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. Gene-targeting 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 anti-malarial 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 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

*For correspondence. Email rmenard@pasteur.fr.

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′

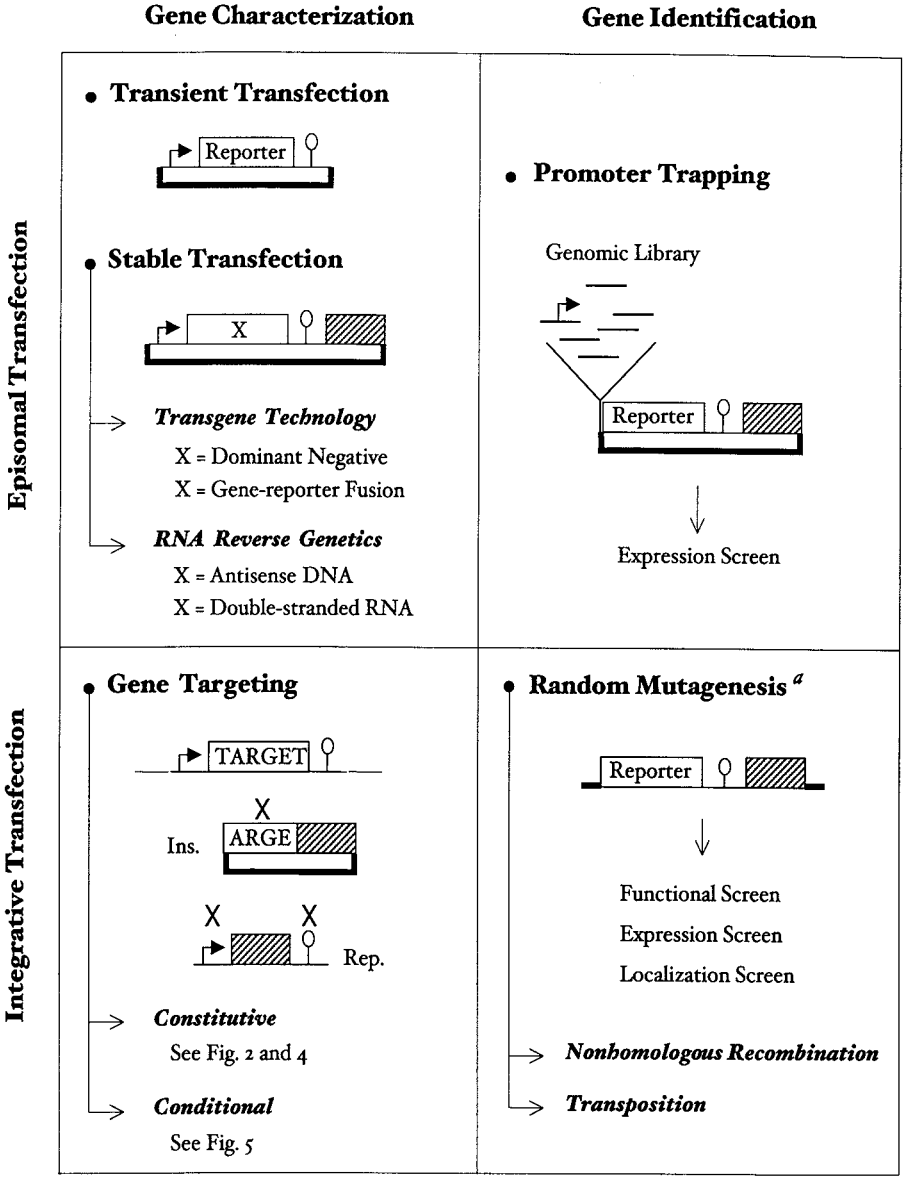

Figure 1. A theoretical view of molecular genetic techniques. The selectable marker and its expression sequences are symbolized by a grey box, the bacterial plasmid by thick lines, the gene promoter by an arrow, and the 3′ untranslated sequences necessary for gene expression by an open circle. Ins, insertion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

[a]Genes 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 factor-binding 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 half-life 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 single-copy 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

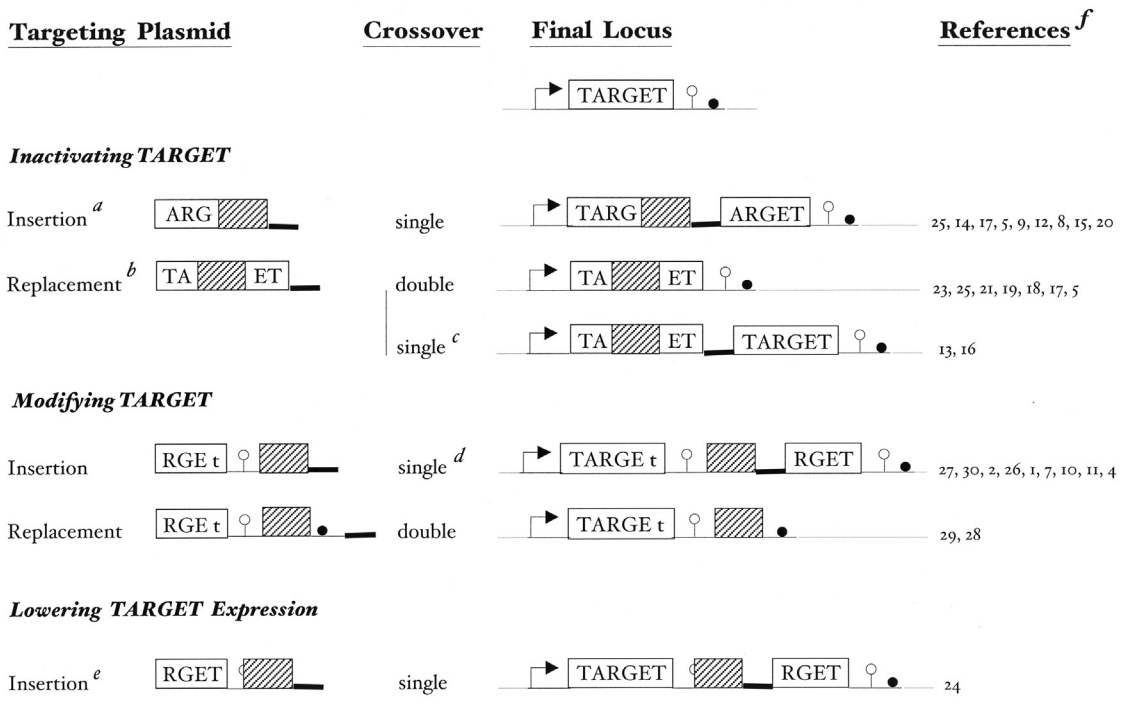

Figure 2. Gene-targeting strategies used in *Plasmodium*. Gene targeting relies on homologous recombination between a genomic sequence (TARGET gene) and its homolog in the targeting construct (shown on the left). The latter can carry a single region of homology (insertion type) or two regions flanking 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 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.
[a]The insertion plasmid must contain an internal fragment of the gene to generate two truncated gene duplicates in the final locus.
[b]The replacement plasmid should be designed to delete part or all of the target coding sequence.
[c]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.
[d]For the gene modification (t) to be recovered in the first, expressed and full-length duplicate, the SCO must occur upstream from the modification.
[e]The 3′ sequence necessary for gene expression is truncated.
[f]See 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 gene-targeting procedures and their outcomes in *P. falciparum* and *P. berghei*. The targets of electroporation are

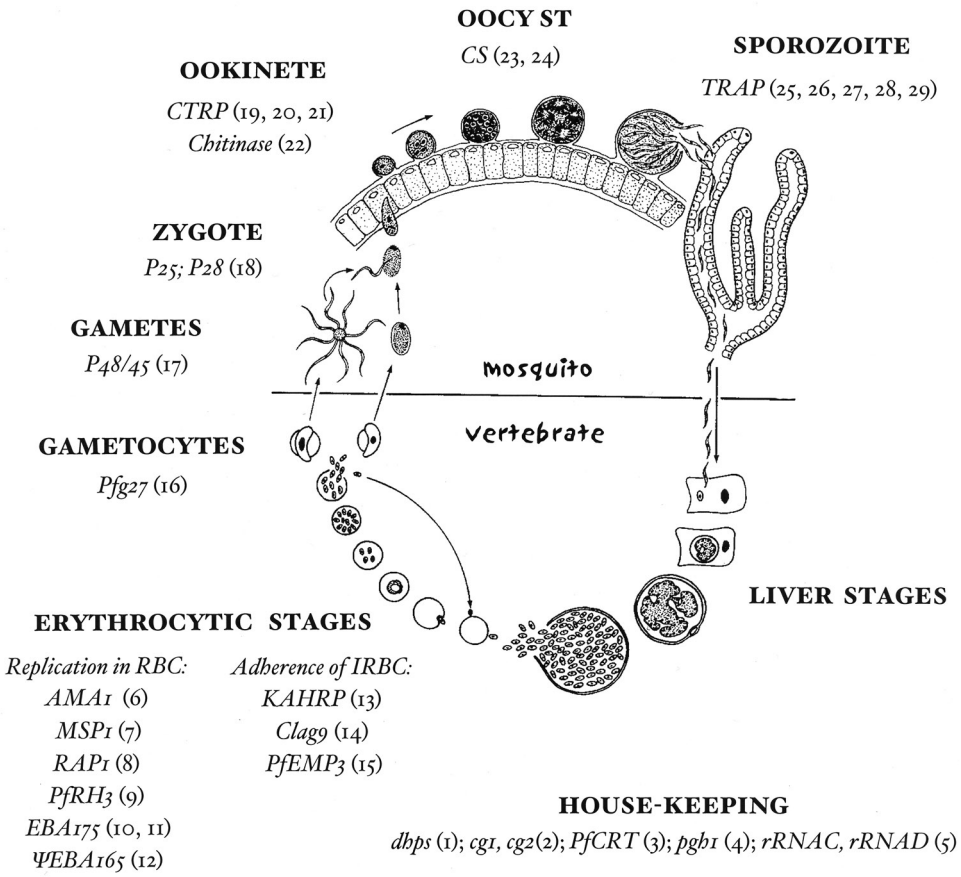

Figure 3. Targeted *Plasmodium* genes (as of February 2002). The *Plasmodium* life cycle in the two hosts, a vertebrate (lower part) and a mosquito (upper part), is shown. The parasite genes that have been manipulated by gene targeting are indicated below the respective parasite stage, with references 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 erythrocytes (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 non-targeted parasites. Therefore, with currently available tools, the best possible evidence that a gene is important for invasion of erythrocytes 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.

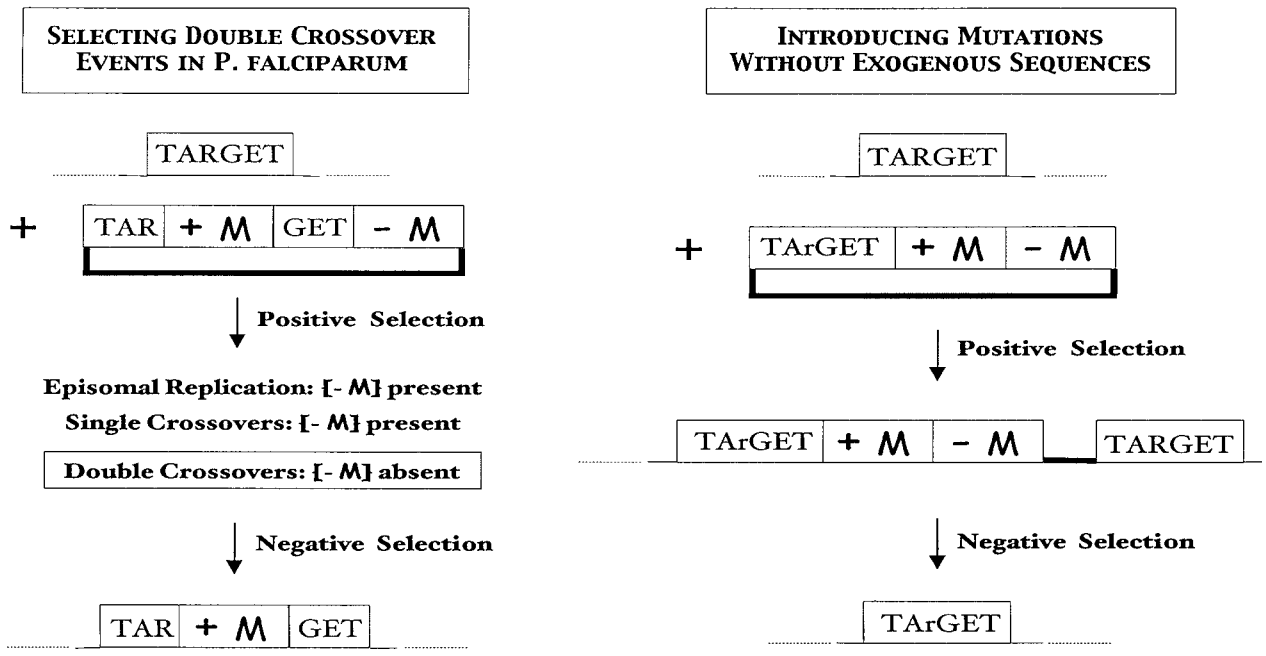

Figure 4. Using negative selectable markers in *Plasmodium* gene targeting. A negative marker allows for selecting parasites that do not express the marker, generally by conferring susceptibility to a drug. The negative marker is symbolized –M, the positive marker by +M, and the bacterial plasmid by 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 on such an integrant clone will recover parasites that undergo intrachromosomal recombination and plasmid excision (run), while leaving the modifcation (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.

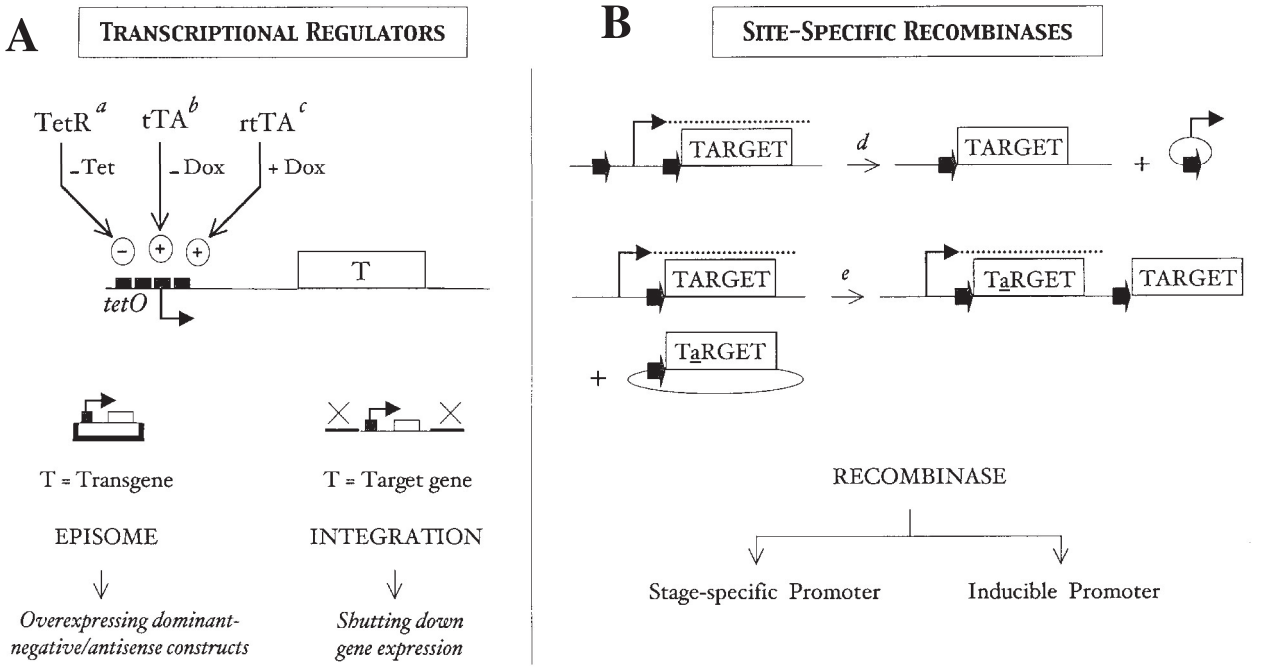

Figure 5. Tools for conditional gene expression. A) A promoter can be made drug-responsive by inserting 2 to 7 copies of 19-bp *tetO* (tetracycline operator) sequences around the transcriptional initiation site of the promoter, which are recognized by a regulator: TetR, tTA or rtTA. Left, a drug-responsive 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.
[a]TetR (tetracycline repressor) is limited by a narrow range of control. TetR dissociates from *tetO* upon tetracycline binding, leading to gene transcription.
[b]tTA (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.
[c]rtTA (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 *lox*P (*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.
[d]intrachromosomal deletion/excision is reversible but is energetically favoured over intermolecular integration.
[e]the 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 Tn*10* (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/*lox*P 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

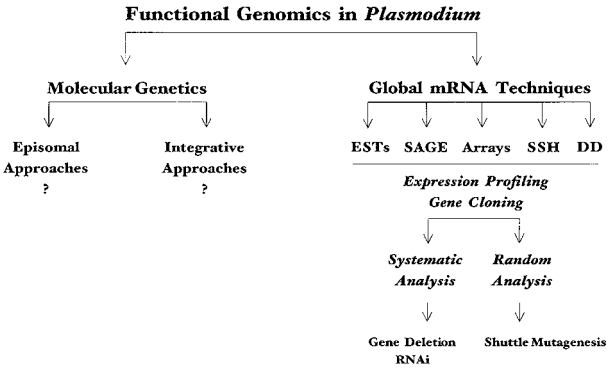

Figure 6. An outlook at functional genomics in *Plasmodium*.

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. coli* 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 Tn*3* or Tn*7* 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. cynomologi*, 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 erythrocytes. 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.

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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.

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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.

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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. berghei* 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-1_{19} 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-1_{19}] is from *P. chabaudi*. Since Pf MSP-1_{19} sequences are conserved among field isolates but highly divergent from that of Pc MSP-1_{19}, 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.

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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.

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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.

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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.

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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 Ψ*EBA165*. This study shows that Ψ*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.

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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.

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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.

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Waters, C.T. and Janse, C.J. 2004. Malaria Parasites: Genomes and Molecular Biology (Wymondham: Caister Academic Press).
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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.

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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.

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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. gondii* 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).

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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.

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Disruption of *CTRP*, which encodes an ookinete-specific product, blocks ookinete invasion and oocyst formation in the mosquito. CTRP(-) or (+) sporozoites (haploid) are generated by mating CTRP(-) and wild-type 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.

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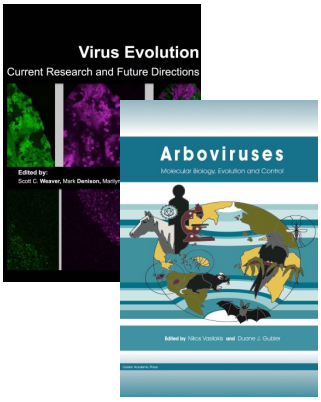

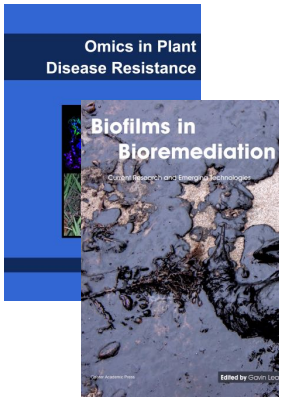

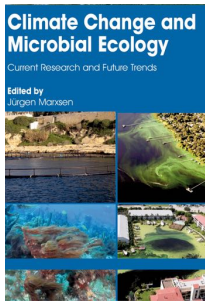

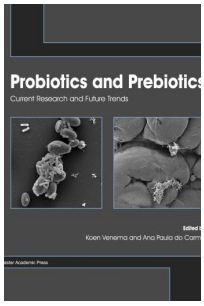

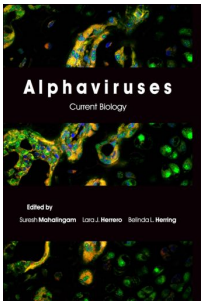

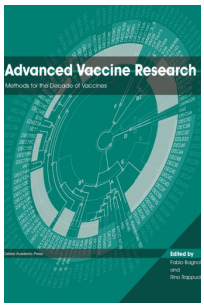

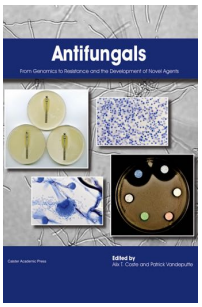

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