Manipulating the Plasmodium Genome

Teresa Gil Carvalho and Robert Ménard*

Unité de Biologie et Génétique du Paludisme, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France

Abstract

Genome manipulation, the primary tool for assigning function to sequence, will be essential for understanding Plasmodium biology and malaria pathogenesis in molecular terms. The first success in transfecting Plasmodium was reported almost ten years ago. Genetargeting studies have since flourished, as Plasmodium is haploid and integrates DNA only by homologous recombination. These studies have shed new light on the function of many proteins, including vaccine candidates and drug resistance factors. However, many essential proteins, including those involved in parasite invasion of erythrocytes, cannot be characterized in the absence of conditional mutagenesis. Proteins also cannot be identified on a functional basis as random DNA integration has not been achieved. We overview here the ways in which the *Plasmodium* genome can be manipulated. We also point to the tools that should be established if our goal is to address parasite infectivity in a systematic way and to conduct refined structure-function analysis of selected products.

Introduction

It is safe to predict that the wealth of information revealed by the sequence of the *Plasmodium falciparum* genome will benefit many areas of malaria research (Waters and Janse, 2004). New drug targets will be identified by capitalizing on the comprehensive view of parasite metabolism, as was already done to demonstrate the antimalarial activities of fosmidomycin and triclosan (Jomaa *et al.*, 1999; Surolia and Surolia, 2001). Another much anticipated impact of the genome sequence is on vaccine development, via the formulation of new 'vaccinomic' approaches (Hoffman *et al.*, 1998; 2002). Comparative genomics will soon be possible as the genome sequence of more *Plasmodium* species and other Apicomplexa is completed, and will provide insights into the evolution of these protozoan parasites and adaptation to their hosts.

To what extent will the sequence help us to understand *Plasmodium* biology? Encompassing 14 chromosomes, the ~25-megabase *Plasmodium* genome is predicted to encode ~5,000 genes. Apicomplexa are part of one of the most ancient eukaryotic lineages, phylogenetically distant from the model organisms already sequenced. They have unique structural features and have evolved distinct solutions to basic problems; for example they divide by multiple fission, locomote by gliding and induce the formation of new membrane

*For correspondence. Email rmenard@pasteur.fr.

compartments in the host cell. Not surprisingly, the proportion of *Plasmodium* products that have homologs in other organisms is the lowest among sequenced genomes. Annotation of P. falciparum chromosome 2 (Gardner et al., 1998) and 3 (Bowman et al., 1999) left about two-thirds of the predicted genes without function, either having no detectable homolog or a Plasmodium/ Apicomplexa-specific homolog for which we have no functional information. Function was tentatively assigned to only a third of the predicted genes, but most of these significant matches remain only partially informative. They may reveal the biochemical activity of the product, inherent to the protein and irrespective of cellular context, for example a kinase or a phosphatase activity. They may also indicate the presence of a domain of known function, but in an otherwise unique molecular context. Obviously, homology searches lead to physiological function only for proteins that are involved in one of the core biological processes common to all eukaryotes.

In studying *Plasmodium* biology, the major questions concern the molecular basis of the features that define Apicomplexa protozoa, the traits that are specific to *Plasmodium*, and the parameters that influence disease such as transmission and virulence. Thus the central challenge is to be able to identify the parasite products that are critical to biological processes of interest. For this, we need molecular genetic tools for manipulating and questioning the genome in a variety of ways.

Plasmodium Transfection: A Brief Account of the First Milestones

Plasmodium was the last protozoan of medical importance to become amenable to molecular genetics. Transfecting Plasmodium was not an easy task, as the parasite spends most of its life located intracellularly within a vacuole, its nucleus being separated from the environment by four membranes. Also, Plasmodium DNA is particularly A/T-rich and unstable in Escherichia coli, which complicates preparation of transforming constructs. The first success in transfecting Plasmodium was reported in 1993, when D. Wirth and collaborators obtained transient gene expression after electroporation of extracellular gametes and zygotes in P. gallinaceum, an avian Plasmodium species (Goonewardene et al., 1993). The decisive breakthroughs came in 1995, when the groups of T. Wellems working on P. falciparum and of C. Janse and A. Waters working on P. berghei, a species that infects rodents, could transfect erythrocytic stages of the parasite. Transfection was transient in P. falciparum (Wu et al., 1995), and stable in P. berghei by means of a pyrimethamine-resistance gene (van Dijk et al, 1995). Using a similar selection system, three studies published the next year described integrative transfection, in P. falciparum (Wu et al., 1996; Crabb and Cowman, 1996) and P. berghei (van Dijk et al., 1996), which indicated a large if not complete dominance of homologous integration in both species. These seminal studies were then rapidly followed by reports on the inactivation of genes of interest (Ménard *et al.*, 1997; Crabb *et al.*, 1997a; Sultan *et al.*, 1997).

Although transfection has since been described in other *Plasmodium* species, *P. falciparum* and *P. berghei* have been the subject of all functional studies. A variety of molecular genetic approaches can now be taken in the two species. Theoretically, episomal or integrative transfection can each be used for either characterizing or identifying genes, as outlined in Figure 1. To propose a complete view of the *Plasmodium* genetic toolbox, we will consider these four situations successively.

Gene Characterization Using Episomal Transfection

Transient Transfection

Since its first use in 1993, transient transfection has been largely used to study gene expression in *Plasmodium*. Transient transfection plasmids only need to contain a reporter gene flanked by the sequences under study (Figure 1), and reporter genes encoding chloramphenicol acetyltransferase, firefly luciferase or green fluorescent protein (GFP) have been used to analyze the untranslated regions of many genes. The goal of most of these studies was to define by deletion mapping the minimal 5' and 3'



Figure 1. A theoretical view of molecular genetic techniques. The selectable marker and its expression sequences are symbolized by a grey box, the bacterial plasmid by thick lines, the gene promoter by an arrow, and the 3' untranslated sequences necessary for gene expression by an open circle. Ins, insertion plasmid; Rep, replacement plasmid. See text for details.

^aGenes of interest can be identified by random insertional mutagenesis using a mutagenizing DNA (plasmid or transposon) that contains a reporter gene at its 5' end, as shown. Screens may then identify gene function (via gene inactivation), gene expression (using promoter-trap constructs, with a reporter lacking a promoter), or localization of the product (using gene-trap constructs, with a reporter lacking a start codon). regions that retained the capacity to efficiently drive gene expression (Wu *et al.*, 1995; Crabb and Cowman, 1996; Crabb *et al.*, 1997b; Horrocks and Kilbey, 1996; Dechering *et al.*, 1997). This information has been particularly valuable for constructing expression or resistance cassettes of minimum size. More recent transient transfection studies have initiated characterization of the DNA elements involved in gene expression (Horrocks and Lanzer, 1999) and stage-specific expression (Dechering *et al.*, 1999), or of the mechanisms that ensure expression of a single member of the *var* gene family (Deitsch *et al.*, 1999; 2001). Another study used transient transfection to demonstrate that readthrough of an internal stop codon was occurring in a *Pf60* gene (Bischoff *et al.*, 2000).

Still, little is known of how gene transcription is controlled in Plasmodium. Plasmodium promoters superficially resemble classical eukaryotic promoters transcribed by RNA polymerase II, consisting of a core promoter region controlled by upstream enhancer elements. However, they are functionally distinct from other eukaryotic promoters, as they do not function in mammalian COS cells and their sequences share no homology with any known transcription factorbinding site of eukaryotes (Horrocks et al., 1998). In addition, promoters of SV40 or other viruses, which are ubiquitously active in higher eukaryotes, fail to drive reporter expression in P. falciparum (Horrocks et al., 1998). What adds stage-specificity to gene expression in Plasmodium is also mysterious, although upstream elements may be involved (Dechering et al., 1999). Unraveling the transcriptional machinery in Plasmodium will be important, as it may reveal new schemes of gene expression and lead to the development of new tools for timely expression of transgenes or mutations.

Stable Transfection

Multiple selectable markers are now available for stable episomal transfection in both P. falciparum and P. berghei. The most commonly used markers remain the original Plasmodium or Toxoplasma DHFR-TS variants that confer resistance to pyrimethamine, present in a variety of resistance cassettes (Wu et al., 1996; Waters et al., 1997; Crabb et al., 1997b). In both Plasmodium species, transfectants can also be selected via resistance to the antifolate drug WR99210 encoded by a human DHFR gene (Fidock and Wellems, 1997; de Koning-Ward et al., 2000a). Derivative markers now exist that confer both drug resistance and fluorescence via a GFP fusion (Sultan et al., 1999b; Kadekoppala et al., 2000). Other selectable markers that act independently from the folate pathway have been developed for stable episomal transfection in P. falciparum (Ben Mamoun et al., 1999; de Koning-Ward et al., 2001).

The fate of stably maintained plasmids is different in *P. berghei* and *P. falciparum*. In *P. berghei*, plasmids replicate as unrearranged monomeric units, with an average copy number of 15 per nucleus (van Dijk *et al.*, 1997). These plasmids appear to be fairly stable, even in the absence of drug pressure. In *P. falciparum*, however, the situation is more complex. Plasmids rapidly form large concatemers (Kadekoppala *et al.*, 2001), which probably emerge from inter plasmid homologous recombination. The structure and properties of these concatemers also appear to change with time (O'Donnell *et al.*, 2001). Initially small and unevenly segregated to the daughter merozoites, they become larger structures that after a few months are stably replicated even in the absence of selective pressure. Recently, a 1.4-kb sequence composed of 21-bp degenerate repeats, Rep20, has been shown to improve plasmid maintenance and to allow efficient segregation of plasmids in *P. falciparum* (O'Donnell *et al.*, 2002).

Transgene Expression

Gene function can be approached using transgene expression in several ways (Figure 1). (*i*) Over-expressing dominant-negative forms of a protein can generate a defective phenotype and thus inform on protein function. This strategy is so far limited to a few well-known protein families, and has the drawback of possible unspecific effects. (*ii*) GFP fusions of a protein can be produced to analyze its secretory pathway, as in the case of the insightful studies on the apicoplast-targeted and KAHRP proteins (Waller *et al.*, 2000; Wickham *et al.*, 2001). (*iii*) Modified versions of a gene can be expressed in a corresponding null mutant, although a serious limitation of episomes for addressing subtle structure-function relationships is the gene dosage effect due to the high number of replicating units.

Whenever possible, the transgene should be expressed from the natural expression regions of the target gene to minimize artifacts due to temporal misexpression of the product (Kocken *et al.*, 1998). In the future, tools for controlled gene expression should greatly help to refine episomal approaches, particularly for studies on the erythrocytic stages of the parasite. Transgene technology may nonetheless be limited by episome instability when studying mosquito stages of the parasite, on which drug pressure cannot easily be applied.

RNA Reverse Genetics

Another tool for probing gene function using episomes is antisense technology. Target gene expression can be suppressed by the annealing of antisense molecules to complementary transcripts, by a poorly understood mechanism that may affect transcript stability, processing, transport and/or translation. Both approaches of electroporating single-stranded antisense oligodeoxynucleotides (Barker et al., 1996; 1998) and stably over-expressing antisense transcripts (Gardiner et al., 2000) have been used with success in Plasmodium to disrupt endogenous mRNA function. Stage-specific or inducible expression of antisense RNAs may thus represent an alternative to gene manipulation for investigating protein function. It remains that the inhibitory activity of a given antisense molecule is difficult to predict, and that antisense approaches face the possible limitations of questionable specificity and incomplete efficacy.

RNA interference (RNAi) has emerged as a powerful alternative to antisense technology for specific degradation of target mRNA. RNAi appears to follow the processing of long, double-stranded RNA into 'short

interfering' RNAs (21-23 nucleotide fragments), which guide the cleavage of homologous mRNA by the silencing complex RISC (Hammond et al., 2001; Sharp, 2001). This evolutionarily conserved pathway, which may be part of a basic surveillance system that degrades transposon or viral messages, has already been harnessed as a reverse genetics tool. Injecting or expressing double-stranded RNAs causes the silencing of the corresponding gene in many systems tested, from protozoa to multicellular organisms. Degradation of target mRNA is specific and efficient, even with low concentrations of double-stranded RNA and regardless of the sequence chosen in the target gene. The silencing effect is particularly stable, which obviates the need for the extensive chemical modifications that are necessary for enhancing the halflife of antisense oligodeoxynucleotides.

The single RNAi study undertaken in *Plasmodium* is encouraging, showing a partial but apparently specific reduction of target mRNA levels (McRobert and McConkey, 2002). One limiting factor might be the low transfection frequencies in *Plasmodium*. Selection of interfered parasites would necessitate expressing double-stranded RNA from a selectable episome. A variety of constructs have already been devised for inducing stable

interference in other systems. For example transcription can occur through inverted DNA repeats, giving rise to hairpin single-stranded RNA mimicking double-stranded RNA (Tavernarakis *et al.*, 2000; Shi *et al.*, 2000; Chuang and Meyerowitz, 2000), or from two opposing promoters, each giving rise to one strand of the double-stranded RNA (Wang *et al.*, 2000). Controllable and stage-specific expression of interfering constructs may thus become a handy tool for probing gene function in *Plasmodium*.

Gene Characterization Using Integrative Transfection

Gene Targeting: Current Status

Gene targeting by homologous recombination is arguably the most informative approach to protein *in vivo* function. It is more reliable and predictable than antisense or dominant-negative approaches, and permits a detailed analysis of protein structure-function relationships. The *Plasmodium* genome is haploid, contains mostly singlecopy genes and integrates exogenous DNA by ~100% homologous recombination. Thus, for most genes a single recombination event is sufficient for generating a modified parasite clone. In fact, despite the relative youth of transfection technology in *Plasmodium*, and its relative



Figure 2. Gene-targeting strategies used in *Plasmodium*. Gene targeting relies on homologous recombination between a genomic sequence (TARGET gene) and its homolog in the targeting construct (shown on the left). The latter can carry a single region of homology (insertion type) or two regions flanking the marker (replacement type). Insertion plasmids insert via a single crossover (SCO) between the pair of homologs (plasmid retained), while replacement fragments replace the target sequence via a double crossover (DCO) between pairs of homologs (plasmid retained), while replacement fragments replace the target sequence via a double crossover (DCO) between pairs of homologs (plasmid lost). In *P. berghei*, these events are favored by linearizing the transformed DNA. In *P. falciparum*, linear DNA does not promote recombination, and circular replacement plasmids insert preferentially via a SCO between one pair of homologous sequences. Symbols are as in Figure 1; downstream 3' untranslated sequences are symbolized by a closed circle. ^aThe insertion plasmid must contain an internal fragment of the gene to generate two truncated gene duplicates in the final locus.

^bThe replacement plasmid should be designed to delete part or all of the target coding sequence.

°The SCO shown involves the 5' regions of homology (TA). In this or the reverse case (a SCO between the 3' regions of homology, ET), a full-length target gene copy is created.

^aFor the gene modification (t) to be recovered in the first, expressed and full-length duplicate, the SCO must occur upstream from the modification. ^aThe 3' sequence necessary for gene expression is truncated.

^fSee legend of Figure 3 for references.

inefficiency (stable transfection frequencies have been evaluated at ~ 10^{-6} in both *P. falciparum* and *P. berghei*), numerous gene-targeting studies have already been performed. They have revealed important insights into such diverse processes as drug resistance, cell invasion by the various invasive stages of the parasite, sexual differentiation, or cytoadherence of infected erythrocytes. Although usually genes have been inactivated, several genes have been modified and in one case expression levels have been diminished. Figure 2 illustrates the strategies that have been used and Figure 3 shows the Plasmodium loci that have been targeted, as of february 2002. Previous reviews have described construct design and selection protocols (Waters et al., 1997; Ménard and Janse, 1997; Tomas et al., 1998; de Koning-Ward et al., 2000b: Ménard and Nussenzweig, 2000).

Homologous recombination provides a versatile system for manipulating the *Plasmodium* genome. On the one hand, double crossovers can span and delete tens of kilobases, and be used to introduce large deletions at chromosome ends (Pace *et al.*, 2000). On the other hand, as few as ~300 bp of homology (and possibly less) are sufficient for crossover formation and plasmid

insertion is associated with short gene conversion tracts (Nunes et al., 1999). Therefore, point mutations can be introduced using small insertion plasmids. Also, strict homology between the targeting and target sequences is not required for productive recombination. For example, transfection in P. berghei with targeting vectors containing sequences from the C-rRNA gene resulted in disruption of the C- as well as the D-rRNA gene, which differ in ~5% of their sequence (van Spaendonk et al., 2001). This implies that a targeting vector may occasionally integrate by homologous recombination elsewhere than at the expected locus, especially when recombination occurs between highly A/T-biased 3' or 5' untranslated sequences. Finally, the multiplicity of selectable markers permits complementation experiments, which provide definitive proof for the involvement of a protein in a defective phenotype (Sultan et al., 1999a; 2001; Thathy et al., 2002). The tools are thus available to perform DNA reverse genetics in Plasmodium according to the molecular Koch postulates of S. Falkow (1988).

There are important differences between genetargeting procedures and their outcomes in *P. falciparum* and *P. berghei*. The targets of electroporation are



Figure 3. Targeted *Plasmodium* genes (as of February 2002). The *Plasmodium* life cycle in the two hosts, a vertebrate (lower part) and a mosquito (upper part), is shown. The parasite genes that have been manipulated by gene targeting are indicated below the respective parasite stage, with references in parentheses. 1, Triglia *et al.*, 1998; 2, Fidock *et al.*, 2000a; 3, Fidock *et al.*, 2000b; 4, Reed *et al.*, 2000b; 5, van Spaendonk *et al.*, 2001; 6, Triglia *et al.*, 2000; 7, O'Donnell *et al.*, 2000; 8, Baldi *et al.*, 2000; 9, Taylor *et al.*, 2001; 10, Kaneko *et al.*, 2000; 11, Reed *et al.*, 2000a; 12, Triglia *et al.*, 2001; 13, Crabb *et al.*, 1997a; 14, Trenholme *et al.*, 2000; 15, Waterkeyn *et al.*, 2000; 16, Lobo *et al.*, 1999; 17, van Dijk *et al.*, 2001; 18, Tomas *et al.*, 2001; 19, Dressens *et al.*, 1999; 20, Templeton *et al.*, 2000; 21, Yuda *et al.*, 1999; 22, Tsai *et al.*, 2001; 23, Ménard *et al.*, 1997; 24, Thathy *et al.*, 2002; 25, Sultan *et al.*, 1997; 26, Matuschewski *et al.*, 2002; 27, Kappe *et al.*, 1999; 28, Ménard and Nussenzweig, 2000; 29, Wengelnik *et al.*, 1999; 30, Nunes *et al.*, 1999.

intraerythrocytic forms of the parasite (rings to schizonts) or extracellular merozoites, and selection occurs *in vitro* or in rodents, respectively. Crucially, linear DNA is the preferred substrate for homologous recombination in *P. berghei*, but not in *P. falciparum*. Linear DNA is presumably degraded in *P. falciparum* when it crosses the four membranes to the parasite nucleus, and this has two important consequences. One is that the time required for selecting integrants is longer in *P. falciparum* (3-4 months versus 2 weeks in *P. berghei*), because circular plasmids preferentially replicate episomally than integrate into the genome. Another consequence is that double crossover events can hardly be selected in *P. falciparum*, because circular replacement plasmids preferentially integrate via single crossovers (see Figure 2).

One way to recover the rare double crossovers that may occur when transfecting circular replacement plasmids is to use a negative marker to counterselect the other transfection products (Figure 4). Two negative selectable markers, cytosine deaminase and thymidine kinase, have already been developed in *P. falciparum* (Duraisingh *et al.*, 2002). They will also serve for conducting more reliable protein structure-function analysis in both *Plasmodium* species. Indeed, so far all subtle gene modifications have been introduced in the presence of a selectable marker, which as a new transcription unit may affect gene expression in the targeted or unlinked loci in unpredictable ways. Figure 4 shows the classical 'hit and run' procedure employed to

circumvent this drawback and to introduce mutations in a final locus devoid of exogenous sequence, based on the sequential use of positive and negative selection.

Limitations of Gene Targeting in Plasmodium

Despite these exciting achievements, there are still numerous genes whose function cannot be properly investigated. This is the case of genes involved in parasite replication in ervthrocytes (on which selection is based), including those important for merozoite invasion of erythrocytes, the most scrutinized step of the parasite life cycle and a primary vaccine target. Loss-of-function mutants in these genes die or are overgrown by nontargeted parasites. Therefore, with currently available tools, the best possible evidence that a gene is important for invasion of ervthrocytes is when it can be targeted with a nondisruptive construct but not with a disruptive construct (Cowman et al., 2000). This was reported for MSP-1 and AMA-1, along with direct evidence for their role in merozoite invasion via gain-of-function mutants created by trans-species exchange between human and rodent homologs (O'Donnell et al., 2000; Triglia et al., 2000). Nonetheless, failure to select loss-of-function mutants may be a misleading criterium for identifying important genes among uncharacterized sequences, given the poor targeting frequencies in Plasmodium. In addition, since impaired mutants cannot be selected, the defective phenotype and actual protein function cannot be studied.



Figure 4. Using negative selectable markers in *Plasmodium* gene targeting. A negative marker allows for selecting parasites that do not express the marker, generally by conferring susceptibility to a drug. The negative marker is symbolized –M, the positive marker by +M, and the bacterial plasmid by a thick line. A) Upon positive selection, circular replacement plasmids will preferentially replicate episomally, rarely integrate via a single crossover (TAR or GET), and should also integrate via a double crossover (TAR and GET). For recovering the latter, a negative marker can be placed in the construct as shown, and negative pressure applied to counterselect episomes and single crossover integrations that all maintain the negative marker. B) Shown here is the modification of a target gene (R to r) via a hit and run procedure. Positive selection recovers integration of the insertion plasmid (hit), which introduces the modification (r, shown here ending in the first gene duplicate after SCO between TA regions). Negative selection (after SCO between GET regions). The reverse situation, i.e., a hit step via SCO between GET regions and a run step via SCO between TA regions also leads to a modified gene.



Figure 5. Tools for conditional gene expression. A) A promoter can be made drug-responsive by inserting 2 to 7 copies of 19-bp *tetO* (tetracycline operator) sequences around the transcriptional initiation site of the promoter, which are recognized by a regulator: TetR, tTA or rtTA. Left, a drug-responsive transgene can be borne by an episome and used to express dominant-negative constructs. Right, a controllable copy of a target gene can also be used to replace its chromosomal copy by double crossover. Ideally, the desired state (overexpression or tight repression) should be obtained by adding rather than removing the effector, because the former situation is associated with more rapid kinetics of expression switch.

^aTetR (tetracycline repressor) is limited by a narrow range of control. TetR dissociates from *tetO* upon tetracycline binding, leading to gene transcription. ^btTA (tetracycline-controlled transactivator) is best suited for rapid repression of gene expression and knock-out approaches. In the absence of doxycycline, the gene is expressed; upon doxycycline addition, the gene is rapidly repressed.

^crtTA (reverse tetracycline-controlled transactivator) is best suited for rapid expression of a transgene and dominant-negative approaches. In the absence of doxycycline, rtTA does not bind to *tetO* and the transgene is not expressed; upon doxycycline addition, it is rapidly expressed.

B) Cre (Flp) catalyses a recombination reaction between two identical 34-bp recognition sites called *loxP* (*FRT*). When the two sites are located on the same molecule (chromosome), recombination will excise (invert) intervening DNA if the sites are in the same (opposite) orientation. Shown here is the deletion of the target gene promoter. When one site is on the linear chromosome and the other on a circular plasmid, recombinase inserts the plasmid at the chromosomal site. Shown here is plasmid integration leading to gene modification. Timely expression of the recombinase may rely on stage-specific or inducible promoters, or other approaches.

^dintrachromosomal deletion/excision is reversible but is energetically favoured over intermolecular integration.

ethe inherently unstable insertion product can be obtained by limited expression of the recombinase or by using mutant sites that are refractory to further excision.

Other genes that cannot be at present fully characterized are those encoding multifunctional proteins. Knocking-out these genes only reveals the earliest non-redundant role of their product. One example is CS, known to be involved in sporozoite adhesion to the mosquito salivary glands and to mammalian hepatocytes, but which is first essential for sporozoite formation in the oocyst (Ménard *et al.*, 1997; Thathy *et al.*, 2002). Thus the role of sporozoite surface-associated CS, the leading vaccine candidate against pre-erythrocytic stages of the parasite, cannot be dissected genetically. It is clear that unrestricted functional analysis of the genome requires the tools for activating or silencing genes at will.

Missing Tools: A Brief Overview of Conditional Mutagenesis

Two types of tools have been widely used for conditional gene expression in eukaryotes: transcriptional regulators and site-specific recombinases. Their basic mechanisms of action and some of the possible applications for studying *Plasmodium* essential genes are illustrated in Figure 5.

The most popular transcriptional regulatory systems are derived from the tetracycline resistance operon of bacterial Tn10 (Gossen and Bujard, 1992; Baron et al., 1999; Urlinger et al., 2000). They have been developed into increasingly efficient tools for controlling gene expression in model organisms from yeast to rodents, and have been used with success in various protozoan parasites (Wirtz and Clayton, 1995; Hamann et al., 1997; Wirtz et al., 1999; Meissner et al., 2001; Yan et al., 2002). They consist of (i) a regulator (repressor or activator), (ii) operator sequences, which must be positioned around the transcriptional start site, and (iii) an effector (tetracycline or derivative) for modulating the regulator-operator interaction and turning 'on' or 'off' gene expression. When a tetracycline-responsive copy of a gene is borne by an episome, it can only be used for timely over-expression of dominant-negative, antisense or interfering constructs. A more direct approach is to insert the controllable copy in place of the endogenous gene by homologous recombination. This enables to shut down gene expression in all recombinants at a chosen

time, and to directly assess the consequences of the gradual loss of the product. Inducible promoters come with the clear advantages of reversibility, in allowing to generate truly conditional 'on' and 'off' states in one clone, and flexibility, in enabling to create intermediary or temporary states that may be as informative on protein function as a constitutively 'off' state. In some situations, however, their efficiency will ultimately depend on whether complete repression can be obtained, as well as on the kinetics of repression after effector addition/removal. Studies on essential products of parasite erythrocytic stages would greatly benefit from these tools, particularly the transactivators suited to conditional gene silencing (see Figure 5 legend). The situation seems ideal for P. falciparum, which replicates in erythrocytes in vitro where effector levels can be more easily controlled.

A second way to inactivate a gene in a temporally restricted manner is offered by site-specific recombinases. Two site-specific recombinases of the λ integrase family have been used for this purpose in a variety of eukaryotes: Cre of bacteriophage P1 and Flp of yeast (Sauer, 1998; Porter, 1998). These enzymes catalyse a reciprocal conservative recombination between two identical 34-bp target sequences and, depending on their position and orientation, recombination will insert, invert, or delete DNA (see the Figure 5 legend). Therefore, these systems offer the primary advantage of enabling not only to inactivate but also to modify or swap genes, and thus to investigate protein structure-function relationships. Recombination occurs regardless of DNA topology and host environment, and the Cre/loxP system has been shown to function in the apicomplexan Toxoplasma gondii (Brecht et al., 1999). Although it should be easy to design modifications and insert the LoxP or FRT site(s) into the Plasmodium genome by homologous recombination, the challenge is to express the recombinase conditionally. A first possibility would be to use natural stage-specific promoters. If these prove not to be leaky before being activated, they would then be useful for truly conditionally inducing gene rearrangements at a defined stage of the parasite life. They would allow studies on essential genes not only in that particular stage, but also in erythrocytic stages after complete cycling of the parasite. A gene important for merozoite invasion could for example be deleted in a mosquito stage of the parasite, and after transmission to the mammalian host its function could be assessed in merozoite formation in the liver and in subsequent merozoite invasion of erythrocytes. Directly applying recombinase systems to parasite erythrocytic stages would require expressing the recombinase from an inducible promoter or using one of the recombinase variants that can be activated by an exogenous factor (Metzger and Chambon, 2001). It may become possible to use, as was recently performed with mammalian cells (Jo et al., 2001), a cell-permeable recombinase that could be directly added to cells/parasites bearing a manipulated ('floxed' or 'flrted') gene. As increasingly sophisticated site-specific recombination systems are being developed, their usefulness in Plasmodium should be evaluated as they would offer virtually unlimited ways of analyzing the function of Plasmodium essential genes.

Gene Identification

Plasmodium has been transfected almost exclusively for testing promoter activity or gene function, and reverse genetic techniques are now well established. On the other hand, attempts to identify genes and develop forward genetic screens have been scarce.

Using Episomal Transfection (Promoter Trapping)

In bacteria, episomal transfection has been widely used to identify genes that are induced in response to defined conditions. The basic method consists in fusing a genomic library to a promoterless reporter gene whose product confers a selectable or easily screenable phenotype, for example antibiotic resistance (Figure 1). A simple variant, called differential fluorescence induction, uses green fluorescent protein as the reporter and relies on fluorescence-activated cell sorting (FACS) to isolate bacteria with active transcriptional fusions (Valdivia and Falkow, 1997). In Plasmodium, a similar promoter-trap strategy could in theory be used for isolating promoters that are active during any step of the parasite life. A Plasmodium genomic library of 1-kb average insert size could be scanned in a few minutes by FACS, and active promoters rescued from fluorescent parasites. However, the tens of thousands of clones necessary to cover the genome still represent many individual transfections, given the low transfection frequencies. Also, although it is clear that stage-specific promoters can be active when carried by episomes (Sultan et al., 1999a; 2001), little is known of their regulation throughout the cycle. In P. falciparum, episomes apparently do not properly assemble chromatin (Horrocks et al., 1998), a requirement for the correct developmental expression of many eukaryotic genes. Another problem is that distinct transfected plasmids may assemble into concatemers (Kadekoppala et al., 2001). Therefore episomal transfection is not presently a suitable approach for identifying Plasmodium genes based on their expression profile.

Using Integrative Transfection (Insertional Mutagenesis) A powerful way to identify genes that mediate biological processes, particularly in haploid organisms, is based on random mutagenesis and screening the resulting mutants for a defect in a phenotype of interest. Mutagenesis is typically induced by nonhomologous integration of a plasmid or insertion of a transposon, two methods that tag the mutated site and facilitate its recovery. In theory, saturation mutagenesis permits identification of the function of any gene whose inactivation is not immediately lethal, and for which an appropriate selection or screen is available (for examples in bacterial pathogenesis studies, see Strauss and Falkow, 1997; Chiang et al., 1999; Wren, 2000). In protozoa, efficient random mutagenesis has been reported only in Toxoplasma and Leishmania, allowing in both cases to select for gene fusions and trap new genes. In Toxoplasma gondii, nonhomologous recombination can be obtained by incorporating discontinuous genomic DNA in transfection constructs, and current screens are targeting parasite genes induced by the transition from the tachyzoite to the bradyzoite stage (Roos et al., 1997). In Leishmania major, the Mos1 element of the mariner/ Tc1 family of transposons, which are ubiquitous elements



Figure 6. An outlook at functional genomics in *Plasmodium*.

of eukaryotic genomes, transposes efficiently (~10⁻⁴; Gueiros-Filho and Beverley, 1997).

Unfortunately, such methods are not in sight in *Plasmodium*. Nonhomologous recombination does not occur using currently used vectors, or with frequencies incompatible with gene discovery. Transposition was reported only once, using the *mariner* element, but with apparently low efficiency (Ben Mamoun *et al.*, 2000). Even if tools for random DNA insertion into the *Plasmodium* genome can be established, their utility for gene discovery would also necessitate increasing frequencies of transfection.

What are the Prospects for Functional Genomics in Plasmodium?

In the absence of appropriate molecular genetic tools, genomic techniques will be crucial for classifying genes according to their pattern of expression (Figure 6). High-redundancy methods can be useful for providing transcriptome snapshots, such as massive cDNA sequencing projects (Carlton et al., 2001) and serial analysis of gene expression (Munasinghe et al., 2000; Patankar et al., 2001). Several genome-wide techniques that compare relative levels of mRNAs in two conditions are also being applied to *Plasmodium*. including DNA microarray hybridization (Hayward et al., 2000; Ben Mamoun et al., 2001), subtractive suppressive hybridization (Dessens et al., 2000), and differential display (Lau et al., 2000; Cui et al., 2001). So far these techniques have been used mainly for analyzing expression profiles in erythrocytic stages of the parasite, the only stages that yield the necessary amounts of mRNA. To facilitate similar studies with mosquito or liver stages, tools are being developed that should help to purify the small available numbers of parasites by FACS (Natarajan et al., 2001) or laser capture microdissection (Sacci et al., 2002). All these technologies will permit to down scale the genome to its expressed portion during a process of interest and to identify stage-specific genes. More focused screens (e.g. involving drug-treated or mutant parasites, or parasites in ex or in vivo conditions) may narrow down to smaller subsets of co-expressed genes and provide sharper leads to investigators. But it is likely that in most cases these global mRNA techniques will leave us with large numbers of differentially expressed genes. As for the entire genome, sequencing will hardly by itself constitute a rationale for further analysis, although sequence may occasionally suggest function. The challenge remains to translate the flow of expression data into biological activities.

Function could be addressed by a systematic, gene-by-gene approach. One possibility would be to systematically delete expressed genes by homologous (double crossover) recombination, and generate null mutants. However, the transition from gene sequence to parasite mutant takes at least 12 weeks in the relatively handy P. berghei system, making such large-scale functional studies impractical in most laboratories. If it proves reliable in Plasmodium, RNAi technology would be a more rapid method for testing the function of many genes. Systematic functional studies using RNAi have been performed against the products encoded by entire chromosomes in Caenorhabditis elegans (Fraser et al., 2000; Gönczy et al., 2000) or the components of complete pathways in Drosophila (Clemens et al., 2000) (reviewed in Kuwabara and Coulson, 2000; Barstead, 2001). RNAi would be an efficient way to mine the Plasmodium genome for potential drug targets, or to screen for important genes that may deserve further analysis by homologous recombination.

Yet for most laboratories, a direct screen for genes of interest or essential genes would be a more appealing prospect than the gene-by-gene approach. New opportunities for generating random mutants in Plasmodium may arise from the construction of (differentially) expressed gene libraries, which reduces the initial pool of genes and allows their mutagenesis in other organisms. Cloned genes could for example be mutagenized in E. coll and mutated alleles subsequently introduced into Plasmodium for replacement of their chromosomal copy by homologous recombination. Such 'shuttle mutagenesis' has been used in yeast, after Tn3 or Tn7 mutagenesis in E. coli (Kumar and Snyder, 2001). There is now a wide choice of mutagenizing agents, including multifunctional transposons which use the same insertion event to determine (i) when the gene is expressed (via reporter fusion), (ii) where the product is localized in the cell (via formation of epitope-tagged products), and (iii) the consequence of the absence of the product (via gene inactivation) (Ross-Macdonald et al., 1997; 1999). To avoid having to screen mutants individually, molecular barcodes have been developed for bacterial pathogenesis studies (Hensel et al., 1995) and yeast functional genomics (Shoemaker et al., 1996; Winzeler et al., 1999). These short sequences serve as clone identifiers and allow large numbers of mutants to be pooled and analyzed simultaneously by comparative hybridization on filters or high-density arrays. In bacteria for example, these tags associated with classical transposition (signature-tagged transposition method, STM) have served to isolate mutants that were unable to survive in the host (Hensel et al., 1995). Establishing such tools in Plasmodium would permit to envisage focused approaches to virtually any aspect of parasite biology.

Animal models of malaria should be particularly valuable for functional genomic studies and tackling basic aspects of parasite biology. Rodent *Plasmodium* species, including *P. berghei* and *P. yoelii* (Mota *et al.*, 2001), are

practical because they can be studied routinely and safely in the laboratory, and in vivo throughout their life cycle. Double crossover recombination is readily obtained with linear DNA, allowing in principle shuttle strategies. An additional attractive feature of rodent systems is that the three actors (parasite, mosquito and vertebrate host) can be genetically manipulated, and the sequence of their genome is, or will soon be known. P. knowlesi and P. cvnomologi, which infect primates and are closely related to the human parasite P. vivax, can also be manipulated by double crossover recombination (van der Wel, 1997; Kocken et al., 1999; 2002). However, their use for large-scale studies is prohibited by ethical and practical reasons. P. falciparum remains the mandatory target for studying specific virulence traits, such as cytoadherence of infected ervthrocytes. This system offers the advantage of an erythrocytic cycle that can be studied in vitro and synchronized, but is limited by the difficulty to produce mosquito stages of the parasite and the time consuming molecular genetic procedures. A precise understanding of malaria pathogenesis will necessitate that each system contributes its part.

Conclusion

The landscape of malaria research has changed dramatically in the last decade. The sequence of the genome of several Plasmodium species is now known, genomic techniques have been developed, and the parasite can be transfected. The molecular genetics toolbox, however, is far from complete. On the one hand, understanding the function of a given gene (reverse genetics) is straightforward, and we should soon have the tools for manipulating any gene. On the other hand, identification of genes based on their function (forward genetics) is still problematic. The powerful genomic techniques will continue to categorize the genome into subsets of interest, and may suggest function of groups of genes, but only constitute a first step. Molecular genetic methods must be adapted to translate the wealth of sequence and expression data into biological functions, and to link them to investigator-driven research addressing specific questions that can only be answered by a reductionist approach.

These are exciting times for the malaria research community. The blending of these new technologies will lead to an increasingly sophisticated view of parasite biology, and uncover the molecular details behind the unique features of this ancient eukaryote. More importantly, they hold great promise to help reducing the burden of malaria in allowing a systematic hunt for drug targets and a rational choice of vaccine candidates, and will certainly lead us to other intervention strategies that are now unforseeable.

Acknowledgements

We thank, Patricia Baldacci, Freddy Frischknecht, Hiroshi Sakamoto, and Sabine Thiberge for their review of the manuscript and many helpful comments.

References

Baldi, D.L., Andrews, K.T., Waller, R.F., Roos, D.S., Howard, R.F., Crabb, B.S., and Cowman, A.F. 2000. RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*. EMBO J. 19: 2435-2443.

Integration of an insertion plasmid at *RAP1* (rhoptry associated protein 1) generates a parasite clone that produces a RAP1 truncate. In these parasites, the components of the rhoptry low molecular weight complex (RAP1-3) fail to associate, indicating that the complex is not important for parasite invasion of red blood cells.

- Barker, R.H.Jr., Metelev, V., Coakley, A., and Zamecnik, P. 1998. *Plasmodium falciparum*: effect of chemical structure on efficacy and specificity of antisense oligonucleotides against malaria *in vitro*. Exp. Parasitol. 88: 51-59.
- Barker, R.H.Jr., Metelev, V., Rapaport, E., and Zamecnik, P. 1996. Inhibition of *P. falciparum* malaria using antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA. 93: 514-518.
- Baron, U., Schnappinger, D., Helbl, V., Gossen, M., Hillen, W., and Bujard, H. 1999. Generation of conditional mutants in higher eukaryotes by switching between the expression of two genes. Proc. Natl. Acad. Sci. USA. 96: 1013-1018.
- Barstead, R. 2001. Genome-wide RNAi. Current Opin. Chem. Biol. 5: 63-66.
- Ben Mamoun, C., Gluzman, I.Y., Beverley, S.M., and Goldberg, D.E. 2000. Transposition of the *Drosophila* element *marinerl* within the human malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. 110: 405-407.

The *Drosophila* transposable element *mariner* is a member of the Tc1 family of transposons, which are ubiquitous elements of eukaryotic genomes capable of mobilization independently of host factors. The transposon was introduced in *P. falciparum* by stable episomal transfection; few insertions in the parasite genome occurred, a number of which were in the same gene (protein kinase A).

Ben Mamoun, C., Gluzman, I.Y., Goyard, S., Beverley, S.M., and Goldberg, D.E. 1999. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA. 96: 8716-8720.

Describes episomal transfection in *P. falciparum* using *BSD* (blasticidin S deaminase) of *Aspergillus terreus*, which confers resistance to blasticidin, and *NEO* (neomycin phosphotransferase II) of transposon Tn5, which confers resistance to geneticin/G418.

Ben Mamoun, C., Gluzman, I.Y., Hott, C., MacMillan, S.K., Amarakone, A.S., Anderson, D.L., Carlton, J.M.-R., Dame, J.B., Chakrabarti, D., Martin, R.K., Brownstein, B.H., and Goldberg, D.E. 2001. Coordinated programme of gene expression during asexual intraerythrocytic development of the human malaria parasite *Plasmodium falciparum* revealed by microarray analysis. Mol. Microbiol. 39: 26-36.

Microarrays were constructed with 944 amplified inserts from *P. falciparum* cDNA expressed sequenced

- Bischoff, E., Guillotte, M., Mercereau-Puijalon, O., and Bonnefoy, S. 2000. A member of the *Plasmodium falciparum Pf60* multigene family codes for a nuclear protein expressed by readthrough of an internal stop codon. Mol. Microbiol. 35: 1005-1016.
- Transient transfection is used to demonstrate that translation occurs through an internal ochre codon in the 6.1 member of the Pf60 gene family, as a luciferase reporter inserted downstream from the internal stop codon can be expressed.
- Bowman, S., *et al.* 1999. The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. Nature. 400: 532-538.
- Brecht, S., Erdhart, H., Soete, M., and Soldati, D. 1999. Genome engineering of *Toxoplasma gondii* using the site-specific recombinase Cre. Gene. 234: 239-247.
- Carlton, J.M.R., *et al.* 2001. Profiling the malaria genome: a gene survey of three species of malaria parasite with comparison to other apicomplexan species. Mol. Biochem. Parasitol. 118: 201-210.

A comparative analysis of six datasets: 5482 *P. berghei* GSSs [genome survey sequences, obtained after mung bean treatment of genomic DNA], 5582 *P. berghei* ESTs [expressed sequenced tags, obtained from cDNA libraries], 10 874 GSSs in two *P. vivax* lines, and 2438 *P. falciparum* ESTs or GSSs present in GenBank. Approximately 1000 putative new *Plasmodium* genes are identified. Their functional categorization using InterPro (a database of protein domains and signatures) is presented.

- Chiang, S.L., Mekalanos, J.J., and Holden, D.W. 1999. *In vivo* genetic analysis of bacterial virulence. Annu. Rev. Microbiol. 53: 129-154.
- Chuang, C.-F., and Meyerowitz, E.M. 2000. Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. 97: 4985-4990.
- Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. 2000. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA. 97: 6499-6503.
- Cowman, A.F., Baldi, D.L., Healer, J., Mills, K.E., O'Donnell, R.A., Reed, M.B., Triglia, T., Wickham, M.E., and Crabb, B.S. 2000. Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells. FEBS Letters. 476: 84-88.

A review that presents conclusions of gene disruption experiments at loci potentially involved in merozoite invasion of red blood cells: *MSP1-5*, *RAP1-2*, *RhopH3*, *Ag512*, *EBA175*, *AMA1*, *S-antigen*, and *ABRA*. They suggest that in the *P. falciparum* D10 line, all these genes except *EBA175* and *RAP* are essential for invasion of red blood cells in *vitro*.

- Crabb, B.S., and Cowman, A.F. 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA. 93: 7289-7294. A second report of stable transfection and plasmid integration via homologous recombination in *P. falciparum* using a *Toxoplasma gondii* DHFR-TS pyrimethamine-resistance gene. The evidence for non homologous recombination is based on a single clone.
- Crabb, B.S., Cooke, B.M., Reeder, J.C., Waller, R.F., Caruana, S.R., Davern, K.M., Wickham, M.E., Brown, G.V., Coppel, R.L., and Cowman, A.F. 1997a. Targeted gene disruption shows that knobs enable malariainfected red cells to cytoadhere under physiological shear stress. Cell. 89: 287-296.

The *KAHRP* gene in *P. falciparum* is inactivated by insertion of a replacement plasmid via a single crossover. KAHRP(-) mutants do not correctly localize PfEMP1 and form knobs on the surface of infected erythrocytes, which do not adhere to CD36 under physiological flow conditions.

- Crabb, B.S., Triglia, T., Waterkeyn, J.G., and Cowman, A.F. 1997b. Stable transgene expression in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 90: 131-144.
- Cui, L., Rzomp, K.A., Fan, Q., Martin, S.K., and Williams, J. 2001. *Plasmodium falciparum*: differential display analysis of gene expression during gametocytogenesis. Exp. Parasitol. 99: 244-254.
- Dechering, K.J., Kaan, A.M., Mbacham, W., Wirth, D.F., Eling, W., Konings, R.N.H., and Stunnenberg, H.G. 1999. Isolation and functional characterization of two distinct sexual-stage-specific promoters of the human malaria parasite *Plasmodium falciparum*. Mol. Cell. Biol. 19: 967-978.
- *Pfs16* and *Pfs25* are activated at the onset of gametocytogenesis (in erythrocytes) and gametogenesis (in mosquitoes) respectively. Their promoters are characterized by transient transfection in *P. falciparum* (blood stages) and *P. gallinaceum* (mosquito stages). An AAGGAATA sequence is identified that is present only in the *Pfs25* promoter region, binds a mosquito stage-specific transcription factor (PAF-1), and is important for *Pfs25* transcription.
- Dechering, K.J., Thompson, J., Dodemont, H.J., Eling, W., and Konings, R.N. 1997. Developmentally regulated expression of *pfs16*, a marker for sexual differentiation of the human malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. 89: 235-244.
- Deitsch, K.W., Calderwood, M.S., and Wellems, T.E. 2001. Cooperative silencing elements in *van* genes. Nature. 412: 875-876.
- Deitsch, K.W., del Pinal, A., and Wellems, T.E. 1999. Intracluster recombination and *var* transcription switches in the antigenic variation of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 101: 107-116.

Transient transfection is used to analyze the promoter of a *var* gene (*var7b*). The promoter can be active when borne by an episome transfected in a clone where its chromosomal version is inactive. This favors a model of epigenetic regulation of *van* gene transcription, involving gene silencing through local changes in chromatin structure. de Koning-Ward, T.F., Fidock, D.A., Thathy, V., Ménard, R., van Spaendonk, R.M.L., Waters, A.P., and Janse, C.J. 2000a. The selectable marker human dihydrofolate reductase enables sequential genetic manipulations of the *Plasmodium berghei* genome. Mol. Biochem. Parasitol. 106: 199-212.

Describes the selection of plasmid integration events using a WR99210-resistant form of human DHFR in a genome already manipulated using the pyrimethamineresistant form of *P. berghei* DHFR-TS.

- de Koning-Ward, T.F., Janse, C.J., and Waters, A.P. 2000b. The development of genetic tools for dissecting the biology of malaria parasites. Annu. Rev. Microbiol. 54: 157-185.
- de Koning-Ward, T.F., Waters, A.P., and Crabb, B.S. 2001. Puromycin-N-acetyltransferase as a selectable marker for use in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 117: 155-160.
- Dessens, J.T., Beetsma, A.L., Dimopoulos, G., Wengelnik, K., Crisanti, A., Kafatos, F.C., and Sinden, R.E. 1999. CTRP is essential for mosquito infection by malaria ookinetes. EMBO J. 18: 6221-6227.

Ookinetes of *P. berghei* in which the *CTRP* gene is disrupted after a double crossover event are not motile, fail to invade the mosquito midgut epithelium and do not develop into oocysts.

- Dessens, J.T., Margos, G., Rodriguez, M.C., and Sinden, R.E. 2000. Identification of differentially regulated genes of *Plasmodium* by suppression subtractive hybridization. Parasitol. Today. 16: 354-356.
- Duraisingh, M.T., Triglia, T., and Cowman, A.F. 2002. Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. Int. J. Parasitol. 32: 81-89.
- Falkow, S. 1988. Molecular Koch's postulates applied to microbial pathogenicity. Rev. Infect. Dis. 10 Suppl. 2: S274-276.
- Fidock, D.A., and Wellems, T.E. 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc. Natl. Acad. Sci. USA. 94: 10931-10936.

P. falciparum transfected episomally with a human, methotrexate-resistant DHFR variant becomes resistant to the anti-folate compounds WR99210 and cycloguanil, but not the cycloguanil precursor proguanil, suggesting that the latter acts independently of DHFR.

- Fidock, D.A., Nomura, T., Cooper, R.A., Su, X.-z., Talley, A.K., and Wellems, T.E. 2000a. Allelic modifications of the *cg2* and *cg1* genes do not alter the chloroquine response of drug-resistant *Plasmodium falciparum*. Mol. Biochem. Parasitol. 110: 1-10.
- Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M.B., Sidhu, A., Naudé, B., Deitsch, K.W., Su, X.-z., Wootton, J.C., Roepe, P.D., and Wellems, T.E. 2000b. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell. 6: 861-871.
- Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. 2000. Functional genomic analysis of *C. elegans*

chromosome I by systematic RNA interference. Nature. 408: 325-330.

- Gardiner, D.L., Holt, D.C., Thomas, E.A., Kemp, D.J., and Trenholme, K.R. 2000. Inhibition of *Plasmodium falciparum clag9* gene function by antisense RNA. Mol. Biochem. Parasitol. 110: 33-41.
- An internal fragment of *clag9* is cloned in an antisense direction under the control of the strong *calmodulin* promoter. Stable expression of the episome strongly decreases production of clag9 protein and reproduces the phenotype obtained after *clag9* disruption (decreased adherence of infected eythrocytes to CD36+ melanoma cells).
- Gardner, M.J., *et al.* 1998. Chromosome 2 sequence of the human malaria parasite *Plasmodium falciparum*. Science. 282: 1126-1132.
- Gueiros-Filho, F.J., and Beverley, S.M. 1997. Transkingdom transposition of the *Drosophila* element *mariner* within the protozoan *Leishmania*. Science. 276: 1716-1719.
- Gönczy, P., *et al.* 2000. Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. Nature. 408: 331-336.
- Goonewardene, R., Daily, J., Kaslow, D., Sullivan, T.J., Duffy, P., Carter, R., Mendis, K., and Wirth, D. 1993. Transfection of the malaria parasite and expression of firefly luciferase. Proc. Natl. Acad. Sci. USA. 90: 5234-5236.

The first demonstration that *Plasmodium* can be transfected. *P. gallinaceum* gametes and zygotes (extracellular) are electroporated with a plasmid that contains the firefly luciferase gene inserted into the *Pgs28* gene and flanking DNA. After 24 hr, luciferase activity is transiently expressed in electroporated parasites.

- Gossen, M., and Bujard, H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA. 89: 5547-5551.
- Hamann, L., Buss, H., and Tannich, E. 1997. Tetracyclinecontrolled gene expression in *Entamoeba histolytica*. Mol. Biochem. Parasitol. 84: 83-91.
- Hammond, S.M., Caudy, A.A., and Hannon, G.J. 2001. Post-transcriptional gene silencing by double-stranded RNA. Nature Rev. Genet. 2: 110-119.
- Hayward, R.E., DeRisi, J.L., Alfadhli, S., Kaslow, D.C., Brown, P.O., and Rathod, P.K. 2000. Shotgun DNA microarrays and stage-specific gene expression in *Plasmodium falciparum* malaria. Mol. Microbiol. 35: 6-14.
 - PCR-amplified inserts (3648 inserts of 1.5-kb average size, thought to represent ~40% of the parasite genes) from a *P. falciparum* mung bean nuclease genomic library (ORF- or exon-enriched) were printed on glass slides. These 'shotgun' microarrays were hybridized with a mixture of Cy3 or Cy5-labelled cDNA from the erythrocytic trophozoite and gametocyte stages, respectively. The 85 arrayed genes showing the highest differential fluorescence (red/green or green/red) were sequenced, and new genes were identified.
- Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. 1995. Simultaneous identification

of bacterial virulence genes by negative selection. Science. 269: 400-403.

- Hoffman, S.L., Rogers, W.O., Carucci, D.J., and Venter, J.C. 1998. From genomics to vaccines: malaria as a model system. Nature Med. 4: 1351-1353.
- Hoffman, S.L., Subramanian, G.M., Collins, F.H., and Venter, J.C. 2002. *Plasmodium*, human and *Anopheles* genomics and malaria. Nature. 415: 702-709.
- Horrocks, P., and Kilbey, B.J. 1996. Physical and functional mapping of the transcriptional start sites of *Plasmodium falciparum* proliferating cell nuclear antigen. Mol. Biochem. Parasitol. 82: 207-215.
- Horrocks, P., and Lanzer, M. 1999. Mutational analysis identifies a five base pair *cis*-acting sequence essential for *GBP130* promoter activity in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 99: 77-87.

This work progressively narrows down a 5 bpsequence, located within a 73 bp-enhancer element, which is crucial for efficient activity of the *GBP130* promoter and specifically binds nuclear factors.

- Horrocks, P., Dechering, K., and Lanzer, M. 1998. Control of gene expression in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 95: 171-181.
- Jo, D., Nashabi, A., Doxsee, C., Lin, Q., Unutmaz, D., Chen, J., and Ruley, H.E. 2001. Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase. Nat. Biotech. 19: 929-933.

Jomaa, H. *et al.* 1999. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science. 285: 1573-1576.

Kadekoppala, M., Cheresh, P., Catron, D., Ji, D., Deitsch,
K., Wellems, T.E., Seifert, H.S., and Haldar, K. 2001.
Rapid recombination among transfected plasmids,
chimeric episome formation and *trans* gene expression
in *Plasmodium falciparum*. Mol. Biochem. Parasitol.
112: 211-218.

Co-transfection with a plasmid that expresses GFP fluoresence and another that confers pyrimethamine resistance allows drug selection of a population where a third of the parasites stably fluoresce. These fluorescent parasites contain drug-selectable, chimeric concatemers of both plasmids, and can only be obtained when both plasmids share a large region of homology of *Plasmodium* DNA (5' *hrp3* UTR sequences).

Kadekoppala, M., Kline, K., Akompong, T., and Haldar, K. 2000. Stable expression of new chimeric fluorescent reporter in the human malaria parasite *Plasmodium falciparum*. Infect. Immun. 68: 2328-2332.

A DHFR-GFPmut2 fusion, which confers methotrexateresistance and fluorescence, is used in *P. falciparum* episomal and integrative transfection.

Kaneko, O., Fidock, D.A., Schwartz, O.M., and Miller, L.H. 2000. Disruption of the C-terminal region of EBA-175 in the Dd2/Nm clone of *Plasmodium falciparum* does not affect erythrocyte invasion. Mol. Biochem. Parasitol. 110: 135-146.

EBA-175 (Erythrocyte-binding antigen) is localized in the merozoite micronemes and binds the red blood cell surface molecule glycophorin A in a sialic aciddependent manner. Its encoding gene is targeted in the *P. falciparum* Dd2/Nm clone, whose merozoites invade erythrocytes mainly via a sialic acid-independent pathway. Parasites expressing a EBA-175 truncate lacking the cytoplasmic domain can be selected, implying that EBA-175 is not involved in the sialic acid-independent pathway of merozoite invasion.

- Kappe, S., Bruderer, T., Gantt, S., Fujioka, H., Nussenzweig, V., and Ménard, R. 1999. Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. J. Cell. Biol. 147: 937-943. An ends-in strategy is used to demonstrate the role of the cytoplasmic tail of TRAP in sporozoite gliding motility and cell invasion. Exchanging the tail of TRAP by that of *Toxoplasma gondii* MIC2 does not affect sporozoite gliding or invasion, suggesting a functional conservation of the motor system that drives these processes in Apicomplexa. Mutations in the TRAP tail lead to a novel form of motility, termed pendulum motility.
- Kocken, C.H.M., Ozwara, H., van der Wel, A., Beetsma, A.L., Mwenda, J.M., and Thomas, A.W. 2002. *Plasmodium knowlesi* provides a rapid *in vitro* and *in vivo* transfection system that enables double-crossover gene knockout studies. Infect. Immun. 70: 655-660.
- Kocken, C.H.M., van der Wel, A.M., and Thomas, A.W. 1999. *Plasmodium cynomolgi*: transfection of bloodstage parasites using heterologous DNA constructs. Exp. Parasitol. 93: 58-60.
- Kocken, C.H.M., van der Wel, A.M., Dubbeld, M.A., Narum, D.L., van de Rijke, F.M., van Gemert, G.J., van der Linde, X., Bannister, L.H., Janse, C., Waters, A.P., and Thomas, A.W. 1998. Precise timing of expression of a *Plasmodium falciparum*-derived transgene in *Plasmodium berghel* is a critical determinant of subsequent subcellular localization. J. Biol. Chem. 273: 15119-15124.
- Kumar, A., and Snyder, M. 2001. Emerging technologies in yeast genomics. Nature Rev. Genet. 2: 302-312.
- Kuwabara, P.E., and Coulson, A. 2000. RNAi Prospects for a general technique for determining gene function. Parasitol. Today. 16: 347-349.
- Lau, A.O.T., Sacci Jr, J.B., and Azad, A.F. 2000. Retrieving parasite specific liver stage gene products in *Plasmodium yoelii* infected livers using differential display. Mol. Biochem. Parasitol. 111: 143-151.
- Lobo, C.-A., Fujioka, H., Aikawa, M., and Kumar, N. 1999. Disruption of the *Pfg27* locus by homologous recombination leads to loss of the sexual phenotype in *P. falciparum*. Mol. Cell. 3: 793-798.

Pfg27 encoding a protein specifically produced at the onset of gametocytogenesis is targeted by single crossover integration of a replacement plasmid. Two mutants, in which full-length Pfg27 lacks its promoter or terminator sequences, have reduced or no Pfg27 transcription and display fewer or no typical gametocytes, respectively.

Matuschewski, K., Nunes, A., Nussenzweig, V., and Ménard, R. 2002. *Plasmodium* sporozoite invasion into insect and mammalian cells is directed by the same dual binding system. EMBO J. 21: 1597-1606.

This paper reports the effect of loss-of-function mutations in the extracellular domain of TRAP, a protein necessary for sporozoite gliding and cell invasion. Results indicate that neither the A-domain nor the

thrombospondin type 1 repeat is necessary for gliding but that both are important for sporozoite invasion into mosquito salivary glands, the rodent liver, and cultured cells.

- McRobert, L., and McConkey, G.A. 2002. RNA interference (RNAi) inhibits growth of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 119: 273-278.
- *P. falciparum*-infected red blood cells are electroporated with ~1-kb long double-stranded (ds) RNA corresponding to a segment of an essential gene (dihydroorotate dehydrogenase, DHODH). This causes a ~50% decrease in DHODH mRNA levels and in parasite growth, detected after 24 h and up to 72 h after electroporation. Electroporation with single-stranded RNA, or with dsRNA corresponding to nonessential genes, do not inhibit parasite growth in red blood cells.
- Meissner, M., Brecht, S., Bujard, H., and Soldati, D. 2001. Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. Nucleic Acids Res. 29: e115.
- Ménard, R., and Janse, C.J. 1997. Gene targeting in malaria parasites. Methods. 13: 148-157.
- Ménard, R., and Nussenzweig, V. 2000. Structurefunction analysis of malaria proteins by gene targeting. Parasitol. Today. 16: 222-224.
- Ménard, R., Sultan, A.A., Cortes, C., Altszuler, R., van Dijk, M.R., Janse, C.J., Waters, A.P., Nussenzweig, R.S., and Nussenzweig, V. 1997. Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes. Nature. 385: 336-340.

The first *Plasmodium* mutant obtained by site-directed mutagenesis. Clones in which the single-copy *CS* gene is disrupted by allelic exchange do not produce sporozoites in the mosquito midgut.

- Metzger, D., and Chambon, P. 2001. Site- and timespecific gene targeting in the mouse. Methods. 24: 71-80.
- Mota, M.M., Thathy, V., Nussenzweig, R.S., and Nussenzweig, V. 2001. Gene targeting in the rodent malaria parasite *Plasmodium yoelii*. Mol. Biochem. Parasitol. 113: 271-278.
- Munasinghe, A., Patankar, S., Cook, B.P., Madden, S.L., Martin, R.K., Kyle, D.E., Shoaibi, A., Cummings, L.M., and Wirth, D.F. 2000. Serial analysis of gene expression (SAGE) in *Plasmodium falciparum*. Application of the technique to A-T rich genomes. Mol. Biochem. Parasitol. 113: 23-34.
- Natarajan, R., Thathy, V., Mota, M.M., Hafalla, J.C.R., Ménard, R., and Vernick, K.D. 2001. Fluorescent *Plasmodium berghei* sporozoites and pre-erythrocytic stages: a new tool to study mosquito and mammalian host interactions with malaria parasites. Cell. Microbiol. 3: 371-379.

The *gfp* gene is introduced at the *CS* locus and placed under the control of *CS* 5' and 3' expression sequences, rendering recombinant parasites fluorescent at the sporozoite and liver stages. This clone, which has a wild-type phenotype, can be used to separate infected cells by FACS.

Nunes, A., Thathy, V., Bruderer, T., Sultan, A.A., Nussenzweig, R.S., and Ménard, R. 1999. Subtle mutagenesis by ends-in recombination in malaria parasites. Mol. Cell. Biol. 19: 2895-2902.

This paper shows that in *P. berghel* insertion plasmids integrate at the homologous genomic locus via the double-strand gap repair model. Short lengths of gene conversion tracts are associated with plasmid integration at the *TRAP* locus, allowing various modifications to be introduced in the gene by a single-step ends-in strategy.

- O'Donnell, R.A., Freitas-Junior, L.H., Preiser, P.R., Williamson, D.H., Duraisingh, M., McElwain, T.F., Scherf, A., Cowman, A.F., and Crabb, B.S. 2002. A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. EMBO J. 21: 1231-1239.
- O'Donnell, R.A., Preiser, P.R., Williamson, D.H., Moore, P.W., Cowman, A.F., and Crabb, B.S. 2001. An alteration in concatemeric structure is associated with efficient segregation of plasmids in transfected *Plasmodium falciparum* parasites. Nucleic Acids Res. 29: 716-724.
- In *P. falciparum*, transfected plasmids are arranged as head-to-tail concatemers. These episomes are first unstable (unstable replicating forms, URFs) and lost if selection is removed. After extended periods (>4 months), they become stable and segregate evenly between daughter merozoites, even in the absence of drug pressure (stably replicating forms, SRFs). These SRFs are more complex structures and may contain single-stranded DNA.
- O'Donnell, R.A., Saul, A., Cowman, A.F., and Crabb, B.S. 2000. Functional conservation of the malaria vaccine antigen MSP-1₁₉ across distantly related *Plasmodium* species. Nature Med. 6: 91-95.

This study in *P. falciparum* shows that *MSP-1* (merozoite surface protein 1) can be targeted but not disrupted, and can be replaced by a gene encoding a hybrid MSP-1 whose C-terminus [MSP-1₁₉] is from *P. chabaudi*. Since Pf MSP-1₁₉ sequences are conserved among field isolates but highly divergent from that of Pc MSP-1₁₉, sequence conservation in *P. falciparum* cannot be explained by a functional constraint.

Pace, T., Scotti, R., Janse, C.J., Waters, A.P., Birago, C., and Ponzi, M. 2000. Targeted terminal deletions as a tool for functional genomics studies in *Plasmodium*. Genome Res. 10: 1414-1420.

This study describes an interesting system based on transfection of linear replacement molecules in *P. berghei* for introducing large (up to 60 kb) and defined deletions at chromosome ends. It provides a means to study the functional consequences of genomic rearrangments, or the loss of specific genes associated with chromosome ends.

Patankar, S., Munasinghe, A., Shoaibi, A., Cummings, L.M., and Wirth, D.F. 2001. Serial analysis of gene expression in *Plasmodium falciparum* reveals the global expression profile of erythrocytic stages and the presence of anti-sense transcripts in the malarial parasite. Mol. Biol. Cell. 12: 3114-3125.

A SAGE library of ~8,000 tags is generated from erythrocytic stages of *P. falciparum*. BLAST analysis of highly abundant tags documents the major metabolic

pathways of the parasite cultured in normal conditions, and reveals the presence of antisense transcription in the parasite.

Porter, A. 1998. Controlling your losses: conditional gene silencing in mammals. Trends Genet. 14: 73-79.

Reed, M.B., Caruana, S.R., Batchelor, A.H., Thompson, J.K., Crabb, B.S., and Cowman, A.F. 2000a. Targeted disruption of an erythrocyte binding antigen in *Plasmodium falciparum* is associated with a switch toward a sialic acid-independent pathway of invasion. Proc. Natl. Acad. Sci. USA. 97: 7509-7514.

An insertion plasmid is used to generate a *P. falciparum* clone that produces an EBA-175 truncate lacking the C-terminus including the cytoplasmic domain. Mutant merozoites still invade erythrocytes, indicating that EBA-175 is not essential for invasion. However, mutants have switched to a sialic acid-independent invasion pathway, showing that the parasite can utilize the sialic acid-independent pathway for invasion.

- Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K., and Cowman, A.F. 2000b. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature. 403: 906-909.
- Roos, D.S., Sullivan, W.J., Striepen, B., Bohne, W., and Donald, R.G.K. 1997. Tagging genes and trapping promoters in *Toxoplasma gondii* by insertional mutagenesis. Methods. 13: 112-122.
- Ross-Macdonald, P., *et al.* 1999. Large-scale analysis of the yeast genome by transposon tagging and gene disruption. Nature. 402: 413-418.
- Ross-Macdonald, P., Sheehan, A., Shirleen Roeder, G., and Snyder, M. 1997. A multipurpose transposon system for analyzing protein production, localization, and function in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA. 94: 190-195.
- Sacci Jr, J.B., Aguiar, J.C., Lau, A.O., Hoffman, S.L. 2002. Laser capture microdissection and molecular analysis of *Plasmodium yoelii* liver-stage parasites. Mol. Biochem. Parasitol. 119: 285-289.
- Sauer, B. 1998. Inducible gene targeting in mice using the Cre/lox system. Methods. 14: 381-392.
- Sharp, P.A. 2001. RNA interference-2001. Genes & Dev. 15: 485-490.
- Shi, H., Djikeng, A., Mark., T., Wirtz, E., Tschudi, C., and Ullu, E. 2000. Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. RNA. 6: 1069-1076.
- Shoemaker, D.D., Lashkari, D.A., Morris, D., Mittmann, M., and Davis, R.W. 1996. Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. Nature Genet. 14: 450-456.
- Strauss, E.J., and Falkow, S. 1997. Microbial pathogenesis: genomics and beyond. Science. 276: 707-712.
- Sultan, A.A., de Koning-Ward, T.F., Fidock, D., Nussenzweig, V., and Ménard, R. 1999a. Complementation of TRAP knockout parasites of *Plasmodium berghei* using human dihydrofolate reductase gene as a selectable marker. Meeting Abstract. Woods Hole, MA, USA. September 1999.

Sultan, A.A., Thathy, V., de Koning-Ward, T.F., and

Nussenzweig, V. 2001. Complementation of *Plasmodium berghei* TRAP knockout parasites using human dihydrofolate reductase gene as a selectable marker. Mol. Biochem. Parasitol. 113: 151-156.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K., Crisanti, A., Nussenzweig, V., Nussenzweig, R.S., Ménard, R. 1997. TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites. Cell. 90: 511-522.

The sporozoite-specific *TRAP* gene is disrupted in *P. berghei* using insertion and replacement plasmids. TRAP appears to be essential for sporozoite infection of mosquito salivary glands and the rat liver, as well as for sporozoite gliding motility, suggesting that host cell invasion and gliding motility of sporozoites have a common molecular basis.

Sultan, A.A., Thathy, V., Nussenzweig, V., Ménard, R. 1999b. Green fluorescent protein as a marker in *Plasmodium berghei* transformation. Infect. Immun. 67: 2602-2606.

A DHFR-TS fusion to the rapidly folding GFPmut2 is used as a new marker for selecting integration events. Pyrimethamine selection is associated with flow cytometry, which can be used to eliminate drug resistant parasites that arise by spontaneous mutations or gene conversion events at the *DHFR-TS* locus, and clone integrants at day 10 post-electroporation.

Surolia, N., and Surolia, A. 2001. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*. Nature Med. 7: 167-173.

Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A., and Driscoll, M. 2000. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. Nature Genet. 24: 180-183.

Taylor, H.M., Triglia, T., Thompson, J., Sajid, M., Fowler, R., Wickham, M.E., Cowman, A.F., and Holder, A.A. 2001. *Plasmodium falciparum* homologue of the genes for *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins, which is transcribed but not translated. Infect. Immun. 69: 3635-3645.

Disruption in *P. falciparum* of a member of the *P. vivax PvRBP* and *P. yoelii Py235* gene family, *PfRH3*, has no consequence on parasite replication in red cells. *PfRH3* appears to be a full-length and transcribed pseudogene.

Templeton, T.J., Kaslow, D.C., and Fidock, D.A. 2000. Developmental arrest of the human malaria parasite *Plasmodium falciparum* within the mosquito midgut via *CTRP* gene disruption. Mol. Microbiol. 36: 1-9.

Thathy, V., Fujioka, H., Gantt, S., Nussenzweig, R.S., Nussenzweig, V., and Ménard, R. 2002. Levels of CS protein in the *Plasmodium* oocyst control sporozoite budding and morphology. EMBO J. 21: 1586-1596.

P. berghei clones producing low amounts of CS are constructed by truncation of the 3' UTR of the CS gene. Comparison of oocyst differentiation between the wild-type, non CS producer, low CS producer and complemented clones indicate that CS has a specific role in establishing polarity in oocysts and in the formation of sporozoites.

Tomas, A.M., Margos, G., Dimopoulos, G., van Lin,

L.H.M., de Koning-Ward, T.F., Sinha, R., Lupetti, P., Beetsma, A.L., Rodriguez, M.C., Karras, M., Hager, A., Mendoza, J., Butcher, G.A., Kafatos, F., Janse, C.J., Waters, A.P., and Sinden, R.E. 2001. P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. EMBO J. 20: 3975-3983.

Parasites lacking either P25 or P28 generate normal numbers of ookinetes and mildly reduced numbers of oocysts, while parasites lacking both proteins (both genes are linked and were deleted by a single event) generate fewer ookinetes and greatly reduced numbers of oocysts. The functions of P25 and P28 are proposed to be multiple and partially redundant.

- Tomas, A.M., van der Wel, A.M., Thomas, A.W., Janse, C.J., and Waters, A.P. 1998. Transfection systems for animal models of malaria. Parasitol. Today. 14: 245-249.
- Trenholme, K.R., Gardiner, D.L., Holt, D.C., Thomas, E.A., Cowman, A.F., and Kemp, D.J. 2000. *Clag9*: a cytoadherence gene in *Plasmodium falciparum* essential for binding of parasitized erythrocytes to CD36. Proc. Natl. Acad. Sci. USA. 97: 4029-4033.
- Triglia, T., Healer, J., Caruana, S.R., Hodder, A.N., Anders, R.F., Crabb, B.S., and Cowman, A.F. 2000. Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. Mol. Microbiol. 38: 706-718.

The involvement in merozoite invasion of erythrocytes of AMA-1 (apical membrane antigen 1), a merozoite surface protein, is suggested by several lines of evidence. (i) Only single crossover events that do not disrupt the gene can be selected at the *PfAMA-1* locus. (ii) When *PcAMA-1* (P. chabaudii) is co-expressed with endogenous *PfAMA-1*, its contribution to merozoite invasion is shown by increased invasion in mouse red blood cells and using antibody inhibition assays. (iii) However, *PfAMA-1* cannot be disrupted despite simultaneous expression of *PcAMA-1*.

Triglia, T., Thompson, J.K., and Cowman, A.F. 2001. An EBA175 homologue which is transcribed but not translated in erythrocytic stages of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 116: 55-63.

Searches of the *P. falciparum* genome databases reveal the presence of an *EBA175* homologue, called $\Psi EBA165$. This study shows that $\Psi EBA165$, which can be disrupted, is a transcribed pseudogene.

Triglia, T., Wang, P., Sims, P.F.G., Hyde, J.E., and Cowman, A.F. 1998. Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. EMBO J. 17: 3807-3815.

Single crossovers are used to generate a series of modified *dhps* genes to assess the contribution of mutations found in sulfadoxine-resistant field isolates of *P. falciparum*. It is shown that high levels of sulfadoxine resistance can be conferred by a stepwise accumulation of mutations around a primary A437G substitution in DHPS.

Tsai, Y.L., Hayward, R.E., Langer, R.C., Fidock, D.A., and Vinetz, J.M. 2001. Disruption of *Plasmodium falciparum* chitinase markedly impairs parasite invasion of mosquito midgut. Infect. Immun. 69: 4048-4054.

- Urlinger, S., Baron, U., Thellmann, M., Hasan, M.T., Bujard, H., and Hillen, W. 2000. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc. Natl. Acad. Sci. USA. 97: 7963-7968.
- Valdivia, R.H., and Falkow, S. 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science. 277: 2007-2011.
- van der Wel, A.M., Tomas, A.M., Kocken, C.H.M., Malhotra, P., Janse, C.J., Waters, A.P., and Thomas, A.W. 1997. Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. J. Exp.Med. 185, 1499-1503.
- van Dijk, M.R., Janse, C.J., and Waters, A.P. 1996. Expression of a *Plasmodium* gene introduced into subtelomeric regions of *Plasmodium berghei* chromosomes. Science. 271: 662-665.

The first evidence of homologous recombination in *P. berghei*. A targeting plasmid, which is linearized in a 2.2 kb-subtelomeric fragment present as 200-300 copies in the parasite genome, integrates at several of the endogenous copies in three different chromosomes.

van Dijk, M.R., Janse, C.J., Thompson, J., Waters, A.P., Braks, J.A.M., Dodemont, H.J., Stunnenberg, H.G., van Gemert, G.-J., Sauerwein, R.W., and Eling, W. 2001. A central role for P48/45 in malaria parasite male gamete fertility. Cell. 104: 153-164.

This gene disruption study in both *P. berghei* and *P. falciparum* shows that P48/45, which is produced on the surface of male and female gametocytes and gametes, is important specifically for zygote formation. Elegant *in vitro* studies using *P. berghei* parasites indicate that only male null gametes are impaired in fertilization.

- van Dijk, M.R., Vinkenoog, R., Ramesar, J., Vervenne, R.A.W., Waters, A.P., and Janse, C.J. 1997. Replication, expression and segregation of plasmid-borne DNA in genetically transformed malaria parasites. Mol. Biochem. Parasitol. 86: 155-162.
- van Dijk, M.R., Waters, A.P., and Janse, C.J. 1995. Stable transfection of malaria parasite blood stages. Science. 268: 1358 –1362.

The first report of stable transfection in *Plasmodium*. A plasmid carrying a pyrimethamine-resistance *DHFR-TS* (dihydrofolate reductase thymidylate synthase) variant is electroporated into *P. berghel* merozoites, and erythrocytic stages of the parasites bearing replicating episomes are selected in pyrimethamine-treated rodents.

van Spaendonk, R.M.L., Ramesar, J., van Wigcheren, A., Eling, W., Beetsma, A.L., van Gemert, G.-J., Hooghof, J., Janse, C.J., and Waters, A.P. 2001. Functional equivalence of structurally distinct ribosomes in the malaria parasite, *Plasmodium berghei*. J. Biol. Chem. 276: 22638-22647.

Disruption of the C- or D- rRNA gene units (S-type, specifically expressed during the sporogonic cycle), using insertion and replacement plasmids, has little effect on parasite development in the mosquito. This excludes the view that *P. berghei* requires two functionally different S-type ribosomes (distinct from the A-type ribosomes of vertebrate stages) to complete its sporogonic cycle.

Waller, R.F., Reed, M.B., Cowman, A.F., and McFadden, G.I. 2000. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. EMBO J. 19: 1794-1802.

In *P. falciparum*, protein targeting to the apicoplast (surrounded by four membranes) is analyzed using GFP fusions expressed from episomes. The bipartite N-terminal pre-sequence on apicoplast-targeted proteins is necessary and sufficient for apicoplast targeting: the signal peptide directs GFP entry into the secretory pathway, and the transit peptide further allows GFP import into the apicoplast.

- Wang, Z., Morris, J.C., Drew, M.E., and Englund, P.T. 2000. Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. 275: 40174-40179.
- Waterkeyn, J.G., Wickham, M.E., Davern, K.M., Cooke, B.M., Coppel, R.L., Reeder, J.C., Culvenor, J.G., Waller, R.F., and Cowman, A.F. 2000. Targeted mutagenesis of *Plasmodium falciparum* erythrocyte membrane protein 3 (PfEMP3) disrupts cytoadherence of malaria-infected red blood cells. EMBO J. 19: 2813-2823.

Inactivation of *PfEMP3* using an insertion plasmid abolishes PfEMP3 production but does not alter cytoadherence of infected red cells to CD36. Production of truncated forms of PfEMP3 reduces cytoadherence, probably by a dominant negative action of the PfEMP3 truncates that block PfEMP1 transfer to the infected red cell surface.

Waters, C.T. and Janse, C.J. 2004. Malaria Parasites: Genomes and Molecular Biology (Wymondham: Caister Academic Press). This book presents sixteen reviews covering the most

important aspects of post-genomic malaria research.

- Waters, A.P., Thomas, A.W., van Dijk, M.R., and Janse, C.J. 1997. Transfection of malaria parasites. Methods. 13: 134-147.
- Wengelnik, K., Spaccapelo, R., Naitza, S., Robson, K.J.H., Janse, C.J., Bistoni, F., Waters, A.P., and Crisanti, A. 1999. The A-domain and the thrombospondin-related motif of *Plasmodium falciparum* TRAP are implicated in the invasion process of mosquito salivary glands. EMBO J. 18: 5195-5204.

A replacement strategy is used in *P. berghei* to exchange the endogenous TRAP by *P. falciparum* TRAP, but the resulting clone is severely impaired in all TRAP-dependent phenotypes.

Wickham, M.E., Rug, M., Ralph, S.A., Klonis, N., McFadden, G.I., Tilley, L., and Cowman, A.F. 2001. Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infected human erythrocytes. EMBO J. 20: 5636-5649.

The use of two KAHRP-GFP fusions expressed from stably maintained episomes reveals that before being

assembled into knobs on the erythrocyte surface, KAHRP is secreted in the parasitophorous vacuole and transiently associates with the Maurer's clefts in the erythrocyte cytoplasm.

- Winzeler, E.A., *et al.* 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science. 285: 901-906.
- Wirtz, E., and Clayton, C. 1995. Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. Science. 268: 1179-1183.
- Wirtz, E., Leal, S., Ochatt, C., and Cross, G.A.M. 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 99: 89-101.
- Wren, B.W. 2000. Microbial genome analysis: insights into virulence, host adaptation and evolution. Nature Rev. Genet. 1: 30-39.
- Wu, Y., Kirkman, L.A., and Wellems, T.E. 1996. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc. Natl. Acad. Sci. USA. 93: 1130-1134.

The first report of homologous integration of exogenous DNA into the *Plasmodium* genome. Selection of integrants with a pyrimethamine-resistant form of *DHFR-TS* from *P. falciparum* or *T. gondil* necessitates a complex procedure during which drug pressure is applied (selecting replicating episomes), removed (allowing plasmid loss), and reapplied (selecting parasites in which the plasmid integrated).

Wu Y., Sifri C.D., Lei H.-H., Su X.-Z., and Wellems, T.E. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc. Natl. Acad. Sci. USA. 92: 973-977.

The first report of transient transfection of red blood cell stages of *Plasmodium*. *P. falciparum*-infected red blood cells are electroporated with plasmids that contain *CAT* flanked by *P. falciparum* expression sequences.

- Yan, S., Martinez-Calvillo, S., Schnaufer, A., Sunkin, S., Myler, P.J., and Stuart, K. 2002. A low-background inducible promoter system in *Leishmania donovani*. Mol. Biochem. Parasitol. 119: 217-223.
- Yuda, M., Sakaida, H., and Chinzei, Y. 1999. Targeted disruption of the *Plasmodium berghei CTRP* gene reveals its essential role in malaria infection of the vector mosquito. J. Exp. Med. 190: 1711-1715.

Disruption of *CTRP*, which encodes an ookinetespecific product, blocks ookinete invasion and oocyst formation in the mosquito. CTRP(-) or (+) sporozoites (haploid) are generated by mating CTRP(-) and wildtype parasites, via ookinete diploidy and invasion of heterozygous ookinetes. As both sporozoite types are infective, CTRP is shown to be essential only for invasion of the ookinete stage.

Further Reading

Caister Academic Press is a leading academic publisher of advanced texts in microbiology, molecular biology and medical research. Full details of all our publications at **caister.com**

- MALDI-TOF Mass Spectrometry in Microbiology Edited by: M Kostrzewa, S Schubert (2016) www.caister.com/malditof
- Aspergillus and Penicillium in the Post-genomic Era Edited by: RP Vries, IB Gelber, MR Andersen (2016) www.caister.com/aspergillus2
- The Bacteriocins: Current Knowledge and Future Prospects Edited by: RL Dorit, SM Roy, MA Riley (2016) www.cajster.com/bacteriocins
- Omics in Plant Disease Resistance Edited by: V Bhadauria (2016) www.caister.com/opdr
- Acidophiles: Life in Extremely Acidic Environments Edited by: R Quatrini, DB Johnson (2016) www.caister.com/acidophiles
- Climate Change and Microbial Ecology: Current Research and Future Trends Edited by: J Marxsen (2016) www.caister.com/climate
- Biofilms in Bioremediation: Current Research and Emerging Technologies Edited by: G Lear (2016) www.caister.com/biorem
- Microalgae: Current Research and Applications Edited by: MN Tsaloglou (2016) www.caister.com/microalgae
- Gas Plasma Sterilization in Microbiology: Theory, Applications, Pitfalls and New Perspectives Edited by: H Shintani, A Sakudo (2016) www.caister.com/gasplasma
- Virus Evolution: Current Research and Future Directions Edited by: SC Weaver, M Denison, M Roossinck, et al. (2016) www.caister.com/virusevol
- Arboviruses: Molecular Biology, Evolution and Control Edited by: N Vasilakis, DJ Gubler (2016) www.caister.com/arbo
- Shigella: Molecular and Cellular Biology Edited by: WD Picking, WL Picking (2016) www.caister.com/shigella
- Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment
 Edited by: AM Romaní, H Guasch, MD Balaguer (2016)
 www.caister.com/aquaticbiofilms
- Alphaviruses: Current Biology Edited by: S Mahalingam, L Herrero, B Herring (2016) www.caister.com/alpha
- Thermophilic Microorganisms Edited by: F Li (2015) www.caister.com/thermophile







Climate Change and















- Flow Cytometry in Microbiology: Technology and Applications Edited by: MG Wilkinson (2015) www.caister.com/flow
- Probiotics and Prebiotics: Current Research and Future Trends Edited by: K Venema, AP Carmo (2015) www.caister.com/probiotics
- Epigenetics: Current Research and Emerging Trends Edited by: BP Chadwick (2015) www.caister.com/epigenetics2015
- Corynebacterium glutamicum: From Systems Biology to Biotechnological Applications Edited by: A Burkovski (2015) www.caister.com/cory2
- Advanced Vaccine Research Methods for the Decade of Vaccines Edited by: F Bagnoli, R Rappuoli (2015) www.caister.com/vaccines
- Antifungals: From Genomics to Resistance and the Development of Novel Agents Edited by: AT Coste, P Vandeputte (2015) www.caister.com/antifungals
- Bacteria-Plant Interactions: Advanced Research and Future Trends Edited by: J Murillo, BA Vinatzer, RW Jackson, et al. (2015) www.caister.com/bacteria-plant
- Aeromonas
 Edited by: J Graf (2015)
 www.caister.com/aeromonas
- Antibiotics: Current Innovations and Future Trends Edited by: S Sánchez, AL Demain (2015) www.caister.com/antibiotics
- Leishmania: Current Biology and Control Edited by: S Adak, R Datta (2015) www.caister.com/leish2
- Acanthamoeba: Biology and Pathogenesis (2nd edition) Author: NA Khan (2015) www.caister.com/acanthamoeba2
- Microarrays: Current Technology, Innovations and Applications Edited by: Z He (2014) www.caister.com/microarrays2
- Metagenomics of the Microbial Nitrogen Cycle: Theory, Methods and Applications Edited by: D Marco (2014) www.caister.com/n2