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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 glycophorin 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 *Eco*RI, 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 *Hin*dIII. 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

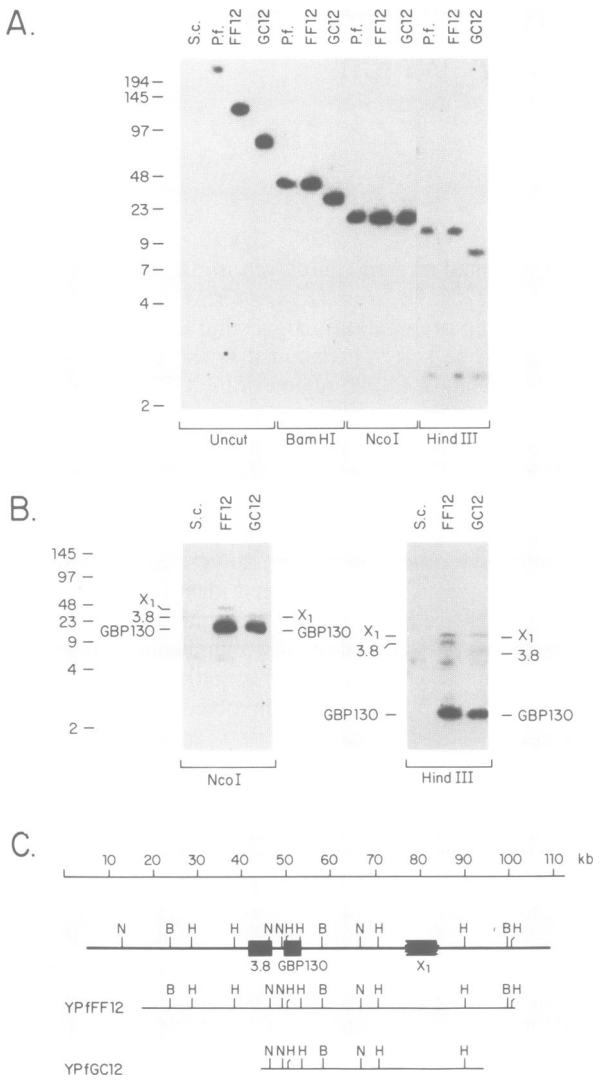

Fig. 1. Structural organization of the GBP130 YAC clones. (**A**) Restriction mapping. *P.falciparum* genomic DNA (lanes marked P.f.), GBP130 YAC clone DNA (lanes marked FF12 and GC12, respectively) and yeast DNA (lane marked S.c.) were digested with the restriction endonucleases indicated, size-fractionated 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 *Nco*I and *Hin*dIII, 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, *Nco*I; B, *Bam*HI; H, *Hin*dIII).

signals for the *Nco*I and *Hin*dIII 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

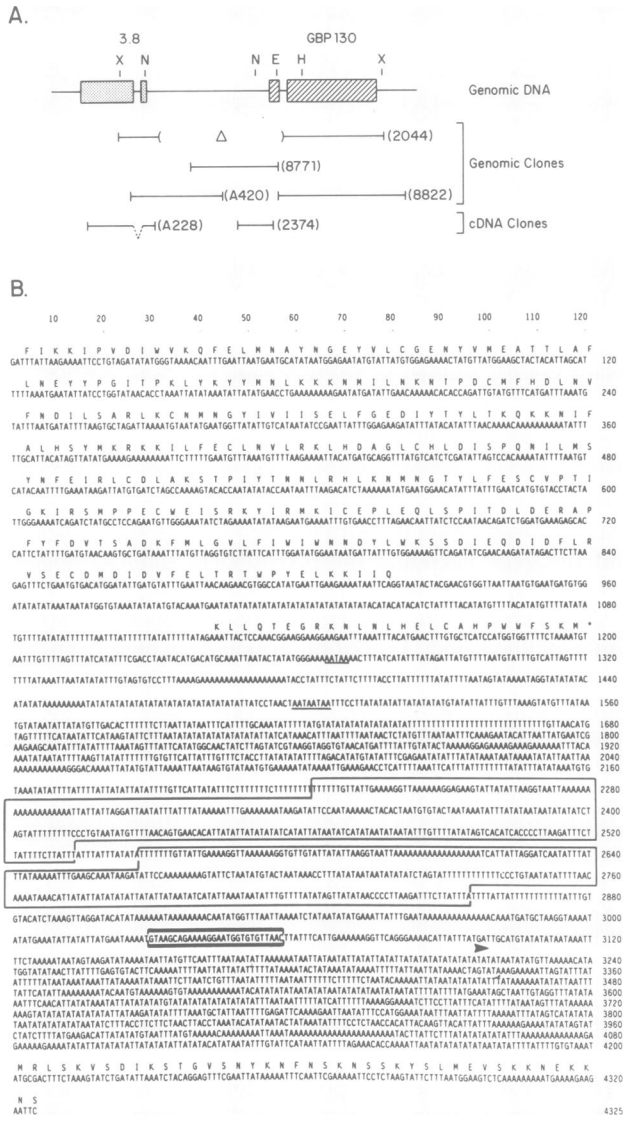

Fig. 2. Genomic organization and the sequence of the 3.8 gene and the GBP130 intergenic region. (**A**) Genomic organization and clones. Open reading frames are indicated by rectangles. Several genomic and cDNA clones are shown. A triangle in the genomic clone 2044 indicates an internal deletion generated 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

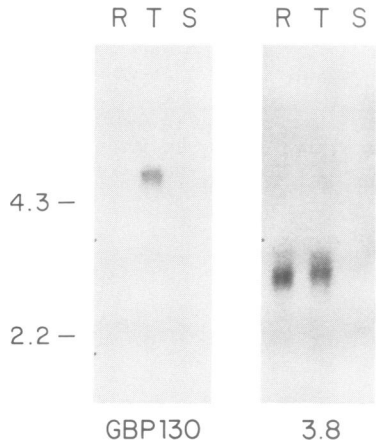

Fig. 3. Stage-specific expression of the GBP130 and 3.8 genes. Stage-specific RNA was isolated from ring (R), trophozoite (T) and schizont (S) parasites, size-fractionated on a 0.8% agarose gel and transferred to nitrocellulose. The nitrocellulose filters were hybridized with a cDNA clone to the GBP130 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).

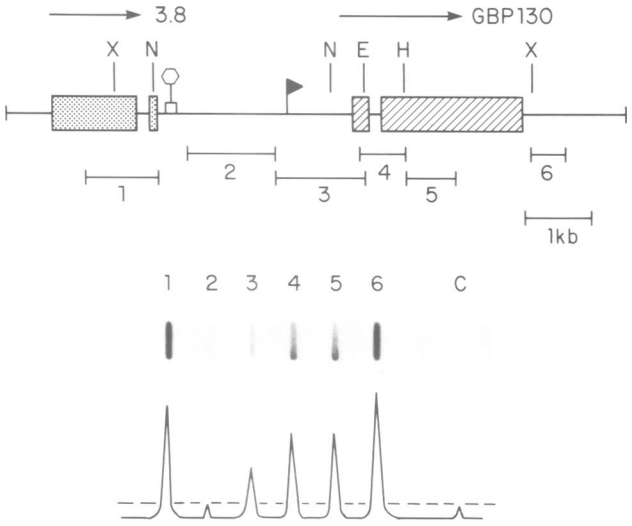

Fig. 4. The 3.8 and the GBP130 genes define independent transcription units. The schematic drawing reveals the organization of the locus and the orientation of the 3.8 and GBP130 genes. Shaded rectangles denote the open reading frames of these genes. The initiation site of the GBP130 gene and the termination 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-plasmodial 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.)

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 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).

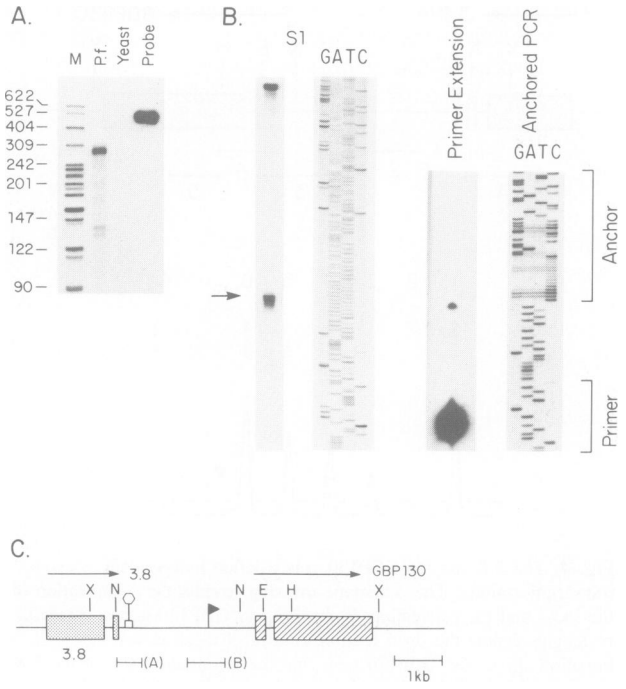

Fig. 5. Mapping of termination and initiation sites in the intergenic region. (**A**) Termination of the 3.8 gene. RNase protection analysis: a single-stranded, radiolabelled probe specific for the 3.8 gene (probe A, position 1179–1582 in Figure 2B) was generated and hybridized to 10 μg of poly(A)$^+$ trophozoite RNA. Upon RNase digestion products were analyzed by gel electrophoresis (lane marked P.f.). 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.

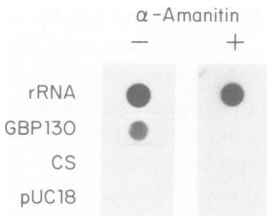

Fig. 6. α-amanitin sensitive transcription of the GBP130 gene. Nuclei were isolated from trophozoite parasites. One aliquot of the nuclei preparation was incubated with 100 μg/ml of α-amanitin prior to transcription. A gene expressed only during the insect stage, the CS gene (Enea *et al.*, 1984) and the ribosomal rRNA genes (Langsley *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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GCTGTGGAATGTGTGTCAG-TTAGGGTGTGGAAAGTCCCC      SV40 ENHANCER
* ****         * *** *** ******* **  ****
GTTGTGAGTAAGCAG-CAGTTTAAGGTGTGGTAACCCCCC      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.

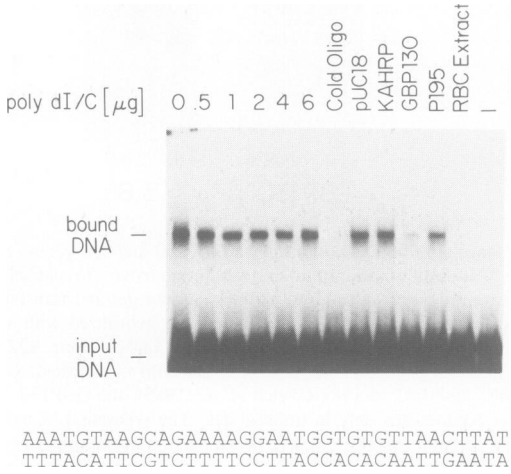

Fig. 8. Interaction of the GBP130 sequence element with nuclear extracts. 2 fmol of double stranded, end labelled oligonucleotides containing the GBP130 sequence element were incubated with 5 μg of crude nuclear extracts derived from asynchronously growing parasite cultures. The sequence of the oligonucleotide is shown at the bottom. The amount of poly d(IC) 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 *Eco*RI, and inserted into the *Eco*RI 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-DRII system [pulse-field conditions: ramped pulse from 2.5 to 10 s over 18 h at 170 V, 1% LE agarose (FMC), 0.5×TBE, 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 $MgCl_2$, 10 mM DTT, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 6 μM dATP, 1 μM [α-^{32}P]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-^{32}P]dCTP using the TAG-IT kit (BIOS) which uses d$(N)_6$(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 1×PBS 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. 5×10^9 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 $MgCl_2$, 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-^{32}P]UTP 3000 Ci/mmol]. Radiolabelled RNA was isolated (Chomcynski and Sacchi, 1987) and purified by TCA precipitation. Usually 2×10^7 c.p.m. were incorporated into nascent RNA, with a specific activity of 9×10^7 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 2×SSC, 0.1% SDS at room temperature, twice at 55°C in 0.1×SSC, 0.1% SDS, followed by one wash at 42°C in 2×SSC, 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 (1.5×10^5 c.p.m.) of end labelled oligonucleotide primer (5′-GAAGTACACTCAAAATAAGTTATATACCATATG-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 $MgCl_2$, 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 *Hinc*II–*Nco*I 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′-TATTAAAAATATTAAA-CAGATTAAG-3′). The single stranded product was purified by gel electrophoresis. 2×10^5 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 *Nco*I–*Hinc*II 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 (Chomcynski 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 Pologe 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 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 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 0.5×TBE).

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