Isolation and Functional Characterization of Two Distinct Sexual-Stage-Specific Promoters of the Human Malaria Parasite Plasmodium falciparum†

KOEN J. DECHERING,1‡ ANITA M. KAAN,1 WILFRED MBACHAM,2 DYANN F. WIRTH,2 WIJNAND ELING,3 RUUD N. H. KONINGS,1 AND HENDRIK G. STUNNENBERG1*  

Department of Molecular Biology, University of Nijmegen, 6525 ED Nijmegen,1 and Department of Medical Parasitology, University of Nijmegen, 6500 HB Nijmegen,3 The Netherlands, and Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts 021152

Received 10 July 1998/Returned for modification 23 September 1998/Accepted 11 November 1998

Transmission of malaria depends on the successful development of the sexual stages of the parasite within the midgut of the mosquito vector. The differentiation process leading to the production of the sexual stages is delineated by several developmental switches. Arresting the progression through this sexual differentiation pathway would effectively block the spread of the disease. The successful development of such transmission-blocking agents is hampered by the lack of a detailed understanding of the program of gene expression that governs sexual differentiation of the parasite. Here we describe the isolation and functional characterization of the Plasmodium falciparum pfs16 and pfs25 promoters, whose activation marks the developmental switches executed during the sexual differentiation process. We have studied the differential activation of the pfs16 and pfs25 promoters during intraerythrocytic development by transfection of P. falciparum and during gametogenesis and early sporogonic development by transfection of the related malarial parasite P. gallinaceum. Our data indicate that the promoter of the pfs16 gene is activated at the onset of gametocytogenesis, while the activity of the pfs25 promoter is induced following the transition to the mosquito vector. Both promoters have unusual DNA compositions and are extremely A/T rich. We have identified the regions in the pfs16 and pfs25 promoters that are essential for high transcriptional activity. Furthermore, we have identified a DNA-binding protein, termed PAF-1, which activates pfs25 transcription in the mosquito midgut. The data presented here shed the first light on the details of processes of gene regulation in the important human pathogen P. falciparum.

Plasmodium falciparum is one of the major debilitating and life-threatening parasitic pathogens of humans. Half of the world’s population lives in areas endemic for malaria, and 2 million to 3 million people are killed annually. Despite years of intensive research, an effective vaccine is still not available and the parasite displays a growing resistance to the currently available drugs. New ways of combatting the disease should be equipped to invade the midgut of the mosquito vector. One approach to control malaria is to prevent sexual development of the parasite and thus transmission to the mosquito (1, 25). Research efforts directed toward such a goal will greatly benefit from a detailed understanding of the gene-regulatory events underlying sexual development.

The process of sexual differentiation is governed by several developmental switches that direct the parasite through a complex series of morphological changes (Fig. 1). First, a subpopulation of asexually reproducing parasites commits to sexual differentiation and undergoes gametocytogenesis, the formation of male and female sex cells. The sexually committed parasites are marked by the expression of the pfs16 gene, which is the earliest event in the sexual differentiation process described to date (13). A second developmental switch defines the sex of the developing gametocyte (39). The next developmental switch is executed following transmission of mature gametocytes to the mosquito midgut. Here, the gametocytes form gametes that fertilize and produce a motile ookinete. This ookinete penetrates the peritrophic membrane surrounding the blood meal and initiates sporogonic development, which leads to the production of new infectious sporozoites. The processes of gametogenesis and fertilization are completed within the 15 to 20 min following the arrival of the gametocytes in the mosquito midgut (1). The onset of gametogenesis is marked by the induction of the pfs25 gene (13, 18), which encodes a membrane protein containing several epidermal growth factor-like domains (27). It has been proposed that Pfs25 is involved in a receptor-ligand interaction required for invasion of the midgut epithelium (40). Antibodies raised against Pfs25 potently block transmission of P. falciparum to...
The significance of the pfs16 and pfs25 genes as specific markers for two important developmental switches executed by the malaria parasite prompted us to investigate the transcriptional regulation of these genes in greater detail. Therefore, we have isolated the promoters of the P. falciparum pfs16 and pfs25 genes. To study the differential activities of these promoters during intraerythrocytic development of the parasite, we have exploited transient transfections of P. falciparum (10, 47). In addition, we have studied the activities of the pfs16 and pfs25 promoters in the parasite stages that develop within the mosquito midgut, using transfections of the related parasite P. gallinaceum. Our data show that the pfs16 and pfs25 promoters exhibit unusual DNA compositions that are extremely biased toward A and T nucleotides. We have identified the regions in these promoters that are essential for high transcriptional activity. Furthermore, we show that distinct mechanisms control the activities of these promoters during development of the parasite. The pfs16 promoter is induced at the very onset of gametocytogenesis and remains active following transmission of the parasite to the mosquito midgut. The activity of the pfs25 promoter is restricted to the parasite stages that develop within the mosquito midgut. We show that the induction of the pfs25 gene partially relies on a DNA-binding protein, termed PAF-1, which activates pfs25 transcription within the mosquito midgut.

**MATERIALS AND METHODS**

**Transfection vectors.** Transfection vectors pHRPCAT, pA0, and pHLH were kindly provided by Y. Wu (Oxford University, Oxford, England). Plasmids pHLH and pA0 are derivatives of pHRCAT and PSOCS2, respectively (47), in which cat (chloramphenical acetyltransferase) reporter genes have been replaced by luciferase reporter genes. A general-purpose transfection vector was constructed by substituting the hrp3 (histidine-rich protein 3 gene) promoter from plasmid pHRCAT with a KpnI/NsiI restriction fragment containing the polylinker of pZERO (Invitrogen). The resulting plasmid was designated pCAT-L.

**Isolation of the pfs16 5’ flanking sequences and pfs16 plasmid construction.** P. falciparum NF54 genomic DNA was digested with EcoRI and additionally sheared by sonication to an average fragment size of 2 kbp. The fragment ends

---

FIG. 1. Life cycle of the human malaria parasite P. falciparum. Infection in humans begins with the introduction of sporozoites in the bloodstream by a bite of an infected Anopheles mosquito (a). The sporozoites are cleared by the liver (b), where cyclic asexual development initiates (c to g). Merozoites invade erythrocytes and transform into a ring-stage parasite (d). Subsequent trophic growth (e) and mitotic divisions lead to the production of up to 32 merozoites in a schizont (f), which bursts and releases new merozoites in the bloodstream. The merozoites can either reinitiate the erythrocytic asexual multiplication cycle (g) or commit to sexual differentiation (h). Initially, sexually committed parasites adapt the typical postinvasion ring-like shape (i) and cannot be discriminated from asexual parasites on a morphological basis. Sexual differentiation becomes morphologically apparent with the appearance of stage II gametocytes (j), which mature into female and male stage V gametocytes (k). The gametocytes are adapted to infect mosquitoes. Following the blood meal of a mosquito, the female gametocyte transforms in a macrogamete (l). The male gametocyte undergoes three rapid nuclear divisions and produces eight microgametes (m) that fertilize (n) the macrogamete, which then transforms in an invasive oocinete (o). This oocinete traverses the midgut epithelium and forms an oocyst at the side of the basal lamina. Sporogonic development (p) subsequently leads to the production of sporozoites that are released from the oocyst, accumulate in the salivary gland of the mosquito, and are infectious upon a new bite (a). DNA synthesis occurs at the transition from ring-stage parasite to schizont (d to f) (23) and at the transition from sexually committed ring-stage parasite to gametocyte (i and j) (24). Accordingly, both transitions are inhibited by pyrimethamine (9). Transcription of the pfs16 gene is induced in sexually committed ring stages, whereas the pfs25 gene is activated immediately following transmission (13).
were made blunt ended with T4 polymerase and introduced in a lambda ZAPI vector (Stratagene) with the aid of EcoRI linkers. The library was propagated in Escherichia coli XL-1 Blue cells (Stratagene) and screened with a pf16-specific CDNA probe. Partial plasmid clones were picked and several were rescued from the phages by an in vivo excision protocol (Stratagene). A plasmid with an insert of 1,461 bp that hybridized to the pf16 probe was isolated. From this plasmid, the pf16 upstream region was amplified by PCR using primers P16.5 (ggctgtacgTACTCAGACTCTTTTGGAC) and P16.6 (GAAC TTTCCAATATCGATTTGG) (lowercase indicates nucleotides not present in the pf16 sequence that introduce restriction sites) and introduced into a pGEM-T vector (Promega) to yield plasmid pK16.3. The pf16 upstream region was introduced in transfection vector pCAT-L by cloning a XhoI/SphI fragment of plasmid pK16.3 in the EcoRV/SphI-digested pCAT-L vector after blunting the XhoI end with Klenow polymerase. A series of 5’ deletions of the pf16 upstream region in plasmid pCAT-L16 was generated. First, one of the two BamHI sites present in pCAT-L16.1 was eliminated by digesting the plasmid with Smal/XhoI and blunting of the fragment ends with Klenow polymerase. Religation of the plasmid then resulted in plasmid pCAT-L16.2. Subsequently, this plasmid was digested with KpnI and BamHI. XmnI/XhoI and SmaI nucleic acid treatment followed by religation of the plasmids then generated a series of incrementing 5’ deletions of the pf16 upstream region. 3’ deletion mutants of the pf16 promoter were generated by cloning the products of a partial XhoI/EcoRI digest of the pf16 upstream region promoter of plasmid pCAT-L16.1 into the SmaI/XhoI site in an in vitro EcoRI/EcoRV-digested pCAT-L vector.

A plasmid containing a luciferase reporter gene under control of the pf16 promoter, a green fluorescent protein (GFP) reporter gene was introduced in plasmid plUC16.1. To this end, a XhoI/SphI fragment of plasmid pKENGFPmut2 (kind gift of B. Cormack, Stanford University, Stanford, Calif.) containing the coding region of GFPmut2 was cloned in plBluescript KS– (Stratagene), and an XhoI restriction site was inserted in the gfp gene by PCR with GFP primer TATA CATATGcataaAGGAGAAG and M13 reverse primer AAcACGCTATGACC ATG. The primer product was digested with XhoI and HindIII and introduced in the XhoI and HindIII sites of plUC16.1

pf16 plasmid construction. The characterization of the pf16 upstream region was based on plasmid pNF4.13, kindly donated by David Kaslow (National Institutes of Health, Bethesda, Md.). The insert of this plasmid is derived from a genomic library of P. gallinaceum clone 3D7 and contains a 3.5-kb genomic DNA region which is directed by the pf16 translation start site of the pfs25 gene. A plasmid with a 1,461-bp insert was obtained. Sequence data have been submitted to the GenBank database under accession no. AF034389 (pfs16).

RESULTS

Structure of the pf16 and pf25 upstream regions. To assess whether the developmental regulation of Pfs16 and Pf25 expression is mediated by DNA elements located upstream of the respective genes, and to enable a subsequent characterization of the elements controlling the expression, we set out to isolate the pf16 and pf25 upstream regions. For the pf25 gene, RACE (rapid amplification of cDNA ends) was performed, the manufacturer’s protocol for the RACE kit (Gibco BRL), and cDNA was synthesized by reverse transcription from 10 μg of P. falciparum gamete RNA with gene-specific primer GCTAAATGGTAAAGGAAG. Amplification products were digested with EcoRI, cloned in an EcoRI/SmaI-digested plBluescript SK– vector (Stratagene), and introduced into electroporated E. coli SURE cells (Stratagene). Ten individual transformants were selected for sequence analysis.

Nucleotide sequence accession numbers. Sequence data have been submitted to the GenBank database under accession no. AF034389 (pfs16) and AF030628 (pf25).

Identification of the transcriptional start site. The RNA of P. falciparum schizonts, gametes, and oocytes was isolated as described previously (13). RNase protection assays were performed with an RPAI kit (Ambion). A 312-nucleotide fragment spanning nucleotides −294 to +17 with respect to the transcriptional start site the pf16 gene was used for the RPAI. The pf16 gene was anchored in oocyte maturation medium (29). Plasmid pACT-L served as a negative control in all transfections. Plasmid pAF0 was used as an internal standard in transfections of blood-stage parasites. Plasmid pAF9,20, which contains a luciferase gene downstream of a truncated p25 promoter (16), served as a control in transfections of mosquito stages. Cells were harvested 48 h after transfection and reporter assays were as described previously (16, 47). Transfected parasites expressing the GFP reporter were visualized on a Bio-Rad MRC600 confocal laser scanning microscope.

Identification of the transcriptional start site of the RNA of P. falciparum schizonts, gametes, and oocytes was isolated as described previously (13). RNase protection assays were performed with an RPAI kit (Ambion). A 312-nucleotide fragment spanning nucleotides −294 to +17 with respect to the transcriptional start site the pf16 gene was used for the RPAI. The pf16 gene was anchored in oocyte maturation medium (29). Plasmid pACT-L served as a negative control in all transfections. Plasmid pAF0 was used as an internal standard in transfections of blood-stage parasites. Plasmid pAF9,20, which contains a luciferase gene downstream of a truncated p25 promoter (16), served as a control in transfections of mosquito stages. Cells were harvested 48 h after transfection and reporter assays were as described previously (16, 47). Transfected parasites expressing the GFP reporter were visualized on a Bio-Rad MRC600 confocal laser scanning microscope.

Identification of the transcriptional start site. The RNA of P. falciparum schizonts, gametes, and oocytes was isolated as described previously (13). RNase protection assays were performed with an RPAI kit (Ambion). A 312-nucleotide fragment spanning nucleotides −294 to +17 with respect to the transcriptional start site the pf16 gene was used for the RPAI. The pf16 gene was anchored in oocyte maturation medium (29). Plasmid pACT-L served as a negative control in all transfections. Plasmid pAF0 was used as an internal standard in transfections of blood-stage parasites. Plasmid pAF9,20, which contains a luciferase gene downstream of a truncated p25 promoter (16), served as a control in transfections of mosquito stages. Cells were harvested 48 h after transfection and reporter assays were as described previously (16, 47). Transfected parasites expressing the GFP reporter were visualized on a Bio-Rad MRC600 confocal laser scanning microscope.

Identification of the transcriptional start site. The RNA of P. falciparum schizonts, gametes, and oocytes was isolated as described previously (13). RNase protection assays were performed with an RPAI kit (Ambion). A 312-nucleotide fragment spanning nucleotides −294 to +17 with respect to the transcriptional start site the pf16 gene was used for the RPAI. The pf16 gene was anchored in oocyte maturation medium (29). Plasmid pACT-L served as a negative control in all transfections. Plasmid pAF0 was used as an internal standard in transfections of blood-stage parasites. Plasmid pAF9,20, which contains a luciferase gene downstream of a truncated p25 promoter (16), served as a control in transfections of mosquito stages. Cells were harvested 48 h after transfection and reporter assays were as described previously (16, 47). Transfected parasites expressing the GFP reporter were visualized on a Bio-Rad MRC600 confocal laser scanning microscope.

Identification of the transcriptional start site. The RNA of P. falciparum schizonts, gametes, and oocytes was isolated as described previously (13). RNase protection assays were performed with an RPAI kit (Ambion). A 312-nucleotide fragment spanning nucleotides −294 to +17 with respect to the transcriptional start site the pf16 gene was used for the RPAI. The pf16 gene was anchored in oocyte maturation medium (29). Plasmid pACT-L served as a negative control in all transfections. Plasmid pAF0 was used as an internal standard in transfections of blood-stage parasites. Plasmid pAF9,20, which contains a luciferase gene downstream of a truncated p25 promoter (16), served as a control in transfections of mosquito stages. Cells were harvested 48 h after transfection and reporter assays were as described previously (16, 47). Transfected parasites expressing the GFP reporter were visualized on a Bio-Rad MRC600 confocal laser scanning microscope.
to sequences present in the EPD database were found in the pfs16 and pfs25 upstream regions.

**Determination of the transcription initiation sites of the pfs16 and pfs25 genes.** As an initial step in the characterization of the pfs16 and pfs25 promoters, we mapped the transcription initiation sites of the respective genes. The initiation site of the pfs16 gene was analyzed by RNase protection. As shown in Fig. 3, hybridization of a pfs16-specific probe to RNA of gametocytes and gametes results in the protection of fragments that cluster in a discrete region that spans approximately 10 nucleotides and that is located 175 nucleotides upstream of the ATG start codon. The protected fragments most likely represent a single transcription initiation site, as the observed minor heterogeneity is frequently associated with this type of experimental approach. No protection of the probe was found when an RNA preparation of the schizont stages was used, which is in agreement with the observation that these stages do not express the pfs16 mRNA (13).

To analyze the transcriptional start sites of the pfs25 gene, we used a RACE procedure. The sequences of the amplified products recovered were colinear with the genomic sequence and heterogeneous in their 5’ ends. Figure 2b shows a compilation of the transcription initiation sites on the basis of the 10 independent RACE products analyzed. The initiation sites cluster in an 18-nucleotide region located 267 nucleotides upstream of the ATG translation initiation codon. Of the 10 RACE products, 5 indicated initiation at position 2267.

**The pfs16 and pfs25 upstream regions contain differentially regulated promoters.** We assessed whether the extremely A/T rich pfs16 and pfs25 upstream regions contain functional promoters. To this end, these sequences were fused to cat reporter genes, the resulting plasmids were introduced into blood- and mosquito-stage parasites by transfection, and CAT activities were determined. As a control, we tested the activity of the hrp3 promoter, which has been described previously (47). For...
transfection of the blood stages of the parasite, preparations of \textit{P. falciparum} were used. As the mosquito stages of \textit{P. falciparum} are neither transfectable nor viable in an in vitro culture, we used \textit{P. gallinaceum} parasites for the transfection of the mosquito stages (45). The data presented in Figure 4A show that the three promoters show distinct patterns of transcriptional activities. Whereas the \textit{pfs16} upstream sequence drives the expression of the \textit{cat} reporter gene in both the mosquito- and blood-stage preparations, the activity of the \textit{pfs25} promoter is restricted to mosquito-stage parasites. Conversely, the activity of the \textit{hrp3} promoter peaks in blood-stage parasites but is not detectable in mosquito stages. A quantitative comparison of the activities of the \textit{pfs16} and \textit{pfs25} promoters in mosquito-stage parasites showed that the \textit{pfs25} promoter exhibits an activity approximately fivefold higher than the transcriptional activity of the \textit{pfs16} promoter (Fig. 4B). Thus, the transcriptional activities of the \textit{pfs16} and \textit{pfs25} promoters are quantitatively and qualitatively distinct. The data suggest that activation of the \textit{pfs25} promoter marks the transition of the parasite to the mosquito midgut whereas transcription from the \textit{pfs16} promoter is activated in the blood stages of the parasite.

The activity of the \textit{pfs16} promoter is restricted to the sexual stages. At the onset of gametocytogenesis, biochemical differentiation precedes morphological differentiation. Initially, sexually committed ring stages are morphologically indistinguishable from asexual ring-stage parasites (Fig. 1) (32). However, they are marked by the induction of the transcriptional activity of the \textit{pfs16} gene, which is the earliest event in the sexual differentiation process described to date (13). Following the accumulation of the \textit{pfs16} mRNA, the sexually committed ring stages transform into the readily recognizable stage II gametocytes (20). Our data indicate that the \textit{pfs16} promoter can drive the expression of a reporter gene in transfection of an asynchronous culture of the blood stages of the parasite (Fig. 4A). Such a culture contains both asexual and sexual parasites, and the experiment depicted in Fig. 4A does not clarify which of the subpopulations is responsible for the \textit{pfs16}-driven expression of the reporter gene. To corroborate and extent the notion that the activity of the \textit{pfs16} gene is restricted to parasites undergoing sexual development, we analyzed the activity of the \textit{pfs16} promoter in cultures undergoing sexual differentiation. Given the finding that the transcriptional activity of the \textit{hrp3} gene is restricted to asexual parasites (43) and that the \textit{hrp3} promoter is silent in the mosquito stages (Fig. 4A), we used the \textit{hrp3} promoter as a specific marker for the asexual population of parasites. \textit{P. falciparum} blood-stage parasite cultures were transfected with \textit{hrp3} and \textit{pfs16} promoter-reporter constructs, and gametocytogenesis was induced by omitting the supply of fresh erythrocytes (36). Figure 5A shows the parasitemias during the course of the experiment together with the temporal activities of the \textit{hrp3} and \textit{pfs16} promoters. The asexual parasitemia initially rises and eventually shows a slight decline. The high asexual parasitemia at day 2 triggers gametocytogenesis, as demonstrated by the appearance of stage 2 gametocytes at day 4. The pattern of activity of the \textit{hrp3} promoter parallels the asexual parasitemia and persists throughout the course of the experiment. The activity of the \textit{pfs16} promoter coincides with the appearance of sexually committed ring-stage parasites in the culture. Thereafter, higher levels of reporter enzyme, driven by the \textit{pfs16} promoter, accompany the increase in numbers of gametocytes.

To assess whether the developing gametocytes are indeed responsible for the observed activity of the \textit{pfs16} promoter,
transfected cultures were treated with pyrimethamine, which eliminates asexual parasites and sexually committed ring stages but leaves gametocytes that have passed developmental stage I unaffected (36). As a consequence, the activity of the \textit{pfs16} promoter is slightly reduced but still persists at a high level (Fig. 5B). By contrast, the pyrimethamine treatment completely abrogates the asexual parasitemia and the activity of the \textit{hrp3} promoter. The sharp contrast in the effect of pyrimethamine on the activities of the \textit{pfs16} and \textit{hrp3} promoters clearly indicates that these reside in different subpopulations of the culture. The pyrimethamine treatment completely abolishes the \textit{hrp3}-driven expression of the reporter gene, indicating that the activity of the \textit{hrp3} promoter is restricted to asexual parasites. The activity of the \textit{pfs16} promoter persists at a high level, indicating that this promoter is active in the developing gametocytes.

Transcriptional activity of the \textit{pfs16} promoter continues in the parasite stages that invade the mosquito midgut. The functional roles of the \textit{Pfs16} protein in the sexual differentiation process and the penetration of the mosquito midgut remain elusive. Moreover, data on the expression pattern of \textit{Pfs16} are ambiguous. Some studies have reported that \textit{Pfs16} is synthesized by gametes and is present on the outer membrane of the gametes (31, 33). Other studies have, however, failed to detect the protein in the gametes and have shown that \textit{Pfs16} apparently associated with the gamete membrane is in fact attached to remainders of the parasitophorous vacuole membrane of the gametocytes (2, 6). These remainders eventually are completely shed from the surface of the gamete. Nuclear run-on analysis has shown that the \textit{pfs16} gene is transcriptionally active in gametes (13). Furthermore, our data show that the \textit{pfs16} promoter is active in the parasite stages that develop within the mosquito midgut (Fig. 4). To specify the pattern of the activity of the \textit{pfs16} promoter in the mosquito stages, we visualized this activity by using the gene encoding the GFP of the jellyfish \textit{Aequorea victoria} as a reporter. Figure 6 shows the GFP expression pattern in the developing midgut stages. The \textit{pfs16} promoter drives GFP expression both in unfertilized gametes and in the developing and mature ookinetes. As the morphological differentiation of ookinetes preceded the appearance of the GFP signal, we conclude that the ookinetes were actively synthesizing the GFP protein and did not show fluorescence as a result of an accumulated pool of GFP originating from the gametes. These results lend support to the notion that the \textit{pfs16} gene is transcriptionally active in the mosquito stages of the parasite and demonstrate that the \textit{pfs16} promoter can drive the expression of foreign genes in the parasite stages that are involved in the invasion of the mosquito midgut epithelium.

\textbf{Deletion mapping of the \textit{psf16} and \textit{psf25} promoters.} To gain insight in the DNA elements involved in the expression of the \textit{pfs16} and \textit{pfs25} genes, plasmids in which the reporter genes were placed under the control of a series of truncated promoter fragments were transfected to \textit{P. gallinaceum} mosquito-stage parasites. The data presented in Figure 7 indicate that the transcriptional activity of the \textit{pfs16} promoter relies on different components. The \(-247\) to \(+1\) region of the promoter is the shortest region tested here that drives transcription of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Activities of the \textit{pfs16} and \textit{hrp3} promoters during sexual differentiation of \textit{P. falciparum}. (A) Asynchronous cultures of \textit{P. falciparum} were transfected at day 0 with plasmids pHLH and pCAT-L16.1, and reporter activities were monitored over time. The upper graph shows the activities of the \textit{pfs16} and \textit{hrp3} promoters; the lower graph shows the parasitemia of the culture, discriminating between asexual parasites, gametocytes (gct), and sexually committed ring stages (cr). The number of sexually committed ring stages was inferred from the number of stage II gametocytes appearing 48 h later (7). (B) Cultures were transfected with plasmid pHLH or pLUC16.1 at day 0 and treated with pyrimethamine from days 4 to 7. Error bars indicate standard deviations calculated from three independent experiments.}
\end{figure}
Deletion of the sequences between nucleotides +1 and 93. However, as this region is located downstream of the transcriptional start site, its deletion affects the transcribed RNA. Hence, the observed lowered CAT activity may be due to a decreased stability or translation efficiency of the mRNA.

The transfection data do not support a functional role for the two elements with homology to the DNA recognition site of the yeast MATa2 repressor that are present in the pfs16 promoter (Fig. 2A). Deletion of either one of these elements does not affect the activity of the promoter (compare the transcriptional activities of exo5 and exo7 and of pCAT-L16.2 and pCAT-L16.3 in Fig. 7). In addition, mutations in the single MAT-like element present in plasmid exo7 do not affect the transcriptional activity of this plasmid in transfections of blood- or mosquito-stage parasites (data not shown).

Mutational analysis of the pfs25 promoter was performed on truncated versions of plasmid pPFS25LUC, which contains a luciferase gene under control of the pfs25 promoter and pfs25 3′ processing signals. The structures of the deletion mutants are schematically depicted in Fig. 7 together with the luciferase activities obtained after transfection of P. gallinaceum mosquito-stage parasites. Deletion from positions −1006 to −959 reduces the activity of the pfs25 promoter to 60% of the activity of the full-length promoter. Further deletions up to position −542 marginally affect the transcriptional activity. The most dramatic effects on the activity of the promoter are seen with next two extended deletions: deletion of the region between positions −542 and −484 results in a 10-fold reduction of the activity of the promoter, and further deletion up to position −386 fully abolishes the activity of the promoter. In conclusion, the region between −484 and +1 is the shortest region tested here that drives transcription of the reporter gene. Equipping this region with the region between −484 and −542 potently activates transcription. Finally, the regions upstream of position −542 contribute to an efficient activity of the pfs25 promoter.

The pfs25 upstream region is the target of protein-DNA interactions. To gain insight in the possible trans-acting factors that play a role in the regulation of transcription of the pfs16 and pfs25 genes, we performed a series of EMSAs. Restriction fragments derived from the pfs16 and pfs25 upstream regions were scanned for the presence of binding sites for proteins present in a nuclear extract of P. gallinaceum gametocytes. Pilot experiments failed to reveal any specific interactions between DNA-binding proteins and the pfs25 promoter. By contrast, three of the four pfs25-specific probes tested yielded DNA-protein complexes when incubated with a nuclear extract, as shown in Fig. 8A and B. Probe C forms a single complex, indicating a single interaction with a DNA-binding protein. Probes B and D form multiple complexes, revealing a more complex set of interactions on these probes.

A cross-competition experiment with probe C as the radiolabeled probe reveals that a 100-fold molar excess of probe B disrupts the interaction of probe C with the nuclear factor, whereas probes A and D cannot prevent the interaction between probe C and the nuclear factor (Fig. 8C). Furthermore, a 100-fold molar excess of probe C prevents the formation of a DNA-protein complex on probe B, whereas probes A and D have no effect on complex formation (Fig. 8C). These observations suggest that the nuclear factor that binds to probe C does not have a preference for generally A/T-rich DNA but obeys more specific sequence constraints that are shared by probe B. Close inspection of the sequences of probes B and C reveals that they indeed share a DNA element, AAGGAATA,
that is found neither in probes A and D or in other regions of the pfs25 promoter.

**AAGGAATA recruits a mosquito-stage-specific transcription factor.** To test whether the AAGGAATA element constitutes a functional promoter element, a mutant version of this element was tested in transfection studies and EMSAs. The data presented in Fig. 9A show that oligonucleotide TFB25, which contains the AAGGAATA sequence, recruits a DNA-binding protein when incubated with a nuclear extract derived from *P. gallinaceum* gametes. The interaction can be disrupted by the addition of a 100-fold molar excess of the unlabeled oligonucleotide but not by the addition of an oligonucleotide containing a mutated version of this sequence motif. Accordingly, oligonucleotide TFB25 can compete for the interaction with the DNA-binding protein.
between probe C and the DNA-binding protein, whereas the mutant oligonucleotide cannot (Fig. 9C). These results indicate that AAGGAATA sequence serves as the recognition site for a DNA-binding protein, which we named PAF-1 (pfs25-activating factor 1 [see below]).

We assessed whether PAF-1 is a developmental-stage-specific protein. To this end, the radiolabeled oligonucleotide TFB25 was incubated with nuclear extracts derived from either P. gallinaceum or P. falciparum gametes or from an asynchronous P. falciparum blood-stage parasite culture that contained both asexual parasites and gametocytes. The results shown in Fig. 9A indicate that extracts derived from P. falciparum or P. gallinaceum gametes contain a DNA-binding protein that specifically interacts with the TFB25 oligonucleotide. In contrast, no PAF-1-like activity is present in the nuclear extract derived from P. falciparum blood-stage parasites. As a control on the activity of the mutant promoter was compared to that of the wild-type promoter. Transfections of P. gallinaceum mosquito-stage parasites showed that the activity of the promoter is significantly reduced by the mutations (Fig. 9D). We conclude, therefore, that the AAGGAATA sequence comprises a cis-acting element involved in the activation of the pfs25 promoter. The correlation between a decline in promoter activity and a decreased affinity for PAF-1 upon mutation of the AAGGAATA sequence indicates that PAF-1 is a transcriptional activator that is recruited to the promoter by the AAGGAATA sequence.

**DISCUSSION**

Sexual differentiation is an obligate part of the life cycle of malaria parasites. It requires a series of developmental decisions to be made during progression from the asexually replicating parasites in the bloodstream of the vertebrate host to the highly specialized cells that invade the mosquito midgut. First, a subpopulation of asexual parasites commits to sexual differentiation; second, the sex of the developing gametocytes is determined; finally, gametogenesis is induced when the ar-
rival of the gametocytes in the mosquito midgut is sensed. A complex network of gene-regulatory events governs the transitions through the sexual differentiation process, as indicated by developmental-stage-specific expression of the \textit{pfs16} and \textit{pfs25} genes. This paper links the regulatory events operating on the \textit{pfs16} and \textit{pfs25} promoters to two important developmental switches exhibited by the parasite. Activation of the \textit{pfs16} promoter marks commitment to sexual differentiation, whereas the induction of the \textit{pfs25} promoter is indicative for the transition to the mosquito midgut. In addition, our data show that the \textit{hrp3} gene, which is expressed in asexual parasites, is shut off when parasites commit to sexual differentiation.

A functional analysis of the mechanisms of gene expression underlying sexual development of the malaria parasite has for long been impeded by the lack of a transfection protocol and by the laborious culture of the sexual stages. Recently, transfection methods for the blood stages of \textit{P. falciparum} have been described (14, 47). However, the in vitro culture and transfection of the mosquito stages of \textit{P. falciparum} is still not feasible. An alternative is offered by the avian malaria parasite \textit{P. gallinaceum}, which has been proven to be a versatile model system for the study of sexual differentiation of malaria parasites (16, 45). As \textit{P. falciparum} and \textit{P. gallinaceum} are very closely related and are thought to have arisen by lateral transfer between the human and the avian host (41), one might assume that the elements that control gene expression are conserved and interchangeable. Functional conservation has been observed for the \textit{P. falciparum} \textit{hrp3} and the \textit{P. berghei} \textit{dhfr} promoters in \textit{P. knowlesi} (42), for the \textit{P. chabaudi} \textit{dhfr} promoter in \textit{P. falciparum} (10), and for the \textit{P. falciparum} \textit{hsp86} and \textit{pcna} promoters in \textit{P. gallinaceum} (11a). \textit{P. gallinaceum} possesses a close homologue of the \textit{Pfs25} protein, termed \textit{Pgs25} (28). The regions flanking the \textit{pfs25} gene have not been cloned, and a direct comparison of the \textit{pfs25} and \textit{pgs25} promoters awaits the isolation of the latter sequence. Nonetheless, the results presented here corroborate the notion that the factors required for basal and activated transcription indeed are conserved between the \textit{Plasmodium} species. Accordingly, the PAF-1 DNA-binding activity was observed in nuclear extracts derived both from \textit{P. gallinaceum} and from \textit{P. falciparum} gametes.

Gene regulation is atypical in many parasitic protozoa. Genes of the Kinetoplastida (e.g., \textit{Leishmania} and \textit{Trypanosoma} species) seem to be transcribed by a class I RNA polymerase and produce long polycistronic RNA precursors, which are processed into mature RNAs via trans-splicing (35). There is no evidence for polycistronic transcription, trans-splicing, or extensive posttranscriptional regulation of gene expression within the phylum of the Apicomplexa (e.g., \textit{Plasmodium} spp. and \textit{Toxoplasma} spp.). Nevertheless, the structure of the gene promoters of organisms of the latter phylum is atypical. Although the deletion mapping of the \textit{pfs16} and \textit{pfs25} promoters suggests that these promoters resemble typical eukaryotic poly-

![Figure 9](http://mcb.asm.org/)

**FIG. 9.** The AAGGAATA elements in the \textit{pfs25} promoter recruit a mosquito stage-specific DNA-binding protein that activates transcription. (A) Oligonucleotide TFB25 (AAATCATAAAGGAATAATAG and its complement AAATCTATCCCTTAG) was incubated with a nuclear extract derived from \textit{P. gallinaceum} (g) or \textit{P. falciparum} (Pf) gametes or from an asynchronous \textit{P. falciparum} blood-stage culture that contained both asexual parasites and gametocytes (Pb). Competitions included either a 100-fold molar excess of the unlabeled oligonucleotide TFB25 or a 100-fold molar excess of an oligonucleotide containing a mutant version of the putative binding site (MUT; AAATCTAGGCGCGGTAG and its complement AAATCTAAGCCGCGTAG). (B) Control on the activity of the \textit{P. falciparum} blood-stage nuclear extract. Radiolabeled oligonucleotide KAHRP (30) was incubated with a nuclear extract of the blood stages of \textit{P. falciparum} parasites. The unlabeled oligonucleotide was added as a competitor DNA as indicated. (C) Radiolabeled probe C was incubated with a nuclear extract derived from \textit{P. gallinaceum} gametes. A batch of nuclear extract different from that used in the experiments depicted in panel A was used, which resulted in the formation of an additional nonspecific complex, indicated by an asterisk. Binding reactions were supplemented with specific competitor DNAs as indicated. (D) Transfection of \textit{P. gallinaceum} mosquito-stage parasites with plasmid pPFS25LUC and a version of the plasmid in which the two AAGGAATA elements were mutated to AAGGCCGC (pPFS25LUC-MUT). Plasmids were cotransfected with pCAT-L16.1SX, and luciferase activities were normalized to CAT activities. Values are from three transfections; error bars indicate standard deviations.
merase II-transcribed promoters, with a core promoter region and more distally located enhancers, the primary structure of the \textit{pfs16} and \textit{pfs25} promoters is atypical. The extreme A/T richness seems to preclude the assignment of a functional TATA box. Accordingly, analysis of promoters of apicomplexan parasites has revealed that a TATA box is not a conserved promoter element in these organisms (4, 11). Conversely, a repeated (A/T)GAGACG element, which acts as a selector of the transcriptional start site, has been identified as a critical element of many \textit{Toxoplasma gondii} promoters (4). This element is absent in the \textit{pfs16} and \textit{pfs25} promoter regions. Our data furthermore indicate that the few elements that do bear homology to known eukaryotic promoter elements, such as the MATA2-like elements in the \textit{pfs16} promoter and the CCAAT box in the \textit{pfs25} promoter, are not essential for a high transcriptional activity. That \textit{P. falciparum} promoters are functionally distinct from other eukaryotic promoters is further substantiated by the observation that the \textit{pfs16}, \textit{pfs25}, and \textit{hrp3} promoters do not function in transfections of mammalian cells (11a). Conversely, viral promoters such as the simian virus 40 promoter do not operate in \textit{P. falciparum} (22).

Although we failed to identify specific cis- and trans-acting factors operating on the \textit{pfs16} promoter, our data show that its mode of action is developmental stage specific. The activity of the \textit{pfs16} promoter persists during pyrimethamine treatment, demonstrating that the gametocytes are responsible for the observed activity of the \textit{pfs16} promoter in transfections of blood-stage parasites. The \textit{pfs16}-driven signal of the reporter gene appears at the time at which sexually committed ring-stage parasites appear in the culture, indicating that the \textit{pfs16} promoter is induced at the very onset of sexual differentiation. Accordingly, elimination of the sexually committed ring-stage parasites from the culture, a consequence of the pyrimethamine treatment (40). The \textit{pfs16} promoter described here fulfills the criteria for a high expression of the \textit{pfs16} promoter is silent during gametocytogenesis, as its activity is not observed under pyrimethamine pressure. These results extend the notion that the commitment to sexual differentiation involves the switching to an alternate program of gene expression (1). Asexual genes such as the \textit{hrp3} gene are shut off during gametocytogenesis, whereas specific sex genes, such as \textit{pfs16}, are turned on. The exact nature of the signal that triggers a parasite to commit to sexual development is unknown (1), although it is well documented that the parasite density is an important determinant (5, 7). Our data substantiate the previous finding that the \textit{pfs16} gene is activated in sexually committed parasites at the very onset of the sexual differentiation process (13) and indicate that the activation of this gene is immediately downstream of the trigger that activates gametocytogenesis. The early expression of \textit{Pfs16} suggests that this protein plays an important role during gametocytogenesis.

Gametocytogenesis ultimately leads to the production of mature gametocytes, which circulate in the bloodstream and remain infective to the mosquito for several weeks (38). Once gametocytes are ingested by a mosquito, their entrance in the midgut induces an array of mutual responses. The drop in temperature following transmission to the poikilothermic mosquito activates gametocytes to produce male and female gametes, a process which is further enhanced by a specific factor in the mosquito midgut (15), recently identified as xanthurenic acid (3). The arrival of the parasites in the mosquito midgut triggers the immune response of the mosquito (37) and induces the expression of mosquito trypsins that help to digest the blood meal (34). The trypsins together with the immune factors provide a hostile environment to the parasite, and ookinetes that do not succeed in penetrating the midgut epithelium are rapidly degraded (17). The key to the escape route through the midgut epithelium is provided by the Pf65 protein, which appears on the surface of the zygote within 30 min following the arrival of the blood meal in the midgut (18). Antibodies against this protein completely block penetration of the epithelium, suggesting a requirement for this molecule for penetration (40). The \textit{pfs25} promoter described here fulfills the need for an immediate expression of the Pf65 protein. Our data indicate, in agreement with previous data (13), that the \textit{pfs25} promoter is silent during asexual growth and gametocytogenesis but activated during gametogenesis. The activation relies in part on the DNA-binding protein PAF-1, which is specific for the mosquito stages of the parasite. Hence, expression of PAF-1 is part of the response of the parasite to the dramatic change in environment following transmission. The recognition site for PAF-1 is novel and is not found in the database of eukaryotic promoter elements (8). Interestingly, the recognition site for PAF-1 is also present in the \textit{P. falciparum} \textit{hrp86} promoter but absent in the \textit{hrp3} promoter. Accordingly, the \textit{hrp3} promoter is active following transmission to the mosquito (11a), whereas the \textit{hrp3} promoter is not (Fig. 4).

The data presented in this paper constitute the first detailed description of cis- and trans-acting elements in \textit{Plasmodium}. Future analysis of the details of the transcriptional activation of the \textit{pfs16} and \textit{pfs25} promoters will yield further insight in the mechanisms underlying the sexual differentiation process and might provide new targets for transmission-blocking agents. In addition, the promoters identified and characterized in this study allow the expression of foreign genes in the parasite stages that invade the mosquito midgut and hence provide invaluable tools for the study of parasite-vector interactions.

**ACKNOWLEDGMENTS**

We gratefully acknowledge Yimin Wu for supplying plasmids pHPR-PCAT, pHLLH, and pA0, Brendan Cormack for providing GFP plasmids, and David Kaslow for the gift of pNF413. We thank Jeffrey VanWye for communicating unpublished results on GFP expression in \textit{Plasmodium} and Patrick van den Boogaard for expert technical assistance.

This investigation received financial support from the Netherlands Ministry for Development Cooperation (grant NLI02701), from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and from the Commission of the European Community for Life Sciences and Technologies for Developing Countries. K.J.D. is grateful to the Harald Quintus Bosz Foundation and the Netherlands Organization for the Advancement of Pure Research (NWO) for gifts that covered travel expenses.

**REFERENCES**


