Species-specific Regulation and Switching of Transcription between Stage-specific Ribosomal RNA Genes in *Plasmodium berghei* *

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Malaria parasites (*Plasmodium spp.*) differentially express structurally distinct sets of rRNA genes in a stage-specific manner. The four rRNA genes of the rodent malaria parasite, *P. berghei*, form two classes of 2 units that are genetically unlinked and termed A-type and S-type. Through Northern analysis and *in situ* hybridization, expression of the units was demonstrated in synchronized parasite preparations covering the developmental pathway from the initiation of the blood-stage asexual cycle to the production of mature ookinetes. A-type units were transcribed in direct response to cell growth in bloodstage asexual parasites yet were differentially regulated during male (inactive) and female (active) gametocytogenesis. S-type expression was not confined solely to the mosquito stages and exhibited a finite period of expression in a subset of bloodstage trophozoites that was significantly elevated in gametocyte-producing parasites. Unlike in the human parasite, *P. falciparum*, there was no evidence for accumulation of precursor forms of the S-type transcripts in gametocytes. No significant rRNA transcription was observed in cultured, fertilized ookinetes until ~20 h of development when S-type transcription was initiated. The results further demonstrate that in *Plasmodium* the expression of the different rRNA units is linked to developmental progression but in a species-specific manner.

One of the remarkable features of *Plasmodium* parasites is the developmentally regulated ribosome characterized by transcription in a stage-specific manner of discrete repertoires of unlinked nuclear rRNA genes, now termed A-type (from asexual) and S-type (from *Sporozoite*) (1). The exceptional pattern of transcription of these genes was initially discovered in the rodent parasite, *Plasmodium berghei*, where it was broadly demonstrated that the asexual blood stages transcribe the A-type units, whereas the S-type units are transcribed in the mosquito stages of the parasite, including the sporozoite (2). A similar pattern of rRNA unit transcription has been confirmed for every species of *Plasmodium* investigated (3–5). Subsequent work in different parasite species demonstrated that the switches in rRNA unit transcription were linked to parasite development associated with transitions between the vector and host. Thus, it was shown in the human parasite, *Plasmodium falciparum*, that the switch from A- to S-type transcription occurred during gametocytogenesis and zygote formation and involved accumulation of S-type rRNA precursor forms (6). In *P. berghei*, the switch back to A-type transcription occurred early in the liver stages following invasion of cultured hepatocytes by sporozoites (7). Recent work in *Plasmodium vivax* has shown that the transition to S-type rRNA occurred much later in the period of development within the mosquito, at the point in oocyst development when sporozoites were being formed and contrasting with the observations in *P. falciparum* (5).

Lately, a third type of developmentally regulated nuclear rRNA gene has been demonstrated in *P. vivax*, a finding that may prompt a re-evaluation of all previous data. Termed O-type, their transcription was first detectable in oocinetes, and their period of maximum transcription was in oocysts separating those of the A- and S-types. O-type genes are structurally significantly different from both the A- and S-type. Transcripts of the O-type appeared to be confined to the oocyst structure and were not detected in salivary gland sporozoites.

These observations and the fact that the O-type has not yet been demonstrated in *P. berghei* indicated that there may be species-specific features of the switch and the types of genes involved. The four rRNA units in the nuclear genome of *P. berghei* are currently divided into two pairs representing the A-type (A and B units) and S-type (C and D units) (8, 9), which somewhat simplifies analysis. We present here a description of the steady-state transcriptional activity of these stage-specific units through the use of probes directed against the relatively rapidly evolving A and C unit external transcribed spacer (ETS)² regions. We found that transcription of the *P. berghei* nuclear rRNA genes was also developmentally regulated. Specific and unexpected periods of rDNA unit transcription were observed, demonstrating a complex pattern of transcriptional regulation that could be linked to the various differentiation pathways of the parasite.

**MATERIALS AND METHODS**

**Parasites**—The following cloned lines of the ANKA strain of *P. berghei* were used in this study: 8417 (gametocyte producer); clone 2.34 (producer); 8548 (low producer); and clone 2.33 (nonproducer). In addition, the nonproducer clone 1 of the K173 strain was used (10).

**Production of the Various Stages of the Parasite**—Synchronous asexual blood stages were obtained from infections in rats that were syn-

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1 J. Li and T. F. McCutchan, unpublished observations cited in Ref. 1.
2 The abbreviations used are: ETS, external transcribed spacer; SSU, small subunit; LSU large subunit; PCR, polymerase chain reaction; kb, kilobase(s); nt, nucleotide(s); hpi, hours post invasion.
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*Chronicized* as has been described (11–13). Parasites synchronized in this way typically have a window of development no greater than 3 h. Blood containing ring forms was collected from the rats, and leukocytes were removed as described and incubated under standard *in vitro* culture conditions (11) for 21–24 h to allow the development of the rings into merozoites. These cultures were taken at the appropriate time points for RNA extraction (see below).

Immature gametocytes were purified from blood collected from rats with synchronous infections as described (14). These immature gametocytes were incubated for a period of 6 h under standard culture conditions (11) for complete maturation. The purified gametocyte preparation consisted of 95% male and female gametocytes in a 1:1 ratio with a 5% mixed with 5% mature schizonts and ring forms.

Zygotes and ookinetes were obtained from *in vitro* cultures as described (15). In this study, we used two different sources of gametocytes to start the cultures: 1) purified gametocytes, which were collected as described above (in these cultures, normally 50–70% of the females were fertilized, and there was a 5% schizont contamination (10)); and 2) infected blood obtained from mice with a high proportion of gametocytes (16). From this material, ookinite smears were prepared for *in situ* hybridization or were purified from unfertilized gametocytes, young gametocytes, and trophozoites by density gradient centrifugation, resulting in ookinete preparations consisting of mature ookinetes, unfertilized cells, and ring forms.

**Nucleic Acid Techniques: RNA Extraction**—RNA was prepared from purified parasites as reported previously (10). RNA was fractionated in formaldehyde containing agarose gels and blotted to nylon membrane (Hybond N+, Amersham Corp.) according to standard protocols (17).

**Definition of the RNA Units and Probes Used in This Study**—The four rRNA gene units of *P. berghei* were originally defined as A–D according to their size upon digestion of genomic DNA with EcoRI (8). This definition has survived the discovery of the specific developmental transcription of the different units, which are now defined as A (Asexual stage), B (C unit), C (A and C rDNA units but also includes the T7 RNA promoter), and D (as described above (15)). The reader is referred to "Materials and Methods" for more details.

**Expression of rRNA Genes in *P. berghei***

Chromosomal classes—The methodologies describing the preparation of *P. berghei* parasites and suitable RNA and DNA probes for *in situ* RNA hybridization and their application have been described thoroughly elsewhere (21–23). Probes were labeled either through the direct incorporation of fluorescein-UTP or the incorporation of digoxigenin-UTP into DNA probes for *in situ* hybridization to chromosomes (24, 25). In the present study, we used A- and C-type SSU rRNA gene probes that have been described previously (18) and were hybridized to chromosomes 5 and 6 (Fig. 2). This demonstrates that the reader is referred to "Materials and Methods" for the definition of the RNA units. The A-type ETS probes (101A and 315) hybridized to chromosomes 5 and 6 (Fig. 2). This demonstrates that the probe directed against the ETS regions of the A and C units was type specific, consistent with the observed similarity of respective restriction maps of the four units (8, 9, 10, 12). The A-type ETS probes (101A and 315) hybridized only to chromosomes 5 and 12, whereas the S-type ETS probe (99S) hybridized to chromosomes 5 and 6 (Fig. 2).

**RESULTS**

**The ETS Probes Distinguish between the A- and S-type rRNA Classes**—The reader is referred to "Materials and Methods" for the definition of the RNA units. The ETS regions of the A and C rDNA units were isolated from the genomic clones described previously (18) by a combination of PCR and subcloning procedures. These and other probes used in this study are illustrated in Fig. 1, which is a schematic representation of the A and C units showing the gene units and the ETS and internal transcribed spacer regions (adapted from Ref. 19). These probes were used to establish the chromosomal location and sequence type of the units and their patterns of transcription in the various stages of the life cycle reported here. A probe for the small subunit rDNA gene hybridized to chromosomes 5, 6, 7, and 12, showing that the four rRNA units are unlinked in the genome. The A-type ETS probes (101A and 315) hybridized only to chromosomes 7 and 12, whereas the S-type ETS probe (99S) hybridized to chromosomes 5 and 6 (Fig. 2). This demonstrates that the probe directed against the ETS regions of the A and C units were type specific, consistent with the observed similarity of respective restriction maps of the four units (8, 9, 10, 12). The A-type ETS probes (101A and 315) hybridized only to chromosomes 5 and 12, whereas the S-type ETS probe (99S) hybridized to chromosomes 5 and 6 (Fig. 2).
Expression of rRNA Genes in P. berghei

Fig. 1. Location of probes used in the study. These are shown superimposed upon a representation and map of the relevant region of the A and C units. Redrawn according to the data of Dame and McCutchan (8, 19, 24) with additional restriction sites added from mapping studies within this laboratory. The map is truncated at its 3' end within the LSU moiety as indicated by the dashed line. E, EcoRI; A, Apal; K, KpnI; RV, EcoRV; N, NdeI; Ns, NotI; Hs, HindIII.

Fig. 2. The 4 rDNA units of P. berghei lie on separate chromosomes but are grouped into two pairs. Chromosomes of different clones of P. berghei (HP, HPE, and K173 as indicated) were separated according to standard field inversion gel electrophoresis methods, photographed, blotted, and probed with the C gene ETS region, probe 99S (left panel); the A gene ETS region, probe 101A (center panel); and the P. falciparum SSU gene (right panel). Final washes were at 65°C in 0.1 x SSC, 0.5% SDS.

The sequence of these regions will be the subject of another report that identifies and characterizes the transcription start site of the rRNA gene units. The internal transcribed spacer region 1 probes (Fig. 1) have the same A- or S-type specificity (data not shown).

Transcriptional Activity of the rRNA Genes in Asexual Blood Stages and Gametocytes—The typical rDNA unit is transcribed as a single large primary transcript, which is then processed through a series of characteristic steps to generate the three mature rRNA species of SSU rRNA (~2050 nt for Plasmodium), 5.8S (~155 nt), and the (naturally nicked in P. berghei) LSU rRNA (~800 and 3000 nt)(8, 18). The ETS probes used in this study will, therefore, detect the primary transcript, and any secondary processed precursor species that contain the ETS but will not hybridize directly to the mature rRNA species. The Northern and in situ hybridization analyses demonstrated that the ETS probes hybridized only to precursor rRNA species and that these were confined to the nucleus (Fig. 3). The processing of rRNA precursor transcripts is a rapid process, and the RNA species have extremely short half-lives. Nuclear run-on analyses using blood-stage parasites confirmed that the A-type units are actively transcribed in these cell types (data not shown). Probes detecting these intermediates could, therefore, be used to measure steady-state rRNA unit transcription. This was performed using synchronous populations of distinct clones of P. berghei, which either could or could not produce gametocytes.

Four time points were investigated representing each of the morphologically distinct phases of the asexual blood-stage cycle (5 hpi, consisting of ring stages/young trophozoites; 16 hpi, old trophozoites; 25 hpi, mature schizonts/young gametocytes; and 28 hpi, mature schizonts/mature gametocytes). RNA from equivalent numbers of parasites were loaded in each lane, which was quantitated by hybridization of an oligonucleotide (TM4) to the mature SSU rRNA species. In the gametocyte-producing clone (8417), the A-type units were transcribed throughout the asexual cycle, and the production of two major precursor species of 2700 and 2900 nt was observed hybridizing to probe 101A (Fig. 3A). A faint band of ~7.0 kb also hybridized to 101A and is interpreted as the size of the primary rRNA transcript of the complete unit or an early processing intermediate (data not shown). The 2900-nt precursor was only observed in the first 16 h after erythrocyte invasion, and both major precursors were equimolar during this period. However, the 2900-nt precursor was not observed during schizogony or in mature gametocytes. In the gametocyte nonproducing clone, the 2900-nt precursor is in the same parasite forms and to the same extent as in the producer clone (Fig. 3A, compare lanes 1, 2, 6, and 7). However, A-type transcription was barely observable in mature schizonts (Fig. 3A, compare lanes 3, 4, 8, and 9). We conclude that the 2700-nt precursor is produced by gametocytes, and these forms are responsible for the signal observed in the schizont samples taken from the gametocyte-producing parasite clone. Mature schizonts do not, therefore, transcribe rRNA genes. This pattern of transcription was observed using both 101A or TM4 (data not shown). Preliminary data indicated that the observed precursors consisted solely of the mature SSU and ETS regions.

These conclusions were confirmed and clarified by in situ hybridization of 101A to blood-stage parasites, which demonstrated that transcripts containing the ETS region were confined to the nucleus, consistent with the exclusive nuclear processing of eukaryotic rRNA primary transcripts (Fig. 3B). A-type transcription was not observed in newly invaded rings (data not shown) but was observed in all asexual mononuclear stages from 5 hpi. Furthermore, A-type transcription decreased with increasing schizont maturity and was barely detectable in fully mature schizonts (16n) and released merozoites (1n). A-type transcription in gametocytes was shown to be sex-specific. Female gametocytes continued to transcribe the A-type units, whereas the males were silent (Fig. 3B).

The S-type units were transcribed in a phased manner in blood-stage parasites (Fig. 3C). S-type precursors of an estimated size of 3000 and 2650 nt, respectively were observed with a peak in production at 16 hpi, when all cells are maturing trophozoites. The same pattern of S-type precursors was observed in both gametocyte producer and nonproducer clones, although the level of transcription in the former was significantly higher (Fig. 3C). The level of S-type transcription appeared to vary between clones between 2- and 10-fold, as estimated by densitometry (Fig. 3C) and appeared to be consistently greater in producer clones. The larger precursor was more abundant than the smaller (ratio, 5:1). A third band of >10 kb was also visible, which is interpreted as being the full-length primary rRNA transcript of the complete unit, comparable to the smaller >7.0 kb transcript of the A-type unit. The transitory appearance of the S-type precursors indicated that

5 R. M. L. van Spaendonk, G. A. McConkey, T. F. McCutchan, C. J. Janse, and A. P. Waters, manuscript in preparation.

6 R. M. L. v. Spaendonk and A. P. Waters, unpublished observations.
FIG. 3. Transcription of the rRNA units in blood-stage parasites. A, Northern analysis of A-type transcription. RNA was isolated from synchronous populations of clone 8417 (gametocyte producer) and from clone 233 (gametocyte nonproducer) and subjected to Northern analysis. Northern blots of the isolated RNA were sequentially hybridized to the A-type ETS probes 315A and 101A and to the oligonucleotide TM4. Lanes 1–5 contain RNA of clone 8417. Lane 1, 5 hpi (called ring stages); lane 2, 16 hpi (trophozoites); lane 3, 25 hpi (maturing schizonts); lane 4, 28 hpi (mature schizonts); lane 5, purified gametocytes 35 hpi. Lanes 6–9 contain RNA of clone 233. Lane 6, 5 hpi; lane 7, 16 hpi; lane 8, 25 hpi; lane 9, 28 hpi. Diagrams of the morphology of the different stages are placed above the appropriate lane of the blot. The small forms illustrated beneath the schizonts in lanes 3 and 4 are gametocytes, which represent about 20% of the population in this parasite clone.

B, in situ RNA hybridization analysis of A-type transcription. Left column, 4,6-diamidino-2-phenylindole-stained nuclei. Right column, in situ RNA hybridization of digoxigenin-labeled probe 101A to asexual parasites. Hybridization was detected with fluorescein-labeled anti-digoxigenin antibodies. 1, trophozoites; 2, schizonts of increasing maturity (increasing number of nuclei; 16n is fully mature) and a unicellular merozoite (Mz); 3, a male (♂) and female (♀)
they did not accumulate as steady-state precursor forms that had been observed in gametocytes of \textit{P. falciparum} \cite{4}, nor was accumulation of the mature transcripts observed through hybridization to the S-type-specific oligonucleotide, TM3 (data not shown). Neither transcription of the S-type units nor accumulation of S-type transcripts in mature gametocytes was evident (Fig. 3C) in clone 8417 illustrated nor in rings, mature trophozoites, nor schizonts from independent nonproducer parasite clones, LK173 and 233 (see “Materials and Methods”; data not shown).

\textbf{In Situ Hybridization of the S\textsubscript{1}—ETS probe to synchronous blood-stage parasites confirmed that the peak of S-type transcription occurred at 16 hpi during trophozoite development.} The peak appeared to be restricted to a subset of mononuclear cells (\textless 1\%). As in Northern analysis, neither S-type transcription nor accumulation was observed in mature gametocytes (Fig. 3D). It is not yet possible to distinguish between 16-hpi trophozoites, which will form schizonts, and those committed to sexual differentiation.

\textbf{Transcription of the Nuclear rRNA Gene Units during Sexual Development.}
Development and Zygote Development—Using the ETS-specific probes, A-type and S-type transcription was examined in mature gametocytes, zygotes, and developing ookinetes (Fig. 4). A-type precursors were still abundant in mature female gametocytes (Fig. 4A, right panel). After fertilization, it appeared that transcription of the A-type units rapidly decreased. In the ookinete preparations, which were derived from purified gametocytes, A-type precursors were hardly visible at 2 and 14 h after fertilization. The strong hybridization at 22 h is due to the presence in this particular preparation of ookinetes of large numbers of asexual ring forms that produce significant amounts of A-type precursors (Fig. 2B). In situ hybridization analysis confirmed that A-type transcription was down-regulated in zygotes and ookinetes during this period and that the nuclear A-type signal resulted solely from asexual parasites present in the culture (Fig. 4B).

In contrast to the rapid decrease in A-type transcription, S-type transcription was up-regulated during ookinete development (Fig. 4A, left and center panels). In mature gametocytes, little if any S-type transcription was seen. S-type transcription could be detected 2 h after fertilization and dramatically increased between 14 and 24 h in the maturing ookinete. Four S-type rRNA precursor bands were observed in ookinetes, the two observed in asexual parasites (3000 and 2650 nt) and two larger forms (3900 and 3400 nt). The 2650-nt precursor was more abundant than the 3000-nt transcript in contrast to the transcription of the S-type units in the bloodstream stages, where the relative abundance of these two precursors was reversed (Fig. 2C). In situ hybridization analysis confirmed that the S-type transcription could be detected in the nucleus of about 10% of 20-h ookinetes and in >70% of mature (>24-h) ookinetes. S-type transcription was not detected with this method in 2-h zygotes or in 4-, 6-, 12-, and 16-h ookinetes (Fig. 4B and data not shown).

DISCUSSION

The pattern of rRNA gene transcription in Plasmodium parasites is complex. This is due not only to the presence of different types of rRNA genes units (A- and S-type) that are transcribed in a stage-specific manner (2–5) but also the presence of multiple and unlinked copies of each type. Each individual unit may be differentially transcribed during the progression of the life cycle, complicating the analysis. The pattern of transcription of the rRNA genes can be established relatively easily in the rodent model malaria parasite, P. berghei, because there are only two copies each of the A-type (the A and B units) and the S-type (the C and D units) (8), and the types can be defined by the ETS probes described here. These probes also further define the extent of pairwise homogeneity between the units. The mechanism that underlies the maintenance of this pairwise homogeneity of units on separate chromosomes involves some level of gene conversion (25), yet it must also discriminate between and maintain the different types.

In Plasmodium, rapid growth periods result in the formation of relatively stable parasite forms (mature schizonts/merozoites, gametocytes, and sporozoites) that only undergo further differentiation after a specific transition, e.g. erythrocyte invasion or passage between the host and vector. The work presented here demonstrates that rRNA transcription in two of these stable forms, schizonts and male gametocytes, is down-regulated and that only the female gametocyte continued to transcribe the rRNA genes. The female gametocyte can accumulate maternal mRNA that is not translated until after fertilization (e.g. Pbs21 ookinete antigen gene (10, 21, 22)) and may, therefore, require protracted ribosome biogenesis during its development and may also accumulate ribosomes for use in the zygote, given the absence of appreciable rRNA transcription in P. berghei at that stage. Electron microscopic examination of mature male gametocytes suggested that ribosomes were undergoing visible degradation, which would be consistent with their cessation of rRNA gene transcription (26, 27).

The steady state pattern of A-type rRNA precursors was complex and probably cell type-specific, implying that the female gametocyte uses a different rRNA processing pathway compared to asexual blood-stage forms, even if the difference only reflects the kinetic stability of the intermediates. Alternatively, the range of rRNA precursors may reflect two specific forms of A-type transcript, one each produced by the A and B units. The currently available probes cannot discriminate between the units, which appear to be highly similar (8).

In most organisms, transcription of rRNA genes occurs during the growth phase of the cell (28). This work has shown that
transcription of the A-type rRNA genes of *P. berghei* is maximal in the rapidly growing blood-stage forms and, therefore, under a similar type of control. In addition, we have shown that at least one of the S-type units, normally mainly transcribed during oocyst growth (2), is also transcribed during the blood-stage development in a small subset of *P. berghei* trophozoites and to a greater extent in parasite clones that produce gametocytes. Bloodstage transcription of the S-type units may indicate a temporary requirement for S-type ribosomes during blood-stage development, implying the existence of a specific mRNA population or cell type. This subset of cells may be committed to sexual development, but in the absence of additional early markers of gametocyte development, this cannot be confirmed. Unlike the observations made in *P. falciparum* (4), however, the *P. berghei* blood-stage S-type transcription did not result in the accumulation of precursor forms in gametocytes; in addition, there was no evidence of mature S-type SSU rRNA in any blood-stage form. This species-specific S-type rRNA gene transcription may well reflect the marked difference in the kinetics of development and morphology of *P. falciparum* gametocytes (26), which take a minimum of 8 days to mature, whereas in other malaria species, gametocyto genesis is of a similar length to asexual blood-stage development. Alternatively, it is possible we observed a general transcriptional event that is so transient that at any time point of analysis, transcription appears to be limited to a subset of the synchronized populations that have a developmental window of about 4 h. The developmental timing of the switch between A- and S-type transcription in the mosquito also differs in populations that have a developmental window of about 4 h. There is thus an urgent need to compare these models results from different types of competitive associations of both transcriptional promoter (e.g. SL1, UBF, TBP, and REB1) and repressor proteins (e.g. Rb) with both enhancer and promoter DNA elements (29, 30). These associations can be affected by the phosphorylation status of protein factors (31). Additionally, the status of the chromatin and the phasing of nucleosomes in the region of the rDNA units can influence transcription (32, 33). All of these mechanisms may play a role in the regulation of the individual rDNA units of *Plasmodium*, and their interplay may then control the relative expression of each unit. The work described here implies that the general transcription of the rDNA units of *Plasmodium* will, however, be regulated in a similar conserved fashion to those of other eukaryotes, and the particular interest will lie in examining the supplementary mechanisms that effect the observed stage-specific control of rRNA transcription.

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