Regulation of gene expression in *Plasmodium falciparum*

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With the ability to adopt an assortment of forms throughout its life cycle, and to thrive in host environments so diverse and challenging, the malaria parasite *Plasmodium falciparum* may well serve as the epitome of the regulation of gene expression. The parasite is replete with mechanisms of control, many of them unique and intriguing, permitting it to transit seamlessly from one defined ecological niche to the next. This review is an attempt to capture the essence of our current understanding of transcriptional, post-transcriptional and translational regulation in *P. falciparum*, and how this works for us in drug development.

Keywords: Gene expression, *Plasmodium falciparum* splicing, transcription regulation, translational machinery.

Introduction

MALARIA has afflicted humans for centuries. Hippocrates, the father of modern medicine, is believed to have described tertian and quartan fevers (the hallmark of malaria infection) as early as the third and fourth centuries BC[1]. Despite this long association of the malaria parasite and its human host, the causative agent of the disease was identified[2] only in the late 1800s by Laveran and Ross. Over the next century, malaria was shown to be caused by protozoan parasites of the species *Plasmodium* and clinical symptoms of the four major human parasites, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* were defined. The life cycle of the parasite in both the human host and mosquito vector was elucidated and shown to include several distinct developmental and morphological stages (Figure 1). Today, malaria is a staple of medical textbooks, particularly in tropical and sub-tropical regions of the world. However, this disease almost never makes it to the pages of textbooks on basic biology. One reason might be that for an understanding of biological phenomena such as replication, transcription, translation, etc., it is essential to be able to culture the organism in the laboratory for further study; indeed one of the most virulent species of *Plasmodium*, *P. falciparum* has been cultured under laboratory conditions[3] since 1976. However, till date it has not been possible to carry out continuous culture of the other *Plasmodium* species that infect humans. New technologies of genomics, transcriptomics and proteomics have certainly opened up avenues of research in *P. vivax* and other human malaria parasites. Nevertheless, *P. falciparum* seems to be the best-understood malaria parasite in many respects and is therefore the focus of this review.

Another reason why *P. falciparum* basic biology is yet to become 'textbook knowledge' could be that some processes in the parasite are different from other eukaryotes, including the human host. For example, the genome of *P. falciparum* consists of 80–90% AT and is one of the most AT-rich genomes sequenced to date[4]. Promoters, translation start sites and splicing choices that depend on DNA consensus sequences are therefore harder to define in this organism.

One important biological process that is unusual in the parasite is regulation of gene expression. A working definition of this term is 'information encoded in DNA being used for synthesis of RNA and finally proteins'. Implicit in this definition is the idea that regulation of gene expression includes transcription, post-transcriptional phenomena such as splicing and capping of mRNA, mRNA stability and translation as well as mechanisms that fine-tune these phenomena. Many of these processes have been studied in *P. falciparum*, particularly after the completion of sequencing of the parasite genome and the advent of genomic and post-genomic technologies such as microarrays, proteomics, etc. These technologies have shown that in different stages of the parasite life cycle, distinct subsets of genes are transcribed and translated (Figure 1). A deeper understanding of the mechanisms by which *P. falciparum* regulates the expression of approximately 6000 genes in its genome is much needed.

This article will review the current status of research on regulation of gene expression in *P. falciparum* with a particular emphasis on transcription, splicing and translation. We will show that much progress has been made in understanding these biological phenomena in *P. falciparum*; however, many unanswered questions remain. Due to the differences between parasite and host biology, some of these processes are also the targets of potential

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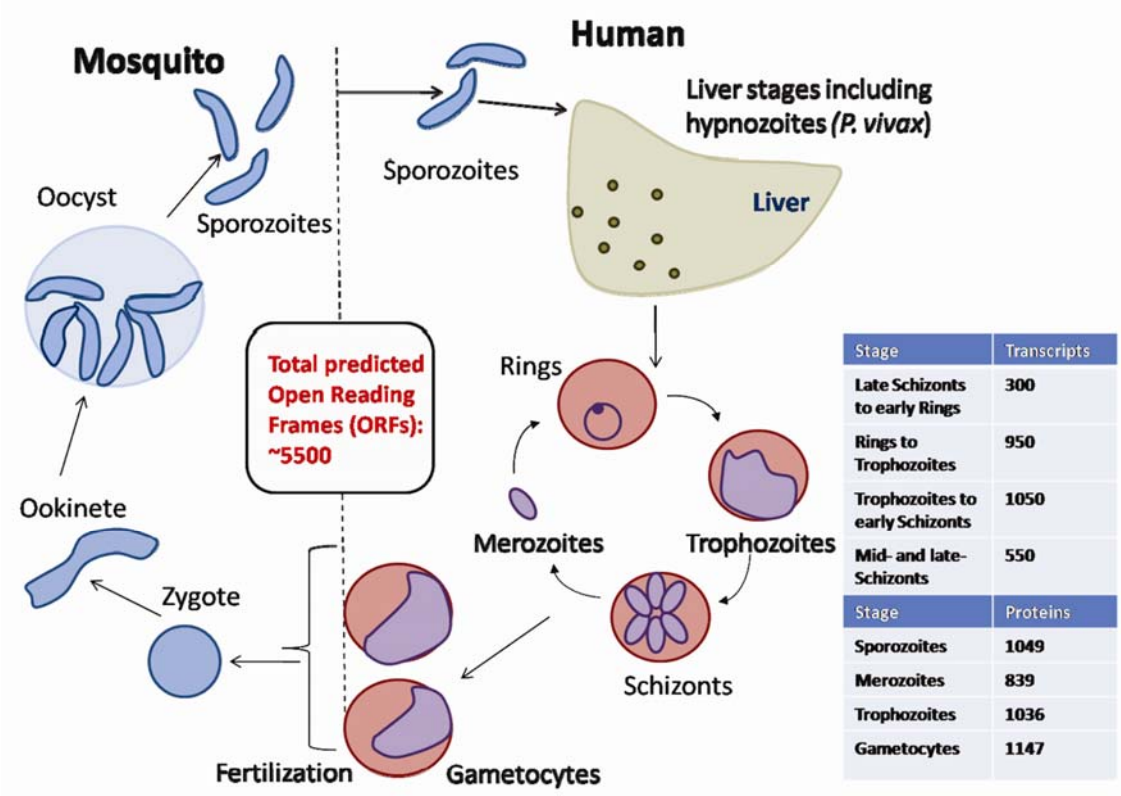

Figure 1. *Plasmodium* life cycle and expression of transcripts and proteins at different stages of the life cycle. The human and mosquito hosts are demarcated and developmental stages of the parasite shown. Note that this is a schematic of the life cycle, is not drawn to scale and merely serves as a representation of the life-cycle stages. Box indicates the number of transcripts and proteins identified at a particular stage. These data are for *P. falciparum* and have been compiled from Bozdech *et al.*[53] and Lasonder *et al.*[121]. Transcriptome and proteome data are available for mosquito stages of the life cycle. However, these data are for other *Plasmodium* species and have not been shown due to paucity of space.

anti-malarial drugs and attempts at drug discovery based on regulation of gene expression in the parasite will be briefly touched upon.

Transcription

P. falciparum follows a complex life cycle involving stages in the mosquito, in the liver and blood cells of humans, with predominantly asexual but also sexual stages. As it passes through these stages, the parasite undergoes a carefully regulated differential expression of its genes, the mechanism of which has now begun to be better understood. This differential expression of the genes results in the synthesis of a unique set of proteins, characteristic of each stage of the parasite. Different proteins are required at different stages to serve specific purposes like binding to host receptors, immune clearance, invasion, etc.

The P. falciparum genome

Pulse-field gel electrophoresis and electron microscopic counts of kinetochore structures have shown that 25–30 Mb of *P. falciparum* nuclear DNA is organized into 14 chromosomes (0.75–3.5 Mb), along with 6 kb linear mitochondrial and 35 kb circular apicoplast genomes. The parasite chromosomes are linear and contain repetitive telomeric ends providing plasticity to the genome due to frequent deletions and insertions[5]. Sequencing the genome of the parasite was a heroic task since the 80–90% AT-rich content resulted in the bacterial plasmids cloned with genomic fragments being unstable. To overcome this problem, libraries of *P. falciparum* genomic DNA were constructed in yeast artificial chromosomes (YACs)[6].

Post-genomic approaches: transcriptome analysis of P. falciparum

As the genome of the parasite was being sequenced, several groups analysed the transcriptome of the parasite to get an idea of gene expression profiles during different life cycle stages. Large-scale microarray analysis was carried out using a mung bean nuclease-generated genomic library[7]. The altered mRNA expression levels between the asexual trophozoite stage and the sexual gametocyte stage allowed for the identification of stage-

specific transcripts. Many parasite genes were found to be expressed during the asexual stages[8,9], with these analyses also resulting in the elucidation of various metabolic pathways and the identification of numerous antisense transcripts. Differences between the transcriptomes of the trophozoite and schizont stages of the asexual cycle were also revealed[10,11].

After the successful sequencing of the *P. falciparum* genome, around 5400 open reading frames (ORFs) distributed across 14 chromosomes were predicted[4]. This enabled scientists to carry out genome-wide analyses to obtain a comprehensive transcription profile of *P. falciparum*[10,11]. Le Roch *et al.*[11] used a high-density custom oligonucleotide microarray of the whole genome (along with the plastid and the mitochondrion) to outline the transcription pattern of several stages, including sporozoites, merozoites, asexual stages and gametocytes. In their study, they found that 88% of the predicted genes were expressed at least once during the life cycle of the parasite; and among them 43% of the genes were regulated. Of these, 32% were in the asexual stages, and 11% in the sporozoite and the gametocytic stages. Further, genes with similar expression profiles were categorized into 15 different clusters, in which only 36% of the genes were found to code for known proteins. The clustering of similar genes helped predict the functions of many genes coding for hypothetical or unknown proteins.

Bozdec[10] and Llinas[12] used a *P. falciparum*-specific DNA microarray representing most of the annotated ORFs. The relative mRNA abundance levels were measured to obtain the expression profile of parasite transcripts across the complete 48 h intra-erythrocytic cycle at 1 h resolution. A striking periodicity in the expression pattern of genes was observed. The transcriptional phaseogram was found to be S-shaped, displaying a cascade of successive expression of genes. This S-shaped transcription profile, which is also called 'just in time' expression, ensures that the induction of a gene occurs at specific points in the life cycle, and exactly when it is required. The transcription wave starts as rings grow to trophozoites, leading to the transcription of around 950 genes involved in general cell functions. During the trophozoite to schizont transition, 1050 genes are maximally transcribed; while transformation from mid to late schizont stages activates an additional 550 genes. The infective ring stage that follows induces another 300 genes. A recent report that simultaneously studied the transcriptome and proteome at 2 h time-points also confirmed these results[13].

Little is known about organellar transcription in *Plasmodium*. The apicoplast carries out transcription of genes in a highly coordinated manner with peak expression levels during the schizont stage. Like chloroplasts, the apicoplast encodes three RNA polymerase subunits (RpoB, RpoC1 and RpoC2) sensitive to rifampin[14]. Mitochondria transcribe metabolic genes, including TCA cycle and electron transport genes. Also transcribed by the mitochondrial genome are ribosomal RNAs, transfer RNA synthetases and elongation factors during the late trophozoite and early schizont stages[10,15].

Mechanisms of transcription regulation

The methods of transcriptional control in *P. falciparum* may be different from other eukaryotes due to high levels of antisense transcription, unique patterns of mRNA decay, periodic transcriptional cascade and due to the lack of transcription-associated regulatory proteins and well-conserved promoters[16].

In addition, histone-modifying enzymes and epigenetic mechanisms also contribute to overall regulation[17]. As mentioned earlier, the parasite synthesizes a unique set of proteins characteristic of each stage. Several studies have supported the hypothesis that one of the important ways of controlling the required gene expression is at the transcriptional level. For instance, it has been shown through nuclear run-on analysis and RNA expression, that the knob-associated histidine-rich protein (KAHRP) gene is transcriptionally regulated in a stage-specific manner[18]. An upstream sequence element was also identified that interacts with nuclear extracts made from different stages, which correlates with the expression pattern of the KAHRP gene.

The monocistronic nature of parasite genes[18], bioinformatics analysis to identify transcription factors[19] and transfection studies[20,21] have garnered support for *P. falciparum* having typical bi-partite eukaryotic promoters, comprising a basal promoter, which is the binding site for RNA polymerase, and upstream elements involved in regulation of this basal promoter.

It has been shown that mRNA synthesis in *P. falciparum* is α-amanitin-sensitive[18], indicating the presence of a typical eukaryotic RNA polymerase II. Bioinformatics approaches predicted the homologues of all the 12 subunits of RNA polymerase II, but failed to identify transcription factors associated with this polymerase since a sequence-based approach was used for the search[19]. This could be attributed to the highly AT-rich genome[4]. Later, secondary structure-based approaches were used to predict the general transcription factors[22], and homologues to most but not all transcription factors were discovered. Recently an Apicomplexan-specific family of putative transcription factors, known as ApiAP2, has been discovered and functionally annotated in the parasite using a bioinformatics approach[23]. These are structurally related to Aptela-2 transcription factors of plants and contain an Aptela-2 (AP2) DNA binding domain. The *Plasmodium* genome has been predicted to contain 27 of these distinct ApiAP2 proteins, and they show a unique expression profile encompassing the entire life cycle of the parasite[23]. Through protein-binding microarrays, it has been shown that the AP2 domains of the two predicted ApiAP2 proteins have a high binding specificity for the upstream

regions of certain co-regulated genes[24,25], and that a majority of them show unique DNA binding preferences. Many of the ApiAP2 proteins have the ability to bind multiple distinct DNA motifs, suggesting complex regulatory networks of transcription. It has recently been reported that a member of the putative ApiAP2 family, PfSIP2 binds to the sub-telomeric SPE2 DNA motif array situated upstream of certain *var* genes[26]. Based on these and other reports, it has been proposed that a unique set of transcription factors is synthesized at each stage and these bind to the upstream elements of promoters leading to either activation or silencing of the downstream gene.

As mentioned earlier, the intergenic regions of *P. falciparum* are highly AT-rich. These AT-rich DNA sequences are quite unique in their physical properties which include increased curvature, more stable hydrogen-bonding patterns and less efficient packaging into nucleosomes[27], and can thus interact with transcription factors in a unique manner that is yet to be understood. For instance, it has been shown that the transcription of a reporter gene driven by the *Plasmodium* calmodulin gene promoter is dependent on poly(dA)–poly(dT) tracts situated within the upstream region[28].

var gene regulation

Gene regulation in *P. falciparum* has been studied extensively in the *var* gene family. The *var* genes are located in sub-telomeric or central positions in the chromosome with a frequency of 2–3 *var* genes on each chromosome[29]. *var* genes have a two-exon structure with 5′ conserved non-coding regions that are a hallmark of the chromosomal location of the *var* gene (Figure 2). There are around 60 *var* genes per haploid parasite genome and out of these only one gene is expressed at a time. The highly diverse *var* gene family undergoes rapid switching

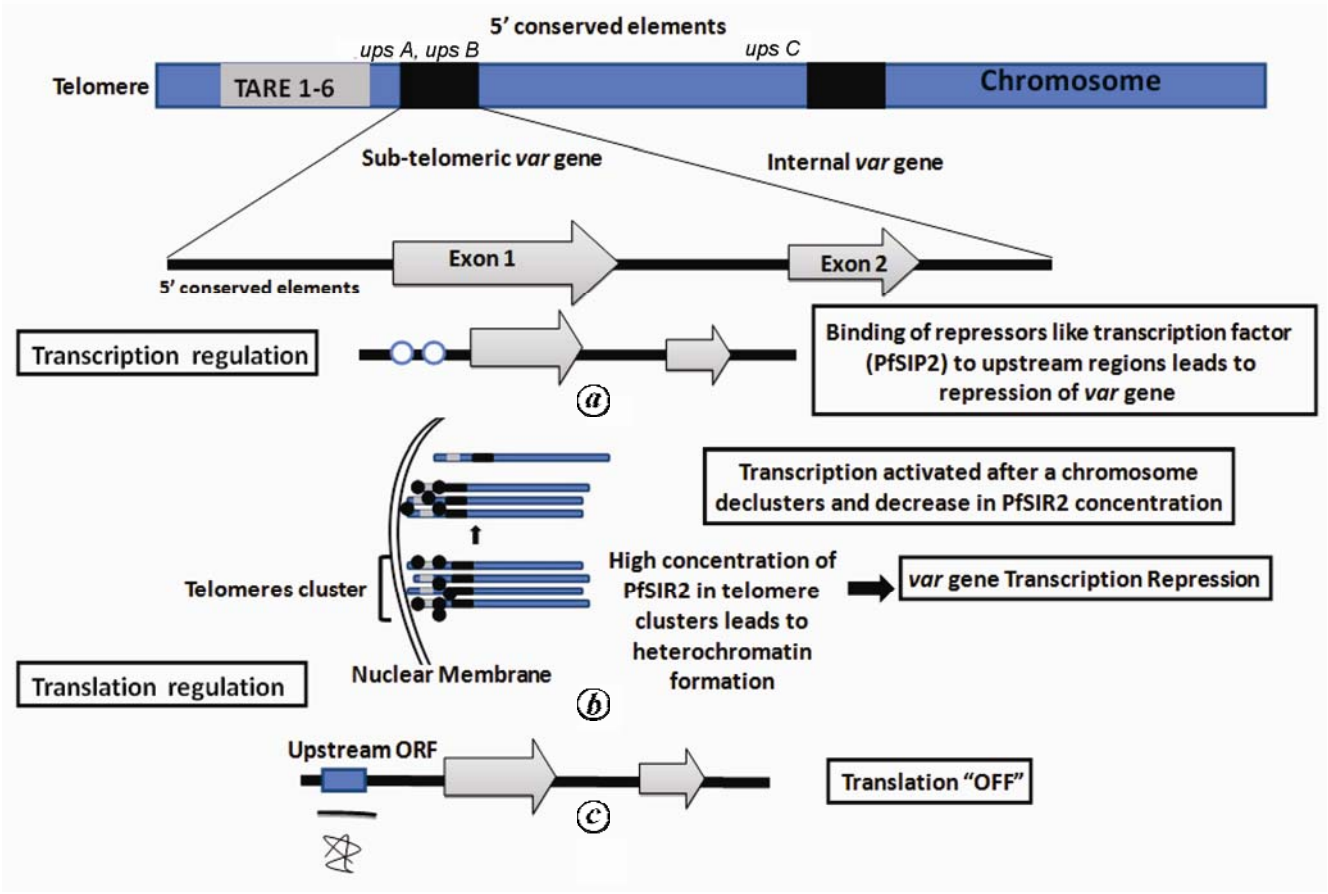

Figure 2. Mechanisms of *var* gene expression regulation in *P. falciparum*. Polymorphic *var* genes are located either at the sub-telomeric or internal positions of the chromosome. They have a two-exon structure along with 5′ upstream elements (*upsA, B, C*). The expression regulation of *var* genes occurs either at transcription or translation level. ***a***, Transcriptional regulation is by binding of repressor elements such as PfSIP2 (sub-telomeric *var* gene promoter element interacting protein, an ApiAp2 transcription factor) to conserved upstream regions. ***b***, Heterochromatin formation due to higher concentration of PfSIR2 (silent information regulator, a histone deacetylase) is also found to be involved in repression of *var* genes at transcription level. Additionally, clustering of telomeres at the nuclear membrane also correlates with expression of *var* genes. ***c***, Translation suppression is due to translation of a small ORF located upstream of the *var* gene.

in the expression of antigenic variants of PfEMP1 proteins to avoid splenic clearance by the human host[30,31]. The mechanism of switching is being elucidated and recent studies have implicated the role of the promoter in the generation of mutually exclusive expression[32,33]. Analysis of the *var* gene transcriptome in the asexual stages shows that most of the transcription is carried out at the trophozoite stage, while there is a leaky expression during the ring stage. Several studies demonstrate that the regulation of expression of the *var* gene family occurs at the transcription initiation level[29,34]. However, the exact mechanism, through which expression of one *var* gene at a time is ensured, is not yet fully understood.

Most *var* genes are located in the sub-telomeric region of each chromosome, whereas some are found in central regions. The sub-telomeric and central *var* genes differ in distinct promoter regions (*upsA*, *B* and *C*)[4,35], as shown in Figure 2. A member of the ApiAP2 family, PfSIR2 has been shown to bind to the sub-telomeric SPE2 DNA motif array situated upstream of a sub-telomeric *var* gene. PfSIR2 is shown to exert *var* gene silencing through heterochromatin formation[26]. Some epigenetic regulatory mechanisms are also involved in controlling *var* gene switching and regulation[36]. SIR2 protein is a histone deacetylase and it induces condensation of chromatin leading to transcriptional repression. PfSIR2 binds to the telomere and the sub-telomeric region leading to transcriptional repression of the *var* gene[37,38]. Histone acetylation has also been associated with *var* gene expression[39]. It has also been suggested that transcriptional activation of the sub-telomeric *var* genes requires exit of the *var* gene loci from a telomeric cluster found at the nuclear periphery[38]. More recently, an upstream ORF of a *var* gene was found to repress the downstream *var* gene[40]. Therefore, multiple checks seem to be regulating the expression of *var* genes, as shown in Figure 2. Other gene families such as *rifin* and *stevor* also code for polymorphic proteins, but their mechanisms of regulation are still not clear[41].

In vivo transcriptome

The work described so far deals with transcription of *P. falciparum* genes in laboratory cultures. Besides this, the *in vivo* transcriptome of the parasite under the influence of host responses has also been studied. Microarrays have been used to reveal the gene expression pattern of *P. falciparum* parasites *in vivo*[42–44]. A recent study describes the *in vivo* transcription profile of *P. falciparum* isolates from 43 Senegalese patients[45]. The analysis proposed that the parasite might exist in previously uncharacterized transcriptional states *in vivo* and utilize different metabolic strategies in the human host depending upon external factors as oxygen, energy source and host environment.

The parasite genome and transcription as drug targets

As mentioned earlier, *P. falciparum* has a highly AT-rich genome. This AT-richness of the genome has been used to target the parasite with chemical agents that show high affinity for AT-rich DNA, for example, distamycin A[46], adozelesin and bizelesin[47]. Adozelesin and bizelesin are toxic to tumour cells as they bind to AT-rich regions of the genome called 'AT islands'[48]. These molecules are under clinical trial as anti-tumour drugs. Recently, it has been shown that adozelesin and bizelesin are also effective against cultures of *P. falciparum* with IC_{50} in the picomolar range[49].

Many evidences in *Plasmodium* point to gene modulation by histone deacetylases (HDACs), resulting in chromosome condensation and transcription repression. The histone deacetylases, PfHDAC1 and PfSIR2, have been characterized in *P. falciparum*[50]. PfHDAC1 was detected throughout the asexual intra-erythrocytic cycle and in the exo-erythrocytic stages. PfSIR2 co-localizes with telomeric clusters generating heterochromatin at chromosome ends. In addition, PfSIR2 binding and deacetylation control the mutually exclusive expression of the surface antigen family encoded by the telomeric *var* genes. The cyclic tetra-peptide drug apicidin that inhibits HDACs, shows de-regulation of transcription repression during the asexual stages of parasite development[51]. This study demonstrated HDACs as potential candidates for chemotherapeutic development in the malaria parasite. Phenylthiazolyl-bearing hydroxamate-based HDAC inhibitors have also been shown to exhibit antimalarial activity in the micromolar range[52] with IC_{50} ranging from 0.0005 to >1 μM.

Post-transcriptional modifications of RNA: capping, splicing and polyadenylation

Transcriptome studies of *P. falciparum* have shown that during the erythrocytic stages, 80% of the total genome is transcriptionally active and in these stages, the transcription of stage-specific genes occurs only when the protein products of these genes are required[53]. This mode of regulation is thought to resemble those of the early developmental stages of *D. melanogaster*, where over 80% of the genome is transcriptionally active and protein expression is controlled post-transcriptionally; *cis*-acting elements in the transcripts regulate the translation of the mRNA in a stage-specific manner[54]. In this regard, studies in *P. berghei* show that U-rich, *cis*-acting elements in the 5′ and 3′ UTRs of mRNAs act as translation repressors[55]. It has been shown that these translation repression elements are important for *P. berghei* and *P. falciparum* zygote development[56,57]. These studies led to the hypothesis that regulation of gene expression in *P. falciparum*

includes mechanisms operating at the interface of transcription and translation. Indeed, post-transcriptional regulation provides an additional layer in the regulatory network of eukaryotes for fine-tuning protein levels required for various cellular processes. Some examples of post-transcriptional modifications of mRNAs include 5′ capping, splicing and poly-adenylation.

When mRNAs are synthesized, they acquire a 7-methyl guanosine (m7G) cap in the nucleus[58]. This methylation has many important roles in mRNA splicing and stability, nuclear export and most importantly, translation initiation[59,60]. *P. falciparum* has similar capping enzymes to that of fungi and plants, where three different enzymes encoded by different genes carry out this m7G capping of mRNAs[61]. Translation initiation of mRNA occurs with the recognition of the m7G cap by a translation initiation factor known as eIF4E. Interestingly, when capped mRNAs was purified using eIF4E protein, a significant amount of mRNAs was found to be under-represented in the purified fraction[62]. This under-representation of some mRNAs in the purified fraction suggests that these mRNAs are preferentially devoid of the m7G cap. An example of such an uncapped mRNA is *Pfmdr1* (*P. falciparum* multi-drug resistance protein 1). An orthologue of *Pfmdr1* in *P. berghei* has been shown to be stored in female gametocytes in a translationally repressed form[56]. These observations suggest that in *Plasmodium* species, uncapped mRNA might be a strategy for mRNAs to remain translationally repressed and translated only when required.

Not only the 5′ end, but also the 3′ end of mRNA participates in translation regulation. For example, in the *Xenopus* oocytes the mRNA of cyclin B1, which is a major regulator of oocyte maturation, is kept in a dormant state by keeping its polyA tail short[63]. Similarly, in malaria parasites, sequences present in the 3′ UTR region of *Pfs25* mRNA have been reported to influence translation[64]. In addition, this study also identified polyadenylation sites in *Pfs25* mRNA, and strangely some of these sites were in the coding region of the gene. Similar to this, when genes of *Bacillus thuringiensis* are expressed in plants, they show polyadenylation in the coding region of the genes. This premature polyadenylation is responsible for the low expression levels of these transgenes[65]. These observations when taken together strongly support the significance of post-transcriptional regulation of gene expression in *P. falciparum*.

Splicing

Splicing is the process whereby introns are removed and exons are joined together to give mature mRNAs that are used by the translation machinery to synthesize functional proteins. Around 54% of the genes of *P. falciparum* are predicted to contain introns, and this number is higher than in budding yeast, where only 5% of the genes have been shown to have introns[4]. The splicing machinery consists of a spliceosome, which is comprised of two types of molecular components, viz. proteins and RNAs. The RNAs that take part in splicing are known as small nuclear RNAs (snRNAs), as they are localized to the nucleus. Together, these snRNAs and proteins form small nuclear ribonucleoproteins (snRNPs), which upon maturation generate functional spliceosomes. In one of the early studies on parasite spliceosomes, snRNPs of *P. falciparum* were immunoprecipitated using sera from patients with an autoimmune disease that causes them to generate antibodies against self snRNPs[66]. Recently, the relative GC-rich nature of snRNAs compared to the AT-biased base composition of the genome was exploited in identifying *P. falciparum* snRNAs[67]. Furthermore, it has been shown that these snRNAs can fold into the same overall conformation as snRNAs from other organisms in order to assemble functional spliceosomes[68].

P. falciparum possesses mRNA capping machinery similar to that present in fungi and plants, and significantly different from the mammalian capping machinery[61]. Additionally, similar to other eukaryotes, snRNAs of *P. falciparum* have 5′ and 3′-end modifications[69,70]. *P. falciparum* snRNAs are capped at the 5′-end with a hypermethylated cap where two more methyl groups are added to the m7G cap[69]. This process of hypermethylation is carried out by an RNA hypermethylase known as trimethylguanosine synthase (TGS1) in budding yeast[71]. It has been shown that TGS1 adds two methyl groups to m7G-capped snRNA using S-adinosyl methionine (S-AdoMet) as a methyl group donor[72]. This RNA hypermethylase is conserved in all eukaryotes and is well-characterized in yeast and humans. A homologue of yeast and human TGS1 is found in *P. falciparum*[73]. The methyltransferase domain of TGS1 in most metazoans is located at the C-terminus of the protein; however the putative *P. falciparum* TGS1 (PfTGS1) has an N-terminal methyltransferase domain with amino acid insertions and an extended C-terminal domain which is predicted to have trans-membrane domains[69]. TGS1 is essential for development in budding yeast and *Drosophila*[74]. Similarly, this protein may play a role in the regulation of splicing in *P. falciparum* development.

The hypermethylation of snRNAs by TGS1 protein is inhibited by sinefungin. Sinefungin, an analogue of S-AdoMet, has been shown to inhibit replication of West Nile virus and Dengue virus[75]. The TGS of Mimivirus, which infects amoebae, has also been shown to be susceptible to sinefungin[76]. As pointed out previously, the parasite TGS1 protein is vastly different from the human TGS1 protein and this difference can be exploited to discover sinefungin analogues that specifically inhibit the methylation of snRNA molecules in parasites. Interestingly, older studies on sinefungin show that this S-AdoMet analogue inhibits methylation reactions in *P. falciparum* also[77], and later studies have shown that the arrest

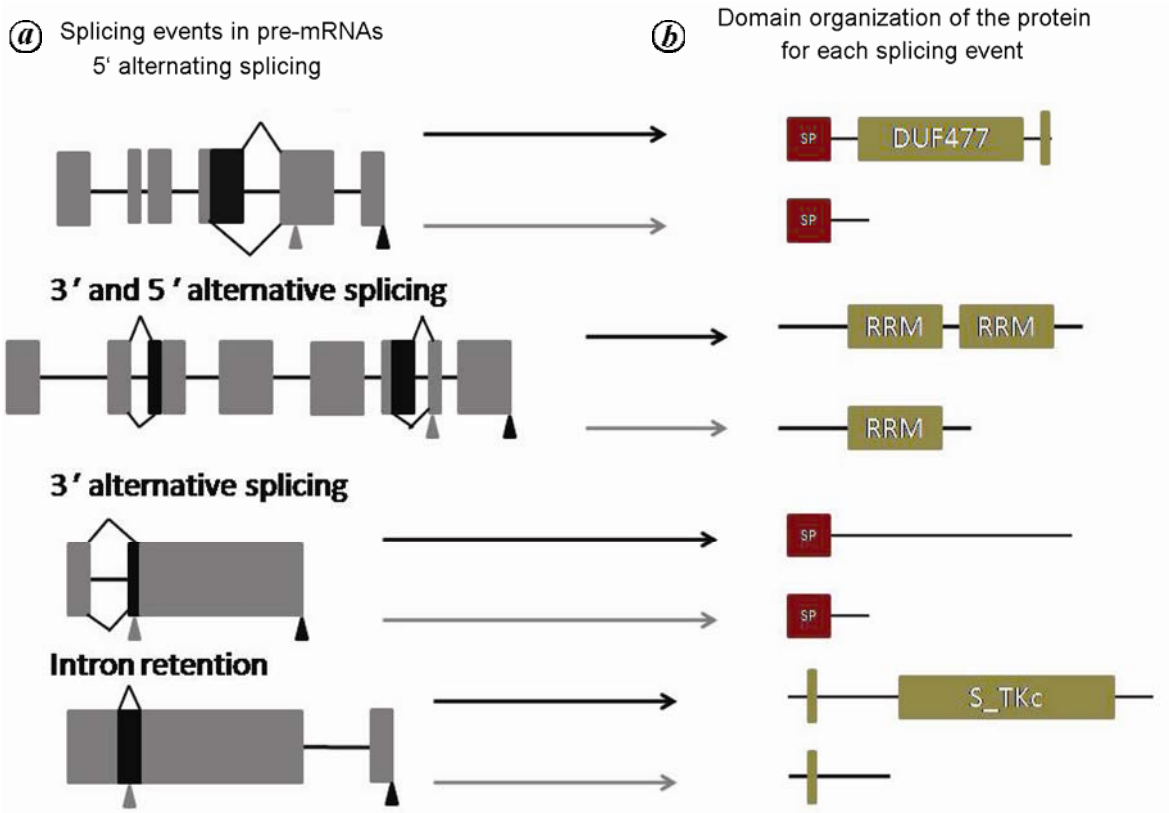

Figure 3. ***a***, Some examples of different types of alternative splicing. Exon–intron boundaries in the genes are shown; grey boxes indicate exons and lines indicate introns. Part shown in black boxes indicates the region which is alternatively spliced out. Black arrows indicate stop codons and grey arrows indicate stop codons generated after alternative splicing. ***b***, Domain architecture of the proteins produced as a result of splicing and alternative splicing. SP, Signal peptide; RRM, RNA recognition motif; S_TKc, Serine/threonine protein kinase; ▮, Transmembrane domain (adapted from Iriko *et al.*[83]).

of *P. falciparum* growth at the trophozoite stage by sinefungin may be due to a block in DNA synthesis[78]. It would be of interest to test whether sinefungin also inhibits parasite TGS1 activity. Inhibitors of methyltransferases, particularly TGS1 that is known to be essential for development in yeast and *Drosophila*, may hold promise for lead compounds that inhibit parasite division and development.

Alternative splicing

Many *P. falciparum* mRNAs are shown to have alternative splice variants[79,80]. Some of these events might also be post-transcriptional control mechanisms, as these alternative splicing events generate premature stop codons in the transcripts in most cases[80]. These alternative splicing events include alternative 5′ splice sites, 3′ splice sites, both 5′ and 3′ splice sites, and intron retention/creation of the analysed genes. Interestingly, along with these splicing events, some genes also show splicing in 5′ and 3′ un-translated regions (UTRs)[79,80].

Serine/arginine (SR)-rich splicing factors, which mediate spliceosome assembly on pre-mRNAs, are important in alternative splicing[81] and one such factor has been identified in *P. falciparum* – PfSR1. It has been demonstrated that when phosphorylated by the PfSRPK1 kinase, PfSR1 loses its affinity for RNA and hence might prevent splicing of pre-mRNAs[82]. Thus, alternative splicing can exercise control over the type of gene product expressed.

Additionally, it has been seen that in *P. falciparum* alternative splicing events commonly result in different domain architectures of the same protein from the same gene, which in turn affect the localization of that protein in different sub-cellular compartments, and/or the functions of that protein[83]. This has been summarized as a schematic in Figure 3. These instances suggest that alternative splicing might be a way of regulating protein activity, in addition to generating protein diversity.

Translation

As we have already seen, gene expression can be regulated to an extent, at the levels of transcription, mRNA stability and splicing; the final step of gene expression is translation which can also be regulated and is highly dissimilar to the biology of the human host[84–86].

In *P. falciparum*, several studies have been carried out to understand the mechanisms of transcriptional regu-

lation; however, much less is known about the mechanisms of translational regulation of gene expression. A recent review by Jackson *et al.*[87] provides a fairly comprehensive view of translation in the parasite. Here, we emphasize regulation at the level of translation, with a take on the distinctive phenomenon of delayed death.

Translation refers to the process of synthesis of protein from mRNA, and involves three major steps – initiation, elongation and termination. The parasite possesses three distinct intracellular compartments believed to house functional protein translation machinery: the apicoplast, mitochondrion and cytosol. The former are organelles thought to be acquired by endosymbiosis events, and they are nestled in the cytosol[88,89]. These organelles harbour prokaryotic components for mRNA translation, whereas the cytosol possesses the eukaryotic counterparts of the same[89,90]. The differences in the origin of the translational machineries present us with three distinct potential targets for inhibition.

Translational machinery in P. falciparum

The translation machinery in the parasite comprises tRNAs, tRNA synthetases, tRNA ligases and translation factors, which together control the initiation, elongation and termination steps. An interesting observation about parasite translation is that only nuclear and apicoplast (and not mitochondrial) genomes code for tRNAs and translation factors[4,91–93]. The nuclear genome of *Plasmodium* codes for a full set of rRNAs, tRNAs, ribosomal proteins and 37 tRNA synthetases, all of which fulfil all the requirements needed for translation in the cytosol[4,94].

Another interesting feature of the parasite translation machinery is efficient translation despite an unusually AT-rich genome[4]. In addition to the AT-rich genome, it has been shown that *P. falciparum* mRNAs have longer untranslated regions (UTRs) than other eukaryotes, with an average length of ~350 bases[95]. Due to these two exceptional features, the frequency of AUG in the 5′ UTRs of *P. falciparum* mRNAs is 6 per 500 bases. Comparatively, in most eukaryotes, half of the mRNAs have no AUG in the 5′ UTRs[94]. Thus, in the parasite, the choice of a translation initiation site (TIS) by the ribosomal machinery is a critical step in translation. A few studies have been attempted to understand the mechanism by which a TIS is decided upon. This includes work by Saul and Battistutta[96], who proposed the optimal sequence context surrounding the TIS (shown as aug) is AAAAaug; Yamauchi[97] has proposed a longer consensus (AAAAUUUUUAAAAUUUAAAaugANAU) for protozoans, including *P. falciparum*. These studies were based on bioinformatics approaches and simple statistical analysis. Recently, Patakottu *et al.*[98] have developed an algorithm to predict TIS in the mRNA of asexual stages of *P. falciparum*. Experimental validations of sequence features have defined the optimal sequences required for translation initiation in the asexual stages of the malaria parasite[99].

Organellar translation in P. falciparum

Translation factors required for protein synthesis in the apicoplast and mitochondrion have been reported in *P. falciparum*. These translation factors include initiation, elongation and termination factors which all are coded by the nuclear genome, with elongation factor Tu (EF-Tu) being the only exception, as it is coded by the apicoplast genome[91]. The cytosolic factors that have been cloned from *P. falciparum* cDNA include eIF4E, eIF4A, eIF4F and poly-A binding protein PABP[62,91,100,101]. Translation factors destined for the organelles show significant differences with respect to their cytosolic counterparts as suggested by their prokaryotic origin.

Unlike the nuclear genome, the apicoplast genome is equipped with genes coding only for rRNAs, tRNAs, ribosomal proteins and the elongation factor EF-Tu, while the nuclear encoded tRNA synthetases and other essential factors required for translation are imported[91,93,102,103]. The identification of polysomes carrying plastid-specific mRNA and rRNAs[104], and the confirmed localization of plastid-origin EF-Tu to the apicoplast[91] support the hypothesis of a translationally active apicoplast.

Another site of translation is the mitochondrion, which has the smallest genome known yet (6 kb) that encodes only three proteins – cytochrome *c* oxidase subunits I, III, cytochrome *b*[92] and some fragmented rRNA genes[92]. Recent experiments show that point mutations in the *cytb* gene of the mitochondrial genome in *P. chabaudi chabaudi* confer resistance to the drug, atovaquone, which targets the cytochrome *bc* 1 complex[105]. This is a sign that the gene products of the mitochondrial genome are almost certainly expressed. To achieve all its translational needs, the mitochondrion relies heavily on nuclear encoded components for translation, and is thought to import all proteins and tRNAs required.

As described earlier, the parasite possesses only 37 amino acid tRNA synthetases, as compared to more than 60 observed in other eukaryotic systems. This suggests that some tRNA synthetases might be operating at more than one sub-cellular location simultaneously, viz. cytosol and organelles (apicoplast and mitochondrion). As expected, 23 out of the 37 amino acid tRNA synthetses have signal peptides possibly targeting them to different sub-cellular localizations[102]. Recently, Kehr *et al.*[106] have shown that alternative translation initiation in *P. falciparum* can generate cellular isoforms of antioxidant proteins. Indeed, many *P. falciparum* proteins have been shown to contain large N-terminal extensions, which might arise from the use of alternative translation start sites. In the same manner, mRNAs belonging to single-copy tRNA synthetases might generate cellular

isoforms[106]. Surprisingly, no amino acid tRNA synthetase has been predicted to be mitochondrially targeted[102]. Several amino acid synthetases have been shown to possess unusual protein domains like Ser–Thr kinase and DNA-binding domains. Additionally, these amino acid tRNA synthetases show evolutionary relatedness to bacterial and plant counterparts[102]. These unusual properties of amino acid tRNA synthetases mark them attractive drug targets.

Organellar translation as a drug target

As discussed earlier, both the mitochondrion and the apicoplast are endosymbiotic organelles believed to be intimately linked, structurally and functionally[107], and are both located in the cytosol. These organelles, true to their proposed origin, possess many features expected in an independent organism, such as self-replicating genetic material and local biosynthetic pathways.

The apicoplast is thought to have been assimilated by the secondary endosymbiosis of a red algal cell[108]. It is accordingly encapsulated in four membranes[109]. It is a relict plastid that has lost its photosynthetic ability. However, the importance of this organelle in the members of the phylum Apicomplexa was definitively established with ciprofloxacin treatment in *Toxoplasma gondii*, which blocked the replication of the apicoplast genome and led to parasite death[110]. We know now that the apicoplast is involved in the synthesis of precursors and metabolites of heme, fatty acid, isoprene and iron–sulphur cluster pathways, involving at least 30 gene products encoded by the 35 kb circular genome of the plastid, and over 540 NEAT (Nuclear Encoded Apicoplast Targeted) gene products[88,93].

The mitochondrion, an organelle we are seemingly better acquainted with, is in fact far less understood in *P. falciparum*. A recent breakthrough indicates that the tricarboxylic acid cycle established in this organelle is possibly divided into oxidative and reductive branches[111]. Also, this organelle shares a joint heme biosynthesis pathway with the apicoplast, where the intermediates shuttle between both organelles[88]. As of now, the mitochondrion is known to play one major role in the metabolism of this organism with its involvement in pyrimidine biosynthesis. Dihydroorotate dehydrogenase, regenerated by coenzyme Q, catalyses a significant reaction in this pathway. It is currently believed that the electron transport chain of the parasite in the asexual stages is maintained at a basal level solely for the regeneration of coenzyme Q[112].

Although progress is slow, and several aspects of translation in the parasite are as yet poorly understood, the awareness that these organelles that house so many intriguing features unique to *Plasmodium* possess translation machinery that is handled by prokaryotic components, obviously makes them lucrative drug targets considering we already have an arsenal of available antibiotics at our disposal. The apicoplast seems particularly interesting considering that no equivalent organelle exists in the human host, and the mechanism of translation is better understood than in the mitochondrion.

Translation inhibitors lead to delayed death

Treatment with a series of prokaryotic translation inhibitors, such as clindamycin and tetracyclines, highlighted a very unique trend. This phenomenon, now universally referred to as ‘delayed death’, had been reported as early as 1985, when Divo *et al.*[113] named a ‘second-cycle effect’ based on a remarkable rise in the sensitivity of parasites upon exposure to certain drugs over a span of 96 hours, while the same parasites had remained morphologically normal during exposure for the first 48 h. The drugs used in this study were inhibitors of the 70S ribosome. We know now that at clinically relevant concentrations, delayed death is observed even if drug exposure lasts only for the first cycle. That is, death occurs in the generation succeeding that of the parasites actually exposed to the drug.

Before the discovery of the apicoplast, the phenomenon was thought to be a result of inhibitor effects on the mitochondrion, that being the only known hub of prokaryotic components in the parasite[114]. This notion was first challenged by Fichera and Roos in 1997, and fully laid to rest in 2001 when He *et al.*[115] demonstrated that apicoplast-deficient *T. gondii* parasites, produced by plastid segregation mutants, showed a classic delayed-death phenotype.

Jackson *et al.*[87] have listed, in their review, known translation inhibitors and their targets in *P. falciparum*. Almost all prokaryotic translation inhibitors are known to cause delayed death. Studies on translation inhibitors that do not cause delayed death invariably indicate the existence of targets other than translation components in the cell. For instance, thiostrepton is a drug that targets the 70S ribosome, but does not cause delayed death[116]. Suspicions that the drug has other targets was confirmed when it was shown to be inhibitory to the 20S proteasome[117].

Although the exact cascade of events leading to delayed death is unknown, relevant hypotheses have been put forward[118]. Briefly, all through the first cycle, that is 0 to 48 h, the apicoplasts already possess functional import machinery distributed to them during the previous cycle. Inhibition of protein synthesis in the apicoplast during this first cycle, by the addition of appropriate antibiotics, may result in the insufficient synthesis of membrane complexes involved in the import of nuclear-encoded proteins. As a result, the daughter apicoplasts that arise from the division of these first-cycle apicoplasts

are deficient in import machinery. Blocked import means essential biosynthetic proteins would not have reached their pathways of action in the apicoplast. This has been observed with PfFabG, a NEAT protein[119]. This causes the arrest of the next generation of parasites in the schizont stage as they fall short of the biosynthetic products required for division.

This hypothesis has been bolstered by the observation that treatment with 15-deoxyspergualin, which inhibits trafficking of NEAT proteins to the apicoplast, results in the formation of dysfunctional apicoplasts in the second cycle, leading to delayed death[119].

There has been a recent explosion in the identification of lead compounds that can be tweaked to function as effective antimalarials. The number of prospective molecules that inhibit translation is most promising at this crucial juncture, where we are just beginning to understand the full potential of organellar translation as a drug target.

Conclusion

P. falciparum parasites have to make choices regarding which gene to express at which point in their complex life cycle. In this review, we have summarized the current knowledge on how this important human pathogen regulates gene expression; it should be apparent that *P. falciparum* uses strategies of transcriptional, post-transcriptional and translational control that show many similarities to those employed by other eukaryotes and also many differences. Genome bias towards AT nucleotides, a 'just in time' mode of transcription over the intra-erythrocytic cycle, proteins with unusual features such as insertions in their active sites and organelles with translation machinery similar to prokaryotes are just some of the differences between the parasite and its human host. In addition to contributing to the understanding of basic biology, these differences are already being exploited to discover anti-malarial compounds that might be able to target the parasite with high specificity and some examples of such compounds (apicidin, sinefungin, tetracyclines and clindamycin) have been discussed in each section of this review. The recent availability of thousands of lead molecules from the GlaxoSmithKline chemical library[120] has already shown the way in identification of antimalarial compounds that target several essential biological processes. We propose that regulation of gene expression may be one such process that could be effectively targeted towards the control of this disease.

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