

Transcriptional Activity of *Plasmodium* Subtilisin-like Protease 2 (*Pf*-Sub2) 5' Untranslated Regions and Its Interaction with Hepatocyte Growth Factor

Shunyao Liao^{1,†}, Yunqiang Liu^{1,†}, Suk-Yul Jung², Pyo Yun Cho¹, Bing Zheng¹ and Hyun Park^{1,*}

¹Department of Infection Biology, Zoonosis Research Center, Wonkwang University School of Medicine, Iksan 570-749, Korea; ²Department of Biomedical Laboratory Science, Namseoul University, Cheonan 331-707, Korea

Abstract: The onset, severity, and ultimate outcome of malaria infection are influenced by parasite-expressed virulence factors and individual host responses to these determinants. In both humans and mice, liver injury is involved after parasite entry, which persists until the erythrocyte stage after infection with the fatal strain *Plasmodium falciparum* (*Pf*). Hepatocyte growth factor (HGF) has strong anti-apoptotic effects in various kinds of cells, and also has diverse metabolic functions. In this work, *Pf*-subtilisin-like protease 2 (*Pf*-Sub2) 5' untranslated region (UTR) was analyzed and its transcriptional activity was estimated by luciferase expression. Fourteen TATA boxes were observed but only one Oct-1 and c-Myb were done. In addition, host HGF interaction with *Pf*-Sub2 was evaluated by co-transfection of HGF- and *Pf*-Sub2-cloned vector. Interestingly, -1,422/+12 UTR exhibited the strongest luciferase activity but -329 to +12 UTR did not exhibit luciferase activity. Moreover, as compared with the control of unexpressed HGF, the HGF protein suppressed luciferase expression driven by the 5' untranslated region of the *Pf*-Sub2 promoter. Taken together, it is suggested that HGF controls and interacts with the promoter region of the *Pf*-Sub2 gene.

Key words: *Plasmodium falciparum*, malaria, subtilisin-like protease 2, hepatocyte growth factor, 5' untranslated region, promoter

INTRODUCTION

Malaria, which is caused by infection with the hemoprotozoan parasite *Plasmodium*, afflicts approximately 500 million people each year, leading to 2.7 million deaths annually worldwide [1]. Malaria results in diverse clinical outcomes presumably because of dynamic relationships between parasite-expressed virulence factors and individual host responses to these determinants [2]. The last few years have seen a rapid growth in the number of reported genetic associations with susceptibility and resistance to malaria in the host; they are mainly vari-

ants in erythrocyte, cytoadherence factor, immune system, and inflammatory genes [3-5]. However, molecular basis of malaria infection remains incompletely defined. Identification of genetic factors in both the host and the parasite involved in malaria susceptibility and resistance would lead not only to a greater understanding of this complex disease, but potentially also to the development of effective medical interventions [2,6].

Interactions between *Plasmodium* and its host hepatocyte factors are of great research interest [7-9]. On one hand, it is well established that malaria sporozoites exploit hepatocyte growth factor (HGF) and its receptor MET signaling during host invasion [10]. On the other hand, *Plasmodium* is a life cycle-specific disease that includes liver injury at the erythrocyte stage of the parasite [11]. Indeed, both humans and mice suffer from liver injury after infection with the fatal strains *Plasmodium falciparum* (*Pf*) and *P. berghei* (*Pb*) [12,13]. Because *Pb* merozoites do not parasitize liver parenchymal cells in mice, it was proposed that the mechanism of *Pb*-induced liver injury is likely to be caused by the local production of cytokines that activate lymphocytes residing in the liver [14]. Pathologically, malaria-induced mouse liver injury is characterized by dense infiltration of lymphocytes, and the presence of apoptotic and necrotic hepatocytes [15].

• Received 22 June 2010, revised 30 September 2010, accepted 3 October 2010.

* Corresponding author (hyunpk@wonkwang.ac.kr)

† S. Liao and Y. Liu contributed equally to this work.

† Present affiliation: S. Liao, Organ Transplantation Center, Sichuan Academy of Medical Sciences & Shichuan Provincial People's Hospital; Y. Liu, Department of Medical Genetics, West China Hospital and State Key Laboratory of Biotherapy, Sichuan University, Chengdu, 610041, P.R. China

© 2010, Korean Society for Parasitology

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Plasmodium subtilisin-like protease 2 (subtilase, Sub2) is an essential integral membrane serine protease in both *Pf* and *Pb*. It functions in the shedding of the ectodomain components of 2 surface proteins, membrane surface protein 1 (MSP1), and apical membrane antigen 1 (AMA1), upon host invasion [16-18]. The importance of the Sub2 gene is evidenced by the demonstration that gene-knock out parasites cannot be recovered [19].

The correlation of HGF and malaria infection is not well understood. In this study, to understand the relationship between the malaria infection-related protein *Pf*-Sub2 and HGF, the *Pf*-Sub2 5' untranslated region (UTR) was analyzed and its promoter activity was estimated. In addition, the influence of HGF on its promoter activity was analyzed.

MATERIALS AND METHODS

Parasite manipulations

Pf 3D7 strain was obtained from Malaria Research and Reference Reagent Resource Center (MR4; Manassas, Virginia, USA; <http://www.mr4.org>). The *Pf* 3D7 culture condition was set according to the MR4 standard protocol. Parasitemia was calculated by counting at least 1,000 cells following staining with Diff Quick (Sysmex, Kobe, Japan).

Cell culture

Cell culture media and components, including fetal bovine serum (FBS), were obtained from Invitrogen (Carlsbad, California, USA). All cells were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. HeLa, Huh-7, HepG2, and 293T cells were used for the experiments.

DNA isolation and plasmid vector construction

All plasmids were purified using JETstar2.0 mini and maxi-prep Kit (GenoMed, St. Louis, Missouri, USA) according to the manufacturer's instructions. *Pf* 3D7 small scale gDNA isolation followed the MR4 protocol. A construct for luciferase activity was based on a pGL3-Basic vector (Promega, Madison, Wisconsin, USA). Two different 5' upstream sequences flanking the *Pf*-Sub2 gene were amplified from *Pf* 3D7 gDNA with primers. The primers used for -2,567/+32 UTR were F (5'-ATAGGTTACCTAGAATAGAATAAAATAAATAACC-3'; *Kpn*I site underlined) and R (5'-ATAAAGCTTATCAAGGAAACCACATAA-3'; *Hind*III

site underlined). The primers used for -1,422/+12 UTR were F (5'-AAGGTACCATGGTATGAGTTCCTTATA-3'; *Kpn*I site underlined) and R (5'-ATGCTAGCAATATTCAGCATTATACGGA-3'; *Nhe*I site underlined). PCR was performed with a T_{GRADIENT} thermocycler (Biometra, Goettingen, Germany) and the amplified UTR sequences were cloned into a pMDT vector (Takara Bio, Shiga, Japan) and subcloned into a pGL3-Basic vector with *Kpn*I and *Nhe*I/*Hind*III sticky end ligation, generating pGL3-Basic-*Pf*-Sub2 5' UTR. The other upstream inserts (-329/+12, -668/+12 and -739/+12) were cloned into a pGL3-Basic vector by blunt restriction sites (*Eco*RV, *Sna*BI and *Swa*I) located in the 1.5 kb region upstream of the luciferase gene. The internal control was pSV- β -galactosidase (Promega). All clones were analyzed by restriction mapping and were sequenced by Macrogen (Seoul, South Korea). To study the interactions between *Pf*-Sub2 5' UTR and HGF, the HGF gene was cloned into pcDNA3 Flag HA vector using the enzymes of BamHI and XhoI.

Transfection and luciferase assay

pGL3-Basic-*Pf*-Sub2 5' UTR and pcDNA3 Flag HA HGF vector were co-transfected at a ratio of 2:1 by a standard calcium phosphate co-precipitation method in 6-well plates. Each sample was analyzed in duplicate and each experiment was repeated twice. Luciferase activity was measured using a model LB96V microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instructions. Luciferase activity was normalized with respect to β -galactosidase activity to correct deviation due to transfection efficiencies and cell numbers.

SDS-PAGE and western blot analysis

Cells were harvested and lysed with RIPA buffer (Pierce, Rockford, Illinois, USA) with freshly added protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA). One hundred nanograms of cell lysate were diluted with a loading buffer that contained a reducing agent, and the proteins were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Immunoblotting was performed with a 1:200 dilution in 3% bovine serum albumin (BSA) of rabbit polyclonal anti-HA (Dako). The secondary antibody was a 1:1,000 dilution in PBS of polyclonal goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark). For proliferating cell nuclear antigen (PCNA) examination, anti-PCNA/HRP monoclonal antibody was used (Dako).

RESULTS

Analysis of *Pf*-Sub2 promoter regions

Transcription elements in *Pf*-Sub2 5' UTR were analyzed. Several transcriptional elements were identified within about 1,453 bp and are listed in Table 1. Most transcription elements were 14 TATA boxes but only 1 Oct-1 and c-Myb were observed.

Different transcription activities of upstream regions of *Pf*-Sub2

We assessed the *Pf*-Sub2 upstream -1,422/+12 UTR 5' promoter region from -1,422, -739, -668 and -329 to +12. The pGL3-Basic vector cloned with *Pf*-Sub2 5' UTR driving the luciferase gene and pGL3-Basic-*Pf*-Sub2 5' UTR was transfected into 293T cells, and its luciferase activities were measured (Fig. 1). In 293T cells, luciferase activities in -1,422/+12, -739/+12 and -668/+12 5' UTR were 4-7-fold higher than in the pGL3-Basic vector ($P < 0.05$). Among them, -1,422/+12 UTR exhibited the strongest luciferase activity but -329 to +12 UTR did not exhibit luciferase activity. Luciferase expression was not derived from transfection efficiency. In fact, transfection efficiency of the pGL3-Basic vector determined by use of the SV40 promoter was 1×10^7 in 293T cells.

Influence of exogenous HGF on *Pf*-Sub2 proximal promoter activity in 293T cells

HGF genes were cloned into pcDNA3 HA vector and confirmed by restriction enzyme digestion (Fig. 2A). Western blotting was performed to analyze whether pcDNA3 Flag HA HGF vector was expressed in 293T cells after it was transfected using FuGENE 6 (Fig. 2B). HGF expression was observed, with about

Table 1. Putative transcriptional elements in upstream region -1,453 bp of *Pf*-Sub2

Transcriptional elements of <i>Pf</i> -Sub2 upstream 1,453 bp	Total number	Trans position	Cis position
TATA	14	132, 169, 896, 913, 1,074, 1,121, 1,204, 1,228	88, 103, 228, 252, 587, 1,204
XFD-3	4	140, 446	294, 1,315
USF	4	117, 1,236	1,150, 1,234
c/EBP	4	782, 1,136	289, 1,235
AP-1	2	1,212	723
Oct-1	1	-	532
c-Myb	1	346	-

a 25 kDa band. To analyze the influence of HGF on the transcriptional activity of 5' UTR of *Pf*-Sub2 in vitro, co-transfection of pGL3-Basic-*Pf*-Sub2 5' UTR (-1,422/+12) and pcDNA3 Flag HA HGF vector was performed in 293T cells. The influence of HGF protein onto *Pf*-Sub2 5' UTR (-1,422/+12) was estimated by the expression of a luciferase gene directed by the *Pf*-Sub2 5' UTR (Fig. 3). As compared with the control of unexpressed HGF, the HGF protein suppressed luciferase expression by a factor of about 6. It is suggested that HGF controls and interacts with the promoter region of *Pf*-Sub2 gene.

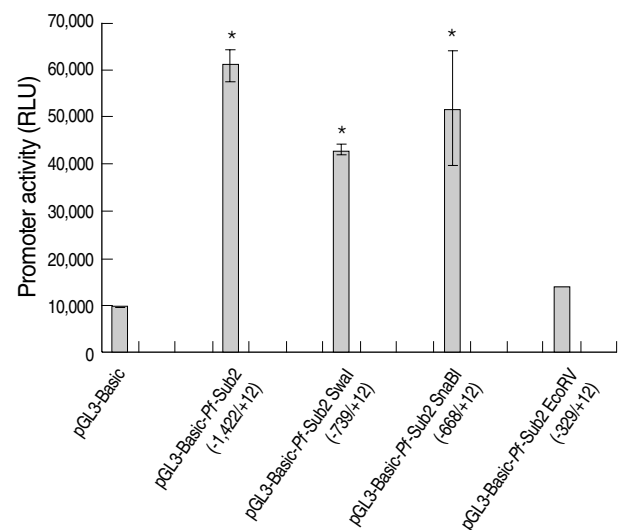


Fig. 1. The promoter activity analysis of pGL3-Basic-*Pf*-Sub2 5' UTR vector. The promoter activities of 4 different pGL3-Basic-*Pf*-Sub2 5' UTR constructs were evaluated by luciferase expression (Relative Luciferase Unit, RLU). β -galactosidase activity was used to normalize transfection efficiency.

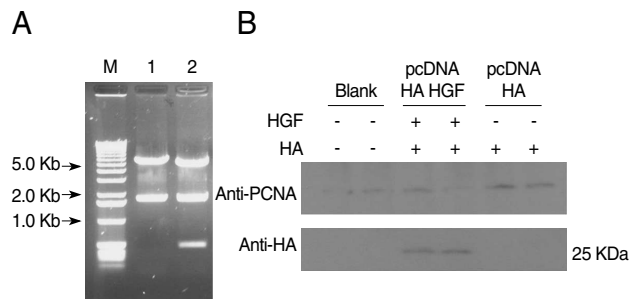


Fig. 2. Construction of pcDNA3 HA HGF vector and HGF-expression. HGF was inserted into pcDNA3 HA vector using *Bam*HI and *Xho*I restriction enzyme sites. DNA fragments were observed for HGF cloning by restriction enzymes digestion (A) Lane 1, *Hind*III; lane 2, *Hind*III + *Xba*I. (B) Shows the expression of 25-kDa-sized HGF. The diagram of pcDNA3 HA HGF vector of about 7.4 kb which contains restriction enzyme sites is provided as supplementary data.

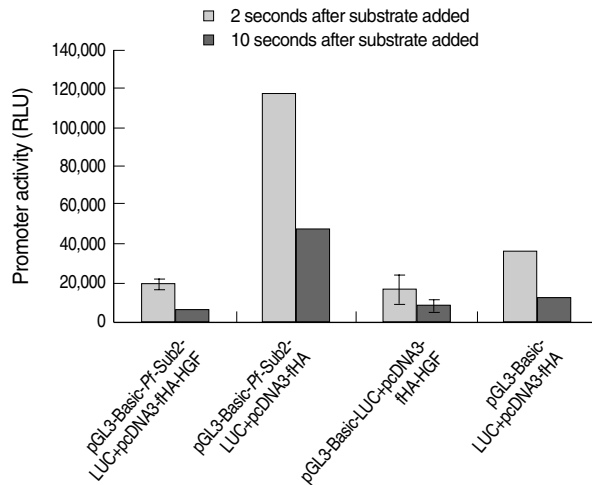


Fig. 3. Influence of HGF onto the promoter activity of pGL3-Basic-*Pf-Sub2* 5' UTR vector. pGL3-Basic-*Pf-Sub2* 5' UTR (-1,422/+12) and pcDNA3 HA HGF vector were co-transfected into 293T cells. Its promoter activity was estimated by luciferase expression (RLU). β -galactosidase activity was used to normalize transfection efficiency.

DISCUSSION

Plasmodium infects liver tissue, so to understand malaria infection it is necessary to understand the relation between hepatocytes and malaria. However, this process is poorly understood. Sporozoites traverse the cytosol of several hepatocytes before invading one by forming a parasitophorous vacuole [20]. The host responds to the traveling parasites by secreting HGF, which renders hepatocytes susceptible to infection [10]. HGF is known to act as an autocrine cytokine in various cancer cells and pre-cancerous cell types [21,22], but it is not normally expressed by hepatocytes or by most other MET-expressing epithelial cells. However, the mechanism of HGF regulation in mammals is not fully understood.

Moreover, clarifying transcription regulation in *Plasmodium* genome is onerous because of the genome's abnormal AT-richness [23,24]. Nonetheless, *Plasmodium* genes are organized similarly to those in eukaryotes and are also monocistronically transcribed [25-27], implying the presence of regulatory sequence elements flanking the coding regions [28,29]. For example, the *Pf* multidrug resistance gene upstream region can be induced at the level of transcription by antimalarial drugs in chloroquine sensitive parasites [30]. Our results obtained from upstream transcriptional activities of *Pf-Sub2* demonstrate that the proximal promoter of *Pf-Sub2* is influenced by the HGF. 5' UTR were analyzed for transcriptional elements of which 14 TATA boxes

and other elements, e.g., one Oct-1 and c-Myb were observed. Deletion mutants of 5' UTR of *Pf-Sub2* were constructed and -1,422/+12 UTR exhibited the strongest promoter activity. To understand the interaction of HGF and 5' UTR of *Pf-Sub2*, 2 vectors of the molecules are cloned and co-transfected into cells. HGF expression was suppressed by 5' untranslated region of the *Pf-Sub2* promoter. In these results, -1,422 to +12 UTR of *Pf-Sub2* was applied and contained all transcriptional elements in Table 1. Therefore, further study will be performed what positions of -1,422 to +12 UTR are important to inhibit the HGF expression. Although the effect of HGF on malaria gene expression in cultured cells may be indirect or atypical, the present results indicate the feasibility of regulation of gene(s) important in malaria invasion through host-parasite interaction. In fact, both recombination in subtelomeric regions and chromosome internal rearrangements under host pressure have proven that the parasite can interact with its vertebrate host [31-33]. Specific upstream regions are important in the activity of the protease. Future studies will be aimed at elucidating the details of the role of HGF in malaria sensitivity/resistance and how *Sub2* gene expression is influenced by the host HGF signaling cascade.

ACKNOWLEDGEMENTS

This research was supported by Wonkwang University in 2009.

REFERENCES

1. Kooij TW, Janse CJ, Waters AP. *Plasmodium* post-genomics: better the bug you know? *Nat Rev Microbiol* 2006; 4: 344-357.
2. Fortin A, Stevenson MM, Gros P. Complex genetic control of susceptibility to malaria in mice. *Genes Immun* 2002; 3: 177-186.
3. Griffith JW, O'Connor C, Bernard K, Town T, Goldstein DR, Bucala R. Toll-like receptor modulation of murine cerebral malaria is dependent on the genetic background of the host. *J Infect Dis* 2007; 196: 1553-1564.
4. Millington OR, Gibson VB, Rush CM, Zinselmeyer BH, Phillips RS, Garside P, et al. Malaria impairs T cell clustering and immune priming despite normal signal 1 from dendritic cells. *PLoS Pathog* 2007; 3: 1380-1387.
5. Min-Oo G, Fortin A, Pitari G, Tam M, Stevenson MM, Gros P. Complex genetic control of susceptibility to malaria: positional cloning of the Char 9 locus. *J Exp Med* 2007; 204: 511-524.
6. Burt RA. Genetics of host response to malaria. *Int J Parasitol* 1999; 29: 973-979.
7. Mikolajczak SA, Kappe SH. A clash to conquer: the malaria para-

- site liver infection. *Mol Microbiol* 2006; 62: 1499-1506.
8. Morosan S, Hez-Deroubaix S, Lunel F, Renia L, Giannini C, Van RN, et al. Liver-Stage Development of *Plasmodium falciparum* in a Humanized Mouse Model. *J Infect Dis* 2006; 193: 996-1004.
 9. Prudêncio M, Rodriguez A, Mota MM. The silent path to thousands of merozoites: the *Plasmodium* liver stage. *Nat Rev Microbiol* 2006; 4: 849-856.
 10. Carolo M, Giordano S, Cabrita-Santos L, Corso S, Vigário AM, Silva S, et al. Hepatocyte growth factor and its receptor are required for malaria infection. *Nat Med* 2003; 9: 1363-1369.
 11. Adachi K, Tsutsui H, Kashiwamura S, Seki E, Nakano H, Takeuchi O, et al. *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and Toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J Immunol* 2001; 167: 5928-5934.
 12. Joshi YK, Tandon BN, Acharya SK, Babu S, Tandon M. Acute hepatic failure due to *Plasmodium falciparum* liver injury. *Liver* 1986; 6: 357-360.
 13. Yoshimoto T, Takahama Y, Wang CR, Yoneto T, Waki S, Nariuchi H. A pathogenic role of IL-12 in blood-stage murine malaria lethal strain *Plasmodium berghei* NK65 infection. *J Immunol* 1998; 160: 5500-5505.
 14. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; 13: 29-60.
 15. Goljan J, Nahorski W, Felczak-Korzybska I, Górski J, Myjak P. Liver injury in the course of malaria. *Int Marit Health* 2000; 51: 30-39.
 16. Howell SA, Well I, Fleck SL, Kettleborough C, Collins CR, Blackman MJ. A single malaria merozoite serine protease mediates shedding of multiple surface proteins by juxtamembrane cleavage. *J Biol Chem* 2003; 278: 23890-23898.
 17. Harris PK, Yeoh S, Dluzewski AR, O'Donnell RA, Withers-Martinez C, Hackett F, et al. Molecular identification of a malaria merozoite surface sheddase. *PLoS Pathog* 2005; 1: 241-251.
 18. O'Donnell RA, Hackett F, Howell SA, Treck M, Struck N, Krnajski Z, et al. Intramembrane proteolysis mediates shedding of a key adhesin during erythrocyte invasion by the malaria parasite. *J Cell Biol* 2006; 174: 1023-1033.
 19. Uzureau P, Barale JC, Janse CJ, Waters AP, Breton CB. Gene targeting demonstrates that the *Plasmodium berghei* subtilisin PbSUB2 is essential for red cell invasion and reveals spontaneous genetic recombination events. *Cell Microbiol* 2004; 6: 65-78.
 20. Mota MM, Pradel G, Vanderberg JP, Hafalla JCR, Frevert U, Nussenzweig RS, et al. Migration of *Plasmodium* Sporozoites Through Cells Before Infection. *Science* 2001; 291: 141-144.
 21. Rong S, Segal S, Anver M, Resau JH, Van Woude GF. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. *Proc Natl Acad Sci USA* 1994; 91: 4731-4735.
 22. Jeffers M, Rong S, Ariver M, Vande Woude GF. Autocrine hepatocyte growth factor/scatter factor-Met signaling induces transformation and the invasive/metastatic phenotype in C127 cells. *Oncogene* 1996; 13: 853-856.
 23. van Noort V, Huynen MA. Combinatorial gene regulation in *Plasmodium falciparum*. *Trends Genet* 2006; 22: 73-78.
 24. Gunasekera AM, Myrick A, Militello KT, Sims JS, Dong CK, Gierahn T, et al. Regulatory motifs uncovered among gene expression clusters in *Plasmodium falciparum*. *Mol Biochem Parasitol* 2007; 153: 19-30.
 25. Lanzer M, Wertheimer SP, de Bruin D, Ravetch JV. *Plasmodium*: control of gene expression in malaria parasites. *Exp Parasitol* 1993; 77: 121-128.
 26. Wickham ME, Thompson JK, Cowman AF. Characterisation of the merozoite surface protein-2 promoter using stable and transient transfection in *Plasmodium falciparum*. *Mol Biochem Parasitol* 2003; 129: 147-156.
 27. Ruvalcaba-Salazar OK, del Carmen Ramírez-Estudillo M, Montiel-Condado D, Recillas-Targa F, Vargas M, Hernández-Rivas R. Recombinant and native *Plasmodium falciparum* TATA-binding-protein binds to a specific TATA box element in promoter regions. *Mol Biochem Parasitol* 2005; 140: 183-196.
 28. Crabb BS, Cowman AF. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. *Proc Natl Acad Sci* 1996; 93: 7289-7294.
 29. Pace T, Olivieri A, Sanchez M, Albanesi V, Picci L, Siden KI, et al. Set regulation in asexual and sexual *Plasmodium* parasites reveals a novel mechanism of stage-specific expression. *Mol Microbiol* 2006; 60: 870-882.
 30. Myrick A, Munasinghe A, Patankar S, Wirth DF. Mapping of the *Plasmodium falciparum* multidrug resistance gene upstream region, and evidence of induction of transcript levels by antimalarial drugs in chloroquine sensitive parasites. *Mol Microbiol* 2003; 49: 671-683.
 31. Calderwood MS, Gannoun-Zaki L, Wellems TE, Deitsch KW. *Plasmodium falciparum* var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J Biol Chem* 2003; 278: 34125-34132.
 32. Voss TS, Healer J, Marty AJ, Duffy MF, Thompson JK, Beeson JG, et al. A var gene promoter controls allelic exclusion of virulence genes in *Plasmodium falciparum* malaria. *Nature* 2006; 439: 1004-1008.
 33. Duffy MF, Tham WH. Transcription and coregulation of multi-gene families in *Plasmodium falciparum*. *Trends Parasitol* 2007; 23: 183-186.

