Journal of Advances in Science and Technology

Vol. V, Issue No. X, August-2013, ISSN 2230-9659

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AN INTERNATIONALLY INDEXED PEER REVIEWED & REFEREED JOURNAL

www.ignited.in

Journal of Advances in Science and Technology
Vol. V, Issue No. X, August-2013, ISSN 2230-9659

Legislation of Gene Appearance in Biology of P. Falciparum: An Integrative Investigation on Coming into the Post-Genomic Period of Malaria Research

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Abstract – The sequencing of the genome of Plasmodium falciparum promises to revolutionize the way in which malaria research will be carried out. Beyond simple gene discovery, the genome sequence will facilitate the comprehensive determination of the parasite's gene expression during its developmental phases, pathology, and in response to environmental variables, such as drug treatment and host genetic background. This article reviews the current status of the P. falciparum genome sequencing project and the unique insights it has generated. We also summarize the application of bioinformatics and analytical tools that have been developed for functional genomics.

The aim of these activities is the rational, information-based identification of new therapeutic strategies and targets, based on a thorough insight into the biology of Plasmodium spp.

Plasmodium falciparum malaria is one of the most lethal infectious diseases afflicting humanity. During development within the erythrocyte, P. falciparum induces significant modifications to the structure and function of the human erythrocyte membrane. This study focused on the identification of new protein-protein interactions between host and parasite.

The intraerythrocytic development of Plasmodium falciparum, the most virulent human malaria parasite involves asexual and gametocyte stages. There has been a significant increase in disparate datasets derived from genomic and post-genomic analysis of the parasite that necessitates delivery of integrated analysis from which biological processes important to the survival of the parasite can be determined.

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, posttranscriptional and translational regulation in P. falciparum, and how this works for us in drug development.

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INTRODUCTION

The development of genome sequencing technologies over the last five years has resulted in a wealth of sequence information, culminating in the recent announcement of a working draft of the human genome. Pathogen genomes, through their smaller size, have been even more tractable to these methodologies and are now well represented in genome science. Although not always suited as ‘‘model’’ organisms, the importance of pathogens in medicine and agriculture has made the exploitation of the sequence databases a high priority. But what does the production of long strings of As, Cs, Gs and Ts actually mean in terms of the alleviation of the burden of disease, particularly in developing countries? So far, genome sequencing has largely been the province of the developed world, where the resources and science infrastructure have allowed the formation of high-throughput sequencing centres. However, the use of sequencing information need not be restricted in this way, provided that resources for training can be met.

Probably the most important aspect of the post-genomic era (i.e. after the sequencing has been carried out) is analysis of the primary sequence data. This is called bioinformatics and embraces a range of theoretical analyses aimed at converting the DNA sequence into biological information. A major part of

this discipline involves the identification of genes through a number of processes, from identifying

similarities with previously identified genes from other organisms, to the use of computer-derived models based on existing data. Subsequently come predictions of biological function and molecular shape (structural genomics), both of which have scope for development.

Knowing the whole sequence of a pathogen genome also allows researchers to investigate the behavior of organisms on a much broader basis than was previously possible. Now, instead of studying the effect of drug treatment or differentiation on one or two genes, it is possible to study variation in all the genes at the same time using global transcriptional analysis. Protein patterns may also be examined, or the organism may be genetically modified (transfection), providing a direct link between these biological effectors and gross phenotype. The technology for these experiments has been developed as a direct consequence of the desire to exploit the genome sequence data.

It is not hard to envisage that the ability to identify and characterize the genetic blueprint of pathogens will help us recognize critical elements in the development and pathogenesis of disease causing organisms and target our research efforts in the production of new therapies. For Plasmodium falciparum, genes and proteins acting at specific stages in the life cycle can be identified, their roles tested by genetic modification and promising candidates used in vaccine production. Parasite metabolic pathways not present in the host could also be targeted with potent inhibitors that are non-toxic to humans; and parasite drug-resistance mechanisms could also be targeted, giving existing drugs a longer effective lifetime. The sequence of the P. falciparum genome will provide many opportunities for research into malaria, but this is only a beginning, with the challenge being to turn those opportunities into effective treatments in the field.

The completion of the *Plasmodium falciparum* (clone 3D7) genome sequence has provided a comprehensive blueprint for analyzing gene expression and protein function in the malaria parasite.1 Biological profiling of the 5,268 predicted *P. falciparum* proteins has resulted in the functional classification of about 30% of the parasite's proteome. Assigning function to parasite proteins with no known protein homology (i.e. hypothetical proteins) and refining the mode of action of putative proteins have been aided by investigating protein-protein interactions involved in malaria cell biology. The association of *P. falciparum* proteins with the erythrocyte membrane has initiated widespread interest in defining new proteins that interact at the host-parasite interface. Despite our current understanding, many questions remain unanswered concerning this dynamic interplay.

Protein 4.1 (4.1R) is a peripheral protein involved in maintaining the structural integrity of the erythrocyte membrane through its binding with spectrum and actin. *P. falciparum* requires 4.1R for optimal parasite growth and viability. Specific 4.1Rparasite protein interactions that play a role include the mature parasite-infected erythrocyte surface antigen (MESA), which binds 4.1R at a 51 residue region within the 30-KDa domain. In addition, Hans pal *et al*. have shown that falcipain-2, a *P. falciparum* cysteine protease, cleaves native and recombinant 4.1R within the spectrin-actin binding domain. Proteolysis of 4.1R results in destabilization of the membrane skeleton, which would assist in the release of merozoite from erythrocytes into the circulation.

The phosphorylation status of 4.1R is altered during erythrocyte parasitism by unidentified parasite kinases, which may affect the biophysical properties of the host membrane and in so doing, support parasite growth.

Malaria is one of the most severe infectious diseases worldwide. It kills 2–3 million young children each year. An effective vaccine is not yet in sight, and resistant parasite strains limit the use of affordable drugs in many endemic areas. The sequencing of the genomes of *Plasmodium falciparum* and of the mosquito vector *Anopheles gambiae* provides the basis for novel molecular approaches to the development of new malaria control strategies. To facilitate an interdisciplinary and concerted effort, a first conference on 'Molecular Approaches to Malaria' was held in Lorne, Australia, in February 2000, attracting more than 250 scientists from all over the world.

Following on from this initial success, a second conference (MAM2004) was held in February 2004 at the same venue attracting 358 scientists, clinicians and epidemiologists, including many from malaria-afflicted countries in Africa, South America and Oceania.

A declared goal of the meeting was to bridge gaps between different disciplines in order to facilitate the development of new tools to combat malaria. In a presentation by David Roos (University of Pennsylvania, Philadelphia, USA) who spoke on behalf of the *Plasmodium* Genome Database Collaborative, it became clear that malaria research has benefited and will continue to benefit from publicly accessible genome, transcript me and proteome databases which form the basis for modern high throughput technologies such as microarray analyses. In the following, we summarize the major topics covered at the meeting, ranging from basic research on the molecular and cellular biology of malaria in animal models and *in vitro*

systems through to applied aspects, including advances in the understanding of the molecular

mechanisms underlying pathogenesis and the identification and characterization of drug targets.

MALARIA has afflicted humans for centuries. Hippocrates, the father of modern medicine, is believed to have described tertian and quart an fevers (the hallmark of malaria infection) as early as the third and fourth centuries BC1. Despite this long association of the malaria parasite and its human host, the causative agent of the disease was identified2 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.

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 conditions3 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 date4. 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. 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 anti-malarial drugs and attempts at drug discovery based on regulation of gene expression in the parasite will be briefly touched upon.

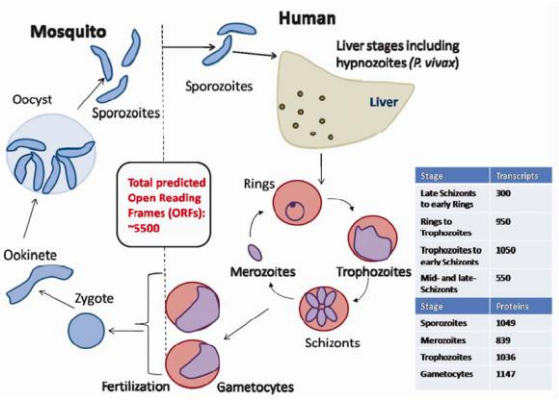

Figure 1. *Plasmodium* life cycle and expression of transcripts and proteins at different stages of the life cycle. The human and mosquito hosts are demarcated and developmental stages of the parasite shown. Note that this is a schematic of the life cycle, is not drawn to scale and merely serves as a 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.

THE PLASMODIUM FALCIPARUM GENOME PROJECT CONSORTIUM

Prior to the advent of yeast artificial chromosome (YAC) technology, there had been relatively little access to the parasite's genome, as its extreme [A+T] content rendered inserts unstable in conventional bacterial plasmid clones. The estimated 80% [A+T]-rich genome could, however, be stably maintained within the pYAC4 construct, as demonstrated by the construction of a number of YAC libraries for different P. falciparum clones . In 1993 a consortium of laboratories distributed throughout the world established the Wellcome Trust Malaria Genome Mapping Project, with the aim of assembling YAC contiguous sequences (contigs) across each chromosome, as well as developing YAC, expressed sequence tag (EST), bioinformatic and genetic mapping technology. This consortium realized that sequencing of the entire nuclear DNA was a real possibility, yet considered the endeavour fraught with difficulties due to the extreme bias in base content.

Genome sequencing has proved to be a powerful and efficient approach in accessing the complete gene complement for organisms as diverse as Mycobacterium tuberculosis , Saccharomyces cerevisiae and Caenorhabditis elegans. The advantages offered by such a tool in the investigation of human malaria eventually resulted in pilot projects being established in 1996 at three high-throughput genome centres, to establish whether sequencing the entire genome was possible: the Sanger Centre (England); The Institute for Genomic Research (TIGR) Malaria Program, Naval Medical Research Centre (NMRC) (USA); and Stanford University (USA). The Wellcome Trust, the Burroughs Wellcome Fund, the National Institute of Allergy and Infectious Diseases and the US Department of Defence provided funding for the pilot projects, and following their success these agencies agreed to fund the entire sequencing effort. Associated with this work are a number of other groups supporting the efforts of the high-throughput centres in a range of activities, including generation of chromosomal material; additional mapping information; testing bacterial strains more tolerant of [A+T]-rich DNA; and the provision of a repository for P. falciparum reagents.

A similar strategy is being used by all of the highthroughput sequencing centres, with individual chromosomes being excised from pulse-field gels, cloned as small inserts into a double-stranded vector, and sequenced in the forward and reverse directions to generate read-pair information which is used in gap filling. Sequence-tagged site and simple sequencelength polymorphism microsatellite markers from the HB3xDd2 genetic cross (7), together with the optical map (8) of ordered restriction fragments, are used to position contigs on each chromosome, or to confirm that sequence data has been assembled correctly. In addition, to help assign and order sequences originating from a particular chromosome region, the groups at the Sanger Centre and Stanford University use a shotgun skim (1–2 fold coverage) of YAC clones selected from the chromosomal YAC contigs, generated by the original P. falciparum mapping project. All three sequencing centres aim for an error rate of less than 1 base in every 10 000 bases.

Once a section of chromosome sequence is finished it is analysed to identify a number of features, including putative protein-coding regions, tRNAs and repetitive sequences. Database searches are performed to identify similarities to protein and EST sequences. Further analyses are performed to determine whether the predictions have protein domains, signal sequences, putative membranespanning regions or any other distinctive features.

A number of computing tools such as Hexamer/ Genefinder (R. Durbin, P. Green, L. Hillier, unpublished software, 1998)), and Glimmer M are available to assist in the identification of protein coding regions, but many other gene prediction programs exist or are currently being developed.

These tools are efficient at identifying single, large, open reading frames (ORFs) or genes with two or three relatively large exons. However, the current generation of gene prediction software produces conflicting data when predicting multi-exon genes, particularly those with small exons. These conflicting gene predictions are currently being tested experimentally by reverse-transcription polymerase chain reaction (RT–PCR) assays. Annotation of current and future P. falciparum sequences is therefore an ongoing process, with predictions and annotations being refined as more information, such as the RT–PCR and EST sequencing data, becomes available.

Chromosome	Size (Mb)	Status[a]
14[b]	3.4	Late Closure
13[c]	3.2	Closure
12[d]	2.4	Late Closure
11[b]	2.4	Closure
10[b]	2.1	Closure
9[c]	1.8	Shotgun in progress
8[c]	1.7	Shotgun in progress
7[c]	1.7	Shotgun in progress
6[c]	1.6	Shotgun complete
5[c]	1.4	Closure
4[c]	1.2	Late Closure
3[c]	1.06	Finished
2[b]	0.95	Finished
1[c]	0.7	Late Closure

Table 1. Progress summary for the Malaria Genome Project.

HOST CELL INVASION

In its human host, *P. falciparum* is an obligatorily intracellular parasite that first invades liver cells and then goes on to invade differentiated erythrocytes. Invasion is an active process that starts with the attachment of the extracellular merozoite to the surface of the erythrocyte, followed by an active reorientation that brings the apical end of the parasite

cell into juxtaposition with the host cell membrane. The parasite then induces an invagination of the erythrocyte membrane and penetrates the erythrocyte.

Inside the erythrocyte, the *Plasmodium* parasite remains within a parasitophorous vacuole, the membrane of which is largely devoid of host cell membrane proteins but which contains lipids derived from the erythrocyte plasma membrane. The biogenesis and the biochemical composition of the vacuole differ markedly from those of the phagocytic compartments in nucleated cells that are inhabited by other pathogens. This distinction has important conceptual implications for our understanding of the vacuole as a parasite–host interface. The identification and functional analyses of the molecules that are involved in each step of the invasion process were covered by several presentations.

There are multiple pathways by which the parasite can invade the erythrocyte (Barnwell and Galinsky, 1998). One of these pathways relies on the presence of sialic acid on the host cell plasma membrane. The erythrocyte binding antigen-175 (EBA175), a ligand that binds to sialic acid on glycophorin A, is expressed in all parasite lines examined so far. A more recently discovered, different family of ligands, called *P. falciparum* reticulocyte binding proteins (PfRBPs) or *P. falciparum* reticulocyte binding protein homologues (PfRh), includes some members that bind to erythrocytes and facilitate sialic acid-independent erythrocyte invasion (Rayner *et al* ., 2001; Duraisingh *et al* ., 2003). Unlike EBA175, these proteins are expressed differently in different parasite lines, raising the interesting possibility that they are involved in switching between sialic acid-dependent and sialic acid-independent invasion pathways. As summarized by Alan Cowman (WEHI, Melbourne, Australia), his group has shown that the disruption of genes encoding either EBA175 or PfRh1 results in a decreased sialic acid dependence for invasion.

These observations provide a plausible and fascinating explanation for how malaria parasites evade an immune response that interferes with a specific invasion pathway. Although the parasite appears to be able to switch between various invasion pathways, it is possible to induce a humoral immune response that inhibits invasion of merozoite stages both *in vitro* and in animal models.

This immune response can be elicited by immunization with the apical membrane protein 1 (AMA1) which is therefore considered an important vaccine candidate (Stowers *et al* ., 2002; Casey *et al* ., 2004). Further evidence for the significant role of AMA1 in the invasion process comes from observations that peptides identified in phage display libraries and which bind to AMA1 are able to block invasion (Keizer *et al* ., 2003). AMA1 is an integral membrane protein and has an ectodomain consisting of three subdomains stabilized by intramolecular disulphide bridges. The correct conformation of the ectodomain is essential for the induction of protective antibodies. Raymond Norton (WEHI, Melbourne, Australia) described a detailed structural analysis of two of the three subdomains as a prerequisite for the production of recombinant AMA1 for immunization studies. This presentation elegantly illustrated the significance of structural information for the design of effective vaccine molecules.

The active mechanism by which *Plasmodium* and other apicomplexan parasites invade cells has been linked to the phenomenon of gliding motility. As described by Dominique Soldati (Imperial College, London, UK) the molecular motor that mediates gliding activity of another apicomplexan, *Toxoplasma gondii* , has been dissected in considerable detail. The actin-based motor consists of a myosin heavy and light chain anchored in the inner membrane complex and linked to the cytoplasmic tail of micromere proteins (MICs) via aldolase, an F-actin binding protein. The MICs are associated into complexes and are involved in binding to host cells. During invasion, the transmembrane MICs are cleaved and released from the parasite surface. Cleavage occurs within the membrane spanning domain and is mediated by a protease, which most probably belongs to the evolutionarily conserved rhomboid family of serine proteases. Several genes encoding members of this family are found in apicomplexan genomes, including that of *P. falciparum.* Information obtained using the more-amenable *T. gondii* system provides a conceptual framework for the identification of functionally equivalent components in *Plasmodium.*

THE PLASMODIUM FALCIPARUM GENOME

The genome of P. falciparum consists of three discrete components: a linear repeat of a 6 kb element located within mitochondria; a 35 kb circle within a plastidlike structure (the apicoplast); and 25–30 Mb of nuclear DNA (genomic DNA). The nuclear DNA is organized into 14 chromosomes, between 0.75–3.5 Mb in size, as determined by pulse-field gel electrophoresis (PFGE) and electron microscopic counts of kinetochore structures. Indirect evidence for the number of chromosomes also comes from genetic studies showing there are 14 linkage groups.

The nuclear genome is organized in a manner typical of eukaryotes, with linear chromosomes being bounded at either end with telomeric sequences. Genome plasticity, seen in many parasite isolates and identified by size polymorphisms on PFGE, is thought to result frequently from deletions and insertions of DNA within subtelomeric sequences, a

region shown to contain ordered repetitive sequence elements.

GENOMICS TECHNOLOGIES AND APPLICATIONS FOR MALARIA RESEARCH

The discovery and characterization of biological processes in the parasite and their potential exploitation to develop improved anti-malarial interventions was the impetus behind the completion of the malaria genome. To complement that of *P. falciparum*, five additional *Plasmodium* genome projects from related species, including another human malaria parasite species, *Plasmodium vivax*, have been initiated and are at various stages of completion. Combined with genome sequences of evolutionarily related *Apicomplexan* parasites, these data provide the foundation of comparative genomics studies—studies that may faciliate the understanding of host–parasite interactions and eventually lead to targeted disruption of those crucial steps in parasite biology.

Follow-on technologies such as high-throughput proteomics, DNA microarrays and systems biology, which rely on having an accurate and complete genome, have come in force to assist malaria biologists in understanding *Plasmodium* . For example, expanding on the recent characterization of large scale protein expression by highthroughput proteomics (Florens *et al*., 2002; Lasonder *et al* ., 2002), several groups have used similar approaches to identify the subcellular location of proteins from parasite organelles, from infected erythrocyte membranes (Florens *et al* ., 2004) and even from the specialized Maurer's clefts structure in the cytosol of these infected erythrocytes (Sam-Yellowe *et al* ., 2004). The proteomics data have been used to select and test new vaccine candidate antigens in T-cell assays from individuals protected by immunization with radiation-attenuated sporozoites which may represent antigens that are more likely to be protective than antigens currently under clinical development (Doolan *et al*., 2003).

Proteomics tools have provided a means to study parasite biology at a level of detail that previously was not easily possible. For example, at the meeting Shahid Khan (LUMC, Leiden, the Netherlands) described a proteomics approach that revealed differences in the proteins expressed in the male and female gametes as well as reporting a proteomic analysis of proteins expressed by merozoites from three different species of *Plasmodium*, each of which has a particular trophism for its hosts erythrocytes.

DESIGN AND METHODS

The *P. falciparum* strain FCR-3 was cultured according to the method of Trager and Jensen. Parasiteinfected erythrocytes were lysed with 0.5% saponin and total RNA isolated using guanidinium isothiocyanate. Messenger RNA was extracted using the Dynal® (Oslo, Norway) mRNA direct kit. cDNA was synthesized and packaged into T7 bacteriophage extracts as previously described. 4.1R was extracted and purified from human erythrocyte membranes and the purity verified using 12% SDS polyacrylamide gels and silver staining (detection limit: 2-5 ng protein). Purified 4.1R was subsequently biotinylated with Dbiotin-N-hydroxysuccinimide ester (Roche, Germany) and immobilized on streptavidin-coated magnetic beads (Roche, Germany). *P. falciparum* phage display libraries were biopanned against 4.1R and insert sequences analyzed as described by Lauterbach *et al*. Sequences were matched to annotated *P. falciparum* genes by performing bioinformatic analyses in the PlasmoDB database version 4.

CONCLUSION

The ability to completely sequence genomes has had a huge impact on the way in which scientific research is conducted. As the number of pathogen genomes that have been completed increases, the impetus to develop techniques that utilize this information has increased. In parallel with the P. falciparum genome project, techniques such as transfection, microarrays and proteomics, as well as bioinformatic analysis, are already being developed and applied to fundamental biological questions. The combination of these tools is expected to provide a predictive platform from which to launch hypothesis-driven biological research.

In this study *P. falciparum* phage display expression libraries have facilitated the identification of novel interactions between the parasite and 4.1R. Addressing the detailed binding kinetics between 4.1R and myosinlike motifs will aid in unravelling the functional importance of these interactions to *P. falciparum* growth and survival. Furthermore, the interaction between 4.1R and parasite proteins containing myosin and neurofilament like sequences may emerge as an important finding for the development of novel drugs against malaria.

The value of integrating a variety of datasets to unravel undiscovered regulation in biological processes during the gametocyte maturation stages of *P. falciparum* was demonstrated. Furthermore, comparative analysis of EST and microarray data was performed on the SERA gene family to advance the knowledge of their gene regulation and additional functional genomics reagents were presented to facilitate their study. Finally, the integrative approach was shown as a means to appraise critically the data quality of the increasing number of post-genomic datasets from malaria parasites.

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, posttranscriptional

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, tetracycline's and clindamycin) have been discussed in each section of this review. The recent availability of thousands of lead molecules from the GlaxoSmithKline chemical library120 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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