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An enhancer-like region regulates *hrp*3 promoter stage-specific gene expression in the human malaria parasite *Plasmodium falciparum*

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Abstract

The asexual blood stage of *Plasmodium falciparum* is comprised of morphologically distinct ring, trophozoite and schizont stages. Each of these developmental stages possesses a distinct pattern of gene expression. Regulation of *P. falciparum* gene expression is thought to occur, at least in part, at the promoter level. Previously, we have found that although the RNA of the *P. falciparum hrp3* gene is only seen in ring-stage parasites, deletion of a specific sequensce in the 5' end of the promoter region decreased ring-stage expression of *hrp3* and enabled detection of its transcripts in trophozoite-stage parasites. In order to investigate this stage specific regulation of gene expression, we employed a series of nested deletions of the 1.7-kb *hrp*3 promoter. Firefly luciferase gene was used as a reporter to evaluate the role of promoter sequences in gene regulation. Using this approach, we identified a ring-stage specific regulatory region on the *hrp*3 promoter located between -1.7-kb and -1.1-kb from the ATG initiation codon. Small 100–150 bp truncations on this enhancer-like region failed to uncover discrete regulatory sequences, suggesting the multipartite nature of this element. The data presented in this study demonstrates that stage specific promoter activity of the *hrp*3 gene in *P. falciparum* blood stage parasites is supported, at least in-part, by a small promoter region that can function in the absence of a larger chromosomal context.

Keywords

Plasmodium; transcription; regulation; histidine-rich protein 3; stage specificity; expression

1. Introduction

Plasmodium falciparum infection remains a major disease burden in the developing world [1,2]. Therefore, it is critically important to understand the details of regulation of gene

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expression during the parasite development. Infection in the human host results in a series of morphologically distinct stages that include ring, trophozoite, schizont and merozoite, during the parasite development within the mature erythrocyte. Transit through developmental stages is linked to strict regulation of distinct stage specific patterns of gene expression. Even housekeeping genes thought to be expressed throughout the cell cycle, exhibit complex patterns of developmental expression [3–7].

In the parasite, regulation of gene expression seems to occur at the transcriptional and post-transcriptional level. While transcriptome and proteome analyses indicate that some genes are regulated post-transcriptionally [8,9], other genes are regulated at the level of transcription. For example, analyses of 5' end upstream regions of the asexual and sexual stages have been shown by transfection studies that they function as promoters by supporting reporter gene expression [10–16]. Moreover, functional and bioinformatic studies of sexual and asexual *Plasmodium* promoters have uncovered putative *cis*-acting regulatory elements possibly involved in gene regulation [17–22]. In addition, it has been recently shown that the expression of parasite variant protein PfEMP1, is regulated at the level of transcription initiation [23]. Taken together, these results indicate the presence of transcriptional gene regulation in the parasite. Although, basal and stage specific *cis*-acting elements in the parasite promoters remain poorly described owing to the high A+T content of its genome.

During our studies on promoter recombination using *hrp*3 nested promoter deletions, we identified a region between -1.7-kb and -1.1-kb, from the ATG initiation codon, involved in ring stage specificity [24]. Unexpectedly, further promoter deletion switched the steady state accumulation of the reporter gene mRNA from ring to trophozoite stage of the parasite development. These results suggest the presence of stage specific *cis*-acting regulatory elements on the *hrp*3 promoter.

P. falciparum histidine-rich protein-2 and 3 (*hrp*2, 3) as well as the Knob associated histidine-rich protein (*kahrp* or *hrp1*) comprise the histidine-rich protein family. The members of this family are expressed at the early stage of the parasite development [25,26]. We took advantage of the previously reported *hrp*3 promoter nested deletions [24] to further characterize the putative ring specific element. The *hrp*3 promoter region between -1.7-kb and -1.1-kb, from the ATG initiation codon, is involved in ring stage specific gene expression [24]. This region was placed upstream of the calmodulin (*cam*) promoter; it specifically increased the reporter activity in ring-stage parasites but had little effect on trophozoite parasites, suggesting that this enhancer-like region contains stage specific promoter elements. This presumptive element did not show homology to other *Plasmodium* promoter regions, suggesting that its function may be regulated through other mechanism rather than being sequence specific, such as transcription initiation or nuclear localization. Although, sequence-dependent regulation cannot be rule out. Here we identified in *P. falciparum* an enhancer-like region, carrying stage-specific *hrp*3 promoter elements. Our findings contribute to the understanding of the regulation of gene expression in the malaria parasite.

2. Experimental procedures

2.1. Plasmid constructs

The *hrp*3 promoter deletions were made using Erase-a-base® (Promega) and pHRPCAT [10] containing the *hrp*3 promoter driving the chloramphenicol acetyl transferase (CAT) gene. *Kpn* I-*Dra* III-digested pHRPCAT was incubated with exonuclease III (exo III) at predetermined times to obtain specific-sized promoters of 1.1-kb and 0.6-kb. The firefly luciferase (FFL) gene was amplified by PCR from the pVLH plasmid [15] using oligonucleotides 5'-GAC**ATGCAT**GAAGACGCCAAAAACATAAAG-3' and 5'-GAC**AAGCTT**GCTTACAATTTGGACTTTCCG-3', (boldfaced nucleotides represent *Nsi* I

and *Hind* III restriction sites, respectively). The FFL was then used to replace the CAT gene in exoIII-digested pHRPCAT to generate pH1.7FL, pH1.1FL and pH0.6FL. Short *hrp*3 promoter deletions were generated by PCR using the specific oligonucleotides (Table S1, supplementary information).

All pcamSluc-derived constructs were engineered by first creating a *Sal* I site at the 5' end of the *cam* promoter in the pcamGFP plasmid [27]. Secondly, the firefly luciferase gene substituted the GFP gene to generate pcamSluc. Subsequently, the *hrp*3 promoter region from -1.7-kb to -1.4-kb (fragment A) was amplified by PCR using the oligonucleotides HAF (5'-ACGC**GTCGACC**GCCCAATCATTATTTTATG-3') and HAR (5'-ACGC**GTCGAC**CCATAAAATATAAAAATAATTTG-3'). In addition, the region A was divided using PCR into fragment A1 from -1.7-kb to -1.1-kb with the oligonucleotides HAF and HA1R (5'-ACGC**GTCGAC**CATTTATATTTATATTAAGAG-3'), and fragment A2 from -1.4-kb to -1.1-kb using the HA2F (5'-ACGC**GTCGAC**GAATATATTCATAATTATAATATTG-3') and HAR oligonucleotides, boldfaced nucleotides in all primers represent the *Sal* I restriction site. These fragments were placed into pcamSluc to generate pAhrpcamSluc, pA1hrpcamSluc and pA2hrpcamSluc. We then amplified the *hrp*3 promoter region from -1.1-kb to -0.6-kb (fragment B) by PCR using the oligonucleotides HBF (5'-ATC**GTCGAC**TATGTATATGTATGTATTTTAAAATATAATAAAATG -3') and HBR (5'-ATC**GTCGAC**GTATGGATAGATTTTATTTTTAAAAAATAATAAATTTTATTATATTC-3'). Subsequently, fragment B was divided by PCR into fragment B1 -1.1-kb to -0.8-kb using HBF and HB1R (5'-ATC**GTCGAC**ATTATGAATATAAGAATATTCCATCTATCTTATG -3') oligonucleotides; and fragment B2 from -0.8-kb to -0.6-kb using HB2F (5'-ATC**GTCGAC**AATATTCAAAAAATAACAGATTTAAACCCTCAAAAATATAG -3') and HBR. A *Sal* I restriction site (boldfaced nucleotides) was engineered at the 5' and 3' end on each PCR product. Fragments B, B1 and B2 were then inserted into *Sal* I-digested pcamSluc to generate pBhrpcamSluc, pB1hrpcamSluc and pB2hrpcamSluc respectively. A *Renilla* luciferase plasmid was constructed as reported by Militello and Wirth [28]. The *Renilla reniformis* luciferase gene was amplified by PCR using the plasmid pRL-null (Promega) as template and forward oligonucleotide 5'-TTA**ATGCAT**ATGCTTCGAAAGTTTATGATC-3' and reverse oligonucleotide 5'-TTC**AAGCTT**ATTGTTCATTTTTGAGAACTCGC-3' containing engineered restriction enzyme sites (*Nsi* I and *Hind* III, boldfaced nucleotides). *Nsi* I/*Hind* III-digested PCR fragment was used to replace the GFP gene in pcamGFP to generate pcamRLuc plasmid.

2.2. Parasite transfection

P. falciparum transient transfections were performed as described in Deitsch et al 2001. Experimental plasmids (100 μg per transfection) were combined with control plasmids, either 20 μg of CAT-containing plasmid (pcamCAT, [27]) or 10 μg of *Renilla* luciferase-containing plasmid (pcamRluc), and electroporated into the red blood cell as described previously [29]. Percoll-purified schizonts were added to DNA-loaded erythrocytes. After 12–14 hours post-invasion (hpi), half of the culture was taken (ring-parasites sample). The remaining half was kept in culture and processed after 32–36 hpi for the trophozoite sample.

2.4. Firefly, *Renilla* luciferase and CAT assays

Transfected parasites were first released from the red cells by 0.01% saponin lysis and released-parasites were lysed (Promega's lysis buffer) 12–14 hpi (ring parasites) or 32–34 hpi (trophozoite parasites). The parasite extract was incubated at 60° C for 10 min., cleared by centrifugation and combined with [^{14}C]chloramphenicol (at 0.05mCi/ml) and n-Butyryl-CoA for 3 hours to determine the CAT activity. After incubation, samples were mixed with xylene

and the aqueous phase containing acetylated [^{14}C]chloramphenicol was quantified in a liquid scintillation counter. CAT activity was used to normalize the firefly luciferase activity (Fig. 1 and 2B). The rest of the experiments were normalized using *Renilla* luciferase as a control. Parasites transfected with luciferase genes were quantified in a single tube Sirius luminometer (Berthold Detection Systems) as recommended by the manufacturer (Promega dual luciferase assay). Firefly and *Renilla* luciferase activities were reported as arbitrary light units.

2.5. Mapping of the *hrp*3 transcription start site

The *hrp*3 transcription start site (TSS) was determined by 5' rapid amplification of cDNA ends (5' RACE, Invitrogen) using total RNA from non-transfected and pH1.7HG-transfected parasites [24]. Total RNA from transfected and non-transfected parasites was isolated using TRIzol® LS reagent (Invitrogen). Five micrograms of total RNA from non-transfected parasites was reverse transcribed using the oligonucleotide 5'-GTA**ATGCAT**TGAATTTTATAAAAAAGCAAAATTATTTA-3' (boldface nucleotides represents a *Nsi* I site) complementary to the region from -350 nt to -320 nt of the *hrp*3 promoter. For RNA from pH1.7HG-transfected parasites, 5 μg of total RNA was reverse transcribed using a specific oligonucleotide to the human DHFR gene, 5'-CCAAATCAATTTCTGGAAAAAACGTGTCAC-3' complementary to the region 434 nt to 464 nt from the ATG initiation codon. cDNA generated from non-transfected and pH1.7HG-transfected parasites was then amplified by PCR using nested reverse oligonucleotide 5'-ATC**ATGCAT**TTTATTATTTTATTTTATTAATATAAGAC-3' (boldface nucleotides represents a *Nsi* I site) complementary to the *hrp*3 promoter region from -543 nt to -514 nt and forward oligonucleotides provided by the manufacturer (Invitrogen). PCR products were cloned into pCR2.1® vector (Invitrogen), DNA was extracted from 15 different recombinant colonies for each group and sequenced to determine the transcription start site.

3. Results

3.1. The *hrp*3 region between -1.7-kb and -1.1-kb regulates ring-stage specific gene expression.

We generated nested deletions on the histidine-rich protein 3 promoter (*hrp*3) to evaluate the role of promoter sequences on gene expression. For this purpose, the CAT gene from pHRPCAT [10] was replaced by the firefly luciferase (FFL) gene in *hrp*3 promoter deletions (pH1.7FL, pH1.1 FL and pH0.6FL, Fig. 1A). These constructs containing FFL and the *hrp*3 promoter deletions were subsequently used to transiently transfect malaria parasites. FFL activity from the *hrp*3 promoter deletion, was normalized to CAT activity by co-transfecting the pcamCAT plasmid [27]. The FFL activity in ring parasites transfected with the 1.1-kb *hrp*3 promoter (pH1.1FL) lacking the enhancer-like region between -1.7-kb and -1.1-kb, was approximately 55 % of that of the full length promoter (Fig. 1B, compare pH1.7FL and pH1.1FL, rings). In contrast, FFL activity remained constant in trophozoite parasites transfected with the 1.7-kb and the 1.1-kb *hrp*3 promoters respectively (Fig. 1B, compare pH1.7FL and pH1.1FL, trophozoites). Further deletion to 0.6-kb greatly decreased FFL activity in both ring and trophozoite parasites (Fig. 1B, pH0.6FL). Taken together, these data indicate the presence of a ring stage specific enhancer-like element between -1.7-kb and -1.1-kb of the *hrp*3 promoter.

3.2. The *hrp*3 region between -1.7-kb and -1.1-kb specifically stimulates ring-stage activity on heterologous *cam* promoter

To investigate whether the *hrp*3 enhancer-like region between -1.7-kb and -1.1-kb contains *bona fide* ring specific elements we performed gain-of-function experiments. In order to test this possibility we selected a 600-nucleotide long calmodulin (*cam*) promoter. This promoter is active at all stages of the parasite growth with a peak of expression at 40 hours post-invasion

[30,31]. Our rationale is that if this region contains ring specific enhancer elements it would specifically increase the *cam* promoter-mediated luciferase activity in ring parasites. Conversely, if this region does not contain an enhancer element, it would not have an effect on *cam*-mediated luciferase activity. It is also possible that gene stage specificity in the *hrp*3 promoter requires the combined action of promoter elements located both in the -1.7-kb and -1.1-kb region and downstream in the *hrp*3 promoter. Therefore, the use of *cam* will test whether or not the -1.7-kb and -1.1-kb region contains a *bona fide* enhancer-like element. For this test, we placed the *hrp*3 region from -1.7-kb to -1.1-kb, fragment A (Fig. 2A and S1) upstream of the *cam* promoter driving the FFL activity. Transient transfection of ring-stage parasites (12–14 hpi) showed that FFL activity driven by the hybrid *cam* promoter containing *hrp*3 fragment A was approximately 55 fold higher (Fig. 2B) than the FFL activity driven by the *cam* promoter alone (Fig. 2B, compare pcamSluc and pAhrpcamSluc, rings). To further refine the location of stage specific elements in the *hrp*3 promoter, we divided fragment A into 5'end fragment, A1 and a 3' end fragment A2 (Fig. 2A and S2, orange and blue areas respectively, supplementary information). These fragments were placed upstream the *cam* promoter and the hybrid promoter used to transfect *P. falciparum*. Neither subfragment stimulated the ring FFL activity to levels similar to that seen when fragment A was used (Fig. 2B, compare pAhrpcamSluc, pA1hrpcamSluc and pA2hrpcamSluc, rings). FFL activity was also measured in trophozoite parasites collected 32–34 hpi to examine the effect of *hrp*3 fragments on *cam*-dependent FFL activity. Figure 2B shows that the presence of the *hrp3* fragment in pAhrpcamSluc and its subfragments in pA1hrpcamSluc and pA2hrpcamSluc had no significant effect on trophozoite stage *cam*-dependent FFL activity when compared with the control (*cam* promoter alone in pcamSluc).

To further confirm that fragment A specifically increased *cam*-dependent promoter activity in ring-stage parasites, we placed a 500 bp *hrp*3 fragment normally located between -1.1-kb and -0.6-kb (or fragment B, Fig. 2C and S1, supplementary information), upstream of the *cam* promoter in the pcamSluc plasmid to generate pBhrpcamSluc. In this and subsequent experiments, FFL activity was normalized to *Renilla* luciferase (RL) activity [28]. Using RL instead of CAT allows a single sample to be used to determine experimental and control luciferase activities. In contrast to region A, region B did not increase luciferase activity driven by the *hrp*3-*cam* hybrid promoter in either ring- or trophozoite-stage parasites (Fig. 2D, compare pcamSLuc and pBhrpcamSLuc). Similarly, the subfragments B1 and B2 (pB1hrpcamSLuc and pB2hrpcamSLuc respectively) that span the *hrp*3 promoter regions from -1.1-kb to -0.8-kb and -0.8-kb to -0.6-kb respectively (Fig. 2C and S2, green and yellow areas respectively, supplementary information) did not stimulate luciferase activity mediated by the *hrp*3-*cam* hybrid promoter (Fig 2D). Together, these results indicate that the *hrp*3 region between -1.7-kb and -1.1-kb contains a *bona fide* ring specific enhancer-like element.

In order to map the putative regulatory elements in the *hrp*3 promoter enhancer region, we generated a series of 100–150 bp nested deletions on the *hrp*3 promoter from -1.7-kb to -0.7-kb. We generated *hrp*3 5' end truncations by using the polymerase chain reaction (PCR) and specific oligonucleotides (Table S1. Supplementary information). Nested truncations were placed upstream of the firefly luciferase gene (Fig. 3) and used to transfect malaria parasites. Luciferase activity was then measured at 12–14 hpi (ring-stage) and normalized by cotransfection with a plasmid carrying *Renilla* luciferase. Figure 3 shows a step-wise decrease of FFL activity in ring-stage parasites as the promoter was shortened. In contrast, promoter truncations had no effect (and remained relatively constant) on trophozoite-stage transient FFL activity (32–34 hpi, Fig. 3). These results strongly suggest that regulation of gene expression in ring-stage parasites in the *hrp*3 promoter is mediated by a regulatory element that may have a multipartite structure.

3.3. Location of the *hrp*3 promoter on an episome did not affect transcription start site (TSS) selection

Previous studies have shown that the location of a promoter on a plasmid rather than in the chromosome can affect its expression. For example, early studies of the regulation of glycophorin binding protein 130 (*gbp130*) showed that the loss of trophozoite stage specificity was correlated with rearrangement of the chromatin structure on the plasmid copy [12]. In contrast, *P. falciparum* stage specific promoters such as histidine-rich protein-3 (*hrp*3) [25], PfEMP1 (*var*) [15] and *P. berghei* apical membrane antigen-1 promoter (Pbama1) [32] used to drive reporter gene expression on plasmids, maintained a strict developmental regulation despite the change in location. Together, these results indicate that regulatory mechanisms in the parasite might not be entirely dependent on chromosomal location. To ensure that the *hrp*3 promoter behaves similarly whether on a chromosome or a plasmid, the *hrp*3 TSS was mapped by 5' rapid amplification of cDNA end (RACE). Our rationale was that if the TSS is preserved, it means episomal location does not affect selection of the TSS. The TSS selection determines the length of the 5' untranslated region (UTR) in the nascent transcripts. Therefore, identification of the TSS will reveal the promoter structure. Because transient transfection is likely to yield low level of mRNA, we decided to map the TSS on a *P. falciparum* stable cell line transfected with pH1.7HG [24]. In this plasmid the *hrp*3 promoter drives the expression of the GFP-hDHFR (HDGFP) fusion gene. Both *hrp*3 and HDGFP mRNAs were characterized by a heterogeneous 5' end, which is consistent with other 5' end termini mapped in *Plasmodium* genes [33]. The TSS (on the basis of sequencing 15 clones) from the endogenous *hrp*3 mapped to a 130 bp long region between -750 bp and -626 bp from the ATG initiation codon (Fig. S3, closed box. Supplementary information). Similarly, all 5' end mRNA termini diagnostic of TSS on the episomal *hrp3* promoter were mapped within this 130 bp region. Our data demonstrate that TSS selection was not significantly modified by episomal context.

As in other *Plasmodium* genes [33], endogenous and episomal transcription start sites start with adenosine (Fig. 4, position 11 in the logo). This is consistent with the preference of purine nucleotides at the 5' end terminus of eukaryotic mRNAs [33]. The analysis of the DNA sequences surrounding each TSS (Fig. 4, 10 nucleotides upstream and downstream the TSS) showed no major difference between the endogenous and plasmid TSS. However, thymine nucleotides were more frequent in the upstream region of the endogenous TSS (Fig. 4). No nucleotide preference is seen at the downstream region, except for thymine at positions 12 and 18 (Fig. 4). This pattern seems to be slightly different in the plasmid TSS, containing predominately adenine at the upstream region and thymine at the downstream region of the TSS (Fig. 4). Further studies will be needed in order to determine the relevance, if any, of this difference on the regulation of gene expression. However, these minor differences on the TSS region are likely not biologically significant.

4. Discussion

In this report we characterized a promoter region responsible for ring stage specific expression in a *P. falciparum* gene. This region between - 1.7-kb and -1.1-kb had a marked effect on stage specific expression independent of the chromosomal context. The structure of this enhancer-like element appears to be multipartite. Consistent with our hypothesis, bioinformatic analysis of *Plasmodium* genome showed that most *Plasmodium* genes seem to have a cluster of *cis*-acting regulatory elements (four or five) associated with or as part of their promoters [21]. van Noort *et al*. 2006 [21] identified a number of motifs that significantly correlated with mRNA expression. None of these motifs were found in the *hrp*3 promoter, suggesting that they might not be relevant for *hrp*3 promoter regulation. The *hrp*3 enhancer region also contains a CCAAT at position -1487 bp to -1483 bp. This was not surprising since the parasite genome encodes the complete CCAAT-box binding complex [34]. However, CCAAT-box containing fragment

A1 (Fig 2A and Fig. S2) failed to stimulate ring luciferase activity in the hybrid *cam* promoter, suggesting that CCAAT-box is not involved in ring stage specificity. Alternatively, CCAAT-box might require other regulatory elements (multipartite structure) that are absent in fragment A1. A TGTATATG motif similar to a schizont/gametocyte motif reported previously [9] is also present in the fragment B1 at position -1109 bp to -1102 bp of the *hrp*3 promoter (Fig. S2. Supplementary information). However, fragment B or B1 that contain the TGTATATG motif failed to stimulate ring luciferase activity in the hybrid *cam* promoter. Conversely, fragment A which lacks this motif still conferred specific ring-stage stimulation on the *cam*-mediated FFL (Fig. 2B and S2). Altogether, these results suggest that, the TGTATATATG motif is not involved in regulation of promoter stage specificity in the parasite.

Comparative analyses of our data with published studies on gene expression in malaria parasites suggest that the *hrp*3 *cis*-acting element between -1.7-kb and -1.1-kb might represent a new mechanism of stage specific regulation. For example, GC-rich promoter elements [35] in *pfhsp*86, GC-rich elements CAGACAGC in the *pgs*28 promoter [18] and CGCACAACAC *P. falciparum* in *rif* genes [20] have been described. These sequences are not found in blood stage promoters such as *hrp*3, *msp*1, *var*7b and knob-associated histidine rich protein (*kahrp*), suggesting that they are not involved in regulation of stage specificity in these promoters. Also, it has been reported that intronic regulatory elements regulate allelic expression in centromeric *var*7b genes [15,36]. Similarly, *hrp*3 5' end sequence contains an intron located 13 bp upstream of the ATG initiation codon [27]. Although, *hrp*3 intron might play a similar regulatory role as the centromeric *var*7b genes intron, previous studies has shown that deletion of this intron in the 5' end *hrp*3 does not affect reporter expression [10]. However, the role of intronic sequence on *hrp*3 stage-specific regulation has not yet been assessed.

Sequence comparison between *hrp*3 and several *var* promoters using bioinformatics analysis (Hiller and Haldar unpublished) as well as comparison to an extended data set obtained from the *Plasmodium* database (www.plasmodb.org) of putative ring and/or trophozoite 5' end regions (Lopez-Estraño, Phan and Garzon unpublished), failed to uncover conserved sequences that may contribute to promoter regulation. The absence of recognizable sequence homology amongst *Plasmodium* promoters suggests that stage specific regulation may require complex mechanisms that could include transcriptional initiation and/or nuclear position amongst others. A wide variety of eukaryotic genes, including those of *Plasmodium*, are regulated by DNA structure such as formation of kinks by poly T tracts flanking the core promoter [37–39] and poly(dA:dT) sequences [40], both of which are very abundant in the *Plasmodium* genome. Other DNA-dependent structures that could play a role in the promoter function such as poly purine-pyrimidine sequences have also been described [41]. A long poly(dA)poly(dT) sequence has been associated with *cam* promoter basal activity [42]. Much shorter poly(dA) poly(dT) tracts are distributed along the *hrp*3 promoter, but whether they are involved in stage or basal promoter regulation needs to be determined.

The *Plasmodium* signals that control gene expression are phylogenetically related and promoters seem to be interchangeable in some degree amongst parasite species [43]. Despite this conservation, *P. falciparum* promoters are quite diverse in terms of their *cis* regulatory elements and questions such as what constitutes a minimal promoter, remain to be addressed. Interestingly, deletion of the *hrp*3 region between -1.7-kb and -1.1-kb turned the truncated promoter constitutive both at the mRNA [24] and at the translation level (luciferase activity, this study). Further deletion to generate the 0.6-kb truncated promoter switched the accumulation of mRNA from ring stage in the 1.7-kb full length promoter to the trophozoite stage in the 0.6-kb truncated promoter [24]. Furthermore, the 0.6-kb promoter did not support luciferase activity in either stage of the parasite development (Fig 1). These results suggest that the region between -1.1 to -0.6-kb might contain transcriptional elements responsible for the basal activity of this promoter. In fact, basal regulatory elements have not been fully

characterized in *Plasmodium* promoters. Although comparisons with other eukaryotic promoter have shown several *cis* acting elements such as SV40 enhancer elements [44], CAAT boxes [10,35], SP1 or octamer and GC box binding sites (GC boxes) [35] in some parasite promoters, but they were not identified in the -1.1 to -0.6-kb *hrp*3 region. In addition, sequences that have been recently described as regulatory elements in the parasite genome [9,18,20,21, 34,35] were not present in the *hrp*3 promoter. Together, these results indicate that basal regulatory *cis* acting elements might also be quite diverse in the malaria parasite.

Conservation of the transcriptional start site on the chromosomally and episomally located *hrp*3 promoter suggests that interaction of transcription factors with their cognate sequences and/or chromatin structure were not altered in the episomal plasmid. This is particularly relevant, since chromatin rearrangement has been associated with the loss of stage specificity in episomally located *gbp* 130 promoter [12]. In the *hrp*3 promoter the TSS was heterogeneous, but restricted to a discrete 130 nucleotide region. In contrast, *var*7b promoter TSS takes place in a specific sequence similar to the *Trichomonas vaginalis* initiator sequence (TCATA). There are four *var*-initiator-like sequences at positions -1572, -1560, -1425 and -870 in the *hrp*3 promoter (Fig. S3. Supplementary information, open box) but none of the HDGFP mRNA 5' end termini mapped, were located at or close to TCATA positions. Therefore, the presence of an initiator sequence does not necessarily indicate function. Our results suggest the presence of different TSS selection mechanisms for *Plasmodium* genes. Luciferase activity driven by the 0.6-kb truncated *hrp*3 promoter was very low compared with the 1.7-kb full length. Analysis of this promoter in stable cell lines by Northern blot [24], showed reporter mRNA in trophozoite but not in ring parasites. Comparison of the 0.6-kb *hrp*3 truncated promoter in a stable cell line [24] with transient luciferase activity (this study), showed no correlation between the mRNA accumulation of reporter gene and luciferase transient activity in *P. falciparum*. This result suggests that mRNA translation driven by the 0.6-kb promoter has been impaired. One possibility is the use of a cryptic TSS in the firefly luciferase mRNA; this in turn generates a mRNA with shortened 5' UTR that might be responsible for the FFL low activity in 0.6-kb *hrp*3 promoter. In fact, the complete TSS region has been truncated in the 0.6-Kb *hrp*3 promoter, suggesting that other TSSs have been used (Fig. S3, closed box and position -624, open circle. Supplementary information). For example, it has been shown in different organisms that elements on the 5' UTR regulate translation of mRNA [53] and therefore changes of the TSS might regulate the translation efficiency on a given gene.

We have identified stage-specific promoter elements that underscore the complex nature of regulation of gene expression in *P. falciparum*. In addition, our results suggest the use of a multipartite ring specific region in the *hrp*3 promoter. Future studies will further characterize the mechanism and identify the regulatory elements at play in the malaria parasite and contribute uncover unique pathways that could be exploited for drug development to fight the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

dhfr-ts, dihydrofolate reductase-thymidylate synthase
bp, base pair
nt, nucleotides
cam, calmodulin
*msp*1, merozoite surface protein 1
*hrp*3, histidine-rich protein 3
*hrp*2, histidine-rich protein 2

PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1
*hsp*86, heat shock protein 86
*pfs*16, *Plasmodium falciparum* sexual-stage antigen 16
*pfs*25, *Plasmodium falciparum* sexual-stage antigen 25
RNA, ribonucleic acid
rRNA, ribosomal ribonucleic acid
CAT, chloramphenicol acetyl transferase
PCR, polymerase chain reaction
hpi, hours post-invasion
mRNA, messenger ribonucleic acid
rif, repetitively Interspersed Family
stevor, subtelomeric variable open reading frame
HDGFP, human dihydrofolate reductase fused to *Aequorea victoria* Green fluorescent protein

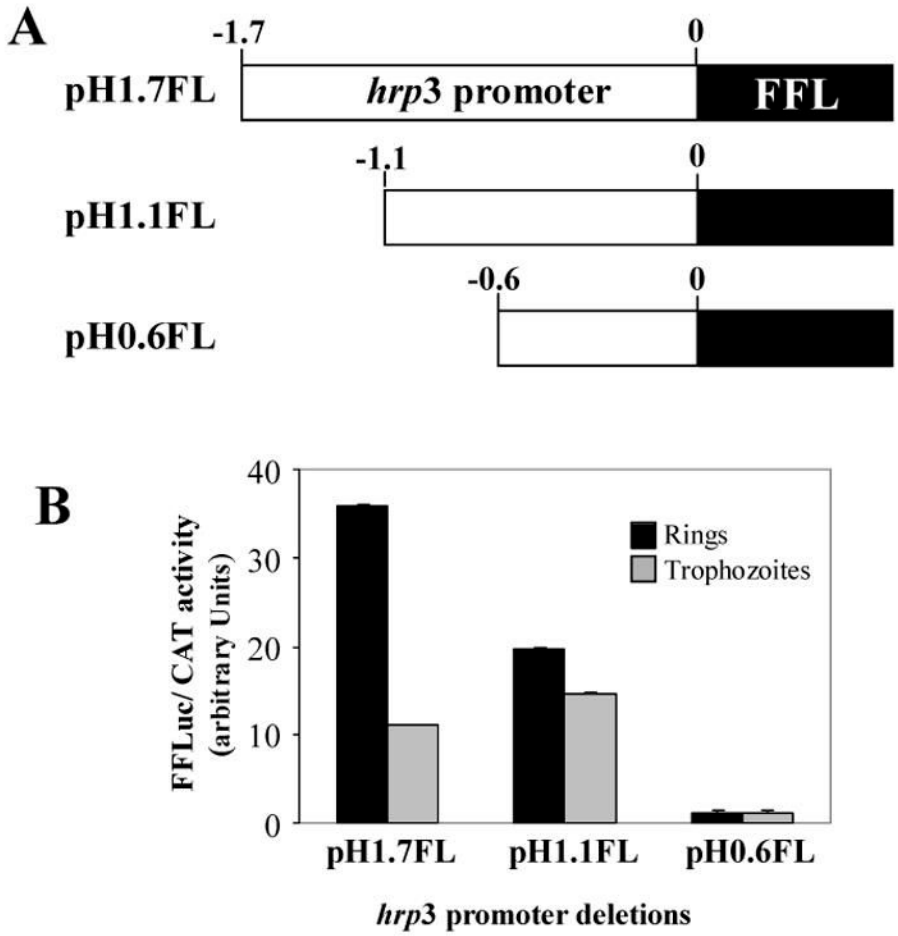
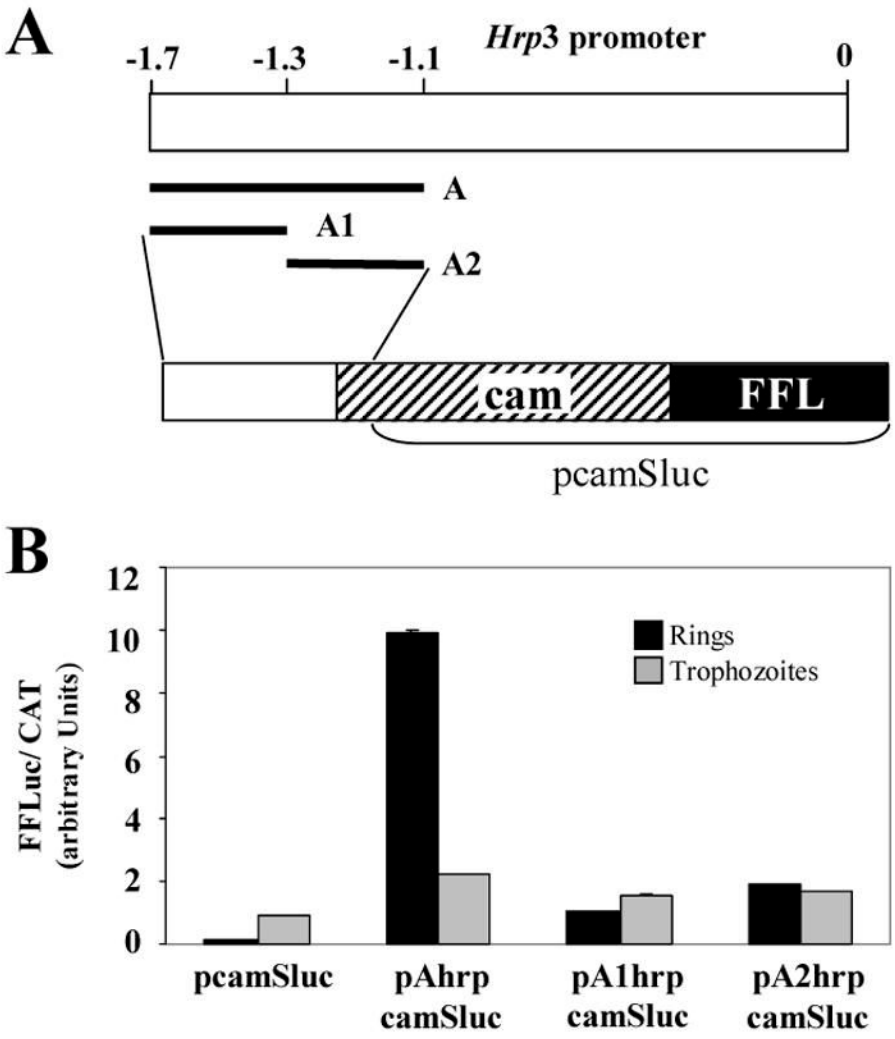


Figure 1.

Deletion of *hrp*3 promoter reduced the ring parasite firefly luciferase activity. A) Schematic representation of *hrp*3 promoter truncations driving firefly luciferase (FFL). B) Transient expression of luciferase gene in ring and trophozoite parasites. FFL activity was normalized by cotransfection using Chloramphenicol acetyltransferase gene (CAT) as described in Material and Methods. Bars are the mean of four independent experiments ± SD.



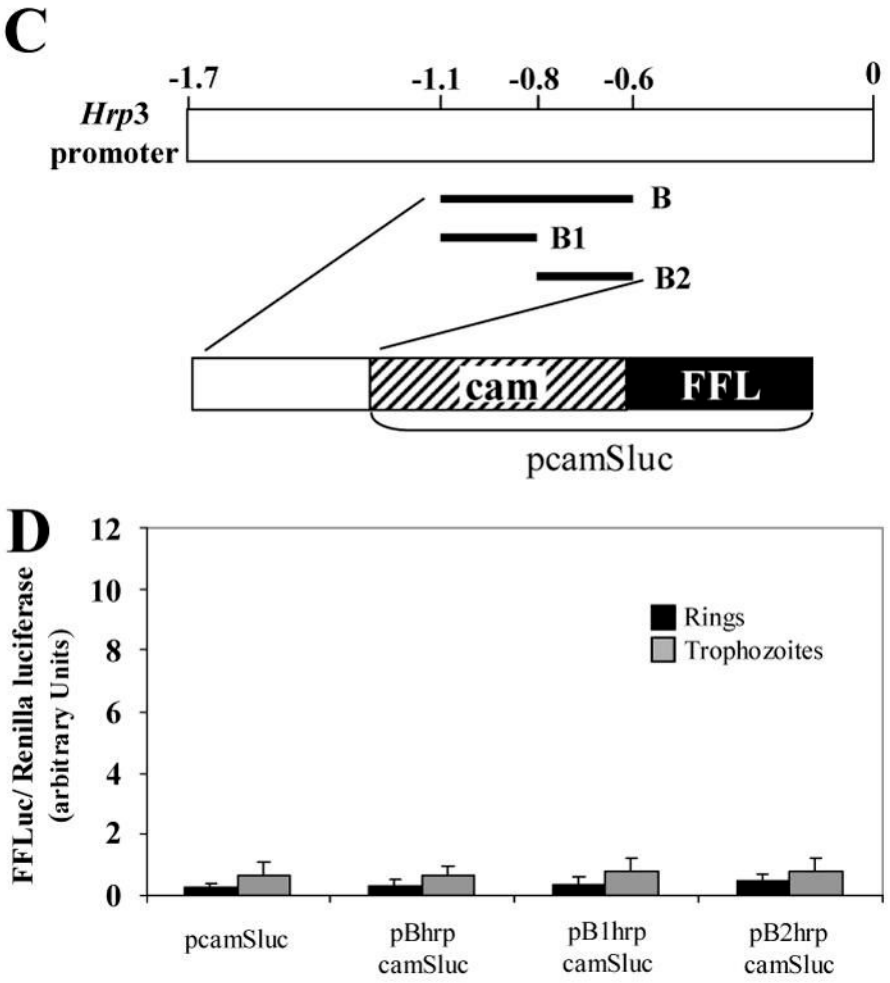


Figure 2.

Ring specific *cam*-dependent luciferase activity is stimulated by *hrp*3 region between -1.7 to -1.1. A) Schematic representation of the 1.7 kb *hrp*3 promoter region depicting the different *hrp*3 fragments placed upstream of the *cam* 5' end region. In addition, the organization of the hybrid promoter is shown. B) Transient luciferase activity was measured in ring and trophozoite parasites and normalized to CAT activity. The control plasmid (pcamSluc) contains *cam* promoter and firefly luciferase gene (FFL). pAhrpcamSluc, pA1hrpcamSluc, pA2hrpcamSluc represent plasmids with *hrp*3 A, A1 and A2 fragments placed upstream of the *cam* promoter respectively. Bars are the mean of three independent experiments ± SD. C) Schematic representation of the 1.7 kb *hrp*3 promoter region depicting the different *hrp*3 fragments placed upstream the *cam* 5' end region. In addition, the general organization of the hybrid promoter is shown. D) Transient luciferase activity was measured in ring and trophozoite parasites and normalized by cotransfection with a plasmid carrying the *Renilla* luciferase gene driven by the *cam* promoter. The control plasmid (pcamSluc) contains the *cam* promoter driving firefly luciferase gene (FFL). pBhrpcamSluc, pB1hrpcamSluc, pB2hrpcamSluc represent plasmids with *hrp*3 B, B1 and B2 fragments placed upstream the *cam* promoter respectively. Bars are the mean of four independent experiments ± SD.

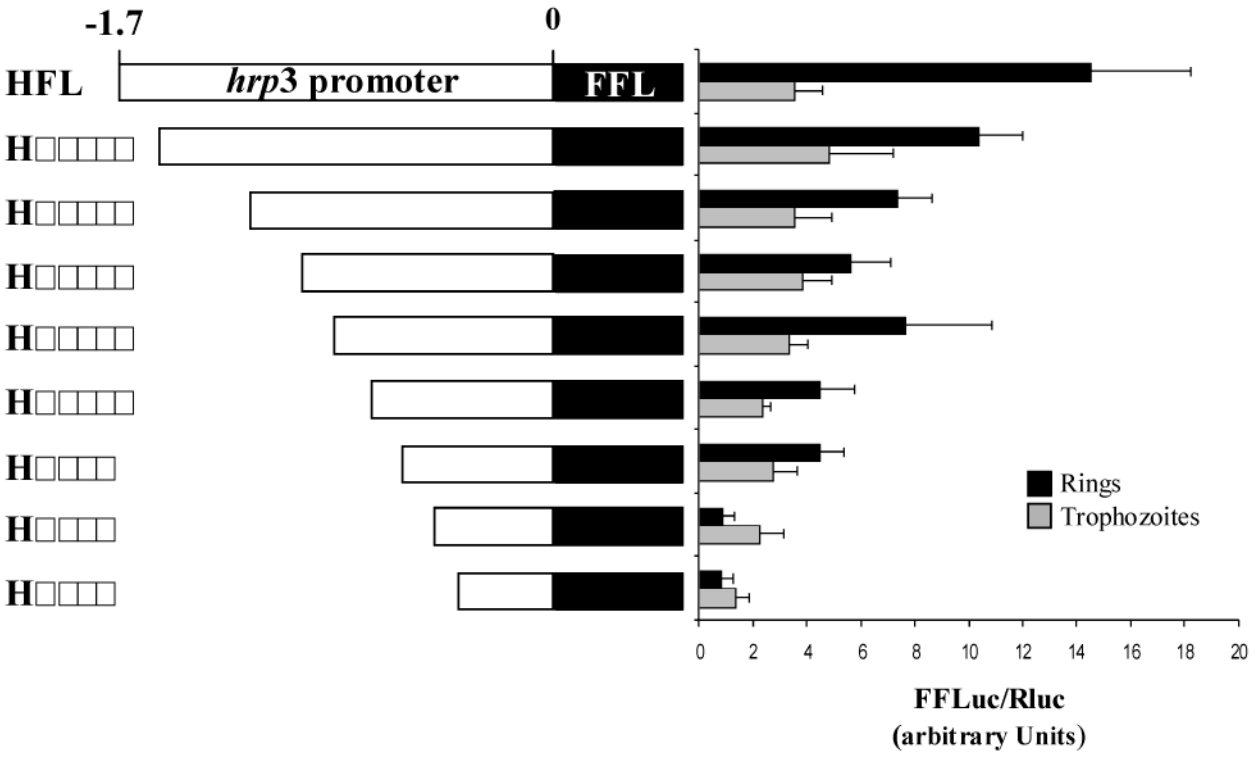


Figure 3.

A putative multipartite element on *hrp*3 promoter controls ring specific expression of a reporter gene. Schematic representation of *hrp*3 deletions (left side of panel). The constructs' name indicates the promoter size after deletion. *hrp*3 promoter was deleted by PCR and placed upstream the firefly luciferase gene. Constructs were transfected into *P. falciparum*. Firefly and *Renilla* luciferase activities were determined 12–14 hours post-invasion (hpi) (ring) and 32–34 hpi (trophozoite). Bars are the mean of four independent experiments ± SD.

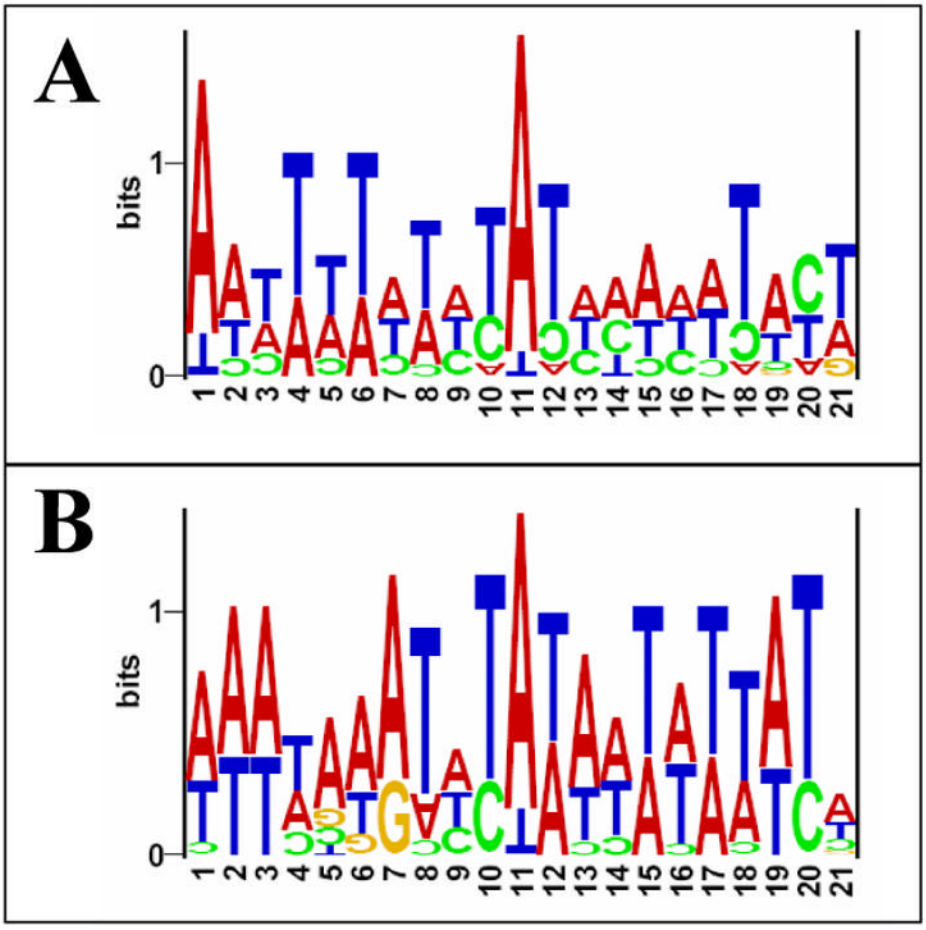


Figure 4.

Logo visualization of *hrp*3 promoter transcription start site. cDNA, from endogenous and episomal located promoter, was cloned in *E. coli* and recombinant plasmids were purified from 15 independent colonies for each group. Recombinant plasmids were then sequenced and used to determine the 5' end of cloned cDNA that represents the TSS. The logo sequence shows alignment of 15 different TSS for endogenous and episomal located *hrp*3 promoter. The first transcribed nucleotide (position 11) was adenosine in most cases. Ten nucleotide upstream and downstream of the TSS in endogenous (A) and episomal (B) were also included. The height of each letter is proportional to the frequency of nucleotides in each position. The total letter height indicates the amount of information (bits) contained in the nucleotides at that position.