Review

Plasmodium gene regulation: far more to factor in

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Gene expression in the malaria parasite has received generous attention over the past several decades, predominantly because of the importance of *var* gene regulation, which is key to antigenic variation and host immune evasion. However, the role of transcriptional regulation in governing other genes expressed during the various stages of development has remained less well characterized. This mostly has been due to the lack of defined transcriptional regulators in *Plasmodium* parasites. Here, we describe recent advances that have become possible by joining traditional biochemistry with new technological innovations. These studies have increased our understanding of the role of transcriptional regulation, not only in the control of gene expression for antigenic variation but also in the coordination of stage-specific parasite development.

Transcriptional regulation in *Plasmodium* parasites

The completion of the *Plasmodium falciparum* genome sequence in 2002 [1] brought the expectation that potential candidate transcription factors would be identified along with *cis*-acting regulatory elements. Unfortunately, despite the wealth of regulatory information available from other eukaryotes, neither of these two hopes were met. Early bioinformatic analysis of the genome identified few proteins with domains typical of eukaryotic transcription factors, although the basal core transcriptional machinery and chromatin-remodeling complexes are highly conserved [2–5]. In light of the observed stage-specific transcription of the majority of *Plasmodium* genes [6–10], there is a strong need to identify *cis*-acting elements and their cognate *trans*-acting factors, to attain a mechanistic understanding of transcriptional regulation in *Plasmodium*. With the emergence of functional genomics approaches, it is now possible to tackle the role of parasite-specific factors and the mechanisms governing gene regulation in this parasite. At the Molecular Approaches to Malaria (MAM2008) meeting, several recent studies revealed novel insights into epigenetic contributions to transcriptional regulation, the role of nuclear structure and a novel family of putative transcription factors, heralding an exciting new era in *Plasmodium* research[*].

Epigenetic regulation of gene expression: the *var* gene example

Epigenetic control of gene expression is a well-studied phenomenon in many eukaryotes, including malaria parasites. Much of the work in *P. falciparum* has focused on the *var* gene family, in particular the mechanisms that underlie mutually exclusive expression (reviewed in Ref. [11]). Although the exact mechanism is still under investigation, the presence of a full complement of chromatin-remodeling proteins in *Plasmodium* parasites indicates that chromatin modifications are certain to play an important part [12–16]. Chromatin modifications have now been assayed directly at *var* gene loci by several groups [17–20]. At MAM2008, Artur Scherf's group presented data from an extensive study of the histone modifications associated with *var* genes in their active or silent states[*]. Many of the modifications are similar to those previously observed in other eukaryotes [21], with two hallmark epigenetic marks, histone 3 lysine 9 tri-methylation (H3K9me3) for silent genes and di- or tri-methylation of histone 3 lysine 4 (H3K4me2/me3) for active genes, being especially enriched at or near the *var* gene transcription start sites. Related work presented by Ron Dzikowski and Kirk Deitsch supports previous evidence for the function of a second promoter found within *var* introns in cooperatively regulating *var* gene expression [22–24][*]. Additional experiments indicated that maintenance of the epigenetic marks found at active *var* genes requires active transcription from the upstream promoter and that briefly preventing an 'on' *var* gene from being transcribed causes it to revert to the silent state, indicating a possible role for transcription complexes in maintaining epigenetic memory in *P. falciparum* [25]. More detailed analysis of *var* upstream regulatory regions has identified several *cis*-acting motifs that interact specifically with nuclear factors [26] and regulate chromatin structure and transcriptional activity [27]. At MAM2008, the laboratory of Till Voss presented a transfection-based approach to dissect the regulatory information encoded in the upsC *var* upstream sequence[*]. Their results provide further evidence on the function and activity of regulatory elements in *var* gene upstream regions involved in silencing, activation and mutually exclusive expression.

Regulation of additional subtelomeric gene families in *Plasmodium*

In other subtelomeric gene families such as rif, stevor or *Pfmc-2tm*, transcriptional regulation is far less well

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understood. Similar to *var* genes, members of these gene families code for proteins that are destined for host cell compartments such as the Maurer's clefts and the infected red blood cell surface [28–33]. Several lines of evidence support clonal variation in their expression [31,34], and although the function of these variant proteins remains elusive, this clearly indicates a role in parasite–host interactions and immune evasion. Owing to their juxtaposition in subtelomeric regions, it is tempting to speculate that expression of subtelomeric gene families might be co-regulated and that epigenetic mechanisms similar to those that govern mutually exclusive *var* gene regulation could be involved. In support of this hypothesis, deletion of the *P. falciparum* silent information regulator 2 gene (*Pfsir2*) resulted in upregulation of a subset of *var* and *rif* genes [18]. However, another study demonstrated that transcription of *var* and *stevor* genes in asexual blood-stage parasites and gametocytes seemed to be unlinked [35]. On the individual gene level, transcriptional timing of subtelomeric gene families does not occur in parallel but occurs in successive waves during the ring stage (*var* genes), early trophozoites (*rifs*) and mature trophozoites (*stevors* and *Pfmc-2tms*) [29–31,34]. This hints at the contribution of family-specific regulatory factors that initiate gene transcription at particular stages of parasite development. Recently, stage-specific binding of nuclear factors to several functional elements in *rif* promoters was demonstrated using gel-shift experiments [34]. Interestingly, two gene families involved in invasion, *eba* and *rhoph1/clag*, were variantly expressed [36] and, at MAM2008, Alfred Cortés presented data linking altered transcriptional states of these genes to differences in nucleosomal structure, again highlighting the potential importance of chromatin structure in regulating gene expression in *Plasmodium* parasites*.

The role of nuclear structure in gene regulation

Several recent publications have described the positioning of *var* genes within the nucleus, particularly with regard to the clustering at the nuclear periphery of both subtelomeric *var* genes and the tandem arrays of *var* genes found in the non-telomeric regions of the chromosomes [18,37–39]. Using florescent *in situ* hybridization (FISH), these groups have shown that the 60 *var* genes present in the parasite genome are not found randomly scattered throughout the nucleus but rather group together into 6–8 perinuclear clusters, potentially tethered to the nuclear envelope. However, some controversy has arisen as to whether the active *var* gene moves away from these clusters into a transcriptionally competent nuclear region, or if genes instead are activated where they reside. At MAM2008, Artur Scherf presented data supporting the view that *var* genes move within the nucleus upon activation*. When parasites are artificially engineered to have two simultaneously active *var* promoters, FISH studies show that both preferentially co-localize within a specific region of the nucleus [23]. Such co-localization was not observed when active and silent *var* promoters were visualized. Together, these results indicate that, in fact, a specific subnuclear *var* expression site does exist, and it will be a major challenge in the future to identify the mechanisms and molecular components involved in this spatial repositioning of *var* gene loci. A more general approach to identify sites of active transcription within the parasite nucleus was reported at MAM2008 by Carolina Moraes and Lucio Freitas-Junior*. Using 5-bromouridine 5′-triphosphate labeling, they showed that there are many sites of active transcription within the nucleus, both at the nuclear periphery and within the inner nuclear space. Further understanding of nuclear structure will undoubtedly cast additional light on gene regulatory pathways in these parasites.

Specific transcriptional regulators during development

Although our understanding of epigenetics in the regulation of subtelomeric gene expression has progressed considerably, the mechanisms of general transcriptional regulation in *Plasmodium* parasites remain an open question. It is clear that, as in other eukaryotes, *P. falciparum* protein-coding genes are transcribed by RNA polymerase II [40], supported by a basal TFIID-based transcription complex [3]. The mRNAs are mono-cistronic and require regulatory information encoded in their promoter regions for proper timing of expression [41–45] (for simplicity, the 5′ untranslated and promoter regions are not distinguished between). The most convincing data that gene expression is regulated by sequence information in 5′-upstream regions is the functional demonstration that the promoters from many genes are necessary and sufficient to drive gene expression stage-specifically [42,46–53]. These sequences have been the driving force toward the first transfection vectors, which are now numerous (reviewed in Refs [54–57]). The first putative regulatory motif described was from the gene coding for glycophorin-binding protein (GBP130) [58]. Since then, several reports demonstrated specific binding of nuclear factors to DNA motifs in *Plasmodium* promoters [26,34,50,53,59] and others predicted important DNA motifs in the regulatory regions of several genes [48,60–62]. However, to date, no DNA-binding protein had been identified other than the relatively non-specific TATA-binding protein [63] and the transcription factor PfMyb1 [64], the role of which in gene regulation remains unclear.

What paucity of transcription factors? The ApiAP2 proteins

The identification of the apicomplexan AP2 (ApiAP2) transcription factor family [65] has changed the landscape with regard to putative transcriptional regulators in the *P. falciparum* genome. Each member of the 26-protein ApiAP2 family contains at least one copy of a small, ~60 amino acid domain that is highly conserved across all Plasmodia. These small domains are related to DNA-binding domains found in the plant AP2/ERF (Apetala2/ethylene response factor) transcription factors, which comprise the second largest family of transcriptional regulators in plants [66]. Between apicomplexan genomes, the AP2 DNA-binding domains are generally well conserved, although lineage-specific expansions are evident, especially in *Toxoplasma gondii* [67]. This is an intriguing family of proteins because their expression is predominantly confined to the red blood cell stages, although at least six

members are not expressed at all during this stage and probably have roles in the mosquito or liver stage [6,7]. At MAM2008, three groups (Manuel Llinás, Masao Yuda and Till Voss) reported specific DNA-binding interactions mediated by AP2 proteins from both *P. falciparum* and *Plasmodium berghei*[*]. Together, these contributions clearly highlighted the importance of ApiAP2 proteins in transcriptional regulation and the development of *Plasmodium* parasites.

Putative AP2 DNA binding of three different *P. falciparum* ApiAP2 proteins (PF14_0633, PFF0200c and PF11_0404) was assayed using a protein-binding microarray (PBM) technique [68] (Figure 1a). This methodology enabled the identification of unique DNA sequence motifs bound by the three *Plasmodium* domains tested (Figure 2). These results demonstrate for the first time that *Plasmodium* parasites contain several *bona fide trans*-acting proteins capable of binding DNA in a sequence-specific manner. This work also demonstrates that AP2 domains bind specific DNA sequence motifs that are located in the upstream regions of co-expressed genes. A highly homologous AP2 domain from *Cryptosporidium parvum*, a distantly related apicomplexan, was characterized as well [68]. Despite only 47% amino acid sequence identity, this protein bound a highly similar DNA motif. Surprisingly, the predicted regulons (genes containing the DNA motif in their 5′-upstream regions) contain no overlap, indicating that although the ApiAP2 proteins in different species contain conserved DNA-binding domains, the activation (or repression) of target genes has diverged dramatically. Remarkably, these motifs had all been previously computationally predicted by the Finding Informative Regulatory Elements algorithm [62], which utilizes mutual information to identify over-represented sequence motifs in genes that are co-expressed by microarray analysis [69]. Similarly, Masao Yuda presented compelling functional

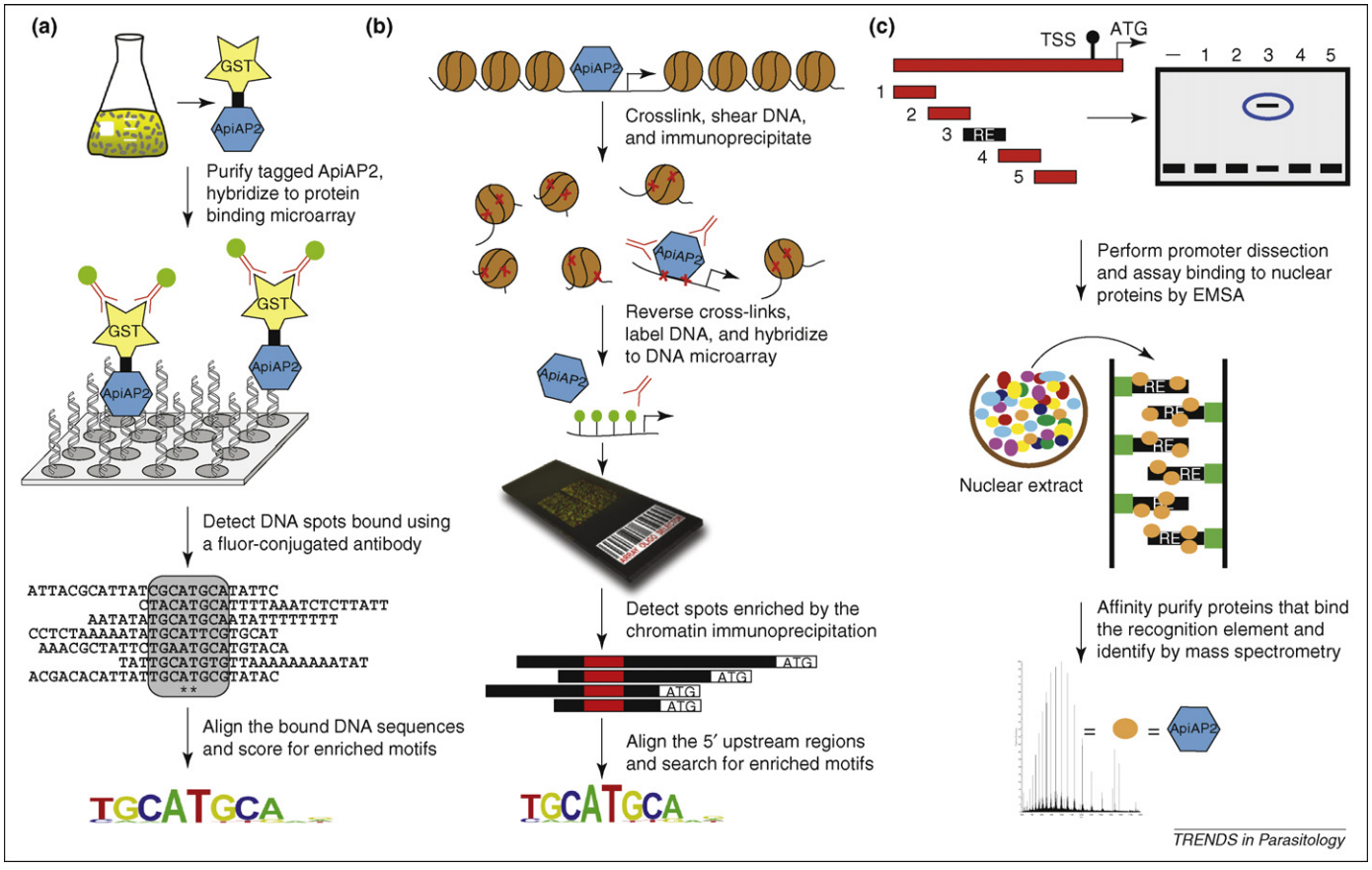

Figure 1. Approaches for characterizing DNA-binding proteins in *P. falciparum*. The three panels demonstrate strategies recently used to determine various properties of DNA-binding proteins. **(a)** Characterization of DNA sequence motifs bound by a putative DNA-binding protein. This *in vitro* method relies on the biochemical purification of a tagged protein, in this case an ApiAP2 protein (blue hexagon) (depicted as glutathione S-transferase [GST]), which is then assayed on a protein-binding microarray (PBM). PBMs are double-stranded DNA microarrays that contain all possible 10-mer DNA sequences [73,74]. Proteins that bind to specific sequences on the PBM array are visualized using an Alexa-fluor-conjugated antibody, which is then scanned using a conventional DNA microarray scanner. The DNA sequences from the spots bound by the protein are computationally analyzed to determine a position-weight matrix to represent the DNA sequence motifs preferred by the protein in question. **(b)** Identification of positions in the genome bound by a known DNA-binding protein. Initially, all protein–DNA interactions within the cell are chemically crosslinked using formaldehyde and the chromosomes are sheared for immunoprecipitation. The protein of interest is assayed by chromatin immunoprecipitation (ChIP) using a DNA microarray (chip) to globally identify all regions of the genome bound by this protein in the native context [75]. Once the regions enriched by ChIP are identified on the microarray, the bound sequences are bioinformatically analyzed for the presence of motifs that are commonly shared between the enriched spots. An alternative version of ChIP–chip couples ChIP with quantitative PCR (qPCR) to directly assay the interaction of a DNA-binding protein to a region suspected of being bound and, therefore, likely to be present in the ChIP reaction. **(c)** Identification of an unknown DNA-binding protein that specifically binds to a known sequence within the 5′-regulatory region of a given gene or gene family. Without any *a priori* knowledge, it becomes much more difficult to characterize DNA-binding proteins. By dissecting the 5′-promoter region upstream of the translation (ATG) and transcription start site (TSS), individual DNA fragments can be assayed by electrophoretic mobility shift assay (EMSA) to determine a region containing a protein-DNA recognition element (RE). Once this DNA region is defined, an affinity purification approach can be applied using the DNA as the bait to isolate proteins from nuclear extracts. Bound proteins can then be identified using current mass-spectrometry-based proteomic approaches. PBM image courtesy of Michael F. Berger.

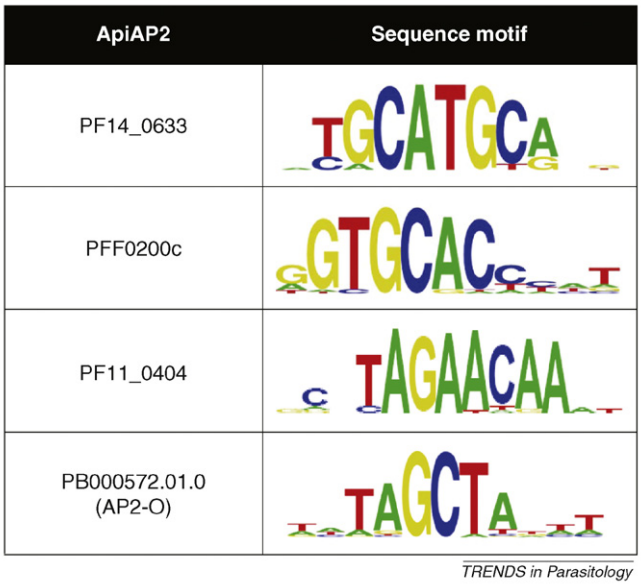

Figure 2. Sequence motifs recognized by ApiAP2 proteins. The sequence motifs recognized by the AP2 domains of PF14_0633, PFF0200c and PF11_0404 were determined *in vitro* using protein-binding microarrays [68]. The sequence motif for PB00572.01.0 was found to be enriched in the 5′-regulatory region of genes, the expression of which was reduced in a knockout line of this ApiAP2 protein, and binding to this motif was confirmed both *in vivo* by ChIP–qPCR and *in vitro* by electrophoretic mobility shift assay. DNA sequence motifs were rendered using the Web-based tool enoLOGOS [76] and are based on position-weight matrices representing the DNA-binding specificities. The larger the height of a nucleotide, the more information there is about this position. In positions with stacked nucleotides, the relative sizes of the different nucleotides represent how frequently they occur. .

evidence for a transcriptional role for an ApiAP2 protein from *P. berghei*[*]. Interestingly, this gene (*pb00572.01.0*, named AP2-O) was found previously to be translationally repressed in late-stage gametocytes [70], already indicating an important role in mosquito-stage development. Several lines of evidence support AP2-O as a transcription factor, including the characterization of an *ap2-o* knockout by DNA microarray analysis, chromatin immunoprecipitation (Figure 1b) with detection by quantitative PCR (ChIP–qPCR) and electrophoretic mobility shift assays.

The DNA sequence motif for PFF0200c (Figure 2) found by PBM [68] is similar to the previously reported subtelomeric *var* promoter element (SPE2) found upstream of UpsB *var* genes [26]. At MAM2008, the group of Till Voss described an affinity enrichment strategy to purify SPE2-binding proteins from parasite nuclear extracts followed by mass spectrometry analysis (Figure 1c)[*]. Interestingly, they identified and confirmed the SPE2-binding protein as PFF0200c, a member of the ApiAP2 family of transcription factors. It is remarkable that three completely different experimental approaches led to the identification of novel parasite transcription factors of the same family (and in one case, the same protein). This convergence underscores the importance of these *cis*-regulatory motifs and their *trans*-acting factors.

Concluding remarks and future directions

These new insights into transcriptional regulation would not be possible without important advances in technology. Three new methods have been successfully applied to characterize transcriptional regulators in *Plasmodium*: PBMs, ChIP and affinity enrichment coupled with mass spectrometry (Figure 1a–c). Furthermore, the ability to integrate recent methods from the past 5–10 years, such as targeted transfection strategies, transcriptional profiling and FISH, is enabling a more rigorous exploration of basic parasite biology and should embolden future endeavors to deepen our understanding of parasite gene regulation. Finally, the identification of *Plasmodium* transcriptional regulators suggests exciting new possibilities to design *Plasmodium*-specific, inducible expression systems. These new insights into transcriptional regulation of gene expression also provide the scientific community with previously unexplored avenues for therapeutic intervention of malaria. For instance, if gene expression is as rigid as has been proposed during asexual parasite development [6], disruption of this cascade by direct targeting of a few key, parasite-specific transcriptional regulators could prove to be sufficient to halt development.

These preliminary studies raise many questions regarding the control of gene expression. How do these DNA-binding proteins interact with the core transcriptional machinery? What mediates this interaction for activation or repression? Are there other specific transcription factors? What roles do chromatin modifications and silencing have in non-subtelomeric regions? Undoubtedly, transcriptional regulation through specific transcription factors will need to be interpreted in the context of other related processes including chromatin remodeling, mRNA decay, post-transcriptional regulation and antisense RNA. For example, there is now strong evidence for a large repertoire of structural noncoding RNAs that might influence patterns of gene expression [71,72], and translational repression has been shown to play a major part in the gametocyte-to-ookinete transition [70]. For now, with candidate genes in hand, it is an exciting time to begin understanding gene regulation in different stages of parasite development, not only in the clinically important erythrocytic stages but also in the transition to gametocytes and the equally relevant mosquito and liver stages.

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