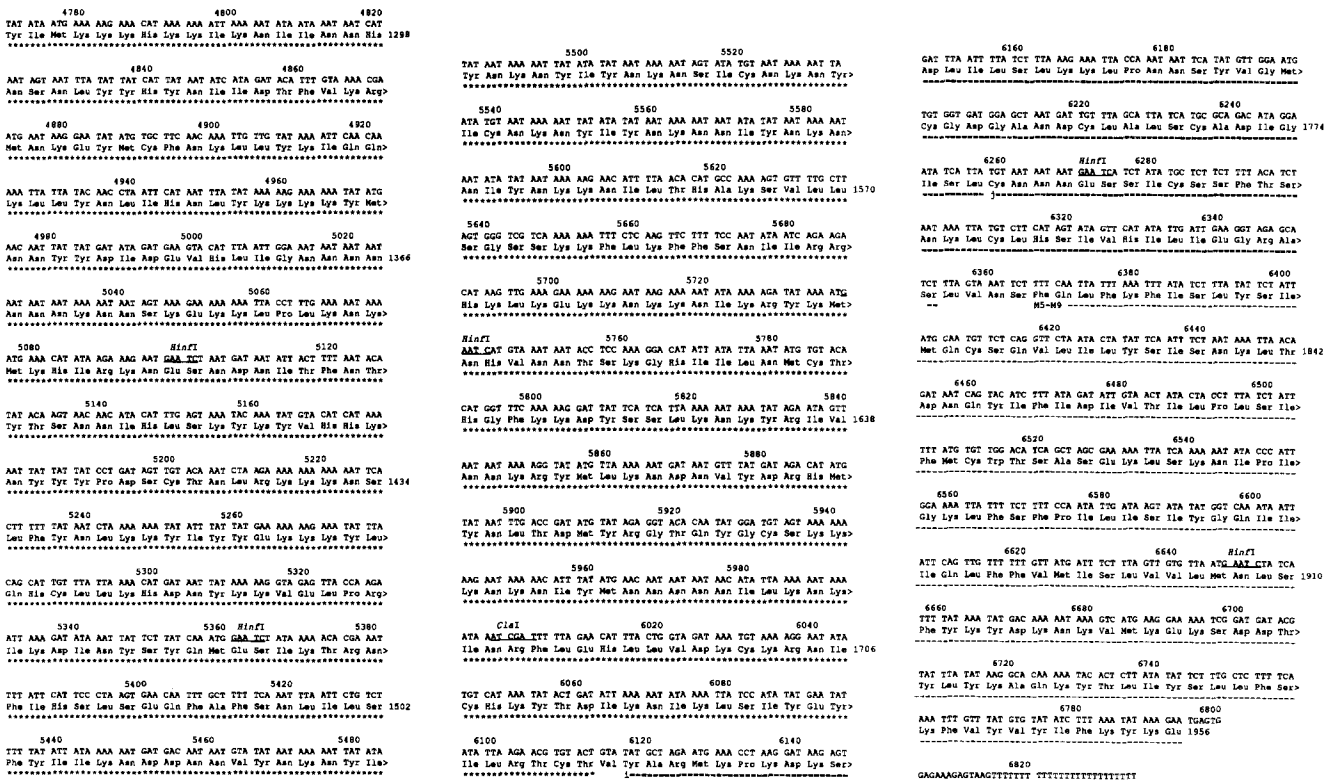

Figure 2.

To explore other structural features of ATPase 1, a distributed array processor–assisted search was carried out. In addition to cation ATPase sequences, similarities between hydrophobic segments in some sodium channel proteins and ATPase 1 were identified. The possible connection between ion channels and cation pumps was investigated further by searches carried out using the complete ATPase 1 sequence at higher stringency (100 PAM) against sequences from published channel proteins. The S3 segment of a potassium channel sequence was similar to a sequence in the terminal transmembrane region of ATPase 1 (present in the sixth membrane-spanning segment, M6, Fig. 3 *b*). A homologous sequence in sodium channels contributes to ion conductance (but not voltage gating, Fig. 3 *b*) (45, 74). Organellar mammalian Ca^{2+} ATPases failed to show this type of similarity in sequence when a search under identical conditions was carried out.

In addition to the conserved elements found in all cation ATPases, ATPase 1 has four novel regions of amino acid sequence we have called inserts (I1–4, shown in Fig. 2). Inserts are rich in amino acids common to malarial proteins, such as asparagine and lysine, and several also contain degenerate versions of tandemly repeated amino acid motifs. Insert 1 (I1, amino acids 171–359) contains 39 Asn in 188 residues (21%), I2 (amino acids 516–884) contains the motif $([S/I]KNELMGK[Y/T])_8$, I3 (amino acids 901–941) is composed mainly of Asn and Asp residues with one Tyr and Gly, and I4 (amino acids 1,114–1,729) contains the repeat $([S/G]DNI[C/Y/N])_{11}$. These inserts are located in regions which are poorly conserved between eukaryotic membrane ATPases. The Ca^{2+} pump from *P. yoelii* is also extended by two sequences similar to inserts (43), e.g., one region (containing a total of 59 residues) follows the conserved cytoplasmic domain f, is rich in Asn, Asp, Lys residues, and corresponds in position to I2 from ATPase 1.

Chromosomal Assignment and Northern Blot Analysis

ATPase 1 was localized to chromosome 5 by pulsed field gel electrophoresis (data not shown) and this assignment was independently confirmed (Dr. T. Wellems, National Institutes of Health, Bethesda, MD, personal communication). Analysis of total RNA from late ring/early trophozoite stages of development by hybridization of Northern blots with a fragment from ATPase 1 (p7CA insert, see Fig. 1 *a*) revealed a single transcript sized at 5.65–6.3 kb (Fig. 4). This mRNA transcript size is consistent with the size of the ORF (5.865 kb) and confirms that ATPase 1 is expressed during the erythrocytic phase of the life cycle of the parasite.

Analysis of Genetic Polymorphisms

The unexpected presence of tandem repeats in ATPase 1 which lacked homologues in ATPases from other organisms led us to investigate them further. Amplification by PCR across a region rich in Asn residues (nucleotide positions 3,377–3,965, including I3, Fig. 2) was undertaken from cDNA (from erythrocytic stage parasites) cloned into vector CDM8 using the primers Var 1 and Var 2 (Table I). The observed amplification product was of the size predicted from sequence analysis (data not shown), confirming that this highly A+T-rich region (>80%) was expressed.

To establish that the cDNA used in this and subsequent experiments was free of contamination with genomic parasite DNA, PCR amplification was undertaken across a region of

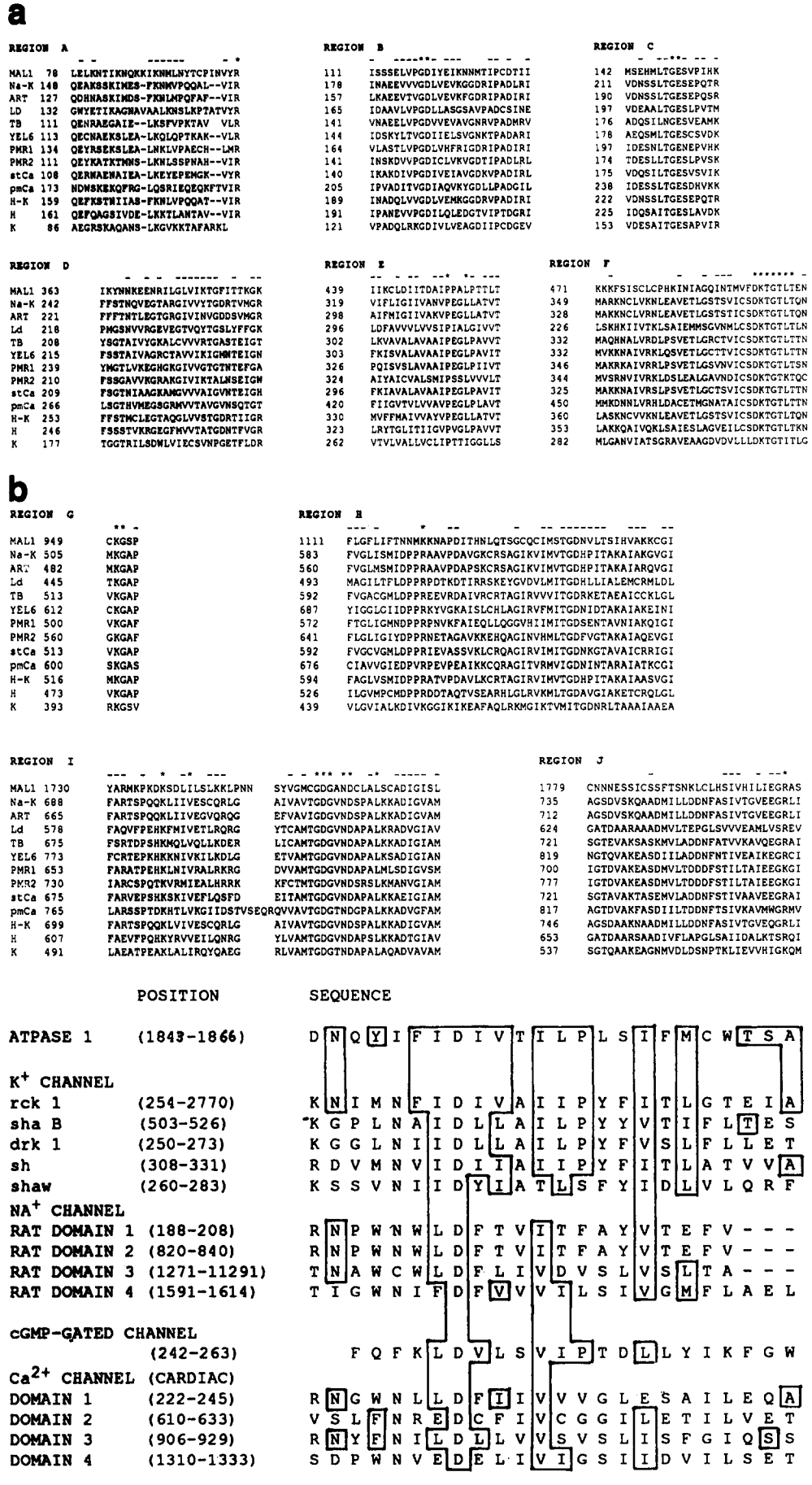

Figure 3. (*a*) Amino acid sequence comparisons of ATPase 1 with published cation ATPase sequences. This figure presents sequence alignments of mainly cytoplasmic conserved regions from ATPase 1 sequenced from *P. falciparum* clone T9/96 and some other published ATPase sequences. Alignments were initially made after comparison by eye with other ATPase sequences. Conservation of a residue (according to Dayhoff rules, i.e., identities or strict semiconservative substitutions, see Table II) in all the compared sequences is indicated by an asterisk, and conservation of a residue in four or more sequences is shown by a dash. Sequences are *MAL1*, ATPase 1 from *P. falciparum*; *Na-K*, the Na^+/K^+ ATPase from sheep (64); *ART*, the Na^+/K^+ ATPase from *Artemia sanfranciscana* (the brine shrimp) (3); *PMR1* and *PMR2*, Ca^{2+} ATPases from yeast (57); *stCa*, the slow twitch Ca^{2+} ATPase from sarcoplasmic reticulum (40); *pmCa* the Ca^{2+} ATPase from plasma membrane (62); *Ld*, the H^+ ATPase from *Leishmania donovani* (41); *TB*, the Ca^{2+} ATPase from *Trypanosoma bruceii* (51). *YEL6*, the Ca^{2+} ATPase from *P. yoelii* (43); *H-K*, the H^+/K^+ ATPase from rat stomach (64); *H*, the H^+ ATPase from *Saccharomyces cerevisiae* (61); and *K*, the K^+ ATPase from *Escherichia coli* (22). (*b*) Alignment of ATPase 1 transmembrane segment 6 with published ion channel sequences. The ATPase 1 sequence is taken as the consensus for these alignments. Identical amino acids and strict semiconservative substitutions (see Table II) are boxed. Alignments to sodium channels were made after searches were carried out on a DAP (see Materials and Methods). *sh* (Shaker), *sha B*, and *shaw* are *Drosophilia* genes, *rck 1* and *drk 1* are sequences obtained from rat brain cDNA (14); the sodium channel alignments have been constructed from sequences in rat sodium channel III (21, 28) and the remaining alignments are from reference 27.

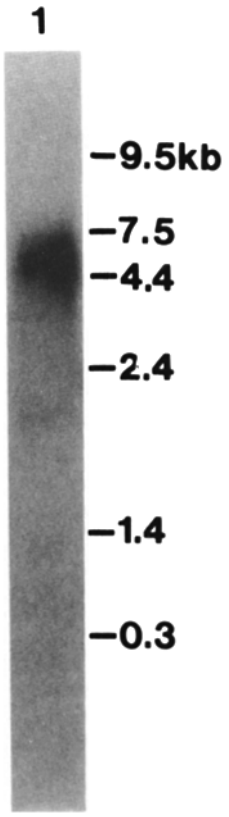

Figure 4. Northern blot analysis of ATPase 1. Total RNA isolated from the late ring/early trophozoite stage of infection (24–26 h after invasion) was transferred to nylon membrane and hybridized with *P. falciparum* ATPase 1 DNA from clone p7CA. After washing (0.2× SSC/0.1% SDS at 65°C for 45 min, followed by 0.1× SSC/0.1% SDS at 65°C for 30 min, autoradiographic exposure was for 72 h. Lane *1* contains RNA from strain T9/96. Molecular weight markers are RNA molecular weight markers (Bethesda Research Laboratories).

the *P. falciparum* β-tubulin gene which contained an intron of 344 bp (Table I, primers BIVS5′, BIVS3′, and BIVSe, reference 10). Genomic and cDNAs subjected to PCR using the same sets of primers yielded product sizes which differed by the size of the intron (Table I).

To examine the nature of tandem repeats within the ATPase 1 gene in different parasite strains, PCR was performed on DNA templates from 12 *P. falciparum* isolates using primers Var 3 and Var 4 to amplify part of I4 (Fig. 2 and Table I; nucleotide sequence 4,134–4,500 bp, corresponding to residues 1,070–1,191). There was clearly variation in the length of products obtained in this experiment (Fig. 5 *a*). The reasons for this length variation were examined by cloning of the PCR products and sequence analysis. This showed that the flanking regions of the degenerate tandem repeats in ATPase 1 were constant in sequence composition. Length increase in this region arose from duplication of one or more repeated elements within the conserved flanking units. There were no examples of variation in the length of the sequence that comprises a single repeat unit (Fig. 6). This presence of repeat units and variation in their numbers and type was a surprising feature of an ion-motive ATPase. To determine if functional constraints may have restricted variation in phylogenetically conserved regions of ATPase 1, a PCR experiment was carried out across regions a–c using primers Var 5 and Var 6 (nucleotide positions 1,089–1,417, Fig. 2 and Table I) from 12 isolates (Fig. 5 *b*). In contrast to the previous experiment, no length variation was seen in this region

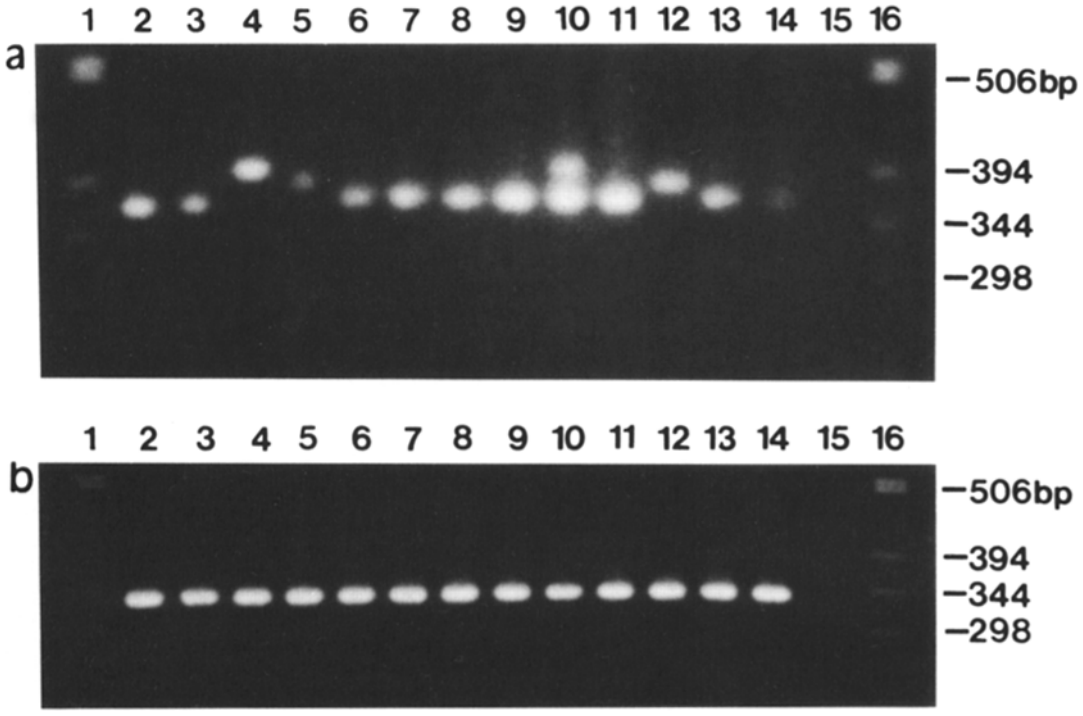

Figure 5. (*a*) PCR across tandem repeats in ATPase 1. PCR using oligonucleotides Var3 and Var4 (positions in Table I) was performed with genomic or cDNA templates from the following parasite strains under conditions described in Table I: (lane *2*) FCB; (lane *3*) FCR3; (lane *4*) 3D7A; (lane *5*) K1; (lane *6*) ITO4; (lane *7*) PAC+; (lane *8*) T9/94; (lane *9*) T9/96K+; (lane *10*) 7901 + T9/96; (lane *11*) T9/96; (lane *12*) HB3A; (lane *13*) Dd2; (lane *14*), cDNA from ITO4; (lane *15*) no template. Molecular weight markers (1-kb ladder) were in lanes *1* and *16*. Resolution was on 3% Nusieve™ agarose gels. (*b*) PCR across conserved regions a–c in ATPase 1. PCR using oligonucleotides Var5 and Var6 (positions in Table I), which flank ATPase 1 regions a–c (Table I), was applied to templates from the same parasites as in *a*. Molecular weight markers and gel running conditions were the same as in Figure 5 *a*.

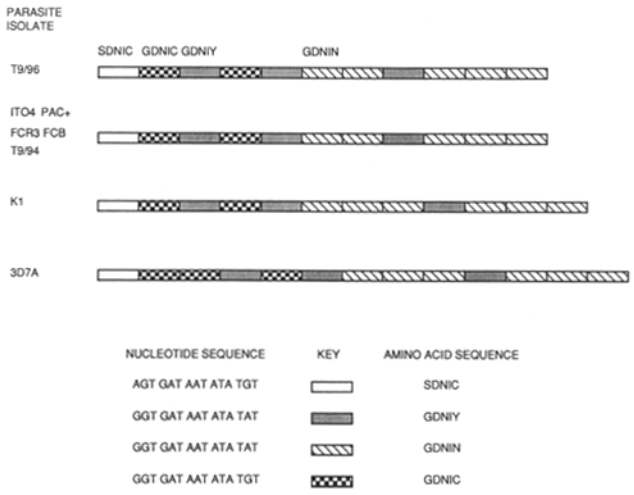

Figure 6. Repeat unit variation from different strains of *P. falciparum*. This schematic diagram illustrates the variation in the number and type of repeat units from different strains of *P. falciparum* in region I4 (Figs. 2 and 5 *a*, and Results). The region represented corresponds to nucleotides 4,288–4,442 (amino acids Ser^{1118} to Asn^{1172}) of the ATPase 1 sequence from T9/96 (Fig. 2). Each box represents one repeat unit, and differences in repeat unit sequence are indicated by differential shading (see key).

of ATPase 1. The degenerate tandem repeats contained in region I4 of ATPase 1 were shown to be expressed by PCR amplification of template from cDNA. The PCR product was identical in size to that obtained by amplification of genomic DNA template (Fig. 5 *a*).

Immunochemical Studies of ATPase 1

Polyclonal antibodies were raised in mice against two synthetic peptides derived from the amino acid sequence of ATPase 1 (Materials and Methods). The regions chosen were designed to minimize the probability of cross-reactivity with other malarial antigens, and were also significantly different between the three malarial ATPases we have isolated (Table II). The two polyclonal reagents were applied to samples on slides prepared for immunofluorescence as described (Materials and Methods). Negative controls did not reveal staining of parasites or erythrocytes (Fig. 7), and a positive control (monoclonal anti-p195) stained parasites as expected (data not shown). Both antipeptide antibodies stain a region around the periphery of the parasite as well as a region next to the pigment body. No staining of the red cell is seen. These findings suggest that ATPase 1 may be associated with the parasite plasma membrane and/or the parasitophorous vacuole.

Discussion

Similarity to Other P-Type ATPases

In eukaryotic cells, the Na^+/K^+ ATPase pump is the primary regulator of cell volume (23). The generation of electrochemical gradients (which includes the membrane potential of *P. falciparum*) and uptake of nutrients also depend upon this or similar enzymes (31). Using oligonucleotide probes we have cloned genes encoding a family of putative P-type ATPases from the most virulent human malarial parasite, *P. falciparum*. Sequencing and comparative analysis of one member of this family (ATPase 1) has shown many interesting features. It is the largest reported cation-ATPase sequence and resembles most closely the α-subunit of mammalian Na^+/K^+ ATPases. Extramembranous loops (between M1, M2 and M3, M4) contain conserved residues common to Na^+/K^+ ATPases, further supporting our suggestion for ion transport (Fig. 2). There is preservation of features shared by cation pumps such as similar hydropathy profiles and critical amino acid residues in conserved cytoplasmic regions (Fig. 3 *a*).

Most Na^+/K^+ ATPase sequences are highly conserved (>90% amino acid identity ranging from *Xenopus* to mammals), although an invertebrate pump (from *Artemia*, the brine shrimp) shares 70% identity with the other pumps (3). The malarial sequence would be the most divergent α-subunit Na^+/K^+ ATPase-like sequence identified to date, raising the possibility that it may no longer function as a P-type ATPase, although we believe that for the reasons discussed before, it retains cation transport capability. The primary structure of ATPase 1 may reflect the general tendency of

Table II. A Comparison of Amino Acid Sequences in Conserved Regions of Published ATPases and ATPase 1

Name of ATPase	Number of amino acids	Region compared (Identities/+semiconservative substitutions) a 27	b 26	c 15	d 26	e 21	f 34	g 5	h 49	i 47	j 32	Total 282	%
Na-K		7/8	11/13	6/7	8/11	7/10	9/11	3/3	15/18	18/24	7/7	91/112	32.3/39.7
ART		4/7	9/10	6/7	7/9	7/8	11/12	3/3	14/17	16/22	7/7	84/94	29.8/33.3
Ld		3/3	7/8	7/8	7/9	5/8	12/14	3/3	10/15	16/20	3/6	73/94	25.9/33.3
TB		5/6	9/14	6/6	6/9	6/6	9/10	3/3	12/15	19/22	6/7	81/97	28.7/34.4
YEL		3/4	10/11	7/8	6/6	6/8	10/10	4/4	12/17	21/23	6/7	85/98	30/34.8
PMR1		4/7	10/11	8/9	6/8	6/7	11/13	2/2	15/19	19/25	4/6	85/107	30/37.9
PMR2		7/9	12/15	8/10	7/8	6/8	8/11	2/2	13/16	16/21	5/7	84/107	29.8/37.9
$stCa^{2+}$		5/6	10/14	7/8	6/9	6/8	10/11	3/3	11/16	18/23	7/7	83/105	29.4/37.2
$pmCa^{2+}$		2/4	8/13	7/8	4/5	6/9	10/10	2/2	13/17	17/21	6/6	75/95	26.6/33.7
H-K		6/7	10/12	6/7	8/10	5/9	9/10	3/3	15/18	18/24	5/5	85/105	30/37.2
H		4/5	11/12	5/6	5/6	5/7	10/12	3/3	9/13	16/18	4/5	72/87	25.5/30.8
K		2/4	8/9	5/7	1/3	3/7	7/12	3/3	5/6	16/19	1/5	51/75	18/26.6

The number of identities and strict semiconservative substitutions (according to Dayhoff rules, I/V, L/M, E/D, K/R, F/Y, and additionally I/L) were calculated for each conserved region using the malarial sequence (ATPase 1) as a template (Fig. 3 *a*). The names of the ATPases listed are the same as for Fig. 3 *a*.

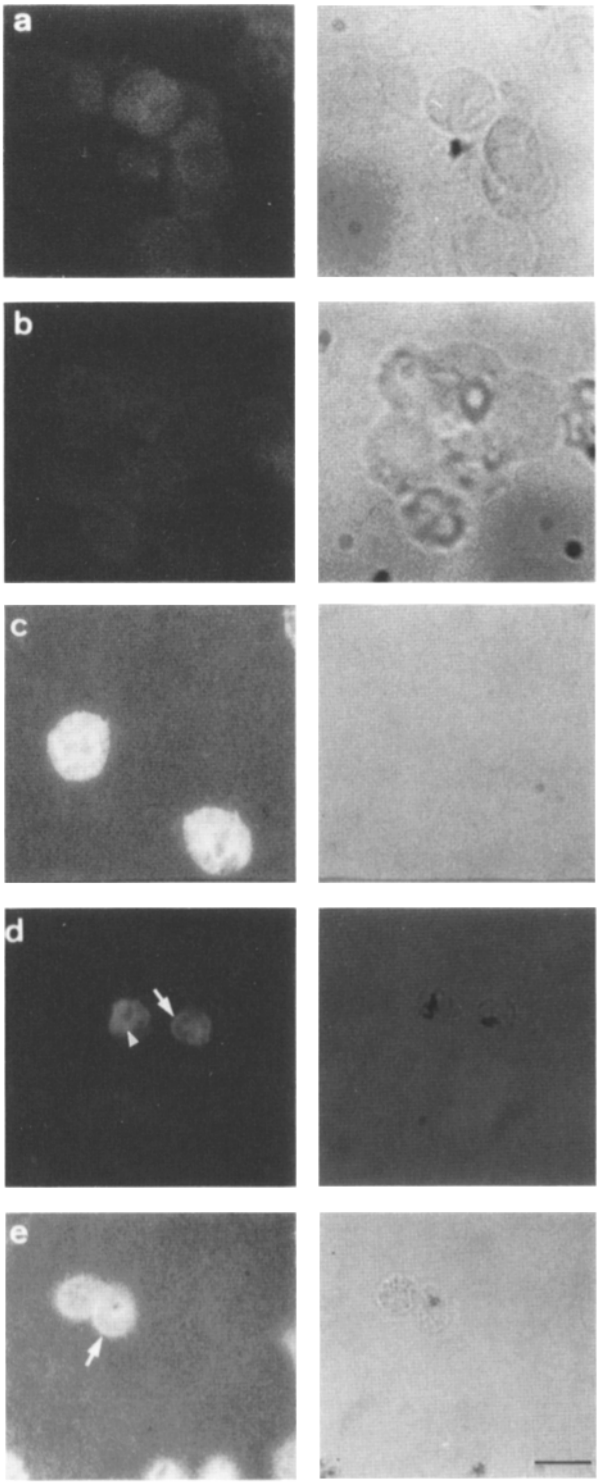

Figure 7. Localization of ATPase 1 by indirect immunofluorescence. Antibodies to two regions of derived amino acid sequence from ATPase 1 (anti-pepl and anti-pep2) were applied to slides from isolate T9/96. Figures show dark-field (fluorescence) and corresponding bright-field views. Haemazoin pigment in bright-field views corresponds to the location of parasitized red blood cells. (*a*) Negative control (no FITC-conjugated antibody) (*b*) negative control (no primary antibody); (*c*) anti-band 3 monoclonal antibody; (*d*) anti-pepl antibody; and (*e*) anti-pep2 antibody. The arrows indicate fluorescence in the region of the parasite plasma membrane/parasitophorous vacuole, and the arrowhead indicates staining internal to the parasite. Bar, 10 μm.

many proteins already sequenced from *P. falciparum* to be larger and less well conserved phylogenetically than from other lower eukaryotes, possibly as a consequence of immune selection pressure (discussed below) or intracellular location. The lipid environment of the pump may also influence the structure and activity of this enzyme (49, 69), as parasite membranes have a markedly different lipid composition compared to uninfected erythrocytes (24).

It has been suggested that P-type cation ATPases arose as an "evolutionary mosaic" when a primitive ATP hydrolyzing domain combined with different types of ion channels (19). The similarities in sequence between the third membrane-spanning sequences in some ion channel proteins (region S3), a voltage-gated G protein receptor, and a sequence in the terminal transmembrane hairpins of ATPase 1 (M6, Fig. 3 *b*) provide the first link between the cation ATPases and the superfamily of ion channels (27).

Erythrocytes infected with *P. falciparum* lose K^+ ions and gain Na^+ ions resulting in an erythrocyte cytosolic ratio (K^+/Na^+) of 0.8 (17), in spite of which the parasite continues to maintain a high (~6:8) K^+/Na^+ ratio (35). ATPase 1 could therefore play a role in regulating the K^+/Na^+ ratio of the parasite cytosol in the face of changes in the ionic composition of host cell cytoplasm. Alternative ion transport specificities for ATPase 1, particularly K^+/H^+ countertransport or H^+ transport, cannot be excluded. The K^+/H^+ ATPase is very closely related to Na^+/K^+ ATPases (69) and could contribute in a similar way to the regulation of cation metabolism in infected erythrocytes by parasites (31). The recent cell-free culture of the erythrocytic stage of *P. falciparum* confirms that the parasite regulates its internal ionic composition independently of the red cell, and may allow direct testing of potential inhibitors of this enzyme (75).

Most transporter systems (such as those involved with sugar, amino acid, and glucose uptake) which have been studied in infected erythrocytes become maximally active first at the late ring/early trophozoite stage of development of the parasite. This is the stage at which most RNA from ATPase 1 is detected (data not shown).

Polymorphism in ATPase 1

Between conserved regions of ATPase 1, there appears to have been interposition of amino acids, some of which are characteristic of malarial antigens (such as Asn and Lys) and tandemly repeated motifs (60). One of the regions containing tandem repeats has been shown to vary in repeat unit type and number between different *P. falciparum* isolates. A simple crossover event at meiosis (perhaps with conversion) could account for the observed variation in numbers of tandem repeats. Differences between repeat units arise from one of two base transitions (G→A and A→G) or one transversion (T→A), each of which changes the repeat structure by one amino acid (Fig. 6). Variation in the number and type of tandem repeat units (between 11–13 in the isolates sequenced) are the means whereby polymorphisms are generated in this region of ATPase 1. The region containing polymorphic tandem repeats and another region rich in Asn and Lys residues are both expressed in mRNA. Partial sequence analysis of ATPases 2 and 3 shows that the unusually divergent nature of ATPase 1 is also a feature of the other two ATPases.

The variations in ATPase 1 are not likely to be due to PCR

artefacts for the following reasons: (*a*) more than one independent clone has been sequenced in all studies on ATPase 1 (strain T9/96); and (*b*) Lockyer et al. (38) have carried out a comparison of polymorphisms in the CSP gene and found PCR across tandem repeats in the malarial genome to be reproduced faithfully in a comparison of at least 10 independent clones. Studies on allelic variation in cDNA from the β-tubulin gene includes information on some of the parasite isolates that we also have examined (76). In coding sequence, the variation results mainly from single nucleotide changes (10 transversions and three transitions in three strains of *P. falciparum*), with one 3-nucleotide insertion/deletion event. In the β-tubulin sequences from three strains that have been examined, the degree of variation appears to be constrained by selection pressures which may result from limits imposed by function (see below).

We suggest that the polymorphism in tandem repeats seen among different isolates in the ATPase 1 gene (region I4) is maintained as a consequence of selection pressure, possibly due to immune responses from the host. As this region has no attributable contribution to ATP hydrolysis or ion transport, the putative transporter domains within ATPase 1 may not be affected by the observed variation. ATP hydrolysis and cation transport remain to be demonstrated in ATPase 1. However, functionally constrained regions present in other ATPases should be conserved in ATPase 1 from *P. falciparum* if it is capable of cation transport. To test this hypothesis, length variation in sequence encoding conserved cytoplasmic regions a–c was examined between 12 strains. As expected, this region was length invariant between isolates, supporting the notion of functionally imposed constraints to variation.

Comparison with Other Malarial Genes and Implications for Antigens

Many malarial antigens with no attributable functions contain tandemly repeated elements, and some are also rich in asparagine and lysine residues (2, 60). Cross-reactivity of antibodies directed both to asparagine-rich regions and tandem repeats has been amply demonstrated. Antigens are also polymorphic in the nature and types of repeats which are encoded. Although the tandem repeats in ATPase 1 have not yet been identified in the antigens isolated, their polymorphic and repetitive nature is consistent with the behavior observed in some antigens sequenced from different isolates.

Malarial gene products vary from 20 to 90% identity in amino acid sequence as compared with mammalian homologues (32). The sequence we have isolated (ATPase 1) is of low similarity to mammalian homologues. Other housekeeping genes share some of the properties of ATPase 1; for example, the RNA POL II gene product from *P. falciparum* is the largest sequenced so far (36) and the recently published RNA POL III (37) sequence is also enlarged by tandemly repeated elements rich in Asn and Lys residues. Likewise, the DHFR (65), pfmdr (13), and *P. yoelii* Ca^{2+} ATPase (43) gene products contain regions rich in Asn residues. However, the phenomenon of polymorphism of tandem repeats and length invariance of other regions has been shown for the first time in a housekeeping gene like ATPase 1.

Immunochemical studies suggest that ATPase 1 is to be found in the region of the parasite plasma membrane and perhaps the food vacuole. The "smokescreen" hypothesis for antigens argues that repeated exposure of the host immune system to antigens serves to delay affinity maturation of antibodies and hence effective immune responses (29). Affinity maturation may be delayed because of the presence of T cell–independent epitopes found in repetitive domains of proteins through a "*cis*-acting" strategy (for review see reference 60). How can this be reconciled with the location of antigens which are not found on the surface of the infected erythrocyte or the merozoite and are not therefore exposed to immunological scrutiny during the normal life cycle of the parasite? We suggest that in most malarial infections exposure of the immune system to (intraparasitic) antigens takes place during the stage of clearance of parasites which do not complete the life cycle (39). Processing of parasite proteins may therefore provide a continuing antigenic stimulus early in infection which subsequently hampers antibody maturation responses, and delays or prevents effective immunological attack at critical stages of the life cycle of the parasite. The maintenance of diversity in many parasite proteins which are not directly exposed to the host's immune system may therefore allow a few proteins with essential functions (such as invasion of red cells or cytoadherence) to perform in a less impeded fashion. This "*trans*-acting" strategy (60) may explain why so many parasite proteins not directly accessible to antibodies are polymorphic (including perhaps ATPase 1). Those proteins which are highly conserved phylogenetically such as parasite calmodulin and tubulin are probably subject to strict functional constraints and therefore cannot tolerate the insertion of novel sequences (including tandemly repeated amino acids).

The recent observations on a connection between the intraerythrocytic parasite and the plasma offer an alternative explanation for polymorphisms in antigens located in the parasitophorous vacuolar membrane or the parasite plasma membrane, but they cannot explain polymorphisms in proteins which are predominantly intraparasitic (48). These observations also offer the intriguing possibility of inhibiting the function of some parasite gene products such as ATPase 1 without the prior requirement of inhibitor penetrating the erythrocyte membrane and cytosol. Many of the observations in this paper are amenable to further genetic and functional studies on this family of sequences.

We are very grateful to Dr. T. Wellems for providing chromosomal Southern blots; to Dr. A Craig for cDNA and some mRNA; to Dr. J. F. Collins for database searches using the distributed array processor; to Dr. H. Dave for peptides; to Dr. A. Willis for peptide purity assays; J. Cordell for anti-band 3 antibody; M. Shepherd and J. Lamb for oligonucleotide synthesis; Drs. N. M. Green and G. G. Germino for valuable discussions; and Sir David Weatherall and Drs. S. Kyes, B. Elford, N. J. White, D. Jackson, and J. Bell for support.

S. Krishna gratefully acknowledges support from the Wellcome Trust and the Medical Research Council and G. Cowan from the United Nations Development Programme/WORLD BANK/World Health Organization Special Programme for Research and Training in Tropical Diseases.

Received for publication 8 May 1992 and in revised form 5 October 1992.

References

1. Allen, D. G., D. A. Eisner, and S. C. Wray. 1985. Birthday present for digitalis. *Nature (Lond.).* 316:674–675.
2. Ardeshir, F., R. F. Howard, S. Viriyakosol, O. Arad, and R. T. Reese. 1990. Cross-reactive asparagine-rich determinants shared between several blood-stage antigens of *Plasmodium falciparum* and the circumsporozoite protein. *Mol. Biochem. Parasitol.* 40:113–126.

3. Baxter-Lowe, L. A., J. Z. Guo, E. E. Bergstrom, and L. E. Hokin. 1989. Molecular cloning of the Na,K-ATPase α-subunit in developing brine shrimp and sequence comparison with higher organisms. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 257:181–187.
4. Brown, C. M., P. A. Stockwell, C. N. A. Trotman, and W. P. Tate. 1990. Sequence analysis suggests that tetra-nucleotides signal the termination of protein synthesis in eukaryotes. *Nucleic Acids Res.* 18:6339–6345.
5. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
6. Choi, I., and J. L. Mego. 1988. Purification of *Plasmodium falciparum* digestive vacuoles and partial characterization of the vacuolar membrane ATPase. *Mol. Biochem. Parasitol.* 3:71–78.
7. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
8. Church, G. M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* 81:1991–1995.
9. Coulson, A. F. W., J. F. Collins, and A. Lyall. 1987. Protein and nucleic acid sequence database searching: a suitable case for parallel processing. *The Computer Journal.* 30:420–424.
10. Delves, C. J., R. G. Ridley, M. Goman, S. P. Holloway, J. E. Hyde, and J. G. Scaife. 1989. Cloning of a β-tubulin gene from *Plasmodium falciparum*. *Mol. Microbiol.* 3:1511–1519.
11. Dluzewski, A. R., I. T. Ling, K. Rangachari, P. A. Bates, and R. J. M. Wilson. 1984. A simple method for initiating viable mature parasites of *Plasmodium falciparum* from cultures. *Trans. R. Soc. Trop. Med. Hyg.* 78:622–624.
12. Ellory, J. C., B. C. Elford, and C. I. Newbold. 1988. Introductory remarks: transport mechanisms across cell membranes. *Parasitology.* 96:S5–S9.
13. Foote, S. J., J. K. Thompson, A. F. Cowman, and D. J. Kemp. 1989. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell.* 57:921–930.
14. Frech, G. C., A. M. J. VanDongen, G. Schuster, A. M. Brown, and R. H. Joho. 1989. A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning. *Nature (Lond.).* 340: 642–645.
15. Gero, A. M., E. M. A. Bugledich, A. R. P. Paterson, and G. P. Jamieson. 1988. Stage-specific alteration of nucleoside membrane permeability and nitrobenzylthioinosine insensitivity in *Plasmodium falciparum* infected erythrocytes. *Mol. Biochem. Parasitol.* 27:159–170.
16. Ghosh, J., M. Ray, S. Sarkar, and A. Bhaduri. 1990. A high affinity Ca^{2+}-ATPase on the surface membrane of *Leishmania donovani* promastigote. *J. Biol. Chem.* 265:11345–11351.
17. Ginsburg, H., S. Handeli, S. Friedman, R. Gorodetsky, and M. Krugliak. 1986. Effect of red blood cell potassium and hypertonicity on the growth of *Plasmodium falciparum* in culture. *Parasitenkunde.* 72:185–199.
18. Green, N. M. 1989. ATP-driven cation pumps: alignment of sequences. *Biochem. Soc. Trans.* 17:972.
19. Green, N. M., and D. H. MacLennan. 1989. ATP driven ion pumps: an evolutionary mosaic. *Biochem. Soc. Trans.* 17:819–822.
20. Greenwood, B. M. 1990. Populations at risk. *Parasitol. Today.* 6:188.
21. Guy, H. R., and F. Conti. 1990. Pursuing the structure and function of voltage-gated channels. *Trends Neurosci.* 13:201–206.
22. Hesse, J. E., L. Wieczork, K. Altendorf, A. S. Reicin, E. Dorus, and W. Epstein. 1984. Sequence homology between two membrane transport ATPases, the Kdp-ATPase of *Escherichia coli* and the Ca^{2+}-ATPase of sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 81:4746–4750.
23. Horisberger, J.-D., V. Lemas, J.-P. Kraehenbuhl, and B. C. Rossier. 1991. Structure-function relationship of Na, K-ATPase. *Annu. Rev. Physiol.* 53:565–584.
24. Hsiao, L. L., R. J. Howard, M. Aikawa, and T. F. Taraschi. 1991. Modification of host cell membrane lipid composition by the intraerythrocytic human malaria parasite *Plasmodium falciparum*. *Biochem. J.* 272: 121–132.
25. Hu, N., and N. Messing. 1982. The making of strand-specific M13 probe. *Gene (Amst.).* 17:271–277.
26. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in λgt11 and λgt11. *In* DNA Cloning. Volume 1. A Practical Approach. D. M. Glover, editor. IRL Press Limited, Oxon, UK. 49–78.
27. Jan, L. Y., and Y. N. Jan. 1990. A superfamily of ion channels. *Nature (Lond.).* 345:672.
28. Kayano, T., M. Noda, V. Flockerzi, H. Takahashi, and S. Numa. 1988. Primary structure of the rat brain sodium channel III deduced from the cDNA sequence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 228:187–194.
29. Kemp, D. J., R. L. Coppel, H. D. Stahl, A. E. Bianco, L. M. Corcoran, P. McIntyre, C. J. Langford, J. M. Favaloro, P. E. Crewther, G. V. Brown, G. F. Mitchell, J. G. Culvenor, and R. Anders. 1986. Genes for antigens of *Plasmodium falciparum*. *Parasitology.* 91:S83–S108.
30. Kramer, R., and H. Ginsburg. 1991. Calcium transport and compartment analysis of free and exchangeable calcium in *Plasmodium falciparum*–infected red blood cells. *J. Protozool.* 38:594–601.
31. Krishna, S., and L. L. Ng. 1989. Cation metabolism in malaria-infected red cells. *Exp. Parasitol.* 69:402–406.
32. Krishna, S., and K. J. H. Robson. 1991. Approaches to investigating cation metabolism in malaria-infected erythrocytes. *In* Biochemical Protozoology. G. M. Coombs and M. J. North, editors. Taylor and Francis Ltd., London, UK. 404–414.
33. Krishna, S., and L. Squire-Pollard. 1990. Calcium metabolism in malaria-infected erythrocytes. *Parasitol. Today.* 6:196–198.
34. Lambros, C., and J. P. Vanderburg. 1979. Synchronisation of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65:418–420.
35. Lee, P., Z. Ye, K. Van Dyke, and R. G. Kirk. 1988. X-ray microanalysis of *Plasmodium falciparum* and infected red blood cells: effects of qinghaosu and chloroquine on potassium, sodium, and phosphorus composition. *Am. J. Trop. Med. Hyg.* 39:157–165.
36. Li, W.-B., D. J. Bzik, H. Gu, M. Tanaka, B. A. Fox, and J. Inselberg. 1989. An enlarged largest subunit of *Plasmodium falciparum* RNA polymerase II defines conserved and variable RNA polymerase domains. *Nucleic Acids Res.* 17:9621–9635.
37. Li, W.-B., D. J. Bzik, M. Tanaka, H. Gu, B. A. Fox, and J. Inselburg. 1991. Characterisation of the gene encoding the largest subunit of *Plasmodium falciparum* RNA polymerase III. *Mol. Biochem. Parasitol.* 46:229–240.
38. Lockyer, M. J., K. Marsh, and C. I. Newbold. 1989. Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein. *Mol. Biochem. Parasitol.* 37:275–280.
39. Looareesuwan, S., T. M. E. Davis, S. Pukrittayakamee, W. Supanaranond, D. Varunee, K. Silamut, S. Krishna, S. Boonamrung, and N. J. White. 1991. Erythrocyte survival in severe falciparum malaria. *Acta Trop.* 48:263–270.
40. MacLennan, D. H., C. J. Brandl, B. Korczak, and N. M. Green. 1985. Amino acid sequence of a Ca^{2+} and Mg^{2+} dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature (Lond.).* 316:696–700.
41. Meade, C. J., J. Shaw, S. Lemaster, G. Gallagher, and J. R. Stringer. 1987. Structure and expression of a tandem gene pair in *Leishmania donovani* that encodes a protein structurally homologous to eucaryotic cation-transporting ATPases. *Mol. Cell. Biol.* 7:3937–3946.
42. Mikkelsen, R. B., D. F. H. Wallach, E. Van Doren, and E. A. Nillini. 1986. Membrane potential of erythrocytic stages of *Plasmodium chabaudi* free of host cell membrane. *Mol. Biochem. Parasitol.* 21:83–92.
43. Murakami, K., K. Tanabe, and S. Takada. 1990. Structure of a *Plasmodium yoelii* gene encoded protein homologous to the Ca^{2+} ATPase of a rabbit skeletal muscle sarcoplasmic reticulum. *J. Cell Sci.* 97:487–495.
44. Nucifora, G., L. Chu, T. K. Mistra, and S. Silver. 1989. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cad A* gene results from a cadmium-efflux ATPase. *Proc. Natl. Acad. Sci. USA.* 86:3544–3545.
45. Oiki, S., W. Danho, and M. Montal. 1988. Channel protein engineering: synthetic 22-mer peptide from the primary structure of the voltage-sensitive sodium channel forms ionic channels in lipid bilayers. *Proc. Natl. Acad. Sci. USA.* 85:2393–2397.
46. Pasvol, G., R. J. M. Wilson, M. E. Smalley, and J. Brown. 1978. Separation of viable schizont-infected red cells of *Plasmodium falciparum* from human blood. *Ann. Trop. Med. Parasitol.* 72:87–88.
47. Peterson, D. S., D. Walliker, and T. Wellems. 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proc. Natl. Acad. Sci. USA.* 85:9114–9118.
48. Pouvelle, B., R. Spiegel, L. Hsiao, R. J. Howard, R. L. Morris, A. P. Thomas, and T. F. Taraschi. 1991. Direct access to serum macromolecules by intraerythrocytic malaria parasites. *Nature (Lond.).* 353: 73–75.
49. Racker, E. 1985. Reconstitutions of Transporters, Receptors, and Pathological States. Academic Press, Inc., Orlando, FL.
50. Rademacher, T. W., R. B. Parekh, and R. A. Dwek. 1988. Glycobiology. *Annu. Rev. Biochem.* 57:785–838.
51. Revelard, P., and E. Pays. 1991. Structure and transcription of a P-ATPase gene from *Trypanasoma bruceii*. *Mol. Biochem. Parasitol.* 46:241–252.
52. Riley, J., R. Butler, D. Ogilvie, R. Finnear, D. Jenner, S. Powell, R. Anand, J. C. Smith, and A. F. Markham. 1990. A novel rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. *Nucleic Acids Res.* 18:2887–2890.
53. Rimm, D. L., D. Horness, J. Kucera, and F. R. Blattner. 1980. Construction of coliphage λ Charon vectors with *BamH*I cloning sites. *Gene (Amst.).* 12:301–309.
54. Robson, K. J. H., and M. W. Jennings. 1991. The calmodulin gene of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 46:19–34.
55. Robson, K. J. H., J. R. S. Hall, M. W. Jennings, T. J. R. Harris, K. Marsh, C. I. Newbold, V. E. Tate, and D. J. Weatherall. 1988. A highly conserved amino acid sequence in thrombospondin, properdin and in proteins from sporozoites and blood stages of a human malaria parasite. *Nature (Lond.).* 335:79–82.
56. Robson, K. J. H., J. R. S. Hall, L. C. Davies, A. Crisanti, A. V. S. Hill, and T. E. Wellems. 1990. Polymorphism of the TRAP gene of *Plasmodium falciparum*. *Proc. R. Soc. Lond.* 242:205–216.
57. Rudolph, H. K., A. Antebi, G. R. Fink, C. M. Buckley, J. LeVitre, L. S. Davidow, J. Mao, and D. T. Moir. 1989. The yeast secretory pathway

is perturbed by mutations in *PMR*1, a member of a Ca^{2+} ATPase family. *Cell.* 58:133–145.
58. Sachs, G., E. Carlsson, P. Lindberg, and B. Wallmark. 1988. Gastric H,K-ATPase as therapeutic target. *Annu. Rev. Pharmacol. Toxicol.* 28:269–284.
59. Sambrook, J. A., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
60. Schofield, L. 1991. On the function of repetitive domains in protein antigens of Plasmodium and other eukaryotic parasites. *Parasitol. Today.* 7:99–105.
61. Serrano, R. 1988. Structure and function of proton translocating ATPases in plasma membranes of plant and fungi. *Biochim. Biophys. Acta.* 947: 1–28.
62. Shull, G. E., and J. Greeb. 1988. Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. *J. Biol. Chem.* 262:8846–8657.
63. Shull, G. E., and J. B. Lingrel. 1986. Molecular cloning of the rat stomach (H^+ and K^+)-ATPase. *J. Biol. Chem.* 261:16788–16791.
64. Shull, G. E., A. Schwartz, and J. B. Lingrel. 1985. Amino acid sequence of the catalytic subunit of the ($Na^+ + K^+$)ATPase deduced from a complementary DNA. *Nature (Lond.).* 316:691–695.
65. Snewin, V. A., S. M. England, P. F. G. Sims, and J. E. Hyde. 1989. Characterisation of the dihydrofolate reductase–thymidylate synthetase gene from human malaria parasites highly resistant to pyrimethamine. *Gene (Amst.).* 76:41–52.
66. Staden, R. 1982. Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. *Nucleic Acids Res.* 10:4731–4751.
67. Staden, R. 1986. The current status and probability of our sequence handling software. *Nucleic Acids Res.* 14:217–231.
68. Sturchler, D. 1989. How much malaria is there worldwide? *Parasitol. Today.* 5:39–40.
69. Sweadner, K. 1989. Isoenzymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta.* 988:185–220.
70. Tanabe, K. 1990. Glucose transport in malaria infected erythrocytes. *Parasitol. Today.* 6:225–229.
71. Tanabe, K., R. B. Mikkelsen, and D. Wallach. 1982. Calcium transport of *Plasmodium chabaudi*-infected erythrocytes. *J. Cell Biol.* 93: 680–684.
72. Tanabe, K., A. Izumo, and K. Kageyama. 1986. Growth of *Plasmodium falciparum* in sodium enriched erythrocyte. *Am. J. Trop. Med. Hyg.* 35:476–478.
73. Tanabe, K., M. Mackay, M. Goman, and J. G. Scaife. 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *J. Mol. Biol.* 195:273–287.
74. Tosteson, M. T., D. S. Auld, and D. C. Tosteson. 1989. Voltage-gated channels formed in lipid bilayers by a positively charged segment of the Na-channel polypeptide. *Proc. Natl. Acad. Sci. USA.* 86:707–710.
75. Trager, W., and J. Williams. 1992. Extracellular (axenic) development *in vitro* of the erythrocytic cycle of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA.* 89:5351–5355.
76. Van Belkum, A., C. Janse, and B. Mons. 1991. Nucleotide sequence variation in the β-tubulin genes from *Plasmodium berghei* and *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 47:251–254.
77. White, N. J. 1986. Pathophysiology. *In* Malaria. G. T. Strickland, editor. W. B. Saunders Company, London, UK. 55–90.
78. Zilberstein, D., and D. M. Dwyer. 1988. Identification of a surface membrane proton-translocating ATPase in promastigotes of the parasitic protozoan *Leishmania donovani*. *Biochem. J.* 256:13–21.