Developmentally regulated expression of \textit{pfs16}, a marker for sexual differentiation of the human malaria parasite \textit{Plasmodium falciparum}\(^1\)

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Abstract

Sexual differentiation is essential for the transmission of \textit{Plasmodium} to mosquitoes and therefore, for the spread of malaria. The molecular mechanisms underlying sexual differentiation are poorly understood but may be elucidated by a detailed study of the regulation of expression of sexual stage specific genes. In the present work we describe the differential expression of the gene encoding the sexual stage specific protein, \textit{Pfs16}. We have conducted a comparative analysis of \textit{pfs16} promoter activity, RNA levels and the rate of de novo protein synthesis during development of \textit{Plasmodium falciparum}. Furthermore, we have determined the pattern of expression of \textit{pfs16} transcripts at the single cell level by in situ hybridisation. We show that the expression of \textit{pfs16} is induced immediately following the invasion of a red blood cell in sexually committed ring stage parasites and continues throughout gametocytogenesis and in macrogametes. The expression of \textit{pfs16} is regulated at the level of transcription initiation and modulated by a post-transcriptional process. These results demonstrate that the expression of the \textit{pfs16} gene is the earliest event in the sexual differentiation process of \textit{P. falciparum} described to date. © 1997 Elsevier Science B.V.

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zoite of the human malaria parasite *Plasmodium falciparum*. It can re-initiate the asexual multiplication cycle from which it originated or it can differentiate into a male or female gametocyte. Thegametocytes are responsible for the transmission of the parasite to the mosquito host and therefore, for the spread of the disease. Little is known about the molecular events that trigger a merozoite to forgo asexual differentiation and undergo gametocytogenesis. The rate at which gametocytes appear in a *P. falciparum* culture is modulated by environmental factors such as the culture density [1] and several compounds, including cyclic adenosine monophosphate, phorbol esters and corticosteroids, have been reported to induce gametocytogenesis (reviewed in [2]). However, the mechanism of action of these compounds is unknown. At the genetic level, a number of observations have indicated that gametocytogenesis is a multifactorial process involving several chromosomal loci. For example, the failure of cultured *P. falciparum* cell lines to undergo sexual differentiation has been linked to a subtelomeric deletion of chromosome 9 [3,4]. Conversely, in other mutants that do not produce gametocytes, chromosome 9 is still intact and rearrangements have occurred at other loci [5]. In addition, Guinet et al. [6] have recently described a *P. falciparum* mutant that is impaired in the production of male gametocytes and have linked the responsible mutation to chromosome 12.

Sexual differentiation is marked by the synthesis of several parasite specific proteins. These include the antigens Pfs16, Pfs25, Pfpg27/25, Pfs48/45 and Pfs230, most of which originally have been characterised as important candidates for the development of a transmission blocking vaccine (reviewed in [7]). It is expected that a detailed study of the mechanisms underlying the stage specific expression of the genes encoding these proteins will deliver important information regarding the signals and mechanisms that trigger a merozoite to develop into a gametocyte.

Here we report on the developmentally regulated expression of *pfs16*, a gene encoding an intrinsic component of the parasitophorous vacuole membrane of gametocytes [8–10]. To examine the regulation of expression of this gene, *pfs16* transcription has been analysed in situ and the changes in *pfs16* promoter activity, and Pfs16 RNA and antigen levels during gametocyte and macrogamete development have been studied. We show that the synthesis of *pfs16* mRNA is the earliest marker of the onset of gametocytogenesis described to date. Furthermore, we demonstrate that the expression of *pfs16* is regulated at the level of transcription and modulated post-translationally by a developmental-stage specific mechanism.

## 2. Materials and Methods

### 2.1. Parasites

Asexual ring and schizont stages of *P. falciparum* isolate NF54 were isolated from sorbitol synchronised cultures as described [11]. For the isolation of sexual stages, cultures producing gametocytes were treated with N-acetyl-glu­cosamine to eliminate asexuals [12]. After 14 days of culture parasites were pelleted, mixed with fetal calf serum and incubated for 1 h at room temperature to allow activation of gametogenesis and the release of gametes. Subsequently, non-activated gametocytes and macrogametes were separated by Nycodenz density gradient centrifugation [13,14]. During this step male gametes are lost and we have, therefore, not assessed *pfs16* expression in microgametes. The qualities of the respective parasite fractions were analysed by microscopic examination of Giemsa stained thin smears and were as follows for the schizont and ring stage preparations: schizonts: 82% schizont, 18% other (ring/trophozoite); rings: > 95% ring stages. For the sexual stages the compositions of the respective parasite preparations typically were as follows: gametocytes: > 95% stage II–IV gameto­cytes (according to the classification proposed by Hawking et al. [15]); gametes: 50–70% macrogametes and 30–50% ghosts (lysed erythro­cytes). To demonstrate that the macrogamete fractions were not contaminated with gameto­cytes, they were subjected to a suspension immuno­fluorescence analysis with a mixture of Pfs25 and Pfs48/45 specific fluorescein isothiocyanate-
conjugated monoclonal antibodies. Simultaneously, nuclei were stained with 10 μg ml⁻¹ Hoechst 33258. After staining the preparations were analysed by fluorescence microscopy on a Bio-Rad MRC600 confocal microscope.

For the nuclear run-on experiments, Northern-blot analysis and protein synthesis studies parasite preparations of comparable qualities have been used.

2.2. RNA analysis

Cytoplasmic RNA and nuclei were isolated via incubation of cells in lysis buffer (10 mM Tris–HCl, pH 7.4/10 mM NaCl/3 mM MgCl₂/0.5% Nonidet P40) for 5 min on ice. Nuclei were pelleted, washed once with wash buffer (lysis buffer without Nonidet P40) and stored in 50 mM Tris–HCl, pH 8.3/5 mM MgCl₂/0.1 mM EDTA/40% glycerol at −80°C until further use. To isolate the RNA from the cytoplasmic fraction, the supernatant was adjusted to 50 mM Tris–HCl, pH 8.0/0.5% Na-sarcosyl/10 mM EDTA and extracted with phenol/chloroform/isoamylalcohol. The RNA was subsequently ethanol precipitated and resuspended in diethyl-pyrocarbonate treated H₂O. Its concentration was subsequently determined spectrophotometrically.

For Northern blot analysis, 3 μg of glyoxylated ring, schizont, gametocyte or macrogamete stage specific RNA was fractionated on an 1.5% agarose gel, transferred to nylon membrane (Hybond N+; Amersham) and probed with a nick-translated pfs₁₆ specific DNA probe [10].

For run-on analysis, 5 x 10⁶ nuclei were metabolically labeled with [α-³²P]UTP and the newly synthesised RNA was isolated as described [16]. Unincorporated label was removed by repeated ethanol precipitations in the presence of 2 M ammoniumacetate and the amount of incorporated label was determined via liquid scintillation counting. Equal amounts of radiolabeled RNA, isolated from nuclei of ring, schizont, gametocyte and macrogamete stages, were hybridised for 72 h at 42°C in 6 x SSC (1 x SSC = 150 mM NaCl/15 mM sodium citrate)/5 x Denhardts/0.5% SDS/100 μg ml⁻¹ yeast RNA and 50% formamide, to pfs₁₆ and pfs₂₅ DNA probes immobilized on Hybond N+ nylon membranes. After hybridisation the filters were washed three times with 2 x SSC/0.1% SDS for 15 min at room temperature, once with 2 x SSC containing 5 μg ml⁻¹ RNase A for 30 min at 37°C and finally with 0.2 x SSC/0.1% SDS for 60 min at 60°C. The filters were then subjected to autoradiography. A quantitative comparison of the expression levels between the different developmental stages was made by scanning the autoradiographs on a Molecular Analyst (Bio-Rad).

2.3. In situ hybridisation

A template for the in vitro synthesis of a pfs₁₆-specific antisense RNA probe was generated by two rounds of amplification by the polymerase chain reaction using the amplification protocol described in Baker et al. [17]. For the first round of amplification primer 1 (CCAACATGAATATTTCGAAAG) was used in conjunction with primer 2 (GACCTCATAAGGGCGGTTCCTCCTGTTTTTAAAC). To generate a 420 bp product consisting of the coding region of gene pfs₁₆ and flanked at its 3’-end by a promoter specific for T₇ RNA polymerase, primer 1 in conjunction with primer 3 (ACAAGCTTCTAATATCGACTCATTAGG) was used. A template for the in vitro synthesis of a 97 nucleotides long P. falciparum small subunit rRNA-specific antisense RNA probe, was generated by two rounds of amplification by the polymerase chain reaction using primer 4 (CCTAACCTAAACTATGCGG) in conjunction with primer 5 (GACCTCATAAGGGCGGTACTGAAGGAAGC) and primer 3 during the first and second rounds of amplification, respectively. In vitro transcription was carried out with phage T7 RNA polymerase (Promega) in the presence of digoxigenin or biotin-labeled UTP (Boehringer-Mannheim) as described by the manufacturer.

Blood films containing sexual-stage parasites were prepared for in situ hybridisation as previously described [17,18]. Hybridisation was performed in the presence of 1–2 ng ml⁻¹ of a digoxigenin-labeled antisense RNA probe specific for pfs₁₆, in conjunction with a biotin-labeled RNA probe complementary to P. falciparum
rRNA. The probes were detected with a rhodamine-conjugated antidi­goxigenin antibody (Boehringer-Mannheim) used at a concentration of 4 µg ml⁻¹ and a fluorescein-conjugated antibiotin antibody (Vector, UK) used at a concentration of 2 µg ml⁻¹, respectively. After hybridisation, slides were mounted in Vector-shield and visualised with a Bio-Rad confocal laser scanning microscope.

2.4. Labeling and immunoprecipitation of antigen Pfs16

Gametocytes and macrogametes purified from N-acetyl-glucosamine treated cultures were metabolically labeled with a mixture of the ³⁵S-la­beled amino acids methionine and cysteine (ICN) for 2 h as described [19]. Cells were lysed in 50 mM Tris–HCl, pH 8.0/150 mM NaCl/1% Nonidet P40 supplemented with protease inhibitors (Complete; Boehringer-Mannheim). After the nuclei and cellular debris were pelleted, the Pfs16 antigen was precipitated from the supernatant with polyclonal antiserum K36A [10] and protein G (Streptococcus sp. suspension; Sigma) as described [20]. Precipitated proteins were analysed by SDS-PAGE and visualised by fluorography.

3. Results

To study the developmental stage specific expression of a particular gene it generally is a prerequisite that this is performed on cells that are not contaminated by other developmental stages. In this study, therefore, we have taken extreme care in the synchronisation and subsequent purifi­cation of the respective developmental stages of the parasite. Asexual parasites were isolated from sorbitol synchronised cultures and sexual stages were isolated via Nycodenz gradient centrifuga­tion from N-acetyl glucosamine treated cultures. Microscopic examination confirmed that the ring stage and gametocyte preparations were more than 95% pure, i.e. consisted for more than 95% of the desired developmental stage. The schizont fraction was less pure (82% schizonts), but, as schizonts are multinucleated cells that contain multiple merozoites, the purity of this fraction in terms of individual merozoites was also greater than 95%. The purity and quality of the macrogamete fractions was judged by staining the nuclei with Hoechst 33258 and by suspension immunofluorescence with a mixture of fluorescein isothiocyanate-conjugated monoclonal antibodies specific for antigens Pfs25 and Pfs48/45. In an unfixed parasite preparation, a reaction with these monoclonals is restricted to those parasites that are not surrounded by a red blood cell membrane and that furthermore contain these antigens at their outer surface, i.e. mature macrogametes. Fluorescence microscopy showed that the macrogamete preparations typically consisted for 50–70% of nucleated cells all showing the typical malaria pigment and for the remaining part of erythrocyte ghosts. As demonstrated in Fig. 1, more than 95% of the nucleated and pigmented cells showed a reaction with the mixture of Pfs25 and Pfs48/45 specific monoclonal antibodies, thus providing compelling evidence that the vast major­ity of cells in the macrogamete preparations consisted of mature female gametes.

To enable a quantitative comparison of promoter activity, RNA steady state level and de novo production of Pfs16, in all experiments equivalent amounts of parasite RNA or protein of the different developmental stages were used. All experiments have been performed more than once and the results of representative examples are presented.

3.1. Sexual-stage specific synthesis of pfs16 RNA

The Northern blot presented in Fig. 2 demonstrates that pfs16 RNA is exclusively synthesised in gametocytes and macrogametes. Scanning of the autoradiograph revealed that the amount of pfs16 RNA present in gametocytes is 2.5 times the amount present in macrogametes. Prolonged exposure of the Northern blot revealed a weak hybridisation signal in ring stage parasites (data not shown). This is consistent with the findings that a sub-population of the early ring stage para­sites present in P. falciparum cultures have made the commitment to the sexual pathway [21,13] and suggests that pfs16 is expressed at the very onset of gametocyto­genesis.
In order to investigate the pattern of \textit{pf\textsubscript{16}} transcription in more detail, we have analysed the expression of \textit{pf\textsubscript{16}} in situ at the individual cell level. In situ hybridisations were performed on infected blood films, containing parasites of all stages of asexual and sexual development, in the presence of a digoxigenin-labeled probe complementary to the \textit{pf\textsubscript{16}} transcript and a biotin-la-

Fig. 1. Suspension immunofluorescence assay on two typical preparations of \textit{P. falciparum} macrogametes. The macrogamete fractions were incubated with a mixture of fluorescein isothiocyanate-conjugated anti-pfs25 and anti-pfs48/45 monoclonal antibodies and assayed by confocal fluorescence microscopy. Left panels: fluorescent image. Right panels: light transmission image. (A) Macrogamete preparation A. In 48% of the cells the typical \textit{P. falciparum} pigment (hemozoin) is clearly visible. As could be confirmed by staining with Hoechst 33258, the other cells are erythrocyte ghosts (not shown). Of the pigmented cells, 93% are recognised by the conjugated monoclonal antibodies. Occasionally a typical banana-shaped gametocyte is observed (indicated by an arrow) that shows no reaction with the monoclonals, indicating that the reaction is specific for mature macrogametes. (B) Macrogamete preparation B. Of the cells, 68% are pigmented and the other 32% are ghosts. Of the pigmented cells, all are recognised by the conjugated monoclonal antibodies.

Fig. 2. Northern blot analysis of the stage specific expression of \textit{pf\textsubscript{16}}. \textit{P. falciparum} RNA (3 µg) from ring stages (lane 1), schizonts (lane 2), gametocytes (lane 3) and macrogametes (lane 4) was fractionated, transferred to nylon membrane and probed with a \textit{pf\textsubscript{16}} cDNA probe.

\textit{pf\textsubscript{16}} RNA is first detected in a sub-population of young ring stage parasites (< 24 h post infection) and thereafter in stage I gametocytes (Fig. 3(1) and (2), respectively) and throughout gametocytogenesis (Fig. 3(3) and (4)) in both male and female gametocytes (data not shown). \textit{pf\textsubscript{16}} RNA is absent in parasites that are undergoing asexual development (Fig. 3). The fact that \textit{pf\textsubscript{16}} is expressed in a sub-population of young ring stage parasites and in all sexual stages, but never in ring stages that have developed into the readily identifiable asexual trophozoites (24 h post infection, [12]), lead us to the conclusion that ring stage parasites that express \textit{pf\textsubscript{16}} have made the commitment to the sexual differentiation route. Expression of the \textit{pf\textsubscript{16}} gene is, therefore, the earliest available marker of the commitment to sexual development.

3.2. Regulation of expression of \textit{pf\textsubscript{16}}

Several research groups have previously demonstrated that antigen Pf\textsubscript{16} is present in gametocyte and gamete preparations of \textit{P. falciparum} [8–10]. The results of immuno-electronmicroscopy studies have led Moelans et al. [10] first to the conclusion that Pf\textsubscript{16} is attached to the surface of gametocytes and macrogametes. More detailed analyses performed by other workers [8,9] have, however,
indicated that this interpretation was not fully correct. In fact it was found that antigen Pfs16 is a component of the parasitophorous vacuole membrane and that the occurrence of Pfs16 in gamete preparations primarily is caused by remnants (whorls) of the parasitophorous vacuole membrane that still are attached to the female gamete. The latter observations do not, however, exclude the possibility that (female) gametes still have retained the capacity to synthesise (and secrete?) antigen Pfs16. An indication that this indeed might be the case is already given by the observations that macrogamete preparations contain abundant amounts of pfs16 specific mRNA (Fig. 2 and Fig. 5; [10]). To obtain an unambiguous answer to this issue, purified gametocytes and macrogametes were metabolically labeled with the amino acids methionine and cysteine. Subsequently it was tested whether among the de novo synthesised proteins antigens were present that specifically reacted with Pfs16 specific antibodies. From the results presented in Fig. 4 it can be

Fig. 3. Visualisation of developmental stage specific pfs16 RNA expression by in situ hybridisation. (A) Hybridisation signals obtained with a small subunit A-type ribosomal RNA (SSU rRNA) probe. (B) Hybridisation signals from a pfs16 specific probe. (1) Young ring stage parasite (<24 h post infection) positive for pfs16 and a schizont showing no pfs16 signal. (2) Ring stage parasite, trophozoite (24–48 h post infection) and stage I gametocyte (~40 h post infection). Only the stage I gametocyte stains with the pfs16 probe. (3) Stage III gametocyte hybridising with the pfs16 probe, negative schizont. (4) Mature gametocyte hybridising to the pfs16 probe, negative schizont.
concluded that macrogametes are still capable of synthesising Pfsl6 antigen. The levels of Pfsl6 synthesis that are observed in gametocytes and macrogametes correspond well with their cognate RNA levels and do not indicate that there is a differential rate of translation of the transcript.

In order to assess whether the Pfsl6 transcripts present in the macrogametes are due to on-going transcription of the Pfsl6 gene or reflect an accumulated pool of Pfsl6 RNA originating from the progenitor gametocytes, we have conducted a nuclear run-on analysis. To this end, nuclei were isolated from macrogametes and gametocytes and for comparison also from asexual ring stages and schizonts, and incubated in the presence of α-32P-labeled UTP. Subsequently, the nascent radiolabeled RNA was isolated and hybridised to Pfsl6 and Pfsl25 specific DNA probes. From the data presented in Fig. 5 it can be concluded that Pfsl6 is transcribed both in gametocytes and macrogametes, but not in asexual ring and schizont stages. A quantitative comparison showed that the frequency of transcription of Pfsl6 is slightly higher in macrogametes than in gametocytes. In contrast, the Northern analysis and immunoprecipitation experiments revealed that Pfsl6 RNA levels and rate of protein synthesis are lower in macrogametes than in gametocytes. These results demonstrate, therefore, that transcription of the Pfsl6 gene is developmentally regulated and strongly suggest that a post transcriptional mechanism, possibly involving increased Pfsl6 mRNA turn-over, operates in macrogametes to modulate the levels of Pfsl6 mRNA and protein.

The level of transcription of the control gene Pfsl25, that was studied in parallel, was found to be very low in gametocytes and to peak after the onset of gametogenesis (Fig. 5). These data are fully consistent with data published previously [22,23,14].
4. Discussion

The data presented here demonstrate that the pfs16 gene is expressed within 24 h following the invasion of a red blood cell in sexually committed ring stage parasites. It is shown that pfs16 is expressed throughout gametocytogenesis, in both female and male gametocytes, and in macrogametes. Furthermore, synthesis of pfs16 RNA occurs in advance of expression of the second early sexual-stage specific gene, pfg27, which initiates at stage II of gametocytogenesis, [24]; (Thompson J, unpublished results). Our results show, therefore, that pfs16 expression is not only a marker for all stages of the sexual development of *P. falciparum* in the vertebrate host, but is also the earliest marker for sexual commitment identified to date. Bruce et al. recorded the expression of Pfs16 at 30–40 h post invasion [9]. The results presented here indicate that pfs16 is expressed even earlier (<24 h post invasion) and indeed marks the very onset of gametocytogenesis.

Previous reports that pfs16 is expressed in asexual parasites [25,10] are not confirmed by the results of our studies and are most likely due to incomplete synchronisation of parasite cultures as already suggested by Moelans et al. [10]. We have shown that pfs16 transcription initiates in sexually committed parasites immediately following the invasion of a red blood cell by a merozoite. As these sexually committed parasites are morphologically indistinguishable from early ring stages, we suggest that their presence accounts for the detection of pfs16 mRNA or protein in cultures which contain no identifiable gametocytes and have, therefore, been termed asexual. Lobo et al. have reported that some clones of *P. falciparum* which are defective in gametocytogenesis, express pfs16 but not pfg27 [24]. In the light of our findings, we would also re-interpret their results to suggest that these clones are not fully "asexual" but show some degree of commitment to the sexual differentiation route as evinced by the expression of pfs16. They are not, however, able to proceed to stage II of gametocytogenesis as they do not express the later sexual-stage marker, pfg27.

The cellular localisation of the Pfs16 antigen has been the subject of some dispute. From immuno-electron-microscopic studies Moelans et al. [10] have concluded that antigen Pfs16 is an integral component of the parasite membrane. Other investigators, however, have not been able to confirm this conclusion, but have obtained convincing evidence that in gametocytes antigen Pfs16 is transported through the parasite membrane and subsequently inserted in the parasitophorous vacuole membrane [8,9]. During gametogenesis part of the parasitophorous membrane remains, for reasons still unknown, attached to the parasite membrane and forms without doubt an important source of the Pfs16 antigen found in (female) gamete preparations [8–10]. Though gametes do not contain an (intact) parasitophorous membrane, the data presented here clearly demonstrate that gene pfs16 is still transcriptionally active in macrogametes and that at this developmental stage de novo antigen Pfs16 is made. Taking the biophysical and biochemical properties of the antigen Pfs16 into consideration [10] (see above) we assume that it now is secreted by the parasite. Whether this hypothesis indeed is correct and whether the protein now fulfils another biological function, or that the activity is residual and that the observed increased turnover of the mRNA in macrogametes anticipates the silencing of the gene, is the subject of current investigations.

The run-on data show that the transcription of pfs16 is initiated early during gametocytogenesis and that the pfs16 promoter remains active at least up to the stage of macrogametes. The appearance of the Pfs16 protein directly reflects induction of the transcriptional activity of the pfs16 gene. Although pfs16 promoter activity does not decrease in macrogametes, however, pfs16 transcript levels and rate of protein synthesis are lower. This suggests that the abundance of pfs16 products is modulated by a developmentally-regulated post-transcriptional mechanism. Differences in mRNA degradation rates during growth and differentiation of eukaryotic cells are a commonly observed phenomenon [26,27]. The pfs16 3' untranslated region contains motifs, including 11 copies of a nearly perfect dodecanucle-
otide repeat (our unpublished results) which may contribute to the differential degradation of the pfs16 transcripts.

The point in the life cycle at which sexual development is triggered is unknown. Carter and Miller [1] have proposed two models for a clonal origin of sexually differentiated parasites. According to the first model, merozoites are released from a schizont and commit to a differentiation route only after re-invasion of a red blood cell. In the second model, the commitment takes place earlier and the entire progeny of a single schizont undergoes either asexual or sexual development. Experiments addressing these models favour the latter model but are not totally conclusive. Inselburg demonstrated that the progeny of a single schizont is to a great extent, but not completely, committed to one of the two available differentiation routes [28]. In an extension of this work, Bruce et al. observed a higher, but still not an absolute degree of commitment of the progeny of a single schizont [21].

Although pfs16 proves to be the earliest expressed marker for sexual development known to date, the pfs16 gene is not expressed in merozoites whilst they are still located within the schizont. This finding indicates, therefore, either that expression of the pfs16 gene first occurs after commitment has taken place or that commitment coincides with the expression of pfs16 but takes place only after re-invasion of a red blood cell by a merozoite. The latter explanation favours the first model proposed by Carter and Miller. Detection of the presence of the pfs16 transcript will be a valuable tool in the further evaluation of processes involved in the sexual commitment of a merozoite. We are currently identifying the mechanisms that lead to the activation of transcription of the pfs16 gene in an attempt to identify the signals that lead to sexual differentiation of P. falciparum.

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