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*Plasmodium falciparum: hrp*3 promoter region is associated with stage-specificity and episomal recombination

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Abstract

The asexual blood stage of *Plasmodium falciparum* in the human host is comprised of morphologically distinct ring, trophozoite and schizont stages, each of which possesses a distinct pattern of gene expression. Episomal promoter recombination has been recently reported in malaria parasites. We aim to investigate the nature of this process, and its relationship with promoter activity, by employing a series of nested deletions of the ring specific *hrp3* promoter. Our results showed a discrete promoter region that is preferentially used for recombination. The *P. falciparum hrp3* RNA gene is only seen in ring-stage parasites but deletion of the recombination region was associated with decreased ring-stage expression and concurrent detection of transcripts in trophozoite-stage parasites. Our results describe a ring-stage specific regulatory region possibly involved in episomal promoter recombination, suggesting that common sequences might mediate both processes.

1. Introduction

Plasmodium falciparum infection kills 2–3 million people every year, mostly children under 5 years of age (Greenwood and Mutabingwa, 2002). The blood stage of the parasite's life cycle is comprised of four distinct morphological forms (rings, trophozoites, schizonts and merozoites) and development through these stages is linked to regulation of gene expression.

Comprehensive evidence for stage specific gene expression comes from microarray expression analysis of distinct global patterns of RNA synthesis at different phases of parasite growth (Ben Mamoun, et al., 2001, Bozdech, et al., 2003, Hayward, et al., 2000, Munasinghe, et al., 2001, Patankar, et al., 2001). The role of *Plasmodium* promoters on regulation of gene expression has been assessed using a small number of asexual and sexual gene promoters. Thus, the structure of basal and stage specific promoters are poorly described. The advent of transfection to the blood stage parasite allowed the use of 5' end regions from asexual genes such as *hsp86*, *cam*, *hrp*3, PfEMP1 (*var*) as well sexual genes such as *pfs16* and *pfs25* to drive reporter gene expression suggesting that they function as promoters (Crabb and Cowman,

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1996, Crabb, et al., 1997, Dechering, et al., 1999, Deitsch, et al., 2001, Horrocks and Lanzer, 1999, Voss, et al., 2000, Wu, et al., 1995).

Transfection efficiency of blood stage malaria parasite remains very low despite recent progress in transfection methodology by several laboratories (Balu, et al., 2005, Nkrumah, et al., 2006). A possible explanation for the low transfection efficiency is the formation of large concatameric extrachromosomal plasmid forms (O'Donnell, et al., 2001). Plasmids appear to integrate into the malaria parasite genome in 1–3 tandem copies, suggesting that they may be present as concatamers prior to integration (Menard, et al., 1997).Episomal plasmid recombination in malaria has been reported to generate large stably replicating forms (O'Donnell, et al., 2001). This recombination appears to occur preferentially at the promoter region suggesting a correlation between promoter activity and promoter recombination (Kadekoppala, et al., 2001). Recently, plasmid recombination at non-promoter region has been reported in *P. falciparum* (Frank, et al., 2006). Plasmid chimeras occurred by intramolecular uneven recombination at the 3' end region of HRP 2 (3'*hrp*2) used as termination region for expression of recombinant protein (Frank, et al., 2006). Together these results suggest the presence of different mechanisms of homologous recombination in the parasite.

The role of malaria parasite promoter and non-promoter sequences on plasmid homologous recombination has not been assessed in detail. Here we investigated promoter-mediated recombination by cotransfecting malaria parasites with two plasmids, one carrying non-selectable marker GFP gene driven by the *hrp*3 promoter, the other carrying a selectable marker (TgDHFR) driven by the same promoter. Successful recombination of these plasmids will generate pyrimethamine-resistant cell lines expressing GFP. Nested deletions on the 1.7-kb ring-specific *hrp3* promoter decreased promoter-mediated recombination and switched promoter stage specificity from ring to trophozoite parasites. Deletion events that switched promoter specificity were associated with low recombination efficiency suggesting that timing of transcription might be important for efficient promoter recombination. In addition, all plasmid chimeras "rescued" from the transfected parasite cell lines conform to at least one of the three chimera types reported earlier (Kadekoppala, et al., 2001). Replacement of *hrp*3 for the trophozoite-specific *msp*1 generated a parasite subpopulation expressing GFP; these two promoters displayed a distinct efficiency of recombination despite their similar length. Together, our results suggest that recombination efficiency might correlate with the promoter's strength and/or stage specificity.

2. Material and Methods

2.1. Plasmid constructs

The *hrp*3 promoter deletions were made using Erase-a-base® (Promega) on pHRPCAT (Wu, et al., 1995) containing the *hrp*3 promoter driving the CAT gene. *Kpn* I-*Dra* III-digested pHRPCAT was incubated with exonuclease III (exoIII) at predetermined times to obtain specific-sized promoters of 1.1-kb, 0.6-kb and 0.5-kb. The CAT gene in exoIII-digested pHRPCAT was substituted with the GFP gene to generate pHRP1.7G (Kadekoppala, et al., 2000), pH1.1G, pH0.6G, pH0.5G. The CAT gene in exoIII-digested pHRPCAT was also substituted with the GFP/hDHFR-fusion gene (HDGFP, (Kadekoppala, et al., 2000)) to generate pH1.7HG (Kadekoppala, et al., 2000), pH1.1HG and pH0.6HG.

2.2. *In vivo* recombination assay and plasmid rescue

Episomal plasmid recombination was assessed by the method described previously (Kadekoppala, et al., 2001). Plasmids containing *hrp*3 1.7-kb and truncated promoters driving GFP expression (pH1.7G, pH1.1G, pH0.6G, pH0.5G), were individually mixed with the pDT.Tg23 plasmid (Wu, et al., 1996) carrying the 1.7-kb *hrp*3 promoter driving *Toxoplasma*

gondii's bifunctional enzyme gene *dhfr-ts* (fig 1A). Plasmids, at 1:1 ratio (50 μg per plasmid) were cotransfected into infected erythrocytes (Fidock and Wellems, 1997). Forty-eight hpi, 100 ng/ml of pyrimethamine was added to the cultures for two days. Subsequently parasite cultures were incubated and maintained at 20 ng/ml of pyrimethamine. The pmspTgDHFR plasmid was generated by digesting the *TgDHFR* gene from pDT.Tg23 with *Nsi* I and *Hind* III. The *Nsi* I-*Hind* III *TgDHFR* fragment used to replace the *GFP* gene in the pMSPGFP (Kadekoppala, et al., 2001). Live parasite cells expressing GFP (gfp^+) and/or pyrimethamine-resistant (pyr^r) were stained with 5 μg/ml of DAPI and used to determine the total parasite number using a epifluorescent Nikon eclipse 400. gfp^+-parasites were scored and reported as a percentage of total pyrimethamine-resistant (pyr^r) cells stained with DAPI.

Total DNA, for plasmid rescue experiments, was isolated from saponin-released gfp^+:pyr^r parasite stable cell lines. Released parasites were lysed with SDS extraction buffer (10mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS) and DNA extracted with buffered phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature. Plasmids from transfected cell lines were recovered by transforming *Escherichia coli* competent cells with purified parasite DNA. *E. coli* recovered plasmid from two independent transfections were digested with *Xho* I and/or *Hpa* I and separated on agarose gel.

2.3. Parasite transfection and Northern blot Analysis

P. falciparum stable cell lines were generated by transfecting parasite-infected erythrocytes (3D7 strain) with 100 μg of experimental plasmids (Fidock and Wellems, 1997). Stable cells were selected with methotrexate (2.2 μM, Sigma) 48 hours post-transfection and maintained until the cell lines were established (3–4 weeks). Parasites were percoll/sorbitol synchronized and monitored by light microscopy of a giemsa stained infected red cells smear. Synchronization was repeated until 99.9% of the parasites were in a specific stage. Total RNA from stable cell lines carrying pH1.7HG, pH1.1HG or pH0.6HG were isolated using TRIzol® LS reagent (Gibco BRL). Ten micrograms of total RNA was electrophoresed in agarose gel and transferred to Hybond N^+ membranes (Amersham) and subsequently hybridized with a ^{32}P-antisense GFP probe generated by asymmetric PCR (Bird, 2005).

3. Results

Recombination of episomal plasmid carrying the *hrp*3 promoter has been previously described (Kadekoppala, et al., 2001). To investigate which promoter region is responsible for *in vivo* recombination, we co-transfected two separate plasmids, one plasmid carrying a non-selectable reporter gene (GFP in pHRPGFP) and the other a drug selection marker from *Toxoplasma gondii* (*dhfr-ts* in pDT.Tg23) (Kadekoppala, et al., 2001). Long-term maintenance of the GFP-containing plasmid (pHRPGFP) will depend upon successful recombination with the selectable plasmid pDT.Tg23. GFP-encoding plasmids were mixed at a 1:1 ratio with the pDT.Tg23 (Fig. 1A). In order to avoid differences in parasite growth due to drug selection, the 1.7-kb *hrp*3 promoter was used to drive the selectable marker *dhfr-ts* gene and truncations were made on the *hrp*3 promoter driving the non-selectable maker GFP. The percentage of *in vivo* recombination at a given time was calculated by counting the number of GFP-expressing parasites and the total parasites were determined by scoring the number of intraerythrocytic DAPI-stained parasite's nuclei in the sample. After pyrimethamine (pyr^r) selection, a subpopulation of the parasite showed gfp^+ expression (Fig. 1B, parasites transfected with pHRP1.7G/pDT.Tg23 plasmids). A sizable number of parasite were pyrimethamine resistant but did not express parasite gfp^+ as evidenced by the DAPI-stained nuclei in fig. 1B. Parasites cotransfected with the *hrp3* 1.7-kb promoter in both GFP and TgDHFR-containing plasmids, showed 8% of green parasites at 12 days post-transfection (Fig. 2, pHRP1.7G). At 24 days post-transfection the gfp^+-expressing parasite reached a peak around 45% and stabilized at

30% after 30 days post-transfection, which is consistent with previous results (Kadekoppala, et al., 2001). The deletion of 600 bp from the 5' end on the *hrp*3 to give rise to the 1.1-kb truncated promoter in the pH1.1G plasmid, reduced green parasites to approximately 15% after 30 days post-transfection (Fig. 2, pHRP1.1G). This reduction in parasites carrying chimeric plasmids, suggests the presence of a recombinogenic element between −1.7-kb and −1.1 kb (Fig. S1 supplementary data, underlined nucleotides). Further deletion of the 1.1-kb *hrp3* promoter to 0.6-kb and 0.5-kb in pH0.6G and pH0.5G respectively, reduced green parasites below 5% (Fig. 2, pHRP0.6G and pHRP0.5G) suggesting a second recombinogenic region located between −1.1-kb and −0.6-kb region (Fig. S1 supplementary data, bold faced nucleotides). Parasites were also transfected with pDT.Tg23 and pMSPGFP in order to evaluate whether recombination was specific to the promoter region. Pyrimethamine-resistant parasites but not gfp^+-expressing parasites were recovered; this result indicates that effective recombination requires homologous promoter as previously shown (Kadekoppala, et al., 2001).

To assess whether the gfp^+ and pyr^r containing plasmids recombined and remained as an episomal chimera, we performed plasmid rescue experiments. This method has been previously used to identify plasmid chimeras in the parasite (Frank, et al., 2006, Kadekoppala, et al., 2001). There are three different types of chimeras depending on the number of concatamers according to (Kadekoppala, et al., 2001); the simplest chimera comprised of two plasmid units, one pDT.Tg23 (1 pyr^r) and one pH1.7G (1 gfp^+) namely type 1. The second class of chimeras contained three concatamers from which two additional types were identified. Type 2 comprised of 1 pyr^r and 2 gfp^+, whereas type 3 comprised 2 pyr^r and 1 gfp^+ (Kadekoppala, et al., 2001). Total DNA purified from gfp^+-pyr^r parasite cell lines, grown in continuous culture for more than a year, was used to transform *E. coli* competent cells. Purified plasmids isolated from individual bacteria colonies (10 colonies for each parasite cell line) were digested with *Xho* I and/or *Hpa* I. These restriction enzymes cut only once in the *dhfr-ts* and *gfp* genes respectively, but not in the plasmid backbone (Kadekoppala, et al., 2001), which allows for the identification of chimera types in the parasites. Plasmid rescued from pDT.Tg23/pH1.7G-transfected parasites digested with *Xho* I or *Hpa* I yielded a single band of 13-kb (Fig. 3, lanes 2 and 3). This result is consistent with a chimera containing one copy of each original plasmid in the parasite. Double digestion with *Xho* I and *Hpa* I restriction enzymes generated bands of 7-kb and 6-kb, consistent with the presence of pDT.Tg23 and pH1.7G plasmids respectively (Fig. 3 lane 4). These results indicate the presence of stable episomal chimeras in *Plasmodium* and demonstrate that chimeras are stably maintained even after long-term culturing of transfected parasites (Kadekoppala, et al., 2001). Episomal chimeras were also rescued from parasite cell lines transfected with pDT.Tg23/pH1.1G, pDT.Tg23/pH0.6G or pDT.Tg23/pH0.5G plasmids. Digestion of these chimeras with *Xho* I generated two bands of 13-kb and 7-kb respectively (Fig. 3, lanes 6, 10 and 14 respectively); a finding that is diagnostic of episomal chimeras comprised of 2 pyr^r and 1 gfp^+ plasmids. Conversely, *Hpa* I digestion generated only one band of approximately 20-kb, confirming the presence of only one gfp^+-containing plasmid in the chimera (Fig. 3, lanes 7, 11 and 15 respectively). Double digestion with *Xho* I/*Hpa* I confirmed the presence of the two plasmid types (pyr^r and gfp^+) in the chimera (Fig. 3, lanes 8, 12 and 16 respectively). In addition, episomal chimeras containing 1 pyr^r-2 gfp^+ (data not shown) were also detected in pyr^r-gfp^+ *Plasmodium* cell lines transfected with pDT.Tg23/pH1.1G, pDT.Tg23/pH0.6G or pDT.Tg23/pH0.5G. The rescue of chimeras indicates the presence of long-term plasmids generated by homologous recombination of the pDT.Tg23 with the pH1.1G, pH0.6G or pH0.5G.

To test whether promoter recombination is a common feature of other malaria promoters, we cotransfected two new plasmids containing the 1.6-kb trophozoite specific *msp*1 promoter. The first plasmid carries the *TgDHFR-ts* and the second plasmid carries the *gfp* gene (pMSPGFP, (Kadekoppala, et al., 2001). Fig. S2, supplemental data). Drug selection of transfected parasites

gave rise to a pyrimethamine-resistant population, consistent with the presence of the selectable marker plasmid pmspTgDHFR. Approximately 1% of the parasites were gfp^+ one week after the start of selection. Thirty days post-transfection the number of gfp^+ parasites remained constant (approximately 1%). No further increase in gfp^+- expressing parasites was observed. These results suggest that homologous recombination also occurs in the trophozoite-specific *msp*1 promoter, suggesting that homologous recombination is a general feature associated with *Plasmodium* promoters. Although the *msp*1 and the *hrp*3 promoters are similar in size, the latter recombined more efficiently suggesting that promoter size might not play a major role on the overall recombination rates.

Because of the putative correlation between promoter activity and recombination, we evaluated the role of *hrp*3 promoter nested deletions on gene expression. The full length (1.7-kb) and deleted (1.1-kb and 0.6-kb) promoters were placed upstream of the HDGFP fusion gene (Kadekoppala, et al., 2000) (Fig. 4A). Methotrexate-resistant stable cell lines were percoll/sorbitol synchronized and used to purify RNA at 12–14 hpi (ring-stage) and subjected to northern blot analysis using a ^{32}P-antisense GFP probe. The 1.7-kb promoter (Fig 4A, pH1.7HG) had the maximum steady state mRNA accumulation (Fig. 4Bi and 4Biii) in the ring-stage of the parasite. Deletions of the *hrp*3 5' end to generate the 1.1-kb and 0.6-kb truncated promoters (Fig 4A, pH1.1HG and pH0.6HG) decreased mRNA accumulation in ring-stage parasites (Fig. 4Bi and 4Biii) with little mRNA detectable in the 0.6-kb promoter truncation. We then investigated the effect of the same 5' deletions on trophozoite-stage promoter activity at 32–34 hpi. Steady state accumulation of HDGFP mRNA from the 1.7-kb promoter cell line pH1.7HG showed a significant reduction of mRNA compared to ring parasites (Fig. 4Bii), consistent with the ring-stage specificity of the 1.7-kb promoter (Cheresh, et al., 2002). HDGFP mRNA from 1.1-kb truncated promoter was also detected in trophozoite-stage parasites (Fig 4Bii, 4Biv). Detection of HDGFP mRNA from the 1.1-kb promoter in both ring and trophozoite stage parasites indicates that the deletion of the −1.7-kb to −1.1-kb region correlates with loss of ring specificity. This truncation may affect the regulation of promoter activity by increasing transcription or HDGFP mRNA stability from trophozoite stage. Unexpectedly, deletion of *hrp3* to a 0.6-kb in pH0.6HG, showed an increase level of HDGFP mRNA accumulation in the trophozoite stage parasites (Fig. 4Bii and 4Biv), but with little transcript in ring stage parasites. Comparison of HDGFP mRNA accumulation in the ring and trophozoite stage parasites clearly shows a switch of promoter stage specificity. One interesting observation was that the 0.6-kb *hrp*3 promoter showed similar recombination efficiency to the *msp*1 promoter despite the size difference (0.6-kb and 1.6-kb respectively). Alignment analysis of these two promoters failed to show a significant homology. However both 0.6-kb *hrp*3 and 1.0-kb *msp*1 promoters have a peak of mRNA accumulation at trophozoite-stage. These data suggest the possibility that homologous recombination is less favored at the trophozoite stage of the parasites. Our results strongly suggest that the region from −1.7-kb to −1.1-kb of *hrp*3 promoter restricts promoter activity to ring stage parasites.

Discussion

In this study we report a correlation between promoter recombination and promoter activity in the human malaria parasite *P. falciparum*. Truncation of a *hrp*3 promoter decreased the number of gfp^+-expressing parasites, suggesting a reduced recombination efficiency of the truncated promoters. Two region of approximate size were associated with the overall recombination efficiency. Truncation of the region between −1.7-kb to −1.1-kb decreased recombination from 30% to 15%. Further truncation of the region between −1.1-kb and −0.6-kb further reduced the number of gfp^+-expressing cells and thus recombination from 15% to 5%. The formation of episomal chimeras due to plasmid recombination was assessed by plasmid rescue experiments on *E. coli* cells. All rescued chimeras conformed the 3 basic structures previously described (Kadekoppala, et al., 2001), indicating that recombination most likely occurred at

the promoter sequences. It was rather unexpected that only “type” 1 chimeras were obtained from the cell line transfected with pDT.Tg23 and pH1.7HG. In contrast, “types” 2 and 3, but not “type” 1, chimeras were obtained from cell lines transfected with pDT.Tg23 and pH1.1HG, pH0.6HG or pH0.5HG. It is possible that long-term cell culture may result in the 1.7-kb promoter forming large self-replicative chimera (O'Donnell, et al., 2001) leaving only “type” 1 chimera available for transforming competent bacterial cells.

Homologous recombination at regions known as hotspots have been described in yeast and human genomes (Chakravarti, et al., 1984, Jeffreys, et al., 2001, McVean, et al., 2004, Petes, 2001) as well as the *Plasmodium* parasite (Mu, et al., 2005). Recombination events in the parasite’s genome appear to be clustered in the middle and near the chromosome end (telomeric regions). Although recombination rates are highly variable, hotspot locations seems to be conserved among distinct population of malaria parasites, suggesting a common mechanism (Mu, et al., 2005). Telomeric regions are packed with genes involve in antigenic variation and cell surface interaction genes such as *var*, *rifin* and *stevor* (Bowman, et al., 1999, Gardner, et al., 2002). Interestingly, *hrp*3 is also located at the end of chromosome 13 (Wellems, et al., 1987), suggesting the possibility that promoter-mediated recombination is a feature of telomeric-located genes. However, whether promoter region and recombination hotspot share the same distribution along *Plasmodium*’s chromosomes has yet to be determined. Homologous recombination on non-promoter regions has been recently reported in episomal plasmid used to study variant *var* gene silencing regulation (Frank, et al., 2006). Specific uneven recombination at the 3’ end of the *hrp*2 region (3*hrp*2) was generated after transfecting the parasite with a double cassette plasmid carrying two copies of 3hrp2. All recombination events detected on the plasmid and into the chromosome has at least one of the two 3*hrp*2 copies immediately adjacent to the *var* intron bidirectional promoter (Calderwood, et al., 2003) used to drive a downstream-located selectable gene (hDHFR) (Frank, et al., 2006). Interestingly, recombination between 3*hrp*2 regions distant from promoter sequences was not detected and thus, it is possible that intron promoter activity might play a role in recombination mediated by 3*hrp*2 in this plasmid. gfp^+-expressing parasites were not detected when parasites were cotransfected with plasmids carrying non homologous promoters (Kadekoppala, et al., 2001), suggesting that chimeric gfp^+pyr^r plasmids were not present in the recovered pyr^r-resistant parasite population. Our results strongly indicate that 3*hrp*2 regions were not involved in chimera formation. However, we cannot rule out the possibility that very low levels of 3*hrp*2-mediated recombination was just not detected in our assays. Recombination efficiency does not appear to be determined exclusively by promoter length. For instance, the *msp*1 promoter displayed lower recombination efficiency than its *hrp*3 counterpart albeit the similar size of these two promoters. In this context, promoter activity might account for the difference in recombination efficiency.

The change in promoter recombination was associated with loss of *hrp*3 promoter stage specificity. The reporter HDGFP mRNA was present in both ring and trophozoite parasite in the promoter lacking the −1.7-kb to −1.1- kb region. This truncation turned the *hrp*3 promoter’s activity constitutive. Further truncation of the *hrp*3 promoter region located between −1.1-kb to −0.6-kb showed mRNA accumulation in trophozoite parasites but not in ring parasites. The pattern of mRNA accumulation was therefore switch from ring to trophozoite after transition from 1.7-kb to 0.6-kb truncated promoter. Interestingly, the *hrp*3 0.6-kb and *msp*1 behave as trophozoite specific promoters with relatively low recombination efficiency compared to the 1.7-kb *hrp*3 promoter, suggesting that recombination in trophozoite parasite might be lees efficient than in ring parasites. Together, these results suggest the presence of common sequences that might regulate both promoter recombination and stage specificity. However, the role of transcription in recombination has to be further evaluated.

Genome wide analysis has been impaired due to the propensity of episomal plasmids to form large chimeras in the parasite, precluding random integration into the genome. The understanding of homologous episomal recombination will facilitate the design of improved vectors for reverse genetic studies in the parasite. To this goal, a more comprehensive analysis of the cis and trans factors mediating episomal recombination is needed. This is stressed by the fact that uneven plasmid recombination has been recently reported (Frank, et al., 2006). This may be undesirable when it results in the loss of plasmid regions required for the proper analysis of biological processes. Questions such as the nature of the DNA sequences involved in these processes, the role of transcription as well as the role of stage specific promoter activity are currently been addressed in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Index descriptors and abbreviations

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

dhfr-ts, dihydrofolate reductase-thymidylate synthase
cam, calmodulin
*msp*1, merozoite surface protein 1
*hrp*3, Histidine-rich protein 3
*hrp*2, Histidine-rich protein 2
GFP, *Aequorea victoria* green fluorescent protein
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
DAPI, 4′-6-Diamidino-2-phenylindole
hpi, hours post-invasion
mRNA, messenger ribonucleic acid
rifin, Repetitively Interspersed Family
stevor, subtelomeric variable open reading frame
HD, Human dihydrofolate reductase (hDHFR)
HDGFP, Human dihydrofolate reductase fused to *Aequorea victoria* Green fluorescent protein
SDS, Sodium dodecyl sulfate

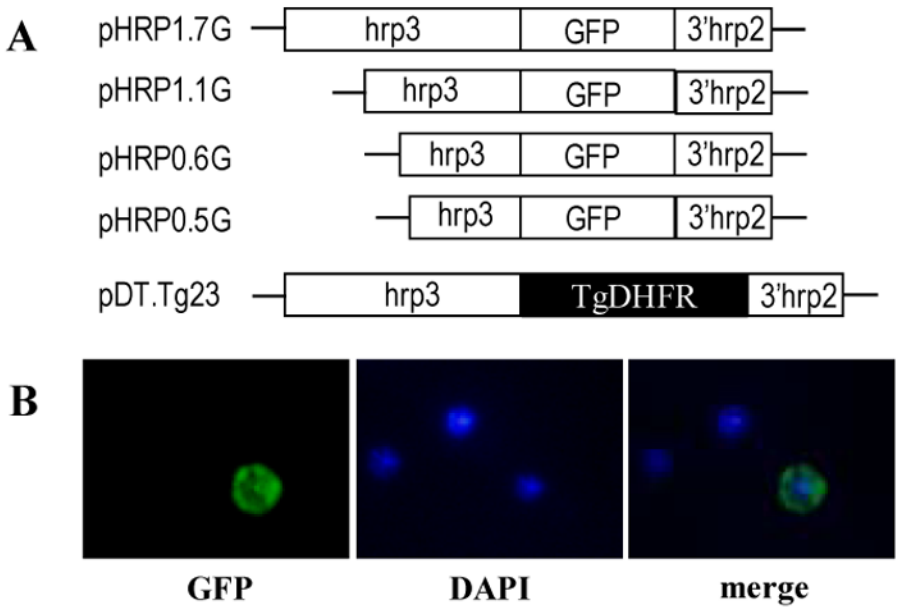

Figure 1. Construction of plasmids used to transfect *P. falciparum* parasites

(**A**) Schematic representation of plasmids containing 1.7-kb promoter (pHRP1.7G), 1.1-kb (pHRP1.1G), 0.6-kb (pHRP10.6G) and 0.5-kb (pHRP0.5G) *hrp*3 truncated promoters driving the GFP gene. The plasmid pDT.Tg23 plasmid is also shown containing the 1.7-kb *hrp*3 promoter driving the *T. gondii dhfr-ts* gene (Kadekoppala, et al., 2001). (**B**) Stable pyr-resistant parasite cultures cotransfected with pHRP1.7G and pDT.Tg23 plasmids show green fluorescent GFP (left panel) in early trophozoite. Nucleic acid staining is shown in blue in the nucleus (Center panel) and the merge image (right panel).

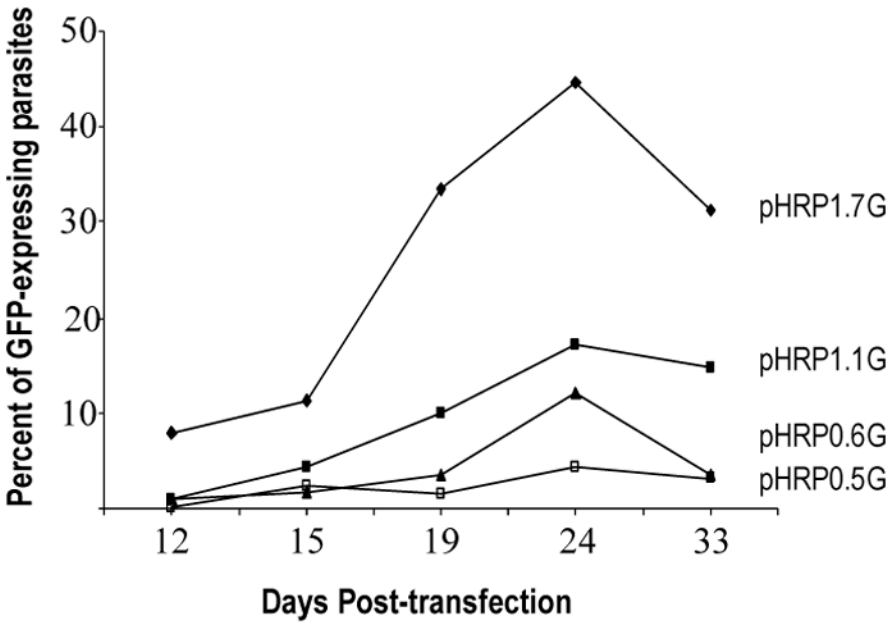

Figure 2. I*n vivo* episomal recombination in *P. falciparum* parasites

Parasite cultures were cotransfected with pHRP1.7G, pHRP1.1G, pHRP10.6G or pHRP0.5G *hrp*3 truncated promoters driving the GFP gene and pDT.Tg23 as shown in figure 1. Parasites were collected at 12, 15, 19, 24 and 33 days post transfection. Green fluorescent parasites were scored and expressed as percent of total.

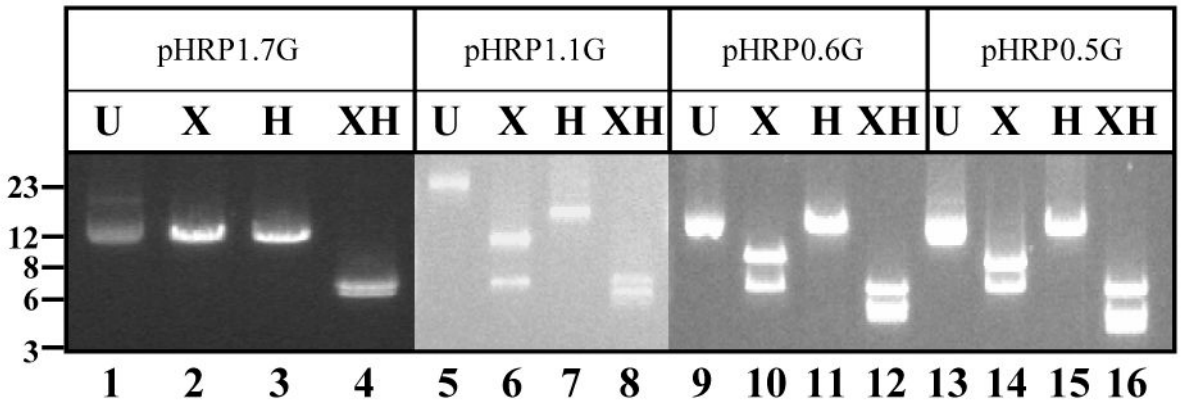

Figure 3. Heteromultimeric plasmids recovered in *E. coli*

(**A**) Schematic representation of heteromultimeric plasmids containing 1pyr^r:1gfp copy of pH1.7G and pDT.Tg23 (type 1); 1pyr^r:2gfp (type 2) or 2pyr^r:1gfp (type 3) (Kadekoppala, et al., 2001). (**B**). *E. coli* competent cells were transformed with total DNA from parasite cell lines. Plasmid recovered in *E. coli* transformed with pDT.Tg23/pH1.7HG (pH1.7HG), pDT.Tg23/pH1.1HG (pH1.1HG), pDT.Tg23/pH0.6HG (pH0.6HG) or pDT.Tg23/pH0.5HG (pH0.5HG) plasmid mixtures were digested with *Hpa* I (H) and/or *Xho* I (X) and separated on agarose gel with undigested (U) plasmid as control. DNA molecular weight marker (in kb) is presented. Detected bands did not change size after long-term culture (more than a year). Plasmid backbone is pBluescript (not shown).

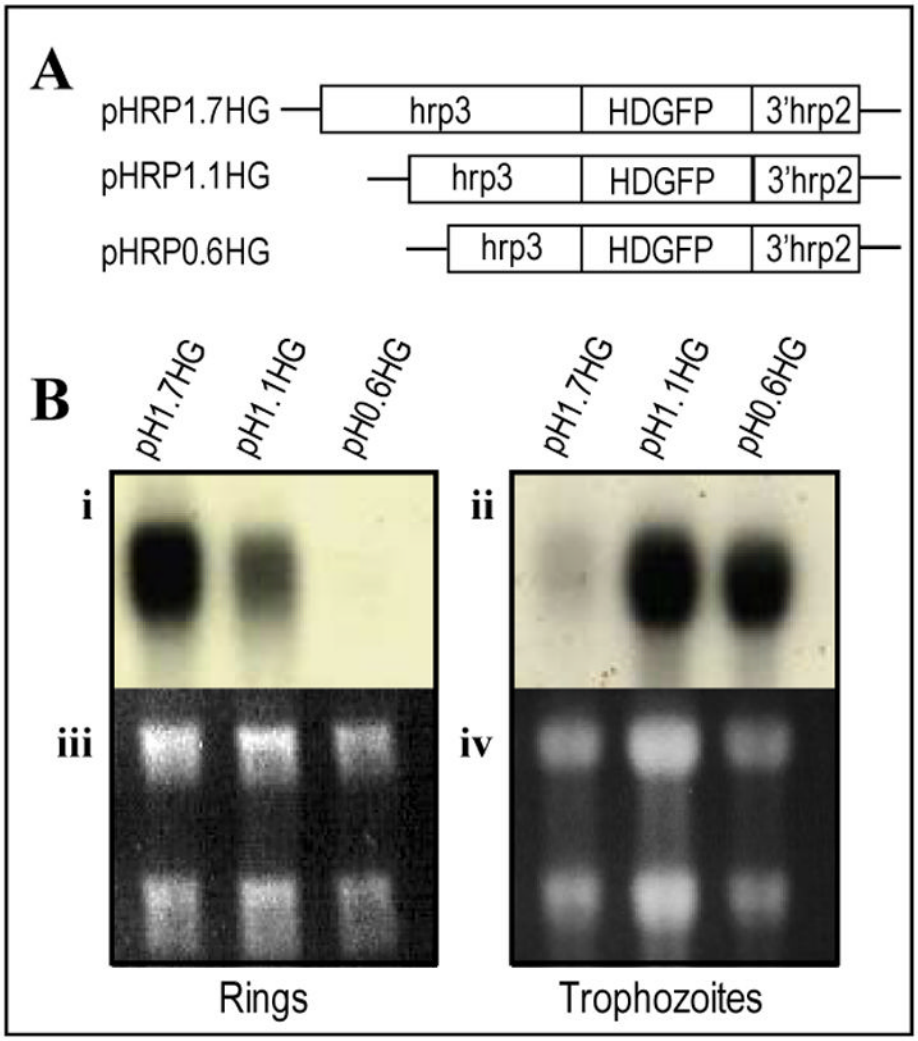

Figure 4. Deletion of the *hrp*3 promoter switched stage specific expression from ring to trophozoite stage

(**A**) Schematic representation of *hrp*3 promoter truncations (diagonal lines) driving human DHFR fused to GFP (HDGFP, filled box) in plasmid pH1.7HG containing the 1.7-kb full length promoter as well as the plasmids pH1.1HG, pH0.6HG carrying the truncated promoters of 1.1-kb and 0.6-kb respectively. (**B**) Total RNA from stable cell lines expressing HDGFP was purified from ring (i, iii) and trophozoite (ii, iv) stages, separated on agarose gels stained with ethidium bromide (iii, iv) and analyzed by northern blot (i, ii) using ^{32}P-GFP antisense probe.