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MOLECULAR GENETIC ANALYSIS OF PARASITE SURVIVAL
IN P. FALCIPARUM MALARIA

ANNUAL REPORT

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<p>The human malaria parasite continues to pose a significant health problem to one half of the world's population. Entry into endemic areas places those individuals at risk of morbidity and mortality from this protozoan parasite. The lack of effective immunoprophalaxis has refocused efforts at defining the unique biology of this parasite in order to develop more effective chemotherapeutic agents. Work in my laboratory has concentrated on defining the mode of gene regulation in this organism and providing insight into the strategies developed by the parasite to overcome innate host immune responses. The long-term goal of this research is to manipulate the genome of the organism by developing approaches enabling the cloning of parasite DNA and reintroduction of those altered genes back into the parasite's genome. Progress in the past year has been achieved in constructing</p>					
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and characterizing yeast artificial chromosome (YAC) libraries from *P. falciparum* and utilizing these sources of stably cloned DNA to define putative promoter elements for asexual intraerythrocytic stage genes. YAC clones have also facilitated the discovery of new transcription units and the relationship of these genes to previously defined chromosomal rearrangements. These studies have resulted in the identification of several DNA sequences which are the sites for nuclear protein binding. These DNA elements have been used in attempts to drive the expression of heterologous reporter genes in DNA transfection studies.

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BODY OF REPORT

BACKGROUND AND SIGNIFICANCE

The complex life-cycle of the malaria parasite results from the ordered expression of specific genes which dictate the pathways of development and differentiation. Despite the central role of these pathways in the physiology of the parasite, little is known of the mechanisms by which gene expression is regulated in this protozoan. Characterization of these pathways requires 1) access to the various stages of the organism, both within its vertebrate and invertebrate hosts, 2) the ability to stably clone and propagate DNA sequences which flank genes of interest and finally, 3) a functional assay for gene regulation. The malaria parasite has proven to pose significant problems for each of these requirements. Although culture methods are available for the asexual, intraerythrocytic stages of *P. falciparum*, gametocyte stages are less reliably obtained in culture and insect stages require infected mosquitoes for isolation of biologically active material. For these reasons, our efforts have focussed initially on the asexual, intra-erythrocytic stages of *P. falciparum*. Although the successful cloning of a *P. falciparum* erythrocytic stage gene was first described in 1985, it rapidly became apparent that the unusually high (>80%) A+T content of *P. falciparum* DNA was unstable in traditional prokaryotic hosts, resulting in large deletion of sequences, particularly in flanking regions. However, it is precisely these flanking regions where regulatory sequences usually reside for eukaryotic genes that made this technical problem so troubling.

Work over the last two years in the laboratory has focussed on developing approaches for the stable cloning of flanking DNA, the identification of putative promoter regions in that DNA and the development of strategies for the reintroduction and expression of DNA back into the erythrocytic stage parasite. The strategy we have adopted is to utilize the fission yeast, *Saccharomyces cerevisiae*, as a host for the DNA of *P. falciparum* in the form of linear, artificial chromosomes. The rationale behind this approach stemmed from the similarity in A+T content of the DNA of these two organisms and the possibility of using yeast artificial chromosome vectors to construct libraries of *P. falciparum* DNA. As will be described below, this strategy proved to be very successful. With YAC clones for a variety of erythrocytic stage genes in hand, the identification of putative promoter regions has been possible. These clones, which average 100kb in length, have also provided us the opportunity to investigate the distribution of genes along the chromosome by developing a method of transcription mapping. The combination of YAC cloning and transcription mapping has led to the identification of several new genes and the definition of intergenic regions where transcriptional termination and re-initiation has been mapped. These structural studies have provided the DNA targets for functional studies into their role in gene regulation.

One approach to defining the functional role of putative promoter regions has been to determine if these sequences are the sites for specific nuclear protein interactions, indicative of the interaction of transcription factors with their promoters. The interactions can be visualized by electrophoretic mobility shift assays (EMSA) using DNA targets and nuclear protein extracts from infected erythrocytes. Since transcription of many erythrocytic genes is stage-specific, some of the complexes defined by EMSA should reflect this stage specificity.

Protein-DNA complexes have been described in the last year which fulfill these criteria and are likely to represent the interaction of promoters with transcription factors.

Finally, the definition of a promoter is ultimately a functional one, describing a DNA element capable of directing the transcription of a reporter sequence. This approach requires the ability to reintroduce exogenous DNA back into a cell type of interest. In the case of the infected erythrocyte, multiple membrane barriers exist which make this problem more complicated than a typical eukaryotic cell. Successful transformation of an intracellular pathogen has not been achieved as of this writing. The laboratory has been approaching this problem by using a variety of physical and chemical approaches for the re-introduction of DNA into the infected erythrocyte, using transient expression of sensitive reporter genes driven by the putative promoters defined in the preceding paragraph. The results of these experiments will be summarized below.

RESULTS

Construction and characterization of a yeast artificial chromosome library from *P. falciparum*

Molecular genetic studies of the human malaria parasite *Plasmodium falciparum* have been hampered in part due to difficulties stably cloning and propagating parasite genomic DNA in bacteria. This is thought to be a result of the unusual A + T bias (>80%) in the parasite's DNA. Pulsed-field gel electrophoretic separation of *P. falciparum* chromosomes has shown that large chromosomal polymorphisms, resulting from the deletion of DNA from chromosome ends, frequently occur. Understanding the biological implications of this chromosomal polymorphism will require the analysis of large regions of genomic, and in particular telomeric, DNA. To overcome the limitations of cloning parasite DNA in bacteria, we have cloned genomic DNA from the *P. falciparum* strain FCR3 in yeast as artificial chromosomes. A pYAC4 library with an average insert size of approximately 100 kb was established and found to have a three to fourfold redundancy for single-copy genes. Unlike bacterial hosts, yeast stably maintain and propagate large tracts of parasite DNA. Long-range restriction enzyme mapping of YAC clones demonstrates that the cloned DNA is contiguous and identical to the native parasite genomic DNA. Since the telomeric ends of chromosomes are underrepresented in YAC libraries, we have enriched for these sequences by cloning *P. falciparum* telomeric DNA fragments (from 40 to 130 kb) as YACs by complementation in yeast.

Ref: de Bruin, D., Lanzer, M. and Ravetch, J.V. (1992). *Characterization of Yeast Artificial Chromosomes from Plasmodium falciparum: Construction of a stable, representative library and cloning telomeric DNA fragments. Genomics, in press.*

Transcription mapping of a 100 kb locus identifying an intergenic region

We have mapped *Plasmodium falciparum* erythrocytic stage transcription units on chromosome 10 in the vicinity of the gene encoding the glycoporphin binding protein (GBP130) using yeast artificial chromosomes (YACs). Three erythrocytic stage transcription

units are clustered in a 40 kb region. Two of these genes are closely linked, separated by <2 kb. Nuclear run-on data demonstrate that transcription of these two genes, though unidirectional, is monocistronic. Within this intergenic region are the sites at which transcription of the upstream gene terminates and the GBP130 gene initiates. These studies represent the first description of the minimal and necessary cis-acting elements for transcription termination and initiation in this protozoan parasite.

Ref: Lanzer, M., de Bruin, D. and Ravetch, J.V. (1992). Transcription mapping of a 100 kb locus of P. falciparum identifies an intergenic region in which transcription terminates and reinitiates, EMBO J., 11:1949-1955.

A putative promoter region is the site of developmentally regulated DNA-protein interactions

The *Plasmodium falciparum* gene encoding the knob associated histidine-rich protein (KAHRP) is shown to be transcriptionally regulated during its expression in the intraerythrocytic cycle as demonstrated by stage specific nuclear run-on analysis. The genomic organization of the KAHRP gene was determined and the structural basis for the stage specific transcription investigated. A sequence motif with two-fold symmetry was found 160 bp upstream of the RNA initiation site. This sequence element interacts with parasite derived nuclear extracts in a stage specific manner that correlates with the transcriptional activity of the KAHRP gene. These studies suggest a functional role for this structural element in the developmental regulation of a *P. falciparum* erythrocytic gene.

Ref: Lanzer, M., de Bruin, D. and Ravetch, J.V. (1992). A sequence element associated with the Plasmodium falciparum KAHRP gene is the site of developmentally regulated protein interactions, Nucl. Acids Res., 20:3051-3056.

Development of transfection strategies for P. falciparum

Our efforts during the past year have focussed on introducing exogenous DNA into the infected erythrocyte using electroporation or biolistic particle bombardment. Initial experiments were aimed at determining the efficiency of introduction of exogenous DNA using these approaches, utilizing labelled DNA. DNA fragments were labelled either by radioisotopic or biotinylated nucleotide triphosphate precursors. The tagged DNA could be reproducibly detected in the isolated parasite pellet of the infected erythrocyte 24 hours after its introduction by electroporation of asynchronous parasites. Control uninfected erythrocytes were routinely negative. Greater efficiencies were seen for schizont stage parasites, when compared to ring or trophozoite enriched stages.

A variety of reporter genes were constructed using DNA sequences from the KAHRP gene, the GBP130 intergenic region and the P195 gene upstream sequences 5' of luciferase or chloramphenicol acetyltransferase reporter genes. These constructs were introduced into schizont-stage parasites using the parameters determined from the labelled DNA experiments. Preliminary results from three independent experiments have suggested that the P195 upstream region when coupled to the luciferase reporter gene results in detectable luciferase activity 24 hours after electroporation of schizont stage parasites. No activity was observed for the GBP130 or KAHRP promoters electroporated into schizonts. These results are encouraging

and consistent with the stage specificity of the three promoters utilized since only the P195 is active during the schizont stage. Confirmation of these results using a second reporter, like CAT, is in progress and will be followed by a detailed analysis of the putative promoter element.

CONCLUSIONS AND FUTURE DIRECTIONS

The success of the YAC cloning strategy has facilitated the analysis of gene organization and regulation in the malaria parasite. These approaches will allow for the cloning of complete chromosomes, thereby identifying genetic loci of biological significance, like drug resistance, cytoadherence, invasion and dimorphism using positional cloning strategies. Novel transcription units can be defined and their regulation studied in intact and rearranged chromosomes. The identification of putative promoters and dominant selectable markers using these strategies will be combined to develop methods for stable transformation of *P. falciparum*. Studies completed during the last year have laid the groundwork for these important extensions.

BIBLIOGRAPHY 1990-1991

- 1) de Bruin, D., Lanzer, M. and Ravetch, J.V. (1992). Characterization of Yeast Artificial Chromosomes from *Plasmodium falciparum*: Construction of a stable, representative library and cloning telomeric DNA fragments. Genomics, in press.
- 2) Lanzer, M., de Bruin, D. and Ravetch, J.V. (1992). Transcription mapping of a 100 kb locus of *P. falciparum* identifies an intergenic region in which transcription terminates and reinitiates, EMBO J., 11:1949-1955.
- 3) Lanzer, M., de Bruin, D. and Ravetch, J.V. (1992). A sequence element associated with the *Plasmodium falciparum* KAHRP gene is the site of developmentally regulated protein interactions, Nucl. Acids Res., 20:3051-3056.

APPENDIX

Characterization of Yeast Artificial Chromosomes From *Plasmodium falciparum*: Construction of a Stable, Representative Library and Cloning of Telomeric DNA Fragments

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Molecular genetic studies of the human malaria parasite *Plasmodium falciparum* have been hampered in part due to difficulties stably cloning and propagating parasite genomic DNA in bacteria. This is thought to be a result of the unusual A + T bias (>80%) in the parasite's DNA. Pulsed-field gel electrophoretic separation of *P. falciparum* chromosomes has shown that large chromosomal polymorphisms, resulting from the deletion of DNA from chromosome ends, frequently occur. Understanding the biological implications of this chromosomal polymorphism will require the analysis of large regions of genomic, and in particular telomeric, DNA. To overcome the limitations of cloning parasite DNA in bacteria, we have cloned genomic DNA from the *P. falciparum* strain FCR3 in yeast as artificial chromosomes. A pYAC4 library with an average insert size of approximately 100 kb was established and found to have a three to fourfold redundancy for single-copy genes. Unlike bacterial hosts, yeast stably maintain and propagate large tracts of parasite DNA. Long-range restriction enzyme mapping of YAC clones demonstrates that the cloned DNA is contiguous and identical to the native parasite genomic DNA. Since the telomeric ends of chromosomes are underrepresented in YAC libraries, we have enriched for these sequences by cloning *P. falciparum* telomeric DNA fragments (from 40 to 130 kb) as YACs by complementation in yeast. © 1992 Academic Press, Inc.

INTRODUCTION

The human malaria parasite *Plasmodium falciparum* infects more than 200 million people and claims over 2 million lives, annually. *P. falciparum* is an obligate intracellular protozoan parasite that requires two hosts, a human and a mosquito, for the completion of its life cycle. In the human host the parasite is haploid and multiplies asexually within hepatocytes and erythrocytes through mitotic divisions. The erythrocytic stages

can be cultured *in vitro* (Trager and Jensen, 1976), providing a source of parasites for study. The sexual phase of the life cycle begins when the mosquito host ingests parasite-laden blood from an infected human. During this phase recombination and independent assortment of chromosomes occurs (Walliker *et al.*, 1987). The complexity of the parasite's life cycle and the difficulties in creating and manipulating mutants have severely restricted the use of classical genetic tools for study of this organism.

Pulsed-field gel electrophoresis studies have revealed that the parasite's haploid genome of 3×10^7 bp contains 14 chromosomes that range in size from 600 kb to 3.5 Mb (Van Der Ploeg *et al.*, 1985; Wellems *et al.*, 1987). Striking polymorphisms have been observed between homologous chromosomes of different geographical isolates. The variations in chromosome size range from 50 to 300 kb, which in some cases corresponds to 15% of the total length of a chromosome (Ravetch, 1989). These chromosomal polymorphisms appear to be the result of large deletions of DNA from chromosome ends. A pathway described in *P. falciparum* that leads to chromosomal polymorphisms involves a process of chromosome breakage followed by the healing of the breakpoint through the *de novo* addition of telomere repeats (Pologe and Ravetch, 1986, 1988; Pologe *et al.*, 1990). This chromosome instability and polymorphism reflects the plasticity of the parasite's genome.

The genomic plasticity of *P. falciparum* is also indicated by the extensive strain-dependent variations seen in antigenic determinants and protein isoforms (see Kemp *et al.*, 1990 for review). Further antigenic differences arise from chromosomal polymorphisms that delete specific antigen genes (Pologe and Ravetch, 1986, 1988; Pologe *et al.*, 1990). We have been interested in determining what additional roles chromosomal polymorphisms play in the parasite's biology and in studying the overall organization and structure of *P. falciparum* chromosomes. Questions concerning the parasite's genome require the analysis of large fragments of genomic

DNA in general and of telomeric fragments in particular.

Molecular biological studies on *P. falciparum* have been hampered by difficulties in stably maintaining large genomic clones of parasite DNA in bacterial hosts (Kochan *et al.*, 1986; Weber, 1988). The problem of clone stability is thought to be a result of the parasite's unusually A + T-rich DNA. The A + T content averages 82% and can approach 90 to 95% in intergenic regions (Pollack *et al.*, 1982; Weber, 1988). As a means of obtaining complete *P. falciparum* genomic clones, we report here the construction and characterization of a stable, representative yeast artificial chromosome (YAC) library and the YAC cloning of *Plasmodium* telomeric DNA fragments by complementation in yeast. The parasite offers a unique eukaryotic system in which to study chromosomal stability and structure, and these YAC clones provide necessary reagents for the analysis of the *P. falciparum* genome.

MATERIALS AND METHODS

Preparation of parasite DNA. Fifty 10-cm plates of the *P. falciparum* strain FCR3 (ATCC 30932) were grown to a parasitemia of 10% (approximately 2.5×10^{10} parasites) as described by Trager and Jensen (1976). DNA was prepared after saponin lysis of the infected erythrocytes by digestions with RNase A and Proteinase K, followed by extraction with buffered phenol:chloroform (1:1) in 50-ml plastic disposable centrifuge tubes (Goman *et al.*, 1982). To minimize shearing, the DNA was slowly mixed by inversion. After centrifugation (1000 rpm for 10 min) to separate the phases, the organic phase was removed by draining it through a needle hole in the bottom of the tube. The aqueous phase was transferred to a fresh tube and extracted again with phenol:chloroform followed by two extractions with chloroform:isoamyl alcohol (24:1). The DNA was then dialyzed extensively against TE (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)).

Digestion of DNA. Parasite DNA for the pYAC4 library was partially digested with *EcoRI* at five different concentrations of enzyme (0.15, 0.10, 0.05, 0.025, and 0.01 U/ μ g DNA) as described in Sambrook *et al.* (1989). For the *Plasmodium* telomere cloning, parasite DNA was digested to completion with *NheI*. YAC vectors pYAC4 (Burke *et al.*, 1987; Burke and Olson, 1991) and pJS97 (Shero *et al.*, 1991) were digested with either *EcoRI* and *BamHI* or *NheI* and *Clal*, respectively. Following digestion, the vectors were treated with calf intestinal phosphatase according to the manufacturer's (Boehringer-Mannheim Biochemicals) specification.

Ligation of DNA. For the pYAC4 library, 1- μ g aliquots from each partial digest were pooled, a 50 \times (14- μ g) molar excess of prepared pYAC4 was added, and the reaction conditions were adjusted to 50 mM Tris (pH 7.5), 10 mM MgCl₂, 25 mM NaCl, 10 mM DTT, 2 mM spermidine, 1 mM ATP, 50 ng/ μ l BSA in a volume of 200 μ l. After the addition of 2400 U of T4 DNA ligase (New England Biolabs), the ligation reaction was incubated at 12°C for 24 h. Ligations for the *Plasmodium* telomere YACs were performed in a similar manner using 5 μ g of *NheI*-digested parasite DNA and 10 μ g of prepared pJS97.

Yeast growth and transformation. The yeast strains used are AB1380 (ATCC 20843, Burke *et al.*, 1987) for the pYAC4 library and YPH274 (Sikorski and Heiter, 1989) for the *Plasmodium* telomere YACs. Methods for the manipulation, growth, and storage of yeast strains are described by Sherman *et al.* (1986). Spheroplast transformation of yeast was performed by the method of Burgess and Percival (1987) using the modifications described by McCormick *et al.* (1990), with the exception that polyamines were excluded. Ten microliters of the ligation mixture was used in the yeast transformations. Transformants were selected on media that lacked either uracil and tryptophan (pYAC4 library) or uracil alone (*Plasmodium* telomere YACs).

***P. falciparum* pYAC4 library organization.** The pYAC4 library was organized and prepared for screening as adapted from Heard *et al.* (1989) and Green and Olson (1990). Individual clones were transferred to microtiter plates containing uracil-deficient media and grown for 2 days. Prior to long-term storage at -70°C, replica plates were made. From one set of replica plates, all the clones on a single plate were pooled and grown for an additional day. Yeast genomic DNA was then prepared (the primary DNA pool) as described by Sherman *et al.* (1986). From the other replica plates, secondary pools of DNA that represented pools of clones in each row and column of a single primary plate were prepared.

PCR screening of pYAC4 library. PCR analysis was used to identify specific clones in the library by first identifying the positive primary DNA pools and then finding the corresponding positive row and column of the microtiter plate by screening the secondary DNA pools. After preliminary identification by PCR analysis, clones were then confirmed by Southern analysis of PFG-separated YACs using gene-specific probes. PCR analysis was performed using a Perkin-Elmer Cetus DNA thermal cycler. One microliter of the DNA pool was used for each 25- μ l PCR reaction (PCR conditions: 50 ng of each PCR primer, 200 μ M of each dNTP, 0.5 U of *Taq* DNA polymerase (Ampli-Taq, Perkin-Elmer Cetus), 50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris (pH 8.3)). Thirty-five cycles of PCR were run for 1 min at 94°C, 2 min at the annealing temperature (5°C less than the calculated melting temperature of the lowest melting primer), and 3 min at 72°C. Primer sets used for the identification of specific YAC clones in the library were as follows: KAHRP (Triglia *et al.*, 1987)—389, TAC CAT CGA CAA CAT TTT CCT; and 407, TAA TCC TCC TAG TAA TGA ACC. PFMDR1 (Foote *et al.*, 1989)—4032, ACA TTA TAT TAA AAA ATG AT; and 4792, TAT AAA TAC ATA TAT ATA TAT ATA. CSP (Dame *et al.*, 1984)—1480—CAA TTC ATG ATG AGA AAA TTA GCT; and 1481, CAT CTT TAC CTT CAC GAC C. CARP (Wahlgren *et al.*, 1986)—1627, GGT CTG TCC ATT CAC TAG GTA TGT GGA; and 1852, ACA ATA GCG AGA ATT TCC AAG G. SERA (Bzik *et al.*, 1988)—1625, TGA ACT TGA ACT AGA ACT TGA ACT TGA ACT; and 1851, CAG GAG GAG GTC AAG CAG GTA ATA C. P-195 (MSA1 or PMMSA; Mackay *et al.*, 1985)—966, GAT CAC TTG TAA ATG TTA ATT G; and 974, GTT AAT GAA ATA TAT ATA ATT ACA CAA CTT AAT AAA ATG. MSA2 (Smythe *et al.*, 1988)—1866, GCA ACA CAT TCA TAA ACA ATG C; and 1867, CAT TTG ATT TAG TTT GAG AGT C. GBP-130 (Kochan *et al.*, 1986)—705, GTA AGC AGA AAA GGA ATG GTG; and 975, GTT GAA ATT TAT ATA AAC CTA CAA TTA GCT ATT TC. EBA-175 (Sim *et al.*, 1990)—5A, GTT AAT ATG AAT GTT GAG AA; and 5B, ACT ATG ATT AAT TTG ACT TC.

Stability of pYAC4 clones. Five milliliters of uracil- and adenine-supplemented AHC media were inoculated with a YAC clone taken from the frozen master plate. AHC is a rich uracil and tryptophan dropout medium containing casein hydrolysate (Brownstein *et al.*, 1989), and yeast cultured in AHC reach much higher cell densities than those in minimal media. The yeast were then grown at 30°C to an OD₆₀₀ > 4 (1 OD₆₀₀ = 3×10^7 cells/ml). Prior to harvesting the YACs for PFG analysis at this "initial" time point, 2.5 μ l of the cell suspension was used to inoculate 25 ml of fresh media. The cells were then grown for 48 h until the OD₆₀₀ was > 7 (below saturation), which is equivalent to 12–14 generations. Again a 2.5- μ l aliquot was removed and used to inoculate 25 ml of fresh media. This was repeated at 48-h intervals and the yeast were harvested for PFG analysis at time points equivalent to 25, 50, and 75 generations.

FOA treatment of YAC clones. Primary transformants from the *Plasmodium* telomere cloning experiments were picked into microtiter wells containing 200 μ l of uracil-deficient medium. After 36 h growth, 7.5- μ l aliquots were replica plated onto 5-fluoro-orotic acid (FOA, PCR Incorporated (Sikorski and Boeke, 1991)) and uracil-deficient plates. The degree of papillation was scored after 4 days growth at 30°C. Colonies that did not grow or papillated at low frequency relative to a yeast strain containing the pJS97 plasmid were chosen for further analysis. Dr. Arthur Lustig (Sloan-Kettering Institute, New York, NY) suggested using FOA as a selection method and generously supplied the compound. A filter replica was made of these colonies onto SUREBLOT nylon membranes (Oncor) as described by

Burke and Olson (1991) with the exceptions that lyticase, 0.1 M sodium citrate, 50 mM EDTA, and 15 mM DTT were replaced with 2.5 µg/ml Zymolyase 20T (ICN Immunobiochemicals), 1 M sorbitol, 10 mM sodium phosphate (pH 7.5), 10 mM EDTA, and 30 mM 2-mercaptoethanol.

Mapping YAC clones and pulsed-field gel electrophoresis. Parasite DNA was prepared for PFG analysis by embedding the infected erythrocytes in an equal volume of 1.25% Incert Agarose (FMC) in TE (10 mM Tris (pH 8.0) and 1 mM EDTA) and then aliquoting 100 µl/block. The embedded parasites were then lysed by adding the blocks to ESP (0.5 M EDTA (pH 8.5), 1% N-lauryl sarcosine, and 2 mg/ml Proteinase K) and incubating at 55°C for 36 h. YACs were grown under selective conditions for 2 days, harvested by centrifugation (3000 rpm for 5 min), and washed once with distilled water followed by 1 M sorbitol. The yeast were resuspended in an equal volume of SPEM (1 M sorbitol, 10 mM sodium phosphate (pH 7.5), 10 mM EDTA, and 30 mM 2-mercaptoethanol) and Zymolyase 20T added to a final concentration of 5 µg/100 µl of cell suspension. The cells were incubated for 1–2 h at 37°C, embedded in an equal volume of 1.25% Incert agarose, and lysed in ESP as described above.

Prior to restriction enzyme digestion of embedded DNA, the agarose blocks were dialyzed extensively against ET (50 mM EDTA, 10 mM Tris (pH 8.0)) with 1 mM PMSF (phenylmethylsulfonyl fluoride), followed by ET alone and TE. Individual blocks were transferred to 2-ml microcentrifuge tubes and equilibrated for 1 h in 1× restriction enzyme buffer. Four hundred microliters of fresh restriction enzyme buffer with 60 U of enzyme was added to the tube, and the digestion was carried out overnight.

Pulsed-field gel electrophoresis analysis was performed using a Bio-Rad CHEF DR-II with 0.5× TBE as the running buffer, 1% LE agarose (FMC) gels, and a constant temperature of 14°C. Pulse times and voltages varied for individual runs and are indicated in the figure legends. Prior to transfer to supported nitrocellulose (Schleicher and Schuell), gels were exposed to UV light (600 µJ of energy from a Stratilinker (Stratagene)).

Hybridization probes. The pBR322 probes for the YAC vector arms are described in Burke *et al.* (1987). The P-195 probe is the *EcoRI*/BglII fragment from the genomic P-195 clone described in Mackay *et al.* (1985), the KAHRP probe (LP-20) is described in Pologe and Ravetch (1986). The GBP-130 probe (8822) is described in Lanzer *et al.* (1992). The PFrep20 probe is the *EcoRI*/*HindIII* fragment described in Patarapotikul and Langsley (1988). The oligonucleotide probe specific for the *P. falciparum* telomere repeat sequence (OL-PFTEL) is 5'-GGGTTTA GGGTTTA GGGTTTA GGGTTTA-3'. DNA fragments were labeled by random priming, and oligonucleotide probes were end labeled as described in Sambrook *et al.* (1989). Hybridization conditions are described in Kochan *et al.* (1986) and Pologe *et al.* (1990).

Bal31 Digestion. Five micrograms of DNA from a putative *Plasmodium* telomere YAC clone was treated with 1 U of Bal31 according to the manufacturer's (New England Biolabs) specification in a volume of 210 µl. At the designated time points, 30-µl aliquots were removed and brought to 25 mM with EGTA, heated at 70°C for 10 min, and then precipitated with ethanol. The DNA samples were then digested with *RsaI*, fractionated on a 1% TBE agarose gel, and transferred to supported nitrocellulose.

End rescue of Plasmodium telomere YAC clone. The end rescue of sequences from YACs in pJS97 is essentially as described by Shero *et al.* (1991) with the following exceptions: after *Bam*HI digestion and ligation, the sample was ethanol precipitated and resuspended in 20 µl of TE. Five microliters was used for electrotransformation of SURE *Escherichia coli* (Stratagene) using a Bio-Rad gene pulser device set at 25 µF, 200 ohm, and 2.5 kV. After selection on ampicillin, plasmid DNA was isolated by a mini-prep procedure (Sambrook *et al.*, 1989), and the end-rescued fragment of 800 bp was used as a probe.

RESULTS

YAC Library Construction

The *P. falciparum* strain FCR3 (Jensen and Trager, 1978) was chosen as the DNA source for the YAC library

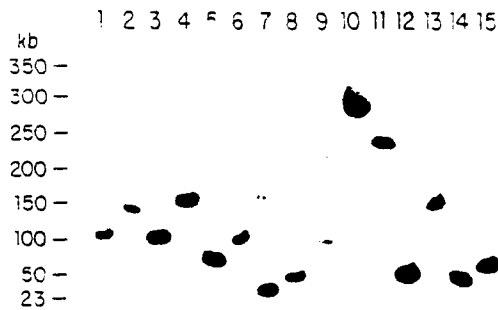


FIG. 1. Size range of *P. falciparum* pYAC4 clones. *EcoRI* partially digested parasite DNA was cloned into pYAC4 and transformed into yeast. Fifteen transformants were picked at random after being color assayed for inserts and prepared for PFG chromosomal analysis. PFG-separated (ramped pulse time from 5 to 30 s over 22 h at 180 V) YACs were transferred to supported nitrocellulose prior to hybridization with a probe to the left arm of pYAC4.

because the strain is well characterized and is readily available to other researchers. Parasite DNA for the YAC library was prepared in aqueous form and care was taken to minimize shearing (see Materials and Methods). The size of the undigested DNA was approximately 650 kb, and partial digestion with *EcoRI* yielded fragments that ranged in size from 30 to 400 kb (data not shown). The parasite DNA was cloned into the *EcoRI* site of the YAC cloning vector pYAC4. Transformants were selected on uracil- and tryptophan-deficient sorbitol agar plates. Random clones were picked and tested for inserts by color assay (Burke *et al.*, 1987). Of 60 colonies assayed, 52 were positive, indicating that approximately 86% of the primary transformants contained inserts.

Random clones were analyzed by PFG to determine the size range of the inserts. After Southern blotting the PFG, the membrane was probed with a pBR322 fragment specific for the YAC vector left arm. Figure 1 shows a typical PFG containing 15 random YACs that range in size from 30 to 290 kb. The largest YAC clone found in the library is 350 kb (data not shown), and the average clone size is approximately 100 kb. To establish a library, primary transformants were picked and color was assayed for inserts. Of 1300 transformants assayed, 1056 positive clones were transferred to 96-well microtiter plates (see Materials and Methods). Since the size of the *P. falciparum* genome is 3×10^7 bp and the average size of the YACs is 100 kb, the library is predicted to have a redundancy of between 3 and 4 for single-copy genes.

Representation of the pYAC4 Library

A PCR-based strategy was used to identify individual genes present in the library (see Materials and Methods). The 11 primary pools of DNA (each pool represents all clones from a single microtiter plate) were screened using oligonucleotide primers specific for the nine loci (representing six different chromosomes)

TABLE 1
Representation of Loci in the YAC Library

Locus	Chromosome	No.	Size (kb)
KAHRP	2	5	80, 75, 50, 40, 30
PFMDR1	5	5	N/D
CSP	3	4	110, 100
CARP	N/D	4	280, 190, 180, 145
SERA	2	3	170, 125, 55
P-195	9	3	75, 60, 55
MSA2	2	2	170, 150
GBP-130	10	2	110, 60
EBA-175	4	1	80

Note. KAHRP, knob-associated histidine-rich protein; PFMDR1, *P. falciparum* multidrug resistance gene 1; CSP, circumsporozoite protein; CARP, clustered asparagine-rich protein; SERA, serine repeat antigen; P-195, merozoite surface antigen 1 (MSA1) or precursor of major merozoite surface antigen (PMMSA); MSA2, merozoite surface antigen 2; GBP-130, glycoprotein binding protein-130; EBA-175, erythrocyte binding antigen-175. Chromosome, parasite chromosome; No., number of primary DNA pools positive for the locus by PCR; and Size, size of YAC clone isolated by a second round of PCR using secondary pools of DNA as determined by PFG. Only two of the four primary YAC clones were isolated for the CSP locus. The sizes of the PFMDR1 YACs were not determined (N/D). The CARP gene has been reported to be located on Chromosome 2 (Wellems *et al.*, 1991). However, probes to CARP and probes recovered from the ends of the CARP YACs hybridize not to Chromosome 2, but to a much larger undetermined chromosome (N/D).

shown in Table 1. All loci examined were represented in the library, and six of the nine were present either at or above the predicted frequency of 3–4 clones per single-copy gene. Individual YAC clones were isolated from eight of these loci by another round of PCR using DNA from secondary pools of clones that represented each row and column of the positive primary microtiter plate. The 22 clones isolated ranged in size from 30 to 280 kb with an average size of 105 kb (Table 1).

Integrity and Stability of Plasmodium YAC Clones

The YAC clones from a representative region of parasite DNA, the P-195 locus (known also as MSA1 or PMMSA; Holder *et al.*, 1985; Mackay *et al.*, 1985), were chosen for detailed mapping studies to determine whether the YAC-cloned DNA was identical to the native parasite DNA. Figure 2 shows a typical PFG mapping experiment for the three P-195 YACs identified. The YACs are 75, 60, and 55 kb in size, and when restriction enzyme maps of the YACs are compared to FCR3 genomic DNA (Fig. 2), the fragments are identical, indicating that no obvious rearrangements have occurred. Additional long-range restriction mapping of YACs from two other loci, the GBP-130 (Lanzer *et al.*, 1992) and the KAHRP (de Bruin *et al.*, manuscript in preparation), gave similar results.

Since the major concern with *P. falciparum* genomic clones is stability, the behavior of six YAC clones in long-term cultures was examined (Fig. 3). Two YACs, each from the GBP-130 locus (Fig. 3A), the KAHRP

locus (Fig. 3B), and the P-195 locus (Fig. 3C), were monitored for gross rearrangements in size over 25, 50, and 75 consecutive generations of growth in selective media. When Southern blot analysis using gene-specific probes against PFG-separated YACs was performed, no obvious changes were detected over this time course. In addition, a comparative *Hind*III restriction analysis between the two P-195 YACs and genomic FCR3 parasite DNA revealed no smaller rearrangements of the YAC-cloned DNA during propagation (Fig. 3D). Together the data presented in Figs. 2 and 3 show that unlike parasite genomic clones in bacterial hosts, *P. falciparum* DNA is stable in yeast.

YAC Cloning *P. falciparum* Telomeric DNA Fragments

Due to the unique structure of the ends of chromosomes, these sequences are underrepresented in standard YAC libraries. Our interest in the telomere-proximal regions of *P. falciparum* chromosomes required a method for obtaining large telomeric DNA fragments. The successes in cloning human telomeres by comple-

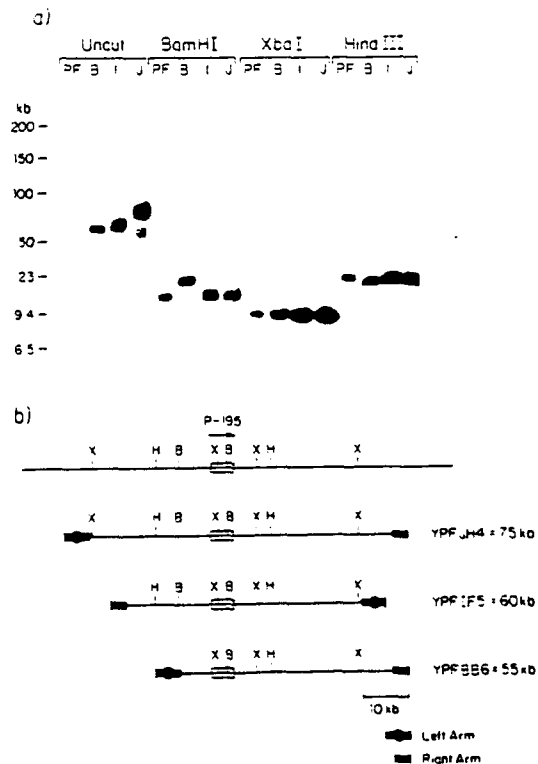


FIG. 2. Integrity of YAC clones from a representative locus. Three YACs were isolated from the library by PCR using oligonucleotide primers specific for the P-195 gene. (a) Comparison of the restriction digests of YAC clones B (YPFBB6), I (YPFIF5), and J (YPFJH4) with genomic FCR3 parasite DNA (PF). PFG-separated (ramped pulse time from 2.5 to 12 s over 20 h at 180 V) restriction enzyme digests of agarose-embedded DNAs were Southern transferred to supported nitrocellulose and hybridized with a probe to the P-195 gene. (b) Long-range restriction maps of the P-195 locus and YAC clones. H, *Hind*III; B, *Bam*HI; X, *Xba*I.

mentation in yeast using YAC-based systems (Riethman *et al.*, 1989; Cross *et al.*, 1989; Brown, 1989) suggested that similar methods for cloning *P. falciparum* telomeric regions could be used. Since the parasite telomere repeat (GGGTT(T/C)A) is similar to the human repeat sequence (GGGTTA), a YAC-based approach to isolating telomeric regions seemed feasible.

After *NheI* restriction enzyme digestion of FCR3 DNA, the sizes of the DNA fragments that hybridize with an oligonucleotide probe specific for the *P. falciparum* telomere repeat sequence (OL-PFTel) are between 30 and 200 kb (data not shown). *NheI*-digested parasite DNA was ligated with a 50× molar excess of the YAC vector pJS97. After transformation into yeast, mitotic stability of the linearized vector requires complementation by parasite sequences that can function as substrates for a putative yeast telomerase.

To enrich for large linear clones and remove any plasmid background, the behavior of the transformants when plated onto 5-fluoro-orotic acid agar plates was observed. FOA is a pyrimidine analog that is toxic to yeast if they contain the *URA3* gene, which encodes an enzyme required for uracil biosynthesis (Boeke *et al.*, 1984). *ura3* cells are resistant to FOA and the loss of *URA3* from a yeast can be monitored as growth on FOA. When *URA3* is contained on a plasmid, the frequency of loss is much higher than that of a wildtype chromosomal

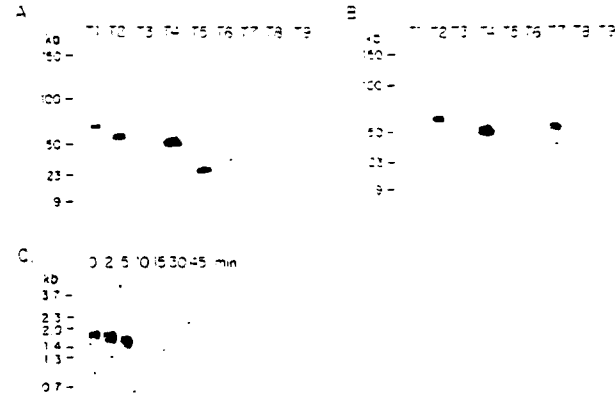


FIG. 4. Analysis of *P. falciparum* telomere YAC clones. Nine putative parasite telomere YACs were PFG separated (ramped pulse time from 5 to 25 s over 22 h at 180 V) and analyzed by Southern analysis: (A) YACs hybridized to a probe specific for the parasite telomere repeat (OL-PFTel) and (B) YACs hybridized to a probe for the *Plasmodium* subtelomeric repeat sequence, PFrep20. The *Bal31* exonuclease sensitivity of one parasite telomere YAC clone (T8) is shown in C. Yeast genomic mini-prep DNA from T8 was treated with *Bal31*. Aliquots were removed at the indicated time points and the reaction was stopped by the addition of EGTA. Samples were digested with *RsaI* prior to Southern analysis using the OL-PFTel probe.

URA3⁺. This frequency is reflected in the degree of papillation of the yeast when plated onto FOA. Our experiments showed that if the *URA3* is contained on a large (>35 kb) *Plasmodium* YAC clone, the degree of papillation is either equivalent to or slightly (two to three times) higher than that seen for the yeast chromosomal *URA3*⁺. In addition, short (<20 kb) linear plasmids (and presumably the smaller YAC clones) behave as circular plasmids and papillate at much higher frequencies (data not shown). These differences may result from the greater mitotic stability of longer linear clones (Murray *et al.*, 1986).

Putative *Plasmodium* telomere YACs were picked from the primary transformants in top agar and replica plated onto uracil-deficient and FOA agar plates. The degree of papillation of the transformants relative to the papillation of a *ura3* yeast strain, a *URA3*⁺ strain, and a strain harboring a plasmid copy of *URA3* (pJS97) was assayed. Transformants that either failed to grow or papillated at low frequency were picked from the uracil minus replica and transferred to microtiter plates for further analysis. The clones were transferred to a nylon filter for colony screening and hybridized against the parasite telomere OL-PFTel probe. Of the 192 clones examined, 66 (34%) hybridized to this probe.

Figure 4A shows Southern analysis of a PFG containing nine putative parasite telomere clones hybridized with the OL-PFTel probe. These clones range in size from 40 to 130 kb. Although equivalent amounts of DNA were loaded on the gel, differences in the intensity of hybridization to OL-PFTel are seen. This may result from variations in the number of parasite telomere repeats present in the clones. To determine if the parasite telomere sequences were at the end of the clones, the

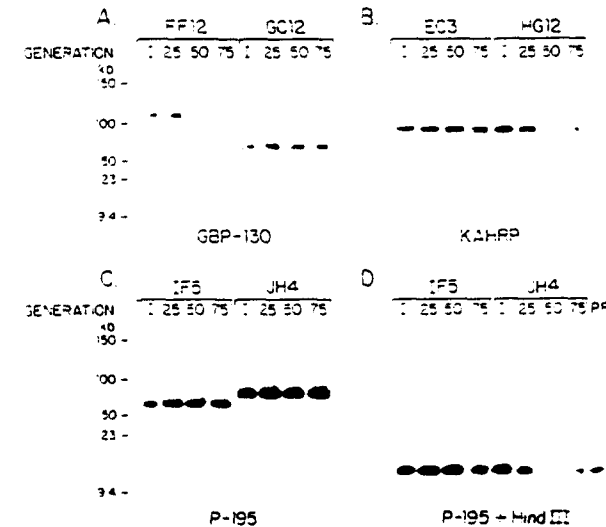


FIG. 3. Stability of *P. falciparum* YAC clones in culture. YAC clones from loci on three different chromosomes, the GBP-130 (YPFGC12 and YPFFF12, A), the KAHRP (YPFEC3 and YPFHG12, B), and the P-195 (YPFIF5 and YPFJH4, C), were grown from the frozen master plates in selective media. PFG sample blocks were prepared from the yeast at the initial time point (Lane I) and at time points representing 25, 50, and 75 consecutive generations from the initial inoculation. (A, B, C) PFG-separated YACs (5 to 20 s over 18 h at 180 V) that have been Southern blotted and hybridized with gene-specific probes. (D) A comparison between *HindIII* digests of the P-195 YACs and FCR3 DNA (PF). The digests were separated as above, and Southern analysis was performed using the P-195-specific probe.

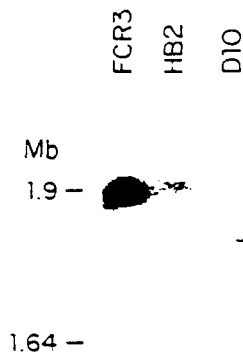


FIG. 5. End-rescued probe from telomeric YAC clone T8 hybridizes to a single parasite chromosome. DNA sequences adjacent to the YAC cloning vector were recovered in *E. coli*. A fragment of 800 bp was recovered and used to probe a nitrocellulose filter of PFG-separated (ramped pulse time from 75 to 330 s over 50 h at 150 V) parasite chromosomes. Parasite strains from three different geographical regions FCR3 (Gambia), HB2 (Honduras), and D10 (New Guinea) are shown.

hybridization to OL-PFTel was examined after treatment with *Bal31* exonuclease. Figure 4C shows an example clone, T8, after digestions with *Bal31* and *RsaI*. Southern blotting and hybridization with OL-PFTel. The diffuse, smeared band seen at time zero is characteristic of DNA fragments from chromosome ends that are associated with variable lengths of telomere repeats. The rapid disappearance of the parasite telomere hybridizing sequence relative to a more telomere-distal sequence, the *URA3* gene on the pJS97 vector (data not shown) demonstrates that this repeat sequence is at the end of the YAC clone.

The chromosome end origin of these *Plasmodium* telomere repeat sequences was determined by examining the clones for hybridization to parasite sequences that are found exclusively in subtelomeric regions. The PFrep20 sequence is a 21-bp repeat that has been found to be associated only with the telomeric DNA fragments of *P. falciparum* chromosomes (Patarapotikul and Lingsley, 1988). In addition, PFrep20 sequences can be lost from either end of a chromosome as a result of the deletion events that lead to chromosomal polymorphisms (Kemp *et al.*, 1990). Figure 4B shows that five of the nine *Plasmodium* telomere YAC clones are positive by hybridization to PFrep20. The data presented in Fig. 4 suggest that true parasite telomeric fragments have been cloned. However, these data cannot rule out the possibility that some internal telomere repeat-like sequences may also have been cloned in this experiment.

The DNA sequences adjacent to the *NheI* cloning site of the T8 telomere clone were recovered in *E. coli* and used as a probe against Southern-blotted, PFG-separated, parasite chromosomes. A fragment of approximately 800 bp was end rescued, and this probe hybridizes to a single large parasite chromosome (possibly Chromosome 10) (Fig. 5). Parasite strains from three different geographical regions, Gambia (FCR3), Honduras (HB2), and New Guinea (D10), are shown in Fig.

5, and hybridization with this end probe demonstrates a typical chromosomal polymorphism.

DISCUSSION

Despite being a major health threat to most of the world's population, little is known about the molecular biology of *P. falciparum*. The unusual plasticity of the parasite's genome offers a unique system in which to study chromosome structure, stability, and polymorphism. Essential methods of molecular biology, such as the cloning of large, stable, tracts of parasite DNA in bacteria, the ability to transfect exogenous DNA into *P. falciparum*, and the *in vitro* culture of the entire parasite life cycle, currently are unavailable to researchers. Furthermore, classical genetic analysis of the parasite is unfeasible on a routine basis due to the complex life cycle of *P. falciparum*, which alternates between vertebrate and invertebrate hosts. Toward overcoming these limitations by providing a stable source of cloned genomic parasite DNA, we have constructed a representative genomic library from *P. falciparum*. Initial studies indicated that parasite DNA could be stably propagated in a yeast host (Triglia and Kemp, 1991). This report details the construction of a stable, representative, and unrearranged yeast artificial chromosome library from *P. falciparum* and the YAC cloning of parasite telomeric DNA fragments.

A pYAC4 library of 1056 clones with an average insert size of 100 kb has been established. Since only 300 clones of 100 kb each are needed to represent the entire 30-Mb parasite genome once, the *Plasmodium* pYAC4 library constructed was predicted to have a three- to fourfold redundancy for single-copy genes. Of the nine loci examined in this report, all were represented in this library by at least one clone and the majority (six out of nine) were present either at or above the expected number. In addition, screening the pYAC4 library with probes that are not associated with any known coding region has shown that unlike bacterial libraries, even intergenic sequences are represented in the YAC library at expected numbers (data not shown). Long-range restriction enzyme mapping of *P. falciparum* YACs has found no obvious differences between the restriction enzyme maps of the YAC-cloned DNA and that of the native genomic parasite DNA (this paper; Lanzer *et al.*, 1992). Data on the long-term stability of six independent YAC clones monitored over 75 generations also support the observation that yeast can stably maintain large tracts of parasite DNA.

The ends of *P. falciparum* chromosomes appear unstable and are frequently deleted both in the wild and during *in vitro* culture. The loss of telomeric regions results in chromosomal polymorphisms through a mechanism of chromosome breakage and healing by *de novo* telomere addition at the breakpoint. Polymorphisms have been studied in detail for parasite Chromosomes 1, 2, and 8 (Pologe and Ravetch, 1986, 1988; Pologe *et al.*, 1990). However, it is not yet known whether such telomeric deletions can occur at the ends of all parasite

chromosomes or if some chromosomes are resistant to these events. Furthermore, the biological implications of such events remain unclear. To study the structure of the chromosome ends and the mechanism responsible for generating polymorphisms, we have cloned parasite telomeric DNA fragments by complementation in yeast. Of nine *Plasmodium* telomere clones examined in detail, all were positive by hybridization to parasite telomere repeat sequences. This hybridization was sensitive to *Bal31* exonuclease activity indicating that the parasite telomere repeats were located at the end of the YAC clone. In addition, five of these nine were positive by hybridization to the *P. falciparum* repeat sequence PFrep20, which is located exclusively in subtelomeric regions. Not all parasite chromosomes contain PFrep20 because breakage and healing events that generate polymorphisms delete these sequences.

These YAC clones will not only provide important reagents for studying the organization and structure of the *P. falciparum* genome, but will also help in the mapping and positional cloning of new loci. We have probed YAC clones from the GPB-130 locus using PCR-amplified, labeled cDNAs as probes (transcription unit mapping) and have identified two new erythrocytic stage genes (Lanzer *et al.*, 1992). Mapping data established a tight linkage (<3 kb) between the GPB-130 and a newly identified locus called the 3.8 gene. Nuclear run-on analysis of the intergenic region has shown that it contains minimal regulatory elements required for transcription initiation and termination. This is a vital first step in the development of a parasite transfection system.

Together the pYAC4 library clones and the *Plasmodium* telomere YACs aid in constructing contig maps for genetically defined loci, such as the chloroquine resistance locus on Chromosome 7 (Wellems *et al.*, 1991), and for entire parasite chromosomes. Chromosomal polymorphism through breakage and telomere healing frequently occurs at the KAHRP locus of Chromosome 2 (Polog and Ravetch, 1986, 1988). Over 300 kb from the end of Chromosome 2 is deleted from some parasite strains, and we have recovered this telomeric region of the chromosome from FCR3 as contiguous YAC clones. With the YAC clones as reagents and using the transcription unit mapping methods to derive probes, we can begin to examine the polymorphic regions of parasite chromosomes for novel sequences and new genes. The data obtained will help in understanding what is unique about the genome organization of the parasite and why it has evolved such great diversity.

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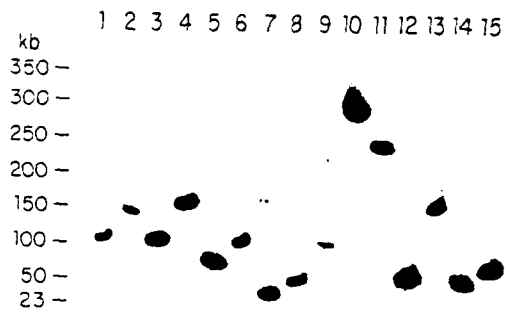
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REFERENCES

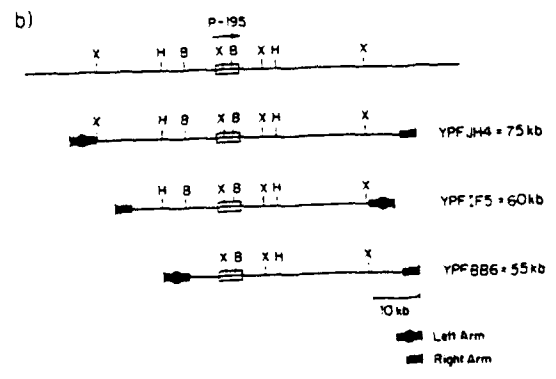
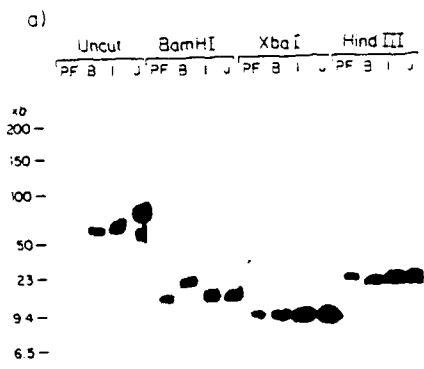
- Boeke, J. D., LaCroute, F., and Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-Fluoro-orotic acid resistance. *Mol. Gen. Genet.* 197: 345-346.
- Brown, W. R. A. (1989). Molecular cloning of human telomeres. *Nature* 338: 774-776.
- Brownstein, B. H., Silverman, G. A., Little, R. D., Burke, D. T., Kros-meyer, S. J., Schlessinger, D., and Olson, M. V. (1989). Isolation of single-copy human genes from a library of yeast artificial chromosome clones. *Science* 244: 1348-1351.
- Burgess, P. M. J., and Percival, K. J. (1987). Transformation of yeast spheroplasts without cell fusion. *Anal. Biochem.* 163: 391-397.
- Burke, D. T., Carle, G. F., and Olson, M. V. (1987). Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* 236: 806-812.
- Burke, D. T., and Olson, M. V. (1991). Preparation of clone libraries in yeast artificial-chromosome vectors. In "Methods in Enzymology" (C. Guthrie and G. R. Fink, Eds.), Vol. 194, pp. 251-270, Academic Press, New York.
- Bzik, D. J., Li, W.-B., Horii, T., and Inselburg, J. (1988). The complete amino acid sequence of the serine-repeat antigen (SERA) of *Plasmodium falciparum* determined from cloned cDNA. *Mol. Biochem. Parasitol.* 30: 279-288.
- Cross, S. H., Allshire, R. C., McKay, S. J., McGill, N. I., and Cooke, H. J. (1989). Cloning of human telomeres by complementation in yeast. *Nature* 338: 771-774.
- Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L., Wirtz, R. A., Hockmeyer, W. T., Maloy, W. L., Haynes, J. D., Schneider, I., Roberts, D., Sanders, G. S., Reddy, E. P., Diggs, C. L., and Miller, L. H. (1984). Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science* 225: 593-599.
- Foote, S. J., Thompson, J. K., Cowman, A. F., and Kemp, D. J. (1989). Amplification of the multidrug resistance gene in some chloroquine resistant isolates of *P. falciparum*. *Cell* 57: 921-930.
- Goman, M., Langsley, G., Hyde, J. E., Yankovsky, N. K., Zolg, J. W., and Scaife, J. G. (1982). The establishment of genomic DNA libraries from the human malaria parasite *Plasmodium falciparum* and the identification of individual clones by hybridization. *Mol. Biochem. Parasitol.* 5: 391-400.
- Green, E. D., and Olson, M. V. (1990). Systematic screening of yeast artificial chromosome libraries by use of the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 87: 1213-1217.
- Heard, E., Davies, B., Feo, S., and Fried, M. (1989). An improved method for the screening of YAC libraries. *Nucleic Acids Res.* 17: 5861.
- Holder, A. A., Lockyer, M. J., Odink, K. G., Sandhu, J. S., Riveros-Moreno, V., Nicholls, S. C., Hillman, Y., Davey, L. S., Tizard, M. L. V., Schwarz, R. T., and Freeman, R. R. (1985). Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. *Nature* 317: 270-273.
- Jensen, J. B., and Traeger, W. (1978). *Plasmodium falciparum* in culture: Establishment of additional strains. *Am. J. Trop. Med. Hyg.* 27: 743-746.
- Kemp, D. J., Cowman, A. F., and Walliker, D. (1990). Genetic diversity in *Plasmodium falciparum*. In "Advances in Parasitology" (J. R. Baker and R. Muller, Eds.), Vol. 29, pp. 75-149, Academic Press, New York.
- Kochan, J., Perkins, M., and Ravetch, J. V. (1986). A tandemly re-

- peated sequence determines the binding domain for an erythrocyte receptor binding protein of *P. falciparum*. *Cell* 44: 659-696.
- Lanzer, M., de Bruin, D., and Ravetch, J. V. (1992). Transcription mapping of a 100 kb locus of *Plasmodium falciparum* identifies an intergenic region in which transcription terminates and reinitiates. *EMBO J.*, in press.
- Mackay, M., Goman, M., Bone, N., Hyde, J. E., Scaife, J., Certa, U., Stunnenberg, H., and Bujard, H. (1985). Polymorphism of the precursor for the major surface antigens of *Plasmodium falciparum* merozoites: Studies at the genetic level. *EMBO J.* 4: 3823-3829.
- McCormick, M. K., Shero, J. H., Connelly, C., Antonarakis, S. E., and Heiter, P. (1990). Methods for cloning large DNA segments as artificial chromosomes in *Saccharomyces cerevisiae*. *Technique* 2: 65-71.
- Murray, A. W., Schultes, N. P., and Szostak, J. W. (1986). Chromosome length controls mitotic chromosome segregation in yeast. *Cell* 45: 529-536.
- Patarapotikul, J., and Langsley, G. (1988). Chromosome size polymorphism in *Plasmodium falciparum* can involve deletions of the subtelomeric pPFP 20 sequence. *Nucleic Acids Res.* 16: 4331-4340.
- Pollack, Y., Katzen, A. L., Spira, D. T., and Golensen, J. (1982). The genome of *Plasmodium falciparum*. I. DNA base composition. *Nucleic Acids Res.* 10: 539-546.
- Pologe, L. G., de Bruin, D., and Ravetch, J. V. (1990). A and T homopolymeric stretches mediate a DNA inversion in *P. falciparum* which results in loss of gene expression. *Mol. Cell. Biol.* 10: 3243-3246.
- Pologe, L. G., and Ravetch, J. V. (1986). A chromosomal rearrangement in a *P. falciparum* histidine-rich protein gene is associated with the knobless phenotype. *Nature* 322: 474-477.
- Pologe, L., and Ravetch, J. V. (1988). Large deletions result from breakage and healing of *P. falciparum* chromosomes. *Cell* 55: 369-374.
- Ravetch, J. V. (1989). Chromosomal Polymorphism and gene expression in *Plasmodium falciparum*. *Exp. Parasitol.* 68: 121-125.
- Riethman, H. C., Moyzis, R. K., Meyne, J., Burke, D. T., and Olson, M. V. (1989). Cloning human telomeric DNA fragments into *Saccharomyces cerevisiae* using a yeast-artificial-chromosome vector. *Proc. Natl. Acad. Sci. USA* 86: 6240-6244.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sherman, F., Fink, G. R., and Hicks, J. B. (1986). "Methods in Yeast Genetics." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shero, J. D., McCormick, M. K., Antonarakis, S. E., and Heiter, P. (1991). Yeast artificial chromosome vectors for efficient clone manipulation and mapping. *Genomics* 10: 505-508.
- Sikorski, R. S., and Boeke, J. D. (1991). *In vitro* mutagenesis and plasmid shuffling: From cloned gene to mutant yeast. In "Methods in Enzymology" (C. Guthrie and G. R. Fink, Eds.), Vol. 194, pp. 302-318. Academic Press, New York.
- Sikorski, R. S., and Heiter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 12: 19-27.
- Sim, B. K. L., Orlandi, P. A., Haynes, J. D., Klotz, F. W., Carter, J. M., Camus, D., Zegans, M. E., and Chulay, J. D. (1990). Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. *J. Cell Biol.* 111: 1877-1884.
- Smythe, J. A., Coppel, R. L., Brown, G. V., Ramasamy, R., Kemp, D. J., and Anders, R. F. (1988). Identification of a novel integral membrane protein of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA* 85: 5195-5199.
- Trager, W., and Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science* 193: 673-675.
- Triglia, T., and Kemp, D. J. (1991). Large fragments of *Plasmodium falciparum* DNA can be stable when cloned in yeast artificial chromosomes. *Mol. Biochem. Parasitol.* 44: 207-212.
- Triglia, T., Stahl, H. D., Crewther, P. E., Scanlon, D., Brown, G. V., Anders, R. F., and Kemp, D. J. (1987). The complete sequence of the gene for the knob-associated histidine-rich protein. *EMBO J.* 6: 1413-1419.
- Van Der Ploeg, L. H., Smits, M., Ponnudurai, T., Vermeulen, A., Meuwissen, J. H., and Langsley, G. (1985). Chromosome-sized DNA molecules of *Plasmodium falciparum*. *Science* 229: 658-661.
- Wahlgren, M., Aslund, L., Franzer, L., Sundvall, M., Wahlin, B., Berzins, K., McNicol, L. A., Bjorkman, A., Wigzell, H., Perimann, P., and Pettersson, U. (1986). A *Plasmodium falciparum* antigen containing clusters of asparagine residues. *Proc. Natl. Acad. Sci. USA* 83: 2677-2681.
- Walliker, D., Quakyi, I. A., Wellems, T. E., McCutchan, T. F., Szariman, A., London, W. T., Corcoran, L. M., Burkot, T. R., and Carter, R. (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* 236: 1651-1666.
- Weber, J. L. (1958). Molecular biology of malaria parasites. *Ex. Parasitol.* 66: 143-170.
- Wellems, T. E., Walker-Jonah, A., and Panton, L. J. (1991). Genetic mapping of the chloroquine resistance locus on *Plasmodium falciparum* chromosome 7. *Proc. Natl. Acad. Sci. USA* 88: 3382-3386.
- Wellems, T. E., Walliker, D., Smith, C. L., de Rosario, V. E., Maloy, W. L., Howard, R. J., Carter, R., and McCutchan, T. F. (1987). A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *Plasmodium falciparum*. *Cell* 49: 633-642.

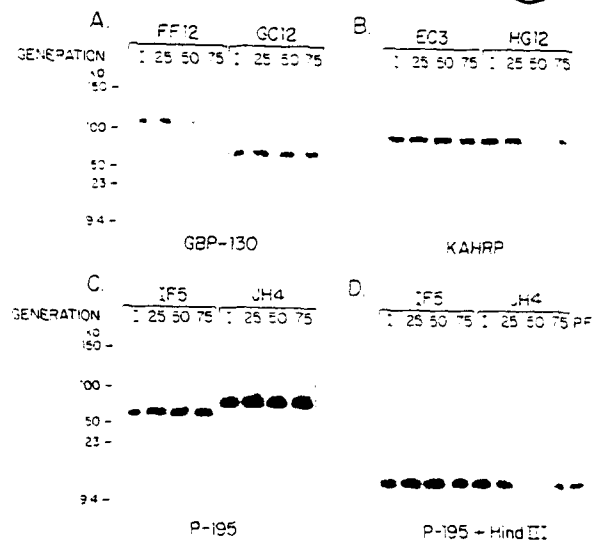
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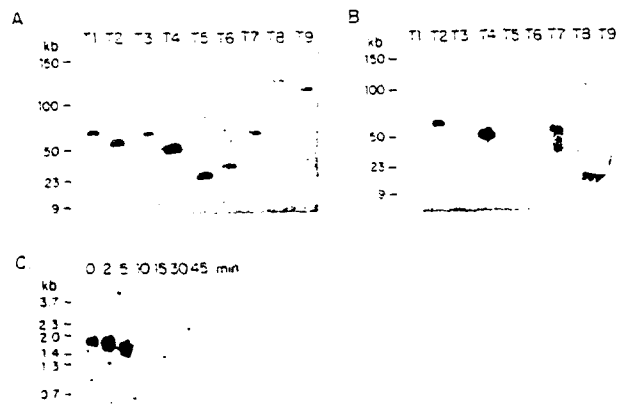
GENOM 1755-2-114



Genom 1755-3-(70)-c



Genom 1755-4-(55)-com.



Genom 1755-5-6

FCR3
HB2
D10

Mb

1.9 -



A

1.64 -

B

Transcription mapping of a 100 kb locus of *Plasmodium falciparum* identifies an intergenic region in which transcription terminates and reinitiates

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We have mapped *Plasmodium falciparum* erythrocytic stage transcription units on chromosome 10 in the vicinity of the gene encoding the glyophorin binding protein (GBP130) using yeast artificial chromosomes (YACs). Three erythrocytic stage transcription units are clustered in a 40 kb region. Two of these genes are closely linked, separated by < 2 kb. Nuclear run-on data demonstrate that transcription of these two genes, though unidirectional, is monocistronic. Within this intergenic region are the sites at which transcription of the upstream gene terminates and the GBP130 gene initiates. These studies represent the first description of the minimal and necessary *cis*-acting elements for transcription termination and initiation in this protozoan parasite.

Key words: malaria parasite/promoter/RNA processing/SV40 enhancer yeast artificial chromosomes

Introduction

The protozoan parasite responsible for the most severe form of human malaria, *Plasmodium falciparum*, alternates between vertebrate and invertebrate hosts. During this complex life cycle gene expression is regulated, as indicated by the accumulation of stage-specific transcripts (Ravetch *et al.*, 1985; Pologe and Ravetch, 1986; Waters *et al.*, 1989; Wesseling *et al.*, 1989). The mechanisms regulating gene expression in this important human pathogen are largely unknown, due in part to the difficulties of cloning and stably maintaining potential regulatory sequences in standard prokaryotic vectors and hosts. Frequent deletion and rearrangement of *P. falciparum* DNA has been observed in *Escherichia coli* hosts (Kochan *et al.*, 1986; Wellems and Howard, 1986; Weber, 1988). This may result from the extreme A + T content of the parasite's genome, which is ~80% overall and approaches 90% in non-coding regions (Goman *et al.*, 1982; Pollack *et al.*, 1982). Thus, our knowledge of the *P. falciparum* genome has been largely restricted to short and isolated fragments of the coding region, with little information on the organization of genes or the elements that regulate transcription. Defining these elements would help in our understanding of the mechanisms regulating gene expression and host switching. Furthermore, a basic understanding of the structural elements involved in transcriptional processes is a necessary first step for the development of a transfection protocol for *Plasmodium*.

Large fragments of *P. falciparum* DNA have been cloned

and propagated as artificial chromosomes in yeast (Triglia and Kemp, 1991; de Bruin, D., Lanzer, M. and Ravetch, J. V., manuscript in preparation), suggesting that DNA from this parasite can be stably maintained in the yeast host. YAC clones spanning a 100 kb region of the GBP130 locus were isolated and erythrocytic stage transcripts were mapped. Two additional transcription units were identified flanking the GBP130 gene. Using nuclear run-on assays, these transcripts were shown to be monocistronic. Sequence analysis revealed that the transcripts are continuous with their DNA. By mapping the termination and initiation sites for these genes a short intergenic region has been identified in which the minimal sequence elements required for these processes must be contained. A structural motif within this intergenic region reveals homologies to another plasmodial upstream region, suggesting common elements involved in transcriptional processes of these genes.

Results

Clustering of blood stage genes on chromosome 10 in the vicinity of the GBP130 gene

A *P. falciparum* YAC library was constructed by cloning genomic DNA, partially digested with *EcoRI*, into the YAC vector pYAC4 (de Bruin, D., Lanzer, M. and Ravetch, J. V., manuscript in preparation). YAC clones containing the GBP130 gene were identified by PCR analysis using oligonucleotides derived from the GBP130 coding region. Two YAC clones, designated FF12 and GC12, with insert sizes of 100 and 50 kb, respectively, were obtained. The two YAC clones were mapped with several restriction enzymes, including *Bam*HI, *Nco*I and *Hind*III. The restriction analysis reveals that the YAC clone GC12 is contained within clone FF12. When compared with total *P. falciparum* genomic DNA, the YAC clones were found to be unrearranged (Figure 1A). These clones have been stably propagated over 50 generations.

To determine the location of additional erythrocytic stage genes surrounding the GBP130 gene, a transcription map was derived. DNA was prepared from yeast cells harboring the GBP130 YACs, digested with the appropriate restriction enzymes, size-fractionated by pulse-field gel electrophoresis and transferred to nitrocellulose. As a control, DNA from untransformed yeast cells was prepared and treated accordingly. The nitrocellulose filter was probed with radiolabelled total cDNA which was prepared from poly(A)⁺ RNA isolated from erythrocytic stage parasites. To increase the hybridization signals the cDNA was amplified by PCR using GC rich, random primers. This choice of primers favored the amplification of coding sequences in *P. falciparum*. Since the distribution of GC rich sequences varies, cDNA species are amplified unequally. Therefore, the intensity of hybridization signals does not necessarily correlate with RNA accumulation or RNA stability (see Figures 3 and 4 for comparison). Hybridization

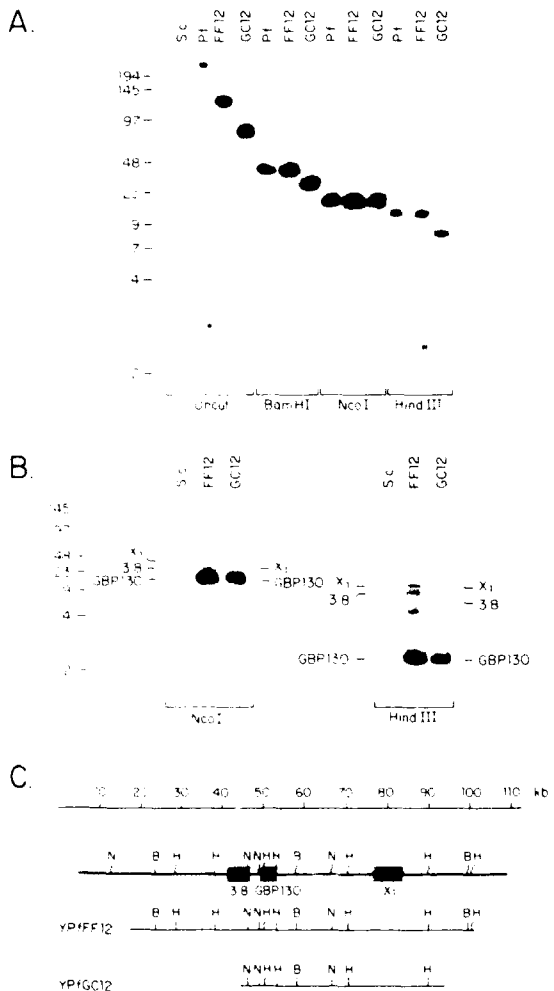


Fig. 1. Structural organization of the GBP130 YAC clones. (A) Restriction mapping *P.falciparum* genomic DNA (lanes marked P.F.), GBP130 YAC clone DNA (lanes marked FF12 and GC12, respectively) and yeast DNA (lane marked S.c.) were digested with the restriction endonucleases indicated, size-fractionated by pulse-field gel electrophoresis and transferred to nitrocellulose. The nitrocellulose filter was probed with a cDNA clone to the GBP130 gene. A DNA size standard is indicated. (B) Transcription mapping. GBP130 YAC clone DNA (FF12 and GC12) and yeast DNA (S.c.) were digested with *NcoI* and *HindIII*, respectively, size-fractionated by pulse-field gel electrophoresis and transferred to nitrocellulose. The nitrocellulose filter was hybridized with a radiolabelled, total cDNA probe. The cDNA was generated from RNA isolated from an asynchronous erythrocytic culture of FCR3 parasites. Hybridization signals specific for plasmodial sequences are identified as GBP130, 3.8 and X1. Additional hybridization signals evident on the autoradiograms were disregarded since they also appear in the yeast control lane. (C) Genomic organization and restriction map of the GBP130 locus. The two GBP130 YAC clones, FF12 and GC12, are indicated. Shaded rectangles indicate the location of transcription units. The precise location of the X1 transcription unit was not determined as denoted by the jagged borders. (N, *NcoI*; B, *BamHI*; H, *HindIII*).

signals for the *NcoI* and *HindIII* digests are shown in Figure 1B. The patterns were related to the restriction map thereby defining a chromosomal transcription map. Bands that were also present in the control lane marked S.c. were disregarded. In addition to the GBP130 gene at least two new erythrocytic transcription units were identified and

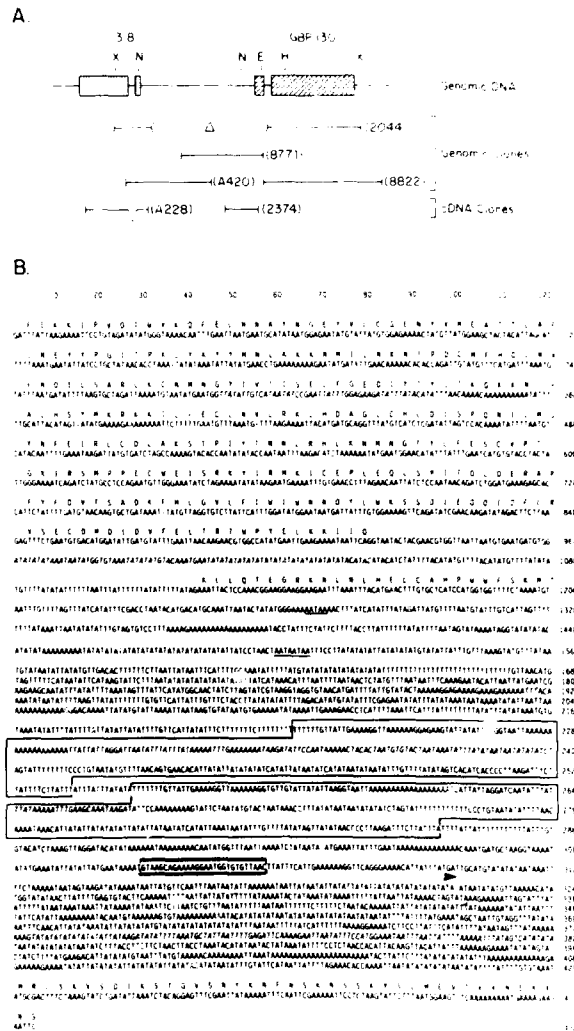


Fig. 2. Genomic organization and the sequence of the 3.8 gene and the GBP130 intergenic region. (A) Genomic organization and clones. Open reading frames are indicated by rectangles. Several genomic and cDNA clones are shown. A triangle in the genomic clone 2044 indicates an internal deletion generated during cloning in *E.coli* (Kochan *et al.*, 1986). (B) Sequence of the GBP130 intergenic region. The intergenic region is flanked by the 3.8 and GBP130 open reading frames as indicated. Two polyadenylation sites for the 3.8 gene are underlined. A duplication of 305 bp is indicated by large boxes. A sequence element with homology to the SV40 core enhancer sequence is highlighted. The GBP130 transcription start site is indicated by an arrowhead.

designated as 3.8 and X1. The chromosomal location of these transcription units is shown in Figure 1C.

Two blood stage transcription units are tightly linked

Restriction mapping of this locus revealed that two of these blood stage genes, the 3.8 and the GBP130 genes, are tightly linked by a short intergenic region of <2 kb. To define this intergenic region the locus was cloned and sequenced, as presented in Figure 2. The sequence reveals the presence of two open reading frames, separated by a 3 kb region of AT rich sequence, characteristic of non-coding sequence in *P.falciparum*. Probes were derived from the 5' open reading frame and used to isolate cDNA clones from a library

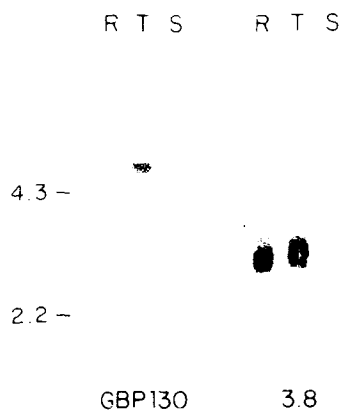


Fig. 3. Stage-specific expression of the GBP130 and 3.8 genes. Stage-specific RNA was isolated from ring (R), trophozoite (T) and schizont (S) parasites, size-fractionated on a 0.8% agarose gel and transferred to nitrocellulose. The nitrocellulose filters were hybridized with a cDNA clone to the GBP130 gene and with the cDNA clone A228 to the 3.8 gene, respectively. An RNA size standard is indicated. Unlike previously published results (Ravetch *et al.*, 1985), the GBP130 transcript accumulates only in trophozoites. The separation of parasite stages by percoll-sorbitol gradient centrifugation enabled us to determine the stage specific transcription of this gene with greater accuracy (Kutner *et al.*, 1985).

generated from asynchronously growing erythrocytic stage parasites (Ravetch *et al.*, 1985). A comparison of the cDNA sequence with the genomic sequence revealed an intron of 201 bp, which is flanked by consensus acceptor and donor sites. Thus, the linkage of two blood stage genes as deduced from the transcription map is confirmed by these structural data.

The erythrocytic stage-specific expression of these two genes was determined by Northern analysis. Total cellular RNA was isolated from the ring, trophozoite and schizont intra-erythrocytic forms of the parasite. When the Northern blot was hybridized with a probe to the 3.8 gene, a single RNA species of 3.8 kb was observed in ring and trophozoite stage parasites (Figure 3). Rehybridization of the same blot with a GBP130 probe revealed the GBP130 transcript of 6.6 kb in trophozoites (Figure 3). A probe from the intergenic region did not hybridize to any RNA species (data not shown).

Transcription of the GBP130 gene is monocistronic and continuous

A nuclear run-on analysis was performed to determine whether transcription of the 3.8 and the GBP130 genes are monocistronic or polycistronic. If transcription of these two genes is monocistronic then the intergenic region should contain regulatory signals. Nuclei were isolated during the trophozoite stage, in which both genes are transcribed. Preformed transcription complexes were allowed to elongate in the presence of labelled nucleotides. The radiolabelled, nascent RNA was used as a probe for DNA fragments spanning this locus (Figure 4). Fragment size and base composition were approximately equivalent for these fragments. Nascent RNA hybridized to fragment 1 which contains the 3.8 gene and to fragments 3–6 which span the GBP130 gene. By contrast, the intergenic region, fragment 2, did not hybridize to nascent RNA, indicating that it is

Transcription units in *P. falciparum*

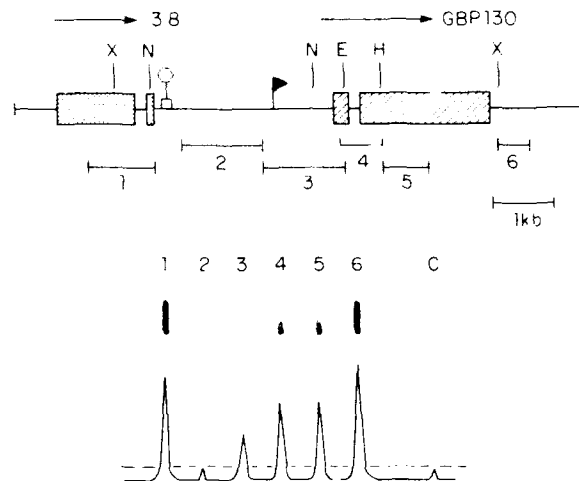


Fig. 4. The 3.8 and the GBP130 genes define independent transcription units. The schematic drawing reveals the organization of the locus and the orientation of the 3.8 and GBP130 genes. Shaded rectangles denote the open reading frames of these genes. The initiation site of the GBP130 gene and the termination site of the 3.8 gene are indicated. DNA fragments spanning the entire locus are presented and numbered. The isolated, single stranded DNA fragments were immobilized on a nitrocellulose filter which was hybridized with labelled, nascent RNA generated in a nuclear run-on analysis. Nuclei were prepared from trophozoite stage parasites. A quantitative analysis obtained by scanning the autoradiogram is shown. C is a non-plasmidial AT rich fragment included as a control. A gradient of signal intensity is seen, reflecting the distribution of labelled transcripts generated during the extension reaction, which hybridize to the single stranded DNA probes used. RNase was included in the washing buffer to remove radiolabelled sequences outside of the hybridization target (Washing conditions: two washes at 55°C in 0.1×SSC, 0.1% SDS for 20 min each; and one wash at 42°C in 2×SSC, 50 µg/ml of RNase A for 1 h.)

not transcribed (Figure 4). Thus, the 3.8 and the GBP130 genes are transcribed independently in a monocistronic fashion. The 3.8 kb transcript is terminated with an efficiency of >90% as calculated from the ratio of radioactivity bound to fragment 1 versus 2.

The precise termination site for the 3.8 transcript was determined by RNase protection experiments (Figure 5A). A single stranded, radiolabelled RNA probe complementary to the 3.8 mRNA was generated (probe A, Figure 5C) and hybridized to poly(A)⁺ trophozoite RNA. Upon RNase digestion a major species of 300 bp was detected, as well as two minor species of 130 and 140 bp in size. The major species maps to the consensus polyadenylation site (AATAA) at position 1500 (see Figure 2B), while the minor species map to the polyadenylation site at position 1270. The polyadenylation site at position 1500 has been confirmed by the isolation of poly(A) containing cDNA clone (A228) which has utilized this site. These data verify the orientation of the 3.8 transcript and its termination site in the intergenic region.

The 5' end of the GBP130 gene was determined by S1 mapping and primer extension (Figure 5B). The primer extended product was recovered from the gel and analyzed by anchored PCR, confirming that the primer used hybridized to and extended the GBP130 RNA. Both primer extension and S1 analysis map the 5' end of the GBP130 RNA to position 3216 (numbering refers to Figure 2B).

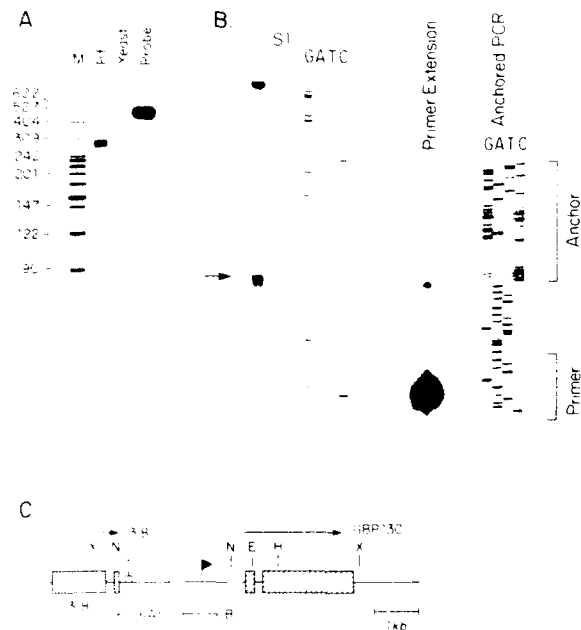


Fig. 5. Mapping of termination and initiation sites in the intergenic region. (A) Termination of the 3.8 gene. RNase protection analysis: a single stranded, radiolabelled probe specific for the 3.8 gene (probe A, position 1179–1582 in Figure 2B) was generated and hybridized to 10 µg of poly(A)⁺ trophozoite RNA. Upon RNase digestion products were analyzed by gel electrophoresis (lane marked P.E.). The sizes of the products were compared with a standard. A control using yeast poly(A)⁺ RNA was analyzed in parallel (lane marked yeast). (B) Initiation of the GBP130 gene. S1 mapping analysis: a single stranded, end labelled probe (probe B, position 3029–3421 in Figure 2B) specific for the GBP130 gene was hybridized to 15 µg of total cellular RNA prepared from trophozoites. Upon digestion with S1 (16°C and 330 U/ml of enzyme for 90 min) products were analyzed by gel electrophoresis. The size of the product (indicated by an arrow) was compared with a sequencing reaction. Primer extension analysis: an end labelled primer (corresponding to position 3236–3269 in Figure 2B) was hybridized to 1 µg of poly(A)⁺ trophozoite RNA. Extension products were analyzed by gel electrophoresis and compared with a sequencing reaction of genomic DNA using the same primer. The primer extended product was recovered from the gel, amplified by anchored PCR technology (Loh *et al.*, 1989), cloned and sequenced. (C) Schematic drawing of the locus. The probes used for RNase protection assay (probe A) and for S1 mapping (probe B) are indicated. The termination site for the 3.8 transcript is indicated by a hexagon and the initiation site for the GBP130 gene by the arrowhead.

	α-Amanitin	
	-	+
rRNA	●	●
GBP130	●	●
CS	●	○
pUC18	○	○

Fig. 6. α-amanitin sensitive transcription of the GBP130 gene. Nuclei were isolated from trophozoite parasites. One aliquot of the nuclei preparation was incubated with 100 µg/ml of α-amanitin prior to transcription. A gene expressed only during the insect stage, the CS gene (Enea *et al.*, 1984) and the ribosomal rRNA genes (Langley *et al.*, 1983) were analyzed in parallel for comparison.

Transcription of the GBP130 gene is sensitive to the RNA polymerase inhibitor α-amanitin as determined by nuclear run-on analysis (Figure 6).

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GTGTGGAAATGTTGTCAC-ITAGGGTGTGGAAATGTCCTT    SV40 ENHANCER
* * * * *
GTGTGGAAATGTTGTCAC-ITAGGGTGTGGAAATGTCCTT    CS
* * * * *
ATAAAATGTAAGCAGAAAAGGAATGGTGTGTTAACTTATT    GBP130
    
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Fig. 7. Sequence analysis. Sequence elements derived from the GBP130 intergenic region and from the upstream region of the *P. knowlesi* CS gene are compared with the SV40 core enhancer region.

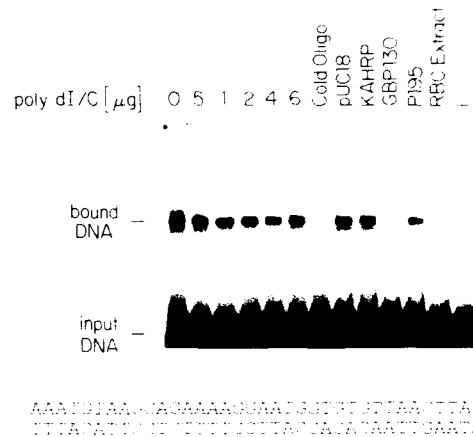


Fig. 8. Interaction of the GBP130 sequence element with nuclear extracts. 2 fmol of double stranded, end labelled oligonucleotides containing the GBP130 sequence element were incubated with 5 µg of crude nuclear extracts derived from asynchronously growing parasite cultures. The sequence of the oligonucleotide is shown at the bottom. The amount of poly dI/C added to the binding assays is indicated. For cross competition experiments 50 ng of unlabelled GBP130 oligonucleotides or 2 µg of DNA fragment containing the GBP130 intergenic region were added. In addition 2 µg of pUC18 DNA and fragments containing the upstream region either of the KAHRP gene (M. Lanzer, D. de Bruin and J.V. Ravetch, manuscript in preparation) or the P195 gene (Myler, 1990) were tested for their ability to compete. A control experiment was performed using extracts from uninfected erythrocytes (lane marked RBC extract).

These data indicate that the GBP130 gene is closely linked to another blood stage gene, which is transcribed in the same orientation. Since transcription of the 3.8 and GBP130 genes is monocistronic, the region between both genes must contain the minimal elements that signal both the termination and initiation of transcription in *P. falciparum* blood stage genes.

Structural analysis of the intergenic region

The intergenic region defined above was examined for sequence elements indicative of eukaryotic promoters. The sequence at position 3029–3063 (highlighted in Figure 2B) shows homology to the core region of the SV40 enhancer sequence (Weiher *et al.*, 1983) and to a sequence element found in the upstream region of the *Plasmodium knowlesi* CS gene (Ruiz i Altaba *et al.*, 1987) (Figure 7). To determine whether this sequence element interacts with nuclear proteins, gel retardation assays were performed (Figure 8). Oligonucleotides containing this element were incubated with nuclear extracts derived from asynchronously growing *P. falciparum* erythrocytic cultures. A stable complex is observed, even in the presence of high concentrations of non-specific competitor DNA. The stability of this complex was analyzed by cross competition experiments. Neither pUC18 DNA nor DNA fragments containing the upstream region of the KAHRP (M. Lanzer,

D. de Bruin and J. V. Ravetch, manuscript in preparation) or the P195 genes (Myler, 1990) can compete for the complex. By contrast, complex formation is not observed in the presence of unlabelled GBP130 oligonucleotides or of DNA containing the GBP130 intergenic region. Extracts prepared from uninfected erythrocytes do not interact with the sequence element tested. These data suggest that the sequence element found in the GBP130 intergenic region is a target for protein-DNA interactions. No further homologies to other known protein binding sites were found. Another prominent feature of the intergenic region is the presence of a 305 bp duplication between positions 2223 and 2855, indicated by the boxed sequences in Figure 2B.

Discussion

During the asexual erythrocytic stage of the malaria parasite *P. falciparum*, three distinct morphological stages have been defined—the ring, trophozoite and schizont. In addition to the morphological distinctions evident during these stages, discrete patterns of gene expression have been observed, both for protein (Hall *et al.*, 1984; Perkins, 1988; Weber 1988; Kemp *et al.*, 1990) and RNA (Pologe and Ravetch, 1986; Waters *et al.*, 1989; Wesseling *et al.*, 1989). In a study characterizing five blood stage genes by nuclear run-on analysis, we have determined that the changes observed in RNA accumulation during the various morphological stages result from the regulation of transcriptional activity (M. Lanzer, D. de Bruin and J. V. Ravetch, manuscript in preparation). Thus, plasmodial genes are regulated during the switch from invertebrate to vertebrate hosts and during differentiation within a single host cell. However, the molecular basis for this stage-specific gene regulation is unknown due to the lack of structural information regarding potential regulatory sequences and a functional assay in which to test these sequences. The isolation and characterization of potential regulatory sequences from *P. falciparum* is a necessary prerequisite for the development of these functional assays. In this study, we have identified a 2 kb region of DNA in which we demonstrate the presence of the minimal elements for transcriptional termination and initiation for blood stage genes.

Structural information regarding large fragments of plasmodial DNA has not been available due to the inability to clone these sequences in an unrearranged and stable form (Weber, 1988). Consequently, only one linkage between plasmodial genes has been established to date (Robson and Jennings, 1991). Long linear fragments of *P. falciparum* DNA were cloned as artificial chromosomes in yeast. Unrearranged sequences for a 100 kb region have been isolated and found to be stably propagated in the yeast host (Figure 1). By probing these YAC clones with labelled, total cDNA, novel transcription units have been identified. The transcription map derived from this study reveals three erythrocytic transcripts contained on a 100 kb region of chromosome 10.

The linkage of the 3.8 and the GBP130 genes defines a short intergenic region of 2 kb. Nuclear run-on analysis indicates that these genes are independent transcription units, with discrete initiation and termination sites. Thus, this observation strongly suggests the presence of signals for transcriptional termination (for the 3.8 gene) and initiation (for the GBP130 gene) within this intergenic region.

The 3' end of the 3.8 gene was found to map to consensus polyadenylation sites which are flanked by long poly(A) and poly(T) tracks. These sequences have the potential to form stem-loop structures in the transcribed RNA which may be associated with the termination of transcription. This region has features characteristic of termination sites defined for the slime mold *Dictyostelium* (Kimmel and Firtel, 1982), in which a consensus polyadenylation signal precedes a genomic poly(A) track of 30 nucleotides. A similar sequence organization has been reported for the termination site of the CS gene of the simian malaria parasite *P. knowlesi* (Ruiz i Altaba *et al.*, 1987). cDNA clones isolated for the 3.8 gene predict an open reading frame encoding a novel plasmodial protein. Comparison of this sequence with the protein database (Dayhoff, December 1991) revealed homology to the family of serine kinases, particularly in the region between amino acids 80 and 170, the enzymatic active site.

A unique initiation site was observed for the GBP130 gene as well as for two other plasmodial genes (the P195 and the KAHRP genes, M. Lanzer, D. de Bruin and J. V. Ravetch, manuscript in preparation). In contrast, multiple initiation sites have been suggested for the three other plasmodial genes investigated to date [the CS gene of the simian malaria parasite *P. knowlesi* (Ruiz i Altaba *et al.*, 1987); the Py230 gene of the rodent parasite *Plasmodium yoelii* (Lewis, 1990) and the P195 gene of *P. falciparum* (Myler, 1990)]. One reason for this difference may be due to the frequent pausing of reverse transcriptase in AT rich regions, which could be misinterpreted as multiple initiation sites. Comparison of genomic and cDNA sequences indicates that the GBP130 gene is continuously transcribed. However, post-transcriptional processing of the transcript occurs through *cis*-splicing and polyadenylation. Transcription of the GBP130 gene is sensitive to the RNA polymerase inhibitor α -amanitin. Similar to other eukaryotic genes transcribed by α -amanitin sensitive polymerases, the sequences immediately upstream of the initiation site for the GBP130 gene contain features suggestive of eukaryotic promoters. A sequence element in the GBP130 intergenic region was found to be homologous to the core region of the SV40 enhancer (Weiher *et al.*, 1983) and to a similar sequence motif in the upstream region of the *P. knowlesi* CS gene (Ruiz i Altaba *et al.*, 1987). The GBP130 sequence element was found to bind to nuclear proteins derived from erythrocytic stage parasites in a sequence-specific manner in mobility shift assays. Although these homologies are suggestive of promoter elements, the lack of a functional assay for putative plasmodial promoters, either *in vitro* or *in vivo*, limits the conclusions that can be drawn regarding the role of this sequence in parasite gene transcription. We would expect that this element is involved in more general transcriptional processes and not in stage-specific regulation, since it is present in genes transcribed at different stages of the parasite's life cycle. Precise stage-specific regulation of the GBP130 gene may be mediated by the large direct duplication that is unique for the upstream sequence of this gene.

Materials and methods

Cultivation of parasites

The *P. falciparum* strains A2 and FCR3 were grown and maintained as described by Trager and Jansen (1976) and by Trager *et al.* (1981). If not stated otherwise the clonal *P. falciparum* strain A2 was used. Parasite cultures

were synchronized by percoll-sorbitol gradient centrifugation (Kutner *et al.*, 1985). No gametocytes were observed in the culture under the growth conditions employed.

Construction of *P. falciparum* YAC library

A *P. falciparum* YAC library was constructed as described (de Bruin, D., Lanzer, M. and Ravetch, J.V., manuscript in preparation). Genomic DNA was prepared from the *P. falciparum* strain FCR3 (Goman *et al.*, 1982), partially digested with *EcoRI*, and inserted into the *EcoRI* cloning site of the YAC vector pYAC4 (Burke *et al.*, 1987). Yeast spheroplasts (strain AB1380, ATCC 20843) were transformed with the ligation mixture as described by McCormick *et al.* (1989) with the exception that polyamines were excluded. Transformants were selected on media lacking either uracil or uracil and tryptophan. The YAC library was screened by PCR analysis (Heard *et al.*, 1989; Green and Olson, 1990).

Mapping of YAC clones

YAC clone DNA imbedded in agarose plugs (Schwartz and Cantor, 1984) was digested with restriction endonucleases and size fractionated by pulse-field gel electrophoresis using a the Bio-Rad CHEF-DR11 system [pulse-field conditions: ramped pulse from 2.5 to 10 s over 18 h at 170 V, 1% LE agarose (FMC), 0.5xTBE, at 14°C]. DNA was transferred to nitrocellulose filters and hybridized with nick translated DNA fragments or with radiolabelled total cDNA. Probes for transcription mapping were generated by the reverse transcription of 1 µg of poly(A)⁺ RNA prepared from an asynchronous erythrocytic culture [50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 6 µM dATP, 1 µM [α -³²P]dATP (3000 Ci/ml), 0.5 µg/ml pd(N)₆, 40 U of rRNasin (Promega) and 600 U of M-MLV H⁻ reverse transcriptase (superscript, BRL) for 60 min at 43°C]. The total cDNA was purified by column chromatography and amplified by PCR in the presence of [α -³²P]dCTP using the TAG-IT kit (BIOS) which uses dt(N)₆(GC)(GC)(GC) as primers. Hybridization conditions (Kochan *et al.*, 1986) included 200 µg/ml of total yeast RNA as competitor.

Nuclear run-on analysis

All steps were carried out on ice. At a parasitemia of ~5% *P. falciparum* cultures were chilled on ice. The contents of 30 10 cm Petri dishes were collected and washed once in 1xPBS buffer. Erythrocytes were lysed by the addition of an equal volume of 0.1% saponin (Wallach, 1982), followed by one wash in solution A (20 mM PIPES pH 7.5, 15 mM NaCl, 60 mM KCl, 14 mM β-mercaptoethanol, 0.5 mM EGTA, 4 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.125 mM PMSF). The parasite pellet was resuspended in 3 ml of solution A and transferred to a dounce homogenizer. 200 µl of a 10% NP-40 solution was added and six strokes with a B pestle were applied. Nuclei were collected (4000 r.p.m. for 10 min in a Sorvall SM24 rotor) and washed once in solution A. 5x10⁹ nuclei were transcribed at 37°C for 10 min in 600 µl of solution B [50 mM HEPES pH 7.9, 50 mM NaCl, 10 mM MgCl₂, 1.2 mM DTT, 10 mM creatine phosphate, 1 mM GTP, 1 mM CTP, 4 mM ATP, 25% glycerol, 125 U/ml rRNasin (Promega), 0.2 mg/ml creatine kinase and 0.5 µM [α -³²P]UTP (3000 Ci/mmol)]. Radiolabelled RNA was isolated (Chomczynski and Sacchi, 1987) and purified by TCA precipitation. Usually 2x10⁷ c.p.m. were incorporated into nascent RNA, with a specific activity of 9x10⁷ c.p.m. µg. The nascent RNA was hybridized to single stranded DNA fragments (0.2 pmol) immobilized on nitrocellulose. The prehybridization and hybridization conditions are described (Nevins, 1987). Filters were washed three times for 20 min in 2xSSC, 0.1% SDS at room temperature, twice at 55°C in 0.1xSSC, 0.1% SDS, followed by one wash at 42°C in 2xSSC, 50 µg/ml of RNase A for 1 h. Filters were dried and exposed overnight at -70°C with an intensifying screen.

Bacterial strains and libraries

To minimize recombination and deletion events plasmid DNA was propagated in the *E. coli* host, SURE (Stratagene). Two libraries of *P. falciparum* (strain A2), a pUC9 plasmid cDNA (Kochan *et al.*, 1986) and a λgt11 genomic library were screened using standard methods (Maniatis *et al.*, 1989). The integrity of all clones and sequences was confirmed by Southern analysis.

Primer extension

0.1 pmol (1.5x10⁵ c.p.m.) of end labelled oligonucleotide primer (5'-GAAGTACTCAAAAATAAGTTATATACCATATG-3') and 1 µg of poly(A)⁺ trophozoite RNA were coprecipitated and hybridized (Maniatis *et al.*, 1989). After ethanol precipitation the primer was extended at 43°C for 90 min [50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM dNTP, 40 U of rRNasin (Promega) and 300 U of M-MLV H⁻ reverse transcriptase (superscript, BRL)]. Products were

analyzed by gel electrophoresis. Primer extension products were recovered from the polyacrylamide gel (Maniatis *et al.*, 1989), tailed with dGTP and amplified (Loh *et al.*, 1989). Amplified DNA fragments were cloned into pUC18 and sequenced by using the universal forward primer.

S1 mapping

A *HincII*-*NcoI* fragment was isolated from the genomic clone 8771. This fragment was used to generate a single stranded DNA probe by PCR amplification using the end labelled primer (5'-TATTAATAATATTAACAGATTAAG-3'). The single stranded product was purified by gel electrophoresis. 2x10⁵ c.p.m. of the probe and 15 µg of total cellular RNA were hybridized (Maniatis *et al.*, 1989). S1 digestion was carried out at 16°C for 90 min with 330 U/ml of S1.

RNase protection assay

A *NcoI*-*HincII* fragment corresponding to position 1179-1582 in Figure 2B was cloned into pGEM3. A single stranded RNA probe complementary to the 3.8 mRNA was generated, gel purified and hybridized to 10 µg of total cellular trophozoite RNA. Hybridization and digestion conditions (0.5 U/ml of RNase A and 100 U/ml of RNase T1 for 30 min at 37°C) were followed as recommended by the manufacturer of the ribonuclease protection assay kit (Ambion).

Northern analysis

Total cellular RNA was isolated by the acidic guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). 5 µg of total cellular RNA were fractionated on a 0.8% agarose-formaldehyde gel, transferred to nitrocellulose and hybridized with nick-translated probes. Hybridization conditions are described by Polog and Ravetch (1986).

Preparation of nuclear extracts

Parasites were prepared by saponin lysis (Wallach, 1982). The following method was adapted from Schreiber *et al.* (1989). About 5x10⁹ parasites were resuspended in 1 ml of lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.65% NP-40). Nuclei were collected by centrifugation and extracted with 100 µl of extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF). After 15 min of vigorous shaking the extract is cleared by centrifugation, yielding a protein concentration of 1-2 µg/µl. 5 µg of crude nuclear extract were incubated with 2 fmol of double-stranded, end labelled oligonucleotides for 20 min at room temperature [20 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.25 mg/ml BSA, 2 µg poly d(IC) or as indicated; final volume: 15 µl]. Binding assays were analyzed by gel electrophoresis (4% polyacrylamide, 5% glycerol and 0.5xTBE).

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References

Burke, D.T., Carle, G.F. and Olson, M.V. (1987) *Science*, **236**, 806-812.
 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156-159.
 Enea, V., Ellis, J., Zavala, F., Arnot, D.E., Asavanich, A., Masuda, A., Quakyi, I. and Nussenzweig, R.S. (1984) *Science*, **225**, 628-629.
 Goman, M., Langsley, G., Hyde, J.E., Yankovsky, N.K., Zolg, J.W. and Scaife, J.G. (1982) *Mol. Biochem. Parasitol.*, **5**, 391-400.
 Green, E.D. and Olson, M.V. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1213-1217.
 Hall, R., Osland, A., Hyde, J.E., Simmons, D.L., Hope, I.A. and Scaife, J.G. (1984) *Mol. Biochem. Parasitol.*, **11**, 61-80.
 Heard, E., Davies, B., Fee, S. and Fried, M. (1989) *Nucleic Acids Res.*, **17**, 5861.
 Kemp, D.J., Cowman, A.F. and Walliker, D. (1990) *Adv. Parasitol.*, **29**, 75-149.
 Kimmel, A.R. and Firtel, R.A. (1982) In Loomis, W. (ed.), *The Development of Dictyostelium discoideum*. Academic Press, New York, pp. 233-244.
 Kochan, J., Perkins, M. and Ravetch, J.V. (1986) *Cell*, **44**, 689-696.
 Kutner, S., Breuer, W.V., Ginsburg, H., Aley, S.B. and Cabantchik, Z.I. (1985) *J. Cell. Physiol.*, **125**, 521-527.

- Langsley, G., Hyde, J. H., Goman, H. and Scaife, J. G. (1983) *Nucleic Acids Rev.*, **11**, 8703–8717.
- Lewis, P. A. (1990) *Mol. Biochem. Parasitol.*, **39**, 285–288.
- Loh, E. Y., Elliot, J. F., Cwirla, S., Lanier, L. L. and Davis, M. M. (1989) *Science*, **243**, 217–220.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- McConmick, M. K., Shew, J. H., Cheung, M. C., Kan, Y. W. and Hieter, P. A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9991–9995.
- Myler, P. J. (1990) In Agabian, N. and Cerami, A. (eds), *Parasites: Molecular Biology, Drugs, and Vaccine Design*. Wiley-Liss, pp. 123–137.
- Nevins, J. R. (1987) *Methods Enzymol.*, **152**, 234–241.
- Perkins, M. (1988) *Exp. Parasitol.*, **65**, 61–68.
- Pollack, Y., Katzen, A. L. and Golenser, J. (1982) *Nucleic Acids Rev.*, **10**, 539–546.
- Pologe, L. G. and Ravetch, J. V. (1986) *Nature*, **322**, 474–477.
- Ravetch, J. V., Kochan, J. and Perkins, M. (1985) *Science*, **227**, 1593–1597.
- Robson, K. J. H. and Jennings, M. W. (1991) *Mol. Biochem. Parasitol.*, **46**, 19–34.
- Ruiz, I., Altaba, A., Ozaki, L. S., Gwadz, R. W. and Godson, G. N. (1987) *Mol. Biochem. Parasitol.*, **23**, 233–245.
- Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) *Nucleic Acids Rev.*, **17**, 6419.
- Schwarz, D. C. and Cantor, C. R. (1984) *Cell*, **37**, 67–75.
- Trager, W. T. and Jansen, J. B. (1976) *Science*, **193**, 673–675.
- Trager, W. T., Tershakovec, M., Lyandvert, L., Stanley, H., Lanners, N. and Grubert, E. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6527–6530.
- Triglia, T. and Kemp, D. J. (1991) *Mol. Biochem. Parasitol.*, **44**, 207–211.
- Waters, A. P., Syn, C. and McCutchan, T. F. (1989) *Nature*, **342**, 438–440.
- Wallach, M. (1982) *Mol. Biochem. Parasitol.*, **6**, 335–342.
- Weber, J. L. (1988) *Exp. Parasitol.*, **66**, 143–170.
- Weither, H., Kong, M. and Gruss, P. (1983) *Science*, **219**, 626–631.
- Wellems, T. E. and Howard, R. J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6065–6069.
- Wesseling, J. G., Snijders, P. J. F., van Someren, P., Jansen, J., Smits, M. A. and Schoenmakers, J. G. G. (1989) *Mol. Biochem. Parasitol.*, **35**, 167–176.

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A sequence element associated with the *Plasmodium falciparum* KAHRP gene is the site of developmentally regulated protein-DNA interactions

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ABSTRACT

The *Plasmodium falciparum* gene encoding the knob associated histidine-rich protein (KAHRP) is shown to be transcriptionally regulated during its expression in the intraerythrocytic cycle as demonstrated by stage specific nuclear run-on analysis. The genomic organization of the KAHRP gene was determined and the structural basis for the stage specific transcription investigated. A sequence motif with two-fold symmetry was found 160 bp upstream of the RNA initiation site. This sequence element interacts with parasite derived nuclear extracts in a stage specific manner that correlates with the transcriptional activity of the KAHRP gene. These studies suggest a functional role for this structural element in the developmental regulation of a *P. falciparum* erythrocytic gene.

INTRODUCTION

Nearly half of the world's population lives in malaria endemic areas. Transmitted to humans by the bite of an infected mosquito, malaria parasites multiply asexually first in hepatocytes then in erythrocytes. The most severe form of human malaria is caused by the protozoan parasite *Plasmodium falciparum*, claiming over three million lives annually. The high mortality associated with *P. falciparum* results from the occlusion of capillaries by infected erythrocytes which adhere to endothelial cells (1, 2, 3, 4). The cytoadherence of infected erythrocytes is dependent upon the interaction of parasite encoded proteins that are translocated to the erythrocytic membrane with receptors expressed on endothelial cells, such as CD36 and ICAM-1 (5, 6). Parasite mutants have been described that exhibit reduced cytoadherence (3, 7, 8). This phenotype was linked to the deletion of a parasite encoded gene, the knob associated histidine-rich protein (KAHRP) (9).

During the asexual intraerythrocytic cycle of parasite development, the KAHRP gene is expressed in a stage specific manner, as indicated by the analysis of steady state RNA (9) and protein accumulation (10, 11). The molecular mechanisms

regulating the expression of this important parasite gene are unknown. To dissect the structural motifs associated with the developmental regulation of *P. falciparum* genes, we have studied the expression and regulation of the KAHRP gene during the intraerythrocytic cycle. A comparison of transcriptional activity as determined by nuclear run-on analysis and RNA accumulation revealed that the KAHRP gene is transcriptionally regulated. A sequence element with two-fold symmetry has been found to interact in a stage specific manner with nuclear extracts. This stage specific interaction correlates with the transcriptional activity of this gene, suggesting that this sequence element may be involved in the developmental expression of the KAHRP gene.

MATERIALS AND METHODS

Cultivation of Parasites

The clonal *P. falciparum* strains FCR3-A2 and FVO⁻ were grown and maintained as described (29). Different intraerythrocytic stages were separated by percoll/sorbitol gradient centrifugation (30). This method allows the separation of these stages with great accuracy as determined in blood smears. Thus, in the trophozoite stage preparation no other stages were detectable. The same is true for the ring and the schizont stage preparation.

Northern analysis

After saponin lysis of infected erythrocytes (31), total cellular RNA was isolated by the acidic guanidinium-phenol chloroform method (32). 1.5 µg of total cellular RNA was fractionated on a 0.8% agarose-formaldehyde gel, transferred to nitrocellulose and hybridized with the nick-translated probe LP20 to the KAHRP gene (9).

Nuclear run-on analysis

All steps were carried out on ice. At a parasitemia of about 5%, cultures were chilled on ice. The contents of thirty 10 cm petri dishes were collected and washed once in 1 × PBS buffer. Erythrocytes were lysed by the addition of an equal volume of

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0.1% saponin (31), followed by one wash in solution A (20 mM PIPES pH 7.5, 15 mM NaCl, 60 mM KCl, 14 mM β -mercaptoethanol, 0.5 mM EGTA, 4 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.125 mM PMSF). The parasite pellet was resuspended in 3 ml of solution A and transferred to a dounce homogenizer. 200 μ l of a 10% NP-40 solution was added and six strokes with a B pestle were applied. Nuclei were collected (4,000 rpm for 10 min, Sorvall SM24 rotor) and washed once in solution A. 5×10^9 nuclei were transcribed at 37°C for 10 min in 600 μ l of solution B (50 mM HEPES pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1.2 mM DTT, 10 mM creatine phosphate, 1 mM GTP, 1 mM CTP, 4 mM ATP, 25% glycerol, 125 units/ml rRNasin (Promega), 0.2 mg/ml creatine kinase, 0.5 μ M [α - ^{32}P]UTP 3000Ci/mmol). Radiolabelled RNA was isolated (32) and purified by TCA precipitation. Typically 2×10^7 cpm were incorporated into nascent RNA, with a specific activity of 9×10^7 cpm/ μ g. The nascent RNA was hybridized to single stranded DNA fragments (0.2 pmol) immobilized on nitrocellulose. The prehybridization and hybridization conditions were as described (33). Filters were washed three times for 20 min in $2 \times$ SSC, 0.1% SDS at room temperature, twice at 55°C in $0.1 \times$ SSC, 0.1% SDS, followed by one wash at 42°C in $2 \times$ SSC, 50 μ g/ml of RNase A for one hour. Filters were dried and exposed overnight at -70°C with an intensify screen.

Bacterial strains and libraries

To minimize recombination and deletion events plasmid DNA was propagated in the *E. coli* host, SURE (Stratagene). A lambda gt11 genomic library of *P. falciparum* (strain A2) was screened using standard methods (34). The integrity of all clones and sequences were confirmed by Southern analysis.

Primer extension

0.1 pmol (1.5×10^5 cpm) of end labelled oligonucleotide primer (5'-CATAATTAATAACAATTAAGTGAAATAAAAAC-3', position 1819 to 1850 in Fig. 3B) and 0.4 μ g of poly A+ ring RNA from the parasite strain FCR3-A2 were coprecipitated and hybridized (34). After ethanol precipitation the primer was extended at 43°C for 90 min (50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 40 units of rRNasin (Promega), and 300 units of M-MLV H reverse transcriptase (superscript, BRL)). Products were analyzed by gel electrophoresis. Primer extension products were recovered from the polyacrylamide gel (34), tailed with dGTP and amplified (35). Amplified DNA fragments were cloned into pUC18 and sequenced by using the universal forward primer.

RNase protection assay

A NsiI/EcoRI fragment corresponding to position 1419 to 2215 in Fig. 3B was cloned into pGEM3. A single stranded RNA probe complementary to the KAHRP mRNA was generated, gel purified and hybridized to 1 μ g of poly A+ ring RNA from the parasite strains FCR3-A2 and FVO respectively. Hybridization and digestion conditions (0.5 units/ml of RNase A, 100 units/ml of RNase T1 for 30 min at 37°C) were followed as recommended by the manufacturer of the ribonuclease protection assay kit (Ambion).

Preparation of nuclear extracts

Parasites were prepared by saponin lysis (31). Nuclear extracts were prepared as described (36). About 5×10^9 parasites were resuspended in 1 ml of lysis buffer (10 mM HEPES pH 7.9,

10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.65% NP-40). Nuclei were collected by centrifugation and extracted with 100 μ l of extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 15 min of vigorously shaking, the extract is cleared by centrifugation and yields a protein concentration of 1–2 μ g/ μ l. Five microgram of total nuclear protein was incubated with 10 fmol of double stranded, end labelled oligonucleotides for 20 min at room temperature (20 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% Glycerol, 0.25 mg/ml BSA, 2 μ g poly d(I/C) or as indicated; final volume: 15 μ l). Binding assays were analyzed by gel electrophoresis (4% polyacrylamide, 5% glycerol, 0.5 \times TBE).

RESULTS

The KAHRP gene is transcriptionally regulated

Three asexual intraerythrocytic stages have been defined for the protozoan parasite *P. falciparum*—the ring, trophozoite and schizont stage. The accumulation of KAHRP RNA during the intraerythrocytic cycle was examined by Northern analysis. The intra-erythrocytic stages were separated by percoll/sorbitol gradient centrifugation (30). Total cellular RNA was isolated from the ring, the trophozoite, and the schizont stages of the clonal isolate FCR3-A2, respectively. Unlike previously published (9), the 4.2 kb KAHRP transcript accumulates during the ring stage and only small amounts are detectable in trophozoites (Fig. 1).

Temporal changes in the KAHRP promoter activity occurring during the intraerythrocytic cycle were studied by nuclear run-on analysis (Fig. 2). For comparison the P195 gene encoding the major merozoite surface antigen (12), the CS gene encoding the circumsporozoite antigen expressed during the insect stage (13), and the ribosomal RNA genes (14) were analyzed in parallel. Nuclei were isolated from synchronized cultures of FCR3-A2 at 18 hrs (ring stage), 30 hrs (trophozoite stage), and 41 hrs (schizont stage) after infection. Preformed transcriptional complexes were allowed to elongate in the presence of labelled UTP. The labelled nascent RNA was used as a probe for DNA fragments specific for the KAHRP, the P195, the CS, and the

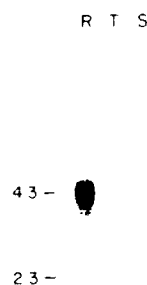


Fig. 1. Stage specific RNA accumulation for the KAHRP gene. Stage specific RNA was isolated from ring (R), trophozoite (T), and schizont (S) parasites, size-fractionated on a 0.8% agarose gel and transferred to nitrocellulose. The nitrocellulose filter was hybridized with the cDNA clone LP20 (9) to the KAHRP gene. Equal amounts of stage specific RNA was loaded as determined by hybridization with probes derived from the actin 1 (17) and 3.8 (25) genes (data not shown). A RNA size standard is indicated. Unlike previously published (9), the KAHRP transcripts accumulates in ring stage parasites. The separation of parasite stages by percoll/sorbitol gradient centrifugation enabled us to determine the stage specific transcription of this gene with greater accuracy (30).

rRNA genes respectively. The KAHRP gene is transcriptionally active only during the ring stage (Fig. 2). The correlation between its promoter activity and RNA accumulation indicates that the KAHRP gene is transcriptionally regulated. In contrast to the erythrocytic stage specific regulation of the KAHRP gene, the P195 gene and the rRNA genes are constitutively transcribed during these stages (Fig. 2). No transcription was observed for the CS gene during the erythrocytic cycle, consistent with its

specificity for the insect stage. Transcription of the KAHRP gene and the P195 gene is sensitive to α -amanitin (Fig. 2), indicating that these genes are transcribed by an α -amanitin-sensitive RNA polymerase.

Structural organization of the KAHRP locus

Genomic sequences were obtained for the KAHRP gene in order to analyze the structural basis for the stage specific regulation observed for this gene. Several overlapping genomic clones spanning the entire locus were obtained and sequenced (Fig. 3). The RNA initiation site of the KAHRP gene was determined by primer extension and RNase protection assays (Fig. 4). The primer extended product was recovered from the gel and amplified by anchored PCR. Both primer extension and RNase protection experiments map the start point of transcription to a single site, 849 bp upstream of the KAHRP initiation codon (Fig. 4). The sequence 5' of the RNA initiation site was examined for homologies to the binding sites of known eukaryotic transcription factors. None were found. However, a novel sequence element with two-fold symmetry was observed 160 bp upstream of the RNA start site (position 1562 to 1573 in Fig. 3B).

Nuclear proteins interact stage-specifically with a sequence motif

To determine whether this motif is a binding site for nuclear proteins, gel retardation assays were performed. Oligonucleotides

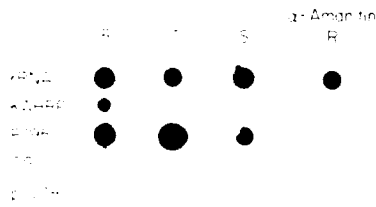


Fig. 2. Stage specific transcriptional activity of the KAHRP gene. The transcriptional activity of the KAHRP gene during the intraerythrocytic cycle was examined by stage-specific nuclear run on analysis. Nuclei were isolated from ring (R), trophozoite (T) and schizont (S) parasite stages, respectively. Nascent, labelled RNA was used as a probe to DNA fragments containing the ribosomal RNA genes, coding sequences of the KAHRP (9), the P195 (12) and the CS (13) genes, respectively. α -amanitin. Prior to transcription nuclei prepared from ring stage parasites were incubated with 100 μ g/ml of α -amanitin for 15 min on ice.

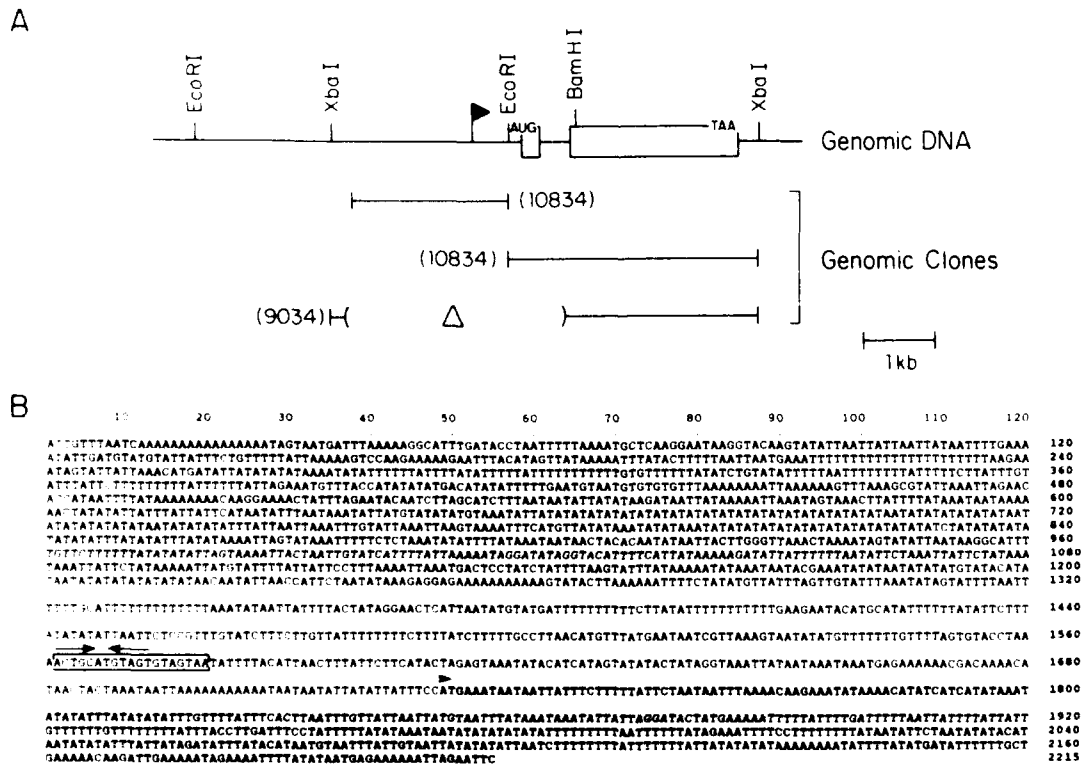


Fig. 3. Genomic organization and sequence KAHRP upstream sequence. **A** Genomic organization and clones. The KAHRP reading frame is indicated by rectangles. The RNA initiation site is indicated by an arrow head. The genomic clone 9034 contains an internal deletion as indicated by a triangle. **B** Sequence of the KAHRP upstream region. The RNA initiation site is indicated by an arrow head. A sequence motif with two-fold symmetry is indicated by arrows. The box indicates the sequence element that is used in gel retardation assays (Fig. 5). Position 2215 (EcoRI site) corresponds to position 248 of the published KAHRP coding sequence (17, accession number J02972).

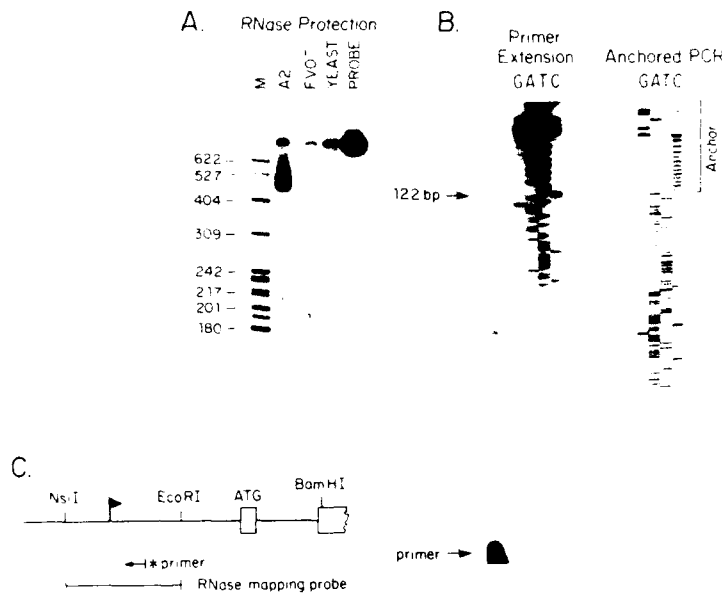


Fig. 4. Mapping the RNA initiation site of the KAHRP gene. RNase protection analysis: A single stranded, radiolabelled RNA probe (position 1419 to 2215 in Fig. 3B) complementary to the KAHRP RNA was generated and hybridized to 1 μ g of poly A+ ring stage RNA derived from the parasite clone FCR3-A2. After RNase digestion products were analyzed by gel electrophoresis. Controls using poly A+ RNA prepared from the mutant KAHRP parasite clone FVO and from yeast were analyzed in parallel. A size standard is indicated. Primer extension: A end labelled primer (corresponding to position 1819 to 1850 in Fig. 3B) was hybridized to 1 μ g of poly A+ ring stage RNA. Extension products were analyzed by gel electrophoresis and compared to a sequencing reaction of genomic DNA using the same primer. Additional primer extended products evident on the autoradiogram were found to be caused by pausing of reverse transcriptase due to the A T richness of the sequence. The primer extended product was recovered from the gel, amplified by anchored PCR technology (35), cloned and sequenced.

containing the KAHRP sequence element were incubated with nuclear extracts prepared from asynchronously growing erythrocytic cultures of the parasite isolate FCR3-A2. Complex formation was analyzed by gel electrophoresis. Three complexes were observed (Fig. 5A). These complexes were found to be stable in the presence of high concentrations of non-specific competitor DNA. Further, neither pUC18 DNA (1 μ g) nor a DNA fragment containing the GBP130 upstream sequence could compete the formation of these complexes (Fig. 5A). By contrast, complex formation is not observed in the presence of unlabelled KAHRP oligonucleotides (100 ng) or of a DNA fragment containing the KAHRP upstream region (1 μ g). Extracts prepared from uninfected red blood cells do not interact with the KAHRP sequence element. To determine the stage specificity of these complexes, the KAHRP oligonucleotide was incubated with extracts prepared from ring and schizont stage parasites, respectively (Fig. 5B). Three complexes, two major and one minor, were observed with nuclear extracts prepared from the ring stage. In contrast, the KAHRP sequence element formed one complex with nuclear extracts from the schizont stage (Fig. 5B). These data indicate that the KAHRP sequence element interacts with nuclear proteins in a stage specific manner.

DISCUSSION

The protozoan parasite *P. falciparum* has a complex life cycle alternating between a vertebrate and an invertebrate host. During the life cycle gene expression is regulated as indicated by distinct patterns of RNA (9, 15, 16, 17) and protein accumulation (10, 11, 18, 19, 20, 21). The mechanisms of gene regulation are not

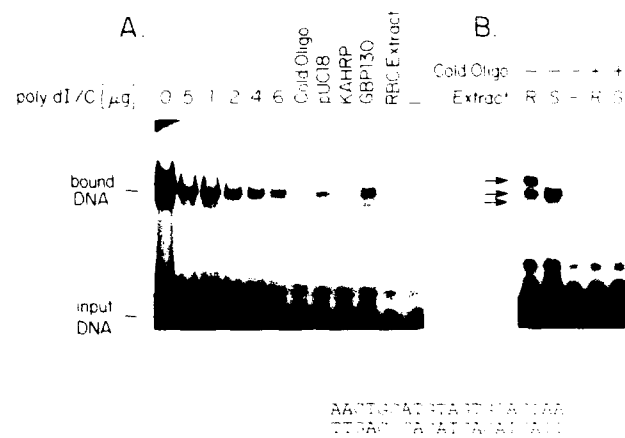


Fig. 5. Stage specific interaction of the KAHRP sequence element with nuclear extracts. A. 10 fmol of double stranded, end labelled oligonucleotides containing the KAHRP sequence element were incubated with 5 μ g of total nuclear protein derived from asynchronously growing parasite cultures. The sequence of the oligonucleotide is shown at the bottom. The amount of poly d(I/C) added to the binding assays is indicated. One μ g of poly d(I/C) equals about 1.5 nmol of non-specific binding sites. For cross competition experiments 100 ng (7.5 pmol) of unlabelled KAHRP oligonucleotides or 1 μ g (1.6 nmol of non-specific binding site) of pUC18 DNA or of DNA fragments containing the upstream regions of the KAHRP, and GBP130 (25) genes, respectively, were tested for their ability to compete. A control experiment was performed using extracts from uninfected erythrocytes (lane marked RBC extract). B. Nuclear extracts were prepared from ring (R), and schizont (S) stage parasites, respectively. The extracts were incubated with ³²P-labelled KAHRP sequence element in the presence of 2 μ g of poly d(I/C). Where indicated 100 ng of unlabelled KAHRP oligonucleotides were added to the binding assay. Complexes formed are indicated by arrows.

well understood for this parasite, mainly, because functional assays for the study of promoter activity, i.e., transfection or a reconstituted *in vitro* transcription system, are not yet available. In addition, structural data are difficult to obtain, since non-coding plasmidial DNA are unstable in *E. coli* (22), which presumably results from the unusually high A/T content of > 90% (23, 24). Consequently, structural elements involved in the developmental expression of *P. falciparum* genes have not been defined. Knowledge of these elements, however, is a prerequisite for the development of a transfection system for *P. falciparum* and will help in our understanding of the mechanisms of gene regulation in this parasite. In this study, we have identified a structural element in the upstream region of the KAHRP gene that, in correlation with the transcriptional activity of this gene during the intraerythrocytic cycle, binds to parasite derived nuclear factors in a stage specific manner.

A single RNA initiation site has been found for the KAHRP gene as determined by both primer extension and RNase protection experiments. A unique transcription start site has also been noted for the erythrocytic stage gene GBP130 of *P. falciparum* (25). In contrast, multiple RNA initiation sites have been reported for the insect stage circumsporozoite gene of the simian parasite *P. knowlesi* (26), the erythrocytic stage major merozoite surface antigen gene P195 from *P. falciparum* (27), and the Py230 gene from the rodent parasite *P. yoelii* (28). The frequent pausing of reverse transcriptase in A/T rich regions can easily be misinterpreted as multiple initiation sites. Hence, it has yet to be determined whether plasmidial genes can have either single or multiple RNA initiation sites. A comparison of genomic and cDNA sequences indicates that the KAHRP gene is continuously transcribed. However, post-transcriptional processing of the KAHRP transcript occurs through cis-splicing and polyadenylation.

A comparison of transcriptional activity as determined by nuclear run-on analysis and RNA accumulation indicates that the KAHRP gene is transcriptionally regulated during the erythrocytic cycle and transcribed only during the ring stage. Like the KAHRP gene, the GBP130 (25) and the HRP II (data not shown) genes are also transcriptionally regulated, while the P195 gene is constitutively transcribed in the erythrocytic stages (Fig. 2).

Transcription of the KAHRP gene is sensitive to the RNA polymerase inhibitor α -amanitin. Eukaryotic genes transcribed by an α -amanitin sensitive RNA polymerase frequently contain regulatory signals immediately upstream of the RNA initiation site. A comparison of the upstream regions of several plasmidial genes including the GBP130 and the CS genes has revealed a common sequence element that is homologous to the core region of the SV40 enhancer (25, 26). This SV40 enhancer-like element is not present in the KAHRP upstream region, nor were there any homologies found to other known binding sites of eukaryotic transcription factors. However, a palindromic sequence element was identified 160 bp upstream of the RNA initiation site of the KAHRP gene. This element was recognized in a specific fashion by parasite derived nuclear extracts. Different complexes were formed with this element depending on the developmental stage of the parasite. Three distinct complexes of different mobility were observed in the ring stage during which the KAHRP promoter is transcriptionally active. During the schizont stage, when the KAHRP promoter is silent, a single complex is formed. It is tempting to conclude that the KAHRP palindromic motif is a promoter component which mediates the developmental expression of the KAHRP gene by interacting with transcriptional factors in a stage specific manner. It remains to be determined

whether the protein-DNA complexes observed define multiple protein binding sites on the KAHRP sequence element or whether a single DNA binding protein is post-transcriptionally modified or binds additional co-factors. It is also possible that this sequence element is recognized both by positive regulatory factors during the ring stage and by negative regulatory factors during the schizont stage. Until a functional promoter assay is developed these conclusions remain speculative.

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REFERENCES

- Müller, I. H., Chien, S. and Usami, S. (1972) *Am. J. trop. Med. Hyg.* **21**, 133-137.
- Aikawa, M., Rabbage, J.R. and Wellde, B.T. (1972) *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, **124**, 722-727.
- Udemba, J.J., Schmidt, J.A., Aikawa, M.A., Miller, I.H. and Green, I. (1981) *Science*, **213**, 555-557.
- Raventos Suarez, C., Kaul, D.K., Macaluso, F. and Nagel, R.L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3829-3833.
- Oquendo, P., Hundt, E., Lawler, J. and Seed, B. (1989) *Cell*, **58**, 95-101.
- Berendt, A.R., Simmons, D.L., Tansey, J., Newbold, C.J. and Marsh, K. (1989) *Nature*, **341**, 57-59.
- Langreth, G.S., Reese, R.T., Moyl, M.R. and Trager, W. (1979) *Exp. Parasitol.* **48**, 213-219.
- Barnwell, J.W., Howard, R.J. and Miller, L.H. (1983) *CIBA Foundation Symposium*, Pitman Press, London, Vol. 94, pp. 117-136.
- Pologe, L.G. and Ravetch, J.V. (1986) *Nature*, **322**, 474-477.
- Kilejian, A. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4650-4653.
- Vernot-Hernandez, J.P. and Heidrich, H.G. (1984) *Molec. Biochem. Parasitol.* **12**, 337-350.
- Holder, A.A., Lockyer, M.J., Odink, K.G., Sandhu, J.S., Riveros-Moreno, Y., Nicholls, S.C., Hillman, Y., Davey, J.S., Tizard, M.L.V., Schwarz, R.T. and Freeman, R.R. (1985) *Nature*, **317**, 270-273.
- Enea, V., Ellis, J., Zavala, F., Arnot, D.E., Asavameh, A., Masuda, A., Quakyi, I. and Nussenzweig, R.S. (1984) *Science*, **225**, 628-630.
- Langsley, G., Hyde, J.H., Goman, H. and Scaife, J.G. (1983) *Nucleic Acids Res.* **11**, 8703-8717.
- Ravetch, J.V., Kochan, J. and Perkins, M. (1985) *Science*, **227**, 1593-1597.
- Waters, A.P., Sym, C., and McCutchan, T.F. (1989) *Nature*, **342**, 438-440.
- Wesseling, J.G., Snijders, P.J.F., Someren van, P., Jansen, J., Smits, M.A. and Schoenmakers, J.G.G. (1989) *Mol. Biochem. Parasitol.* **35**, 167-176.
- Hall, R., Osland, A., Hyde, J.E., Simmons, D.L., Hope, I.A. and Scaife, J.G. (1984) *Mol. Biochem. Parasitol.* **11**, 61-80.
- Perkins, M. (1988) *Exp. Parasitol.* **65**, 61-68.
- Weber, J.L. (1988) *Exp. Parasitol.* **66**, 143-170.
- Kemp, D.J., Cowman, A.F. and Walliker, D. (1990) *Advan. in Parasitol.* **29**, 75-149.
- Weber, J.L. (1987) *Gene* **52**, 103-109.
- Goman, M., Langsley, G., Hyde, J.E., Yankovsky, N.K., Zolg, J.W. and Scaife, J.G. (1982) *Mol. Biochem. Parasitol.* **5**, 391-400.
- Pollack, Y., Katzen, A.L. and Golenser, J. (1982) *Nucleic Acids Res.* **10**, 539-546.
- Lanzer, M., deBruin, D. and Ravetch, J.V. (1992) *EMBO J.*, in press.
- Ruiz, Altaba, A., Ozaki, L.S., Gwadz, R.W. and Godson, G.N. (1987) *Mol. Biochem. Parasitol.* **23**, 233-245.
- Myler, P.J. (1990) In Agabian, N. and Cerami, A. (ed.) *Parasites: Molecular Biology, Drugs, and Vaccine Design*. Wiley-Liss, pp. 123-137.
- Lewis, P.A. (1990) *Mol. Biochem. Parasitol.* **39**, 285-288.
- Trager, W.T. and Jansen, J.B. (1976) *Science*, **193**, 673-675.

- 30 Kutner, S., Breuer, W.V., Ginsburg, H., Aley, S.B. and Cabantchik, Z.I. (1985) *J. Cellular Physiol.*, **125**, 521-527.
- 31 Wallach, M. (1982) *Mol. Biochem. Parasitol.*, **6**, 335-342.
- 32 Chomezynski, P. and Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156-159.
- 33 Navin, J.R., In Abelson, J.N. and Simon, M.I. (ed.), *Methods in Enzymol.*, **152**, 234-241.
- 34 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor.
- 35 Loh, E.Y., Elliot, J.F., Cwirla, S., Lanier, L.L. and Davis, M.M. (1989) *Science*, **243**, 217-220.
- 36 Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. (1989) *Nucleic Acids Res.*, **17**, 6419.
- 37 Pologe, L.G., Pavlovic, A. and Ravetch, J.V. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7139-7143.