The importance of reverse genetics in determining gene function in apicomplexan parasites

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SUMMARY

The phylum Apixomplexa includes obligate intracellular parasites that are of enormous medical and veterinary significance, as they are responsible for a wide variety of diseases including malaria, toxoplasmosis, coccidiosis, cryptosporidiosis, theileriosis and babesiosis. The EST sequencing projects in *Toxoplasma gondii* and the *Plasmodium falciparum* genome sequencing project have greatly accelerated gene discovery, revealing for example novel coding sequences restricted to the Apicomplexa. However, easy acquisition of sequence is almost useless if the function of any given gene cannot be tested. The establishment of transfection systems in *Toxoplasma gondii*, *Neospora* and in several *Plasmodium* species has provided us with the reverse genetics methods appropriate to the functional analysis of genes. Over the past few years, the discovery of novel genes coupled to the ability to introduce or modify genes has already contributed to a better understanding of cell biology and pathogenesis of these obligate intracellular parasites. Some insights into the complex processes of parasite invasion, differentiation, regulation of gene expression and protein trafficking are emerging although identification of the exact functional roles for many molecules is still awaiting more investigation. This review summarizes progress in this area. It also emphasises the tight link and synergy between *Toxoplasma* and malaria research. The use of reverse genetics does not guarantee the answer to gene function, so we can learn from both failed and successful experiments about how better and more efficiently to use 'genomics' to accelerate discoveries relevant to the understanding of parasitism by Apicomplexa.

Key words: Apicomplexa, *Toxoplasma*, *Plasmodium*, ESTs, reverse genetics, gene expression

APICOMPLEXA: AN ACCELERATED PATH TO GENE DISCOVERY

Toxoplasma gondii is responsible for severe disease in humans and animals and has emerged in the last decades as an important opportunistic pathogen for AIDS patients. The complex life cycle of this parasite includes a rapidly-dividing asexual stage known as a 'tachyzoite' and a latent form called 'bradyzoite'. Part of the very successful strategy adopted by the parasite resides in its ability to differentiate into bradyzoites establishing a chronic infection. The molecular signals and triggering factors involved in this differentiation process are not known. Tachyzoites are rapidly dividing within a parasitophorous vacuole whereas bradyzoites have a reduced metabolism and develop into a cyst. The regulatory mechanisms governing stage interconversion are also unknown but differentiation of tachyzoites into bradyzoites can be induced experimentally using stress conditions such as high pH (Soete, Camus & Dubremetz, 1994).

Until recently, a limited number of genes was known and characterized in tachyzoites and only a couple in bradyzoites. Partial cDNA sequences or Expressed Sequence Tags (ESTs) have proven to be an economical way to gain information about expressed genes in a variety of organisms. In 1995, Ajioka and coworkers conducted a pilot experiment aiming at discovering novel genes in *T. gondii* by analysing the DNA sequences from the 5′ end of 239 directionally cloned *T. gondii* RH strain tachyzoite-derived cDNAs (Wan, Blackwell & Ajioka, 1996). This first EST data-set of *T. gondii* led to the identification of genes sharing significant similarity to several classes of genes including ribosomal proteins, metabolic enzymes, cell-cycle regulators and previously cloned abundant surface and secreted antigens. An EST showing extensive homology to the malarial adhesive protein TRAP and *Eimeria tenella* protein Etp100 could be rapidly identified as the previously characterized micronemal protein MIC2 (Wan *et al*. 1997). This informative small-scale project stimulated a joint effort from several laboratories that allowed generation of about 7400 ESTs from tachyzoites (RH and ME49 strains) (Ajioka *et al*. 1998). In parallel, 2500 ESTs from bradyzoites purified from the cysts of infected mice and 760 ESTs from parasites induced to differentiate from tachyzoites to bradyzoites *in vitro* were sequenced (Manger *et al*. 1998). This approach turned out to be very cost-effective in providing a wealth of novel genes and, in particular, sequences phylogenetically restricted to the Apicomplexa. Sequence comparison of ESTs with the databases revealed that more than 50% of the ESTs code for genes of unknown function. A potential function could be assigned to over 500 ESTs including factors

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involved in transcription, translation, metabolism, protein secretion, signal transduction, cytoskeleton organization, and families of apicomplex-specific proteins (Manger, Hehl & Boothroyd, 1998). Analysis of ESTs from the bradyzoite stage of *T. gondii* aimed at identifying developmentally-regulated genes that had been difficult to isolate previously. Although the sample size is still relatively small, examination of stage-specific ESTs provides valuable insights into the metabolism and growth of the parasite in the encysted form and represents useful basal information for further study of differentiation.

In general, the generated ESTs do not cover exhaustively the repertoire of genes in *T. gondii*. Under-representation of stage-specifically regulated genes and over-abundance of some heavily transcribed genes are important technical barriers and often one cannot avoid the tedious screening of libraries to complete sequencing of a given gene. In sharp contrast, the funding for international malaria sequencing project allowed the complete sequencing of the *P. falciparum* genome. This project is progressing unexpectedly rapidly as reflected by the recent report of the complete sequencing of chromosome 2. This accomplishment demonstrates that the barrier of A+T richness of *P. falciparum* genome has been overcome (Gardner *et al*. 1998).

A MORE TORTUOUS PATH: FROM GENES TO FUNCTION

Among the apicomplexan parasites, *T. gondii* is particularly well suited to laboratory study. Tachyzoites invade and replicate in a wide range of host cells and can be cloned easily by plaquing or limited dilution. Transient and stable transformation systems have been established for this parasite and the strategies for DNA transfection have been extensively reviewed (Boothroyd *et al*. 1994; Roos *et al*. 1994; Roos, 1996; Soldati, 1996). The list of reverse genetic tools is in constant expansion and the most recent acquisitions include the establishment of a promoter trap system, the development of a episomal vector, and the successful expression of the green fluorescent protein.

The high efficiency of transient transfection has facilitated a functional analysis of transcriptional processes whereas stable transformation offers the opportunity to express foreign genes or to delete or modify endogenous genes of interest. This latter methodology has already permitted exploration of many aspects of cellular biology such as protein trafficking, mechanisms underlying drug resistance and the process of host cell invasion. As for *T. gondii*, the severe limitation caused by the inability to introduce or modify genes has also been overcome in *Plasmodium* species (Table 1). Transient DNA transfection was first obtained in *P. gallinaceum* (Goonewardene *et al*. 1993) and then in *P. falciparum* (Wu *et al*. 1995). More recently stable transfection in *P. berghei* (van Dijk, Waters & Janse, 1995) and in *P. falciparum* (Crabbe & Cowman, 1996; Wu, Kirkman & Wellems, 1996) have been achieved by using a *T. gondii* dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) gene, modified to confer resistance to pyrimethamine, as a selectable marker. Plasmids are maintained episomally for several generations while under selection until parasite clones containing plasmid integrated in the chromosomes can be isolated. Depending upon the particular construct used for transformation, plasmids integrate either by homologous or non-homologous recombination. The *DHFR-TS* is currently the only selectable marker gene available to transform malaria parasites. The methodology is now established in several laboratories (Waters *et al*. 1997). The number of *Plasmodium* genes that have been analyzed by taking advantage of reverse genetics is increasing fast and has already advanced our understanding of these parasites. Although homologous integration is favoured in malaria parasites, it is possible to introduce and express a gene without potentially altering an endogenous gene by, for example, targeting it within the subtelomeres (van Dijk, Janse & Waters, 1996).

TRAPPING OF STAGE-SPECIFIC PROMOTERS

In *T. gondii*, vectors lacking long stretches of contiguous genomic DNA typically integrate into chromosomal DNA by non-homologous recombination. Random integration turned out to be a very efficient strategy to mutagenize the entire genome of *T. gondii* within one single electroporation cuvette by using high-frequency transformation vectors. Such genomic-scale tagging allows identification of any gene whose inactivation is not lethal to the haploid tachyzoite form of *T. gondii* and for which a suitable functional selection or screen is available. The *T. gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase (*HXGPRT*) gene, which was cloned by such random insertion mutagenesis (Donald *et al*. 1996), encodes a practical genetic marker suitable for both positive and negative selection.

Recent studies have demonstrated that developmentally-regulated genes from *T. gondii* can be isolated by a gene trap strategy based on the versatile selectable *HXGPRT* marker gene in combination with random integration (Knoll & Boothroyd, 1998; Donald, R. G. K. and Roos, D. S., unpublished). In this approach, promoterless *HXGPRT* was randomly integrated into the *T. gondii* genome. A negative selection with 6-thioxanthine was used to eliminate all recombinants fused to constitutive or tachyzoite-specific genes. After induction of the

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surviving tachyzoites to switch into bradyzoites *in vitro*, recombinant parasites were selected for resistance to mycophenolic acid (MPA). These recombinants express *HXGPRT* in a bradyzoite-specific manner, and the recovery of the DNA sequences flanking the integration site led to the identification of bradyzoite-specific genes. Among these genes, the *BRS4* gene, which encodes for the bradyzoite-specific surface antigen P36 has been identified. Interestingly, *BRS4* expression appears to be controlled at the post-transcriptional level by signal located in the 5′UTR or in the coding region of *BRS4*.

AN EPISOMAL VECTOR FOR *T. GONDII*

Insertional mutagenesis is virtually restricted to the study of non-essential genes and mutation in essential genes can only be achieved by generation of chemically-mutagenized conditional mutants. If the identification of genes mutated by insertion is straightforward, the identification of chemically-mutated genes is far more laborious. Functional complementation is the optimal corresponding tool to chemical mutagenesis but requires the use of a stable episomal shuttle vector to facilitate the isolation of the function-rescuing gene. A sequence that permits the episomal maintenance of bacterial plasmids has been selected in *T. gondii* (Black & Boothroyd, 1998). A 500 base-pair fragment was isolated from the *Toxoplasma* genome through selection for episomal stability of a plasmid in the absence of a selectable marker. This fragment shows the characteristics of an episomal maintenance sequence (EMS). The ability to generate EMS-containing vectors bearing large fragments of the parasite genome which can be autonomously replicated will offer the potential to achieve functional complementation in *T. gondii*.

CONTROL OF GENE EXPRESSION IN *T. GONDII*

Very little is known about the regulation of gene expression in protozoan parasites in general and in Apicomplexa in particular. *T. gondii* genes are monocistronic and cis-splicing is not rare since a profusion of genes containing numerous introns has been characterized. Capping of transcripts and α-amanitin sensitivity of transcription have not been investigated in *T. gondii*. Examination of the region upstream of the transcription start site in *T. gondii* genes has not been very informative. The promoters appear to lack canonical eukaryotic promoter elements such as the conserved TATA and CAAT boxes. Transient transfection technology has been used to map promoter elements in several abundantly expressed and stage-specific *T. gondii* genes using chloramphenicol acetyltransferase (CAT) as reporter gene.

Nested deletion analysis of the 5′flanking sequence of *SAG1* linked to a reporter gene (CAT) defined an essential transcriptional cis-acting element necessary for efficient promoter activity. This element is composed of a stretch of six repeated 27 bp sequences which is essential for efficient and accurate initiation of transcription (Soldati & Boothroyd, 1995). Interestingly, this element not only functions as selector of transcriptional initiation, but also acts as an enhancer in both orientations when introduced in the minimal promoter of either a constitutive *TUB1* gene (Soldati & Boothroyd, 1995) or a bradyzoite-specific *SAG4* gene (Soete & Soldati unpublished). A cis-acting motif (A/TGAGACG) present within the 27 bp repeats of *SAG1* has also been recognized as critical cis-acting element for the optimal expression of several highly expressed genes encoding dense granule proteins (*GRAs*) (Mercier *et al*. 1996).

In order to identify sequences involved in regulation of stage-specific genes, transfection was combined with *in vitro* culture conditions (based on alkaline pH shift) that induce differentiation of tachyzoites into parasites resembling bradyzoites from *in vivo* cysts (Soete *et al*. 1994). A minimal promoter region has been mapped in the bradyzoite-specific small heat-shock protein coding gene, *BAG1*. The 5′flanking sequence immediately upstream of the initiation start site of *BAG1* is sufficient to confer bradyzoite expression of CAT and an influence of the 3′flanking sequences of *BAG1* could be excluded (Bohne, Wirsing & Gross, 1997). This stage-specific gene is controlled at the transcriptional level. *LDH1* and *LDH2* encode polypeptides similar to the enzyme lactate dehydrogenase, which accumulate in tachyzoites and bradyzoites respectively. *LDH2* transcripts are only detected in the bradyzoite stage, while the mRNA of *LDH1* accumulates in both the bradyzoite and tachyzoite stages. This observation implies that expression of the tachyzoite form of lactate dehydrogenase (LDH1) is post-transcriptionally controlled. The analysis of the promoter regions showed that *LDH2* promoter upregulates CAT expression during differentiation whereas the promoter sequence of *LDH1* significantly down-regulates reporter gene expression (Yang & Parmley, 1997).

NTP1 and *NTP3* are very actively transcribed genes coding for nucleoside triphosphate hydrolases secreted into the parasitophorous vacuole of tachyzoites. These proteins have an apyrase activity which is presumed to salvage purines from the host cell, for use in survival and replication of the intracellular parasites. The *NTP3* 5′flanking sequences contain one of the strongest promoters analysed so far in tachyzoites, and carry sufficient information for developmentally-regulated expression of CAT activity when the actively-replicating

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tachyzoite differentiates into the dormant bradyzoite. The upstream elements required for expression of *NTP1* and *NTP3* genes have been identified (Nakaar *et al*. 1998). This study brought to light the presence of an essential sequence which resembles an initiator element, TCAGTTT (Inr). The Inrs are known to regulate the expression of a wide variety of RNA polymerase II genes lacking TATA elements. Like a TATA box, this element acts to recruit components of the basal transcription complex and selects the start of transcription. The Inr appears also to be the only recognized core element in the promoters of the flagellated parasitic protist *Trichomonas vaginalis* (Liston & Johnston, 1998).

In *P. falciparum*, the current knowledge of what constitutes a promoter and what parameters regulate gene expression has been recently reviewed (Horrocks, Dechering & Lanzer, 1998). In the past year, the role of chromatin has emerged at the forefront of transcription research (Li, Wrange & Eriksson, 1997; Gregory & Horz, 1998*a*, *b*). The chromatin-modifying machinery has been shown to drastically influence promoter activity in other systems and is likely to be important in protozoan parasites as well. Components of the chromatin remodelling pathway such as the members of histone acetyltransferases and deacetylases families are present in apicomplexan parasites, underscoring the importance of the nucleosome in the regulation of transcription in these lower eukaryotes. We have cloned and characterized a histone acetyltransferase in *T. gondii*, which is homologous to GCN5 and can complement the acetyltransferase activity in the yeast gcn5^{-} mutant (Hettman & Soldati, unpublished).

PROTEIN TRAFFICKING AND SORTING INTO SECRETORY ORGANELLES AND THE VACUOLAR SPACE

Apicomplexan parasites are highly active secretory cells containing three morphologically distinct secretory organelles called rhoptries, dense granules and micronemes. Protein sorting events into these multiple compartments, and even to the surface of the infected host cells in case of blood stage malaria parasites, are likely to be complex.

Sorting of secreted soluble and membrane proteins was examined in *T. gondii* using reverse genetic methods (Karsten *et al*. 1998). This study revealed that most or all soluble proteins are delivered to dense granules and accumulate efficiently in the vacuolar space whereas a GPI anchor is sufficient to target proteins predominantly to the plasma membrane of the parasites. A possible role for the GPI anchor in the formation of high molecular weight complexes and stabilization of proteins has been proposed (Seeber, Dubremetz & Boothroyd, 1998). However, fusion of a heterologous protein to the transmembrane domain and cytoplasmic tail from the endogenous dense granule protein GRA4 localized the protein to the dense granules. Targeting of secreted proteins beyond the boundaries of the parasite involves unusual sorting events. Examination of the post-secretory targeting of GRA2 to the network of membranous tubules that connect with the vacuolar membrane revealed that the combination of two amphipathic alpha helices on GRA2 are necessary for correct localization and stable membrane association (Mercier *et al*. 1998). Rhoptry proteins are subjected to a proteolytic processing that takes place in the nascent rhoptries and does not appear to be essential for sorting of ROP1 to the appropriate organelles (Soldati *et al*. 1998).

Protein trafficking studies involving transfection have also been undertaken with *P. falciparum*. Expression of the rhoptry protein AMA-1 in the rodent parasite *P. berghei* under control of both ama-1 and dhfr-ts promoters illustrated that timing of expression was highly promoter-dependent and was critical for subsequent subcellular localization. Whereas AMA-1 expression and localization in *P. berghei* were limited to the rhoptries of mature schizonts when controlled by its own promoter, the dhfr-ts promoter allowed expression throughout schizogony as well as in gametocytes and gametes. Constitutive expression led to aberrant localization at merozoite and gamete surface (Kocken *et al*. 1998).

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has attracted great interest because it is intrinsically fluorescent. GFP has recently been added to the arsenal of genetic markers in *P. falciparum* (VanWye & Haldar, 1997) and in *T. gondii* (Striepen *et al*. 1998) allowing visualization of protein traffic in living cells. A GFP fusion to the P30 major surface antigen was detected in dense granules of living cells, and was efficiently secreted into the parasitophorous vacuole, whereas a GFP fusion to the rhoptry protein ROP1 was targeted to rhoptries. Recently, GFP has been used to delineate the minimal targeting signal necessary to sort proteins into the apicoplast (Waller *et al*. 1998). The putatively apicoplast-targeted proteins bear a bipartite targeting signal composed of a leader peptide followed by a transit peptide consistent with plastid targeting. The acyl carrier protein (ACP) presequence is sufficient to target a recombinant green fluorescent protein reporter to the organelle in transgenic *T. gondii*.

GFP is not only useful for localization and dynamics of protein trafficking; we have also exploited it as a simple genetic marker to monitor gene expression directly during differentiation. We have engineered a mutant version of GFP which is faithfully expressed as non-fusion and single-copy

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genes in tachyzoites and selectively in bradyzoites when controlled by the flanking sequences derived from the bradyzoite-specific gene *SAG4* (Soete, Hettmann & Soldati, unpublished). In this case, we have used a *gfp* gene mutant in which the codon usage has been extensively modified to restore proper expression in *Arabidopsis* and which turned out to be well suited for expression in *T. gondii* (Haseloff *et al*. 1997). Transgenic parasites transformed with psag4GFPsag4 were cultivated under switching conditions for 6 days and analysed for the expression of the bradyzoite-specific marker P36 and GFP (Fig. 1). GFP is a convenient marker to study promoter activity during the maturation of the cysts *in vitro*. Green bradyzoites can be purified easily by FACS providing with a new approach to trap stage-specific genes without relying on positive and negative drug selection. The fact that we found a mutant of GFP able to fluoresce as non-fusion and as single copy gene in *T. gondii*, offers the possibility to use this marker in combination with insertional mutagenesis to identify proteins targeted precise compartment of the cell (Garraway *et al*. 1997).

USE OF REVERSE GENETICS TO STUDY INVASION

Host cell invasion is a prerequisite for the survival of obligate intracellular parasites. Thus, in the absence of an inducible gene expression system, reverse genetic approaches to study this process are severely limited. Nevertheless, exploiting the versatility of *T. gondii* genetic system by combining chemical mutagenesis and stable transfection, it has been possible to demonstrate that host cell invasion is actively powered by an actin-based contractile system in *T. gondii* (Dobrowolski & Sibley, 1996). In addition, the rhoptry protein ROP1 was characterized as a potential enhancer of host-cell penetration. However, disruption of the *ROP1* gene did not affect either the ability of tachyzoites to invade host cell *in vitro* or virulence *in vivo* (Soldati *et al*. 1995).

In the case of *Plasmodium* sporozoites, an essential role of the thrombospondin-related anonymous protein (TRAP) in invasion of the salivary glands of the mosquito vector and the liver of the vertebrate host was demonstrated by gene disruption. It was possible to take advantage of the stage specificity of TRAP, which made it possible to generate of knockout mutants in blood-stage parasites (Sultan *et al*. 1997). The main limitations of gene targeting in malaria parasites result from their intracellular growth and slow replication. Nevertheless, the technology is facilitated by the very high rate of homologous recombination following transformation with targeting constructs. Disruption of several genes in *Plasmodium* species has allowed confirmation of the presumed role of some genes and to reveal unexpected roles for some others. The knobs and the knob-associated histidine-rich protein (KAHRP) were presumed to play a key role in adherence of parasitized-erythrocytes to microvascular endothelium. The knockout of the gene encoding KAHRP has shown that it is essential for knob formation; and the binding to CD36 under flow conditions was dramatically reduced (Crabb *et al*. 1997). In contrast, the contribution of the circumsporozoite protein (CS) in the attachment of sporozoites to hepatocytes could not be investigated after gene replacement since CS appears to have a pleiotropic role and is essential for sporozoite development within oocysts in the mosquitos (Menard *et al*. 1997).

ILLEGITIMATE GENE EXPRESSION

The surface protein of the ookinete of *P. berghei*, Pbs21 has been illegitimately and stably expressed in the blood-stages, in order to assess its ability to induce a potent transmission-blocking immune response. This was achieved by stable transformation in blood-stage parasites of an episomal vector carrying a minimal Pbs21 gene lacking the elements controlling the stage-specific transcription (Margos *et al*. 1998). As mentioned above, the expression of AMA-1 under the control of a constitutive promoter alters the patterns of expression as well as the subcellular targeting of the protein.

We have expressed the bradyzoite-specific surface antigen SAG4 under the control of *NTP3*, a gene strongly expressed in tachyzoite (Fig. 2). The bradyzoite-specific protein does not appear to be related to the pepsin resistance, a typical characteristic of bradyzoites but we can examine if the expression of SAG4 during acute infection can affect the establishment of chronic infection in the mouse model. (Soete, Soldati & Dubremetz, unpublished).

VIRULENCE AND DIFFERENTIATION

The ability of *T. gondii* to build cysts is a determinant factor in pathogenicity. The 30kDa small heat shock protein BAG1 is restricted to the bradyzoites and first appears approximately 2–3 days after the initiation of bradyzoite differentiation. Targeted disruption of the *BAG1* gene in the cyst-forming strain ME49 does not prevent tissue cyst formation *in vitro* and *in vivo*. The mutant was found to express several bradyzoite-specific markers with the same kinetics and frequency as the parental strain. The morphology and frequency of cysts formed in mice is like wild type and the ability to convert back into tachyzoites is unaffected.

An interesting phenotype was observed with GRA2 gene was disrupted by replacement with the Ble marker gene conferring resistance to phleomycin.

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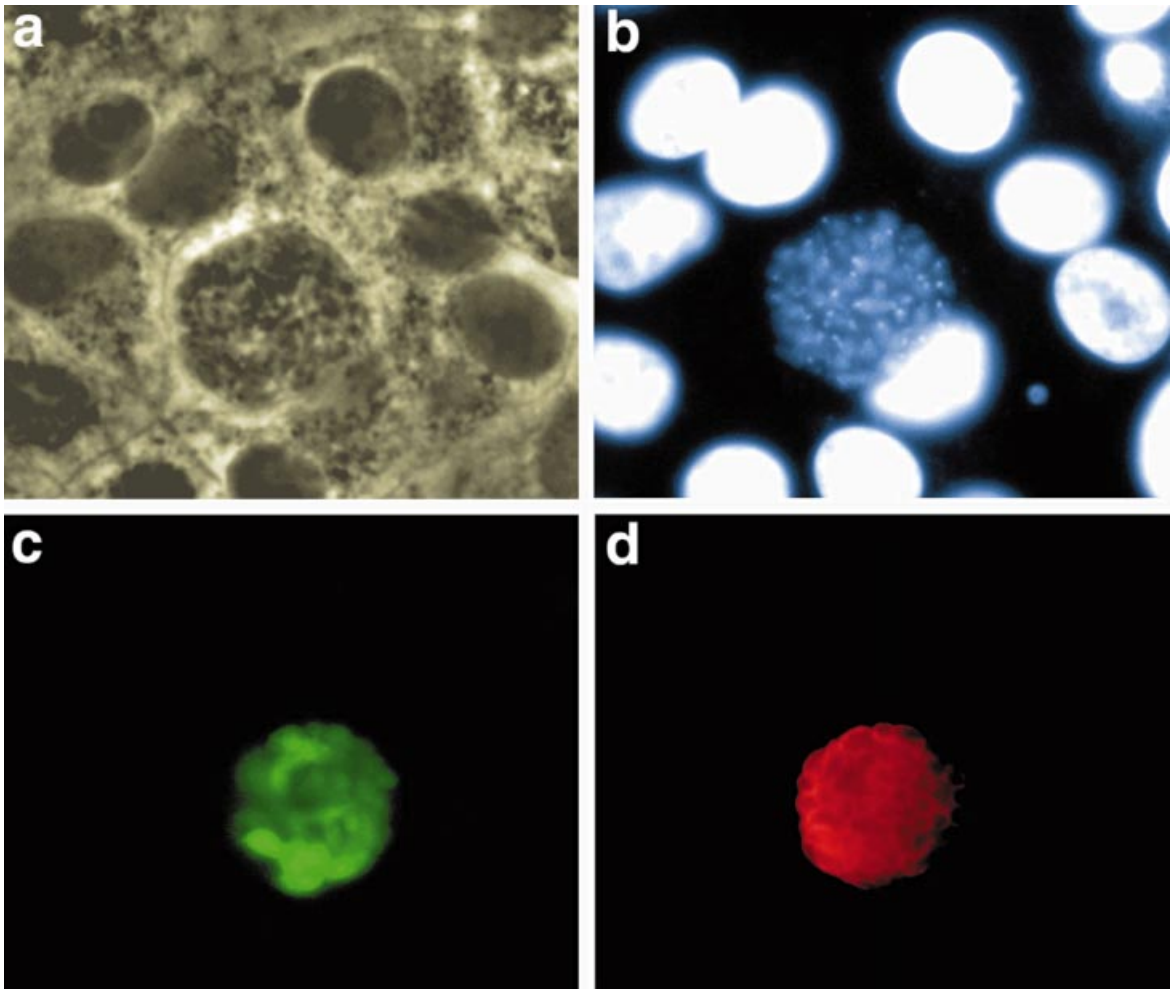


Fig. 1. Expression of a non-fusion Green Fluorescent Protein in a stage-specific fashion. Bradyzoite cyst induced *in vitro* after 6 days in culture at high pH. Transgenic Prugniaud strain stably transformed with psag4GFPsag4. Panel A, phase contrast; panel B, DAPI staining; panel C, GFP analyzed by direct fluorescence using standard FITC filter; panel D direct immunofluorescence using monoclonal antibody against the surface antigen P36.

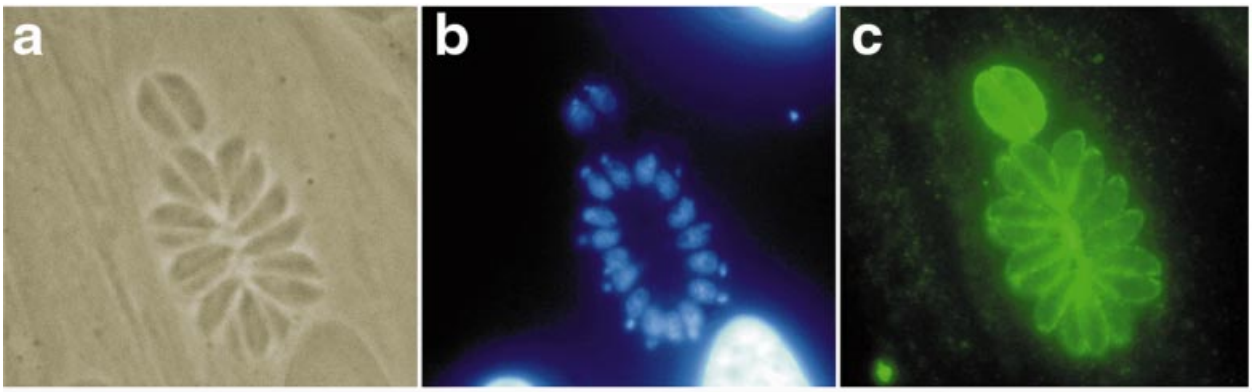


Fig. 2. Illegitimate expression of the bradyzoite surface antigen SAG4 in *T. gondii* tachyzoites. Attenuated ts-4 mutant of RH stably co-transformed with pT70CAT and pntp3SAG4ntp3 vectors. Panel A, phase contrast; panel B, DAPI and panel C, indirect immunofluorescence using monoclonal antibodies against SAG4.

The 28kDa dense granule protein GRA2 is specifically targeted to the tubulovesicular network which forms connections with the vacuolar membrane. Although the mutant invades and grows normally in both fibroblasts and macrophages in culture, it manifests a partial attenuation of the virulence during the acute phase of infection. The absence of GRA2 leads to the establishment of chronic infection by the otherwise highly virulent RH strain (Mercier *et al*. 1998).

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Table 1. Effects of gene disruption in Apicomplexa

Targeted disruption *T. gondii* genes by homologous recombination and knockout phenotypes	
ROP1	Morphological alteration of the rhoptries (Soldati *et al*. 1995)
HXGPRT	Resistance to 6-thioxanthine, sensitivity to MPA (Donald *et al*. 1996)
UPRT	Resistance to 5-fluorouracil (Donald & Roos, 1995)
BAG1	No phenotype (Bohne *et al*. 1998)
GRA2	Reduced virulence during acute infection in mice (Mercier *et al*. 1998)
SAG3	Reduced invasion (Tomavo, S. unpublished)
SAG1	Not determined (Grigg, M. & Boothroyd, J. C. unpublished)
Targeted disruption of *Plasmodium* genes by homologous recombination and knockout phenotypes	
Pf KAHRP	Essential for knob formation (Crabb *et al*. 1997)
Pb *CS*	Inhibition of sporozoite formation (Menard *et al*. 1997)
Pb*TRAP*	Essential for sporozoite host cell invasion and motility (Sultan *et al*. 1997)
Expression of mutated genes	
Dhfr-ts	Resistance to pyrimethamine or cycloguanil (Reynolds & Roos, 1998)
act1	Resistance to cytochalasin D (Dobrowolski & Sibley, 1997)
Allelic replacement	
Pf*dhps*	Resistance to sulfadoxine (Triglia *et al*. 1998)
Illegitimate gene expression	
Pb*s21*	Expression of a mosquito midgut-stage antigen in blood-stage parasites (Margos *et al*. 1998)
Pf *AMA-1*	Controlled by dhfr-ts promoter (Kocken *et al*. 1998)
Tg*SAG4*	Expression of a bradyzoite-specific antigen into tachyzoites (Soete, Solati and Dubremetz, unpublished)

MECHANISMS OF DRUG RESISTANCE

The fact that apicomplexan parasites share not only structural but also functional features, offers the possibility to exploit *T. gondii* to address specific questions relating to malaria. Such an approach has been applied to investigate the influence of specific dihydrofolate reductase polymorphisms identified in field isolates of drug-resistant malaria. The ease of generating transgenic *T. gondii* parasites expressing various mutants coupled to biochemical analysis facilitated the investigation of the mechanism of pyrimethamine and cycloguanil resistance in *P. falciparum* (Reynolds & Roos, 1998).

Transfection of *P. falciparum* was also used to investigate the role of polymorphisms in the enzyme dihydropteroate synthase (DHPS), identified in sulfadoxine-resistant field isolates, consistent with its occurrence in most dhps alleles from resistant isolates. These studies underscored that the mechanism of resistance to sulfadoxine in *P. falciparum* involves mutations in the *dhps* gene and allowed to evaluate the relative contribution of these mutations to the phenotype (Triglia *et al*. 1998).

CONCLUSIONS

The last few years have witnessed an impressive increase in our understanding of the biochemistry and molecular biology of apicomplexan parasites, with attention focused on specific parasite molecules that are keys to parasite metabolism, resistance to drugs, life cycle, host cell invasion and pathogenicity. There is no doubt that the genetic manipulation of apicomplexan parasites has revolutionized the study of this group of pathogens and should reveal new opportunities of intervention. As described here, targeted integration of exogenous DNA into the genome of parasites permits alteration of any given gene in a predetermined way by homologous recombination, or expression of a foreign or mutated gene. Identification of sequences containing important transcriptional control elements have significantly advanced our knowledge of transcriptional processes. The acquisition of information regarding the control of gene expression and the sorting of proteins offers the opportunity to alter the temporal and spatial patterns of expression of a particular gene. The other determinant in this revolution is the accessibility to genetic information. Sequencing projects have been initiated for most relevant parasites and is already very advanced in *P. falciparum*.

Even though the transfection technologies are now being established in malaria parasites, the ease with which *T. gondii* can be manipulated will continue to be instrumental in the investigation of questions in malaria research and will also assist studies on other apicomplexan parasites that are not genetically tractable. The repertoire of molecular tools is constantly expanding and among the novel tools emerging, RNA antisense strategy will definitely constitute a valuable alternative to gene knock-outs. Recent studies report that the incorporation of a ribozyme within a full-length antisense construct for *T. gondii* coding genes (*HXGPRT*, *NTP3*) can cause an effective down-regulation of the corresponding protein production (Nakaar and Joiner, unpublished). Eventually, an inducible gene expression system will be established as in *Trypanosoma brucei* (Wirtz & Clayton, 1995).

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