Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using *Toxoplasma gondii* transactivators

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Genetic manipulation has revolutionized research in the Apicomplexan parasite *Plasmodium falciparum*, the most important causative agent of malaria. However, to date no techniques have been established that allow modifications that are deleterious to blood-stage growth, such as the disruption of essential genes or the expression of dominant-negative transgenes. The recent establishment of a screen for functional transactivators in the related parasite *Toxoplasma gondii* prompted us to identify transactivators in *T. gondii* and to examine their functionality in *P. falciparum*. Tetracycline-responsive minimal promoters were generated based on the characterized *P. falciparum* calmodulin promoter and used to assess transactivators in *P. falciparum*. We demonstrate that artificial tetracycline-regulated transactivators isolated in *T. gondii* are also functional in *P. falciparum*. By using the tetracycline analogue anhydrotetracycline, efficient, stage-specific gene regulation was achieved in *P. falciparum*. This regulatable expression technology has clear potential for the study of essential gene function in *P. falciparum* blood stages. On the other hand, the identified transactivators are not functional in mammalian cells, consistent with the fundamental differences in the mechanism of gene regulation between Apicomplexan parasites and their human hosts.

Apicomplexa | transcription | gene regulation | anhydrotetracycline | inducible expression

Plasmodium falciparum is the causative agent of the most severe form of human malaria. In the last few years, several powerful new tools and strategies associated with DNA transformation have been developed in the clinically relevant blood stage of this organism (for reviews, see refs. 1 and 2). However, for a number of reasons, most notably the low efficiency of transfection (3), genetic manipulations that have even a slight deleterious effect on erythrocyte-stage growth have been very difficult to perform. These include the expression of transgenes that negatively impact growth and the deletion of genes that play a crucial role in maintenance of the blood-stage cycle, which is especially problematic given the haploid nature of the genome during this stage. This technical deficiency is a major roadblock in the functional analysis of important blood-stage *P. falciparum* proteins, many of which display potential as drug and blood-stage vaccine targets.

For most other eukaryotes, if a gene of interest fulfills a critical role for survival, targeted gene disruption can be replaced by a knockdown approach. Use of RNA interference (RNAi) can specifically lower the level of an mRNA and consequently the level of its corresponding protein. For the most part, however, RNAi-based approaches have proved unsuccessful in Apicomplexa possibly because these parasites lack some of the key enzymes involved in the process (4). Another widely used approach to studying essential genes is the tetracycline (Tet)-based transcriptional regulation system. Recently, we succeeded

in establishing anhydrotetracycline (ATc)-regulated gene expression systems in *Toxoplasma gondii*, a distant relative of *P. falciparum* (5, 6).

The most effective of the two approaches developed in *T. gondii* is based on the Tet-transactivator system. In mammalian cells, this system uses a fusion protein of the Tet repressor (TetR) with a C-terminal transactivating domain from the herpes simplex virus VP16 protein, a process that converts the repressor into an efficient Tet-controlled transactivator (tTA) (7). Our first attempts to adapt this system in *T. gondii* failed because tTA was not capable of activating minimal promoters derived from this organism (5). Therefore, a genetic screen based on random insertion was designed to identify a functional transcriptional activating domain in *T. gondii* and to establish an ATc transactivator-based regulation system. This new system permitted the creation of a conditional knockout of an essential gene in *T. gondii* (6).

Here, we report the reversible expression of genes in *P. falciparum*, using a system that employs the ATc-regulated transactivators identified in *T. gondii* to activate *P. falciparum* minimal promoters. We observed >50-fold regulation of the GFP reporter gene when using ATc in the course of a single 48-h growth cycle, whereas 10- to 20-fold regulation was achieved with a chloramphenicol acetyltransferase (CAT) reporter over two growth cycles. This system should have broad applicability for the analysis of important gene functions in *P. falciparum* blood stages.

Materials and Methods

Plasmids. The *T. gondii* TATi-2 expression plasmid was generated as previously described for pTTATi-1-HX (6) by using a poly(T) primer with a *Bam*HI restriction site at the 3' end and Rep-4 (5'-CGGAATTCCTTTTCGACAAAATGTCGCGCCTG-GACAAGAGCAAAGTCATCAACTCTGC-3') as primers for amplification. For the generation of a plasmid allowing stable expression of TATi-3, oligonucleotides encoding the transactivating domain were generated (TATi-3-sense, 5'-TTATCTC-CTGCCAACGTGCATCCCTTAAT-3', and TATi-3-as, 5'-

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Abbreviations: Tet, tetracycline; ATc, anhydrotetracycline; TetR, Tet repressor; tTA, Tet-controlled transactivator; CAT, chloramphenicol acetyltransferase; TATi, transactivator of *Toxoplasma gondii*; CAM, calmodulin; GPI, glycosylphosphatidylinositol.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY860671).

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TAAGGGGTACACTTGGGCAGGAGATAATGCA-3'), yielding double-stranded oligonucleotides with *Nsi*I- and *Pac*I-compatible overhanging ends, allowing insertion into the corresponding sites of pTetR-DHFRTS (6). In a second step, the DHFR-TS-selectable marker was exchanged for HXGPRT by *Sac*II digestion. For expression of TATi-1 and TATi-2 in HeLa cells, we amplified the respective genes using primers TATi-6 (5'-CCGGAATTCACCATGTCGCGCCTGGACAAGAGC-3') and TATi-7 (5'-CCGGGATCCCGTGATTAATTAAGCGTTAATATTTTGTAAATTCGCG-3'); we used the PCR fragments that were generated to replace tTA²⁸ in the vector pUHD16-3 by using *Eco*RI and *Bam*HI, respectively. For stable expression of TATi-1 and TATi-2 in *P. falciparum*, we amplified each gene using primers TATi-4 (5'-CCGCTC-GAGAATGTCGCGCCTGGACAAGAGC-3') and TATi-5 (5'-AACCTAGGTGATTAATTAAGCGTTAATATTTTGTAAATTCGCG-3') and inserted each resulting PCR fragment between the *Xho*I and *Avr*II sites in the vector pHHI to produce pHH-TATi-1 and pHH-TATi-2, respectively. CAT reporter plasmids were generated by inserting a *Hind*III-*Sph*I PCR fragment encoding seven TetO sequences upstream of the minimal calmodulin (CAM) promoters in the constructs pCAM5.4/3, pCAM5.5/3, and pCAM5.6/3 (8), resulting in plasmids pTOCAM5.4/3, pTOCAM5.5/3, and pTOCAM5.6/3.

To create constructs for the generation of parasites stably expressing TATi-1 or TATi-2 and the regulatable *CAT* reporter gene, PCR fragments encoding the seven TetO minimal CAM promoters of pTOCAM5.4/3 or pTOCAM5.6/3 were inserted into the *Not*I sites of pHH-TATi-1 or pHH-TATi-2. The primers used were Tet-s (5'-AAGCGGCCGCTTTCGATACCGTC-GACCTCGAGTTTAC-3') and CAM-as (5'-AAGCGGCCG-CATCGATAACTGCAGCTGGCGATCCTGATATATT-TCTATTAGG-3'). *Kpn*I-digested DNA fragments from pTOCAM5.4/3 and pTOCAM5.6/3 encoding the respective minimal CAM promoters and *CAT* reporter were introduced into pTO5.6/TATi-2. This generated the plasmids pTC5.4/TATi-1, pTC5.6/TATi-1, pTC5.4/TATi-2, and pTC5.6/TATi-2.

To construct pTGPI-GFP, the 5' and 3' ends of EGFP were flanked with the sequence encoding the signal peptide and the glycosylphosphatidylinositol (GPI)-anchor signal of *P. falciparum* MSP-1, respectively, by using MSP-1ss (5'-AGGATCGCT-GCAGGTCAAATGAAGATCATATCTTTTATGT-3' and 5'-GTTCTTCTCCTTACTCATTTGATGTGTAC-ACATTGTGTA-3'), EGFP (5'-TACACAATGTGTAACAC-ATGAAATGAGTAAAGGAGAAGAAC-3' and 5'-TCCG-GAAGAACTACAGAATACAATTGCTTTTAAATC ACT-3'), and MSP-1gs (5'-AGTGATTTAAAGCAATTGTAT-TCTGTAGTTCTTCCGGA-3' and 5'-TGAAGAACT-AGTTTAAATAAATTAATAC-3') primers in PCRs. The products were then sewn together in subsequent rounds of PCR to produce a GPI-GFP fragment. A PCR fragment comprising the CAM 3' UTR (8) was amplified by using the primers 5'-GCAGAACTGCAGCGTATTATATTACTAGTTTAAATATGCAGA-3' and 5'-GATTCGATCGATCACTTA-ATAAAAAAGAGGA-3', digested with *Pst*I and *Cla*I, and ligated downstream of the CAM minimal promoter in the intermediate vector pTO5.6/TATi-2 (see above). This new plasmid was then digested with *Pst*I and *Spe*I and the GPI-GFP fragment was ligated between the CAM minimal promoter and 3' UTR. The HSP86 promoter driving TATi-2 expression was excised and replaced with the MSP-2 promoter (9) via *Bgl*II and *Xho*I sites. Finally, a *Bgl*II fragment comprising the Rep-20 repeat sequence (3) was inserted into a *Bgl*II site between the MSP-2 and CAM promoters controlling the expression of human dihydrofolate reductase (hDHFR). To generate pHH/TetR, the complete TetR sequence was amplified by PCR from pHD360 (10) by using the oligonucleotides TetR.For (5'-CGAAGCTC-GAGTATGTCAAGATTAGATAAAAAGTA) and TetR.Rev

(5'-TAAATCTCGAGGATCCTTAAGACCCACTT), and the resulting product was inserted into the *Xho*I site of pHH1 (11).

Parasites and Transfection. Stable transformants of *T. gondii* were derived as described (12). The recipient strain p7TetOS1LacZ-p7TetOS1HXGPRT-CAT has been described (6). For the random integration screening, 10⁹ recipient parasites were transfected with pTRep-DHFRTS and selected for resistance to pyrimethamine and to mycophenolic acid/xanthine in the absence of ATc (see also ref. 6). TATi-1-, TATi-2-, and TATi-3-expressing parasites were obtained by stable integration of the respective expression vector in parasites lacking *HXGPRT* (RH-hxgprt). Positive clones were examined for transactivator expression by Western blotting and indirect immunofluorescence assay (IFA) (data not shown). Blood stages of *P. falciparum* strain D10 were cultivated and synchronized as described (13, 14). *P. falciparum* ring-stage parasites were transiently or stably transfected with 50–100 µg of purified plasmid DNA (Plasmid Maxi Kit, Qiagen, Valencia, CA) under modified electroporation conditions (2, 8, 15). HeLa cells (XL1.6) were transiently transfected as described (16).

Results

Isolation and Characterization of Functional Transactivators in *T. gondii*. Recently, we described an insertion mutagenesis approach to identifying functional transactivating domains in *T. gondii* by integrating at random a linear DNA fragment encoding a resistance marker and the gene for TetR without a stop codon into the genome of a recipient strain of *T. gondii* (6). This strain includes a reporter gene (*LacZ*) and a selectable marker gene (*HXGPRT*) both under control of a TetO-minimal promoter (p7TetOS1). This genetic screen led to the isolation of a functional TetR transactivator, termed TATi-1 (6). Using the same strategy here, we isolated a second transactivator, termed TATi-2. Surprisingly, the sequence of TATi-2 matched almost perfectly that of TATi-1, indicating that the insertion had occurred at the same locus. Further analysis of the DNA sequence showed that the integration was not random throughout the *T. gondii* genome as had been anticipated but had occurred within the plasmid backbone of the previously integrated plasmids (data not shown). However, the sequence of TATi-2 is different than that of TATi-1; this difference is due to integration in different positions, resulting in a shorter TetR fusion protein (Fig. 1A). Note that for TATi-1 and TATi-2, the short C-terminal deletions observed in TetR are not predicted to influence DNA binding or induction by Tet (17). Hence, TATi-1 and TATi-2 should be considered artificial (not endogenous) *T. gondii* transactivators.

Although the primary sequences of TATi-1 and TATi-2 differ, the amino acid composition of both transactivating domains is very similar, each having a predominance of hydrophobic and polar residues (Fig. 1B). A screen for artificial transactivating domains in yeast retrieved a transactivator (referred to here as TATi-3) with a minimal transactivating domain of only eight amino acids (18). As for TATi-1 and TATi-2, the sequences of their transactivating domains are relatively hydrophobic and polar. On the other hand, the minimal transactivating domain present in the tTA, which is not functional in *T. gondii*, is highly acidic and contains no polar amino acids (Fig. 1B).

To compare the strength of these artificial transactivating domains, TATi-1, TATi-2, and TATi-3 were stably expressed in *T. gondii* tachyzoites and tested for their ability to transactivate a minimal Tet-responsive promoter (6). As expected, the original tTA (7) did not transactivate the TetO-minimal promoter in *T. gondii*, whereas TATi-1 and TATi-2 demonstrated strong transactivating capacity in this parasite (Fig. 1C). ATc reversed the transactivation by both TATi-1 and TATi-2; however, the background activity was substantially higher in the case of TATi-2

Fig. 1. Functional transactivators in *T. gondii*. (A) An alignment of the transactivating domains (boxed) compared with the C terminus of TetR. The italicized residues arose from an introduced restriction site. (B) Amino acid composition of transactivating domains. The percentages of acidic (DE), basic (KR), charged (RKHYCDE), polar (NCQSTY), and hydrophobic (AILFWV) amino acids in the transactivation domains of tTA, TATI-1, TATI-2, and TATI-3 were determined by using the PROTEAN program (DNASTAR, Madison, WI). (C) *T. gondii* transactivators show different efficiencies. *T. gondii* parasites expressing tTA, TATI-1, TATI-2, or TATI-3 were transiently transfected with the reporter plasmid T7S1lacZ-CAT and incubated in the presence or absence of ATc for ≈ 48 h. A summary of three independent experiments is shown. (D) *T. gondii* transactivators are not functional in HeLa cells. The HeLa line XL1/6, which contains the gene for luciferase under the control of the target promoter P_{HCMV}^* (7), was transiently transfected with expression plasmids for tTA, TATI-1, and TATI-2. Cells were grown for 24 h in the presence or absence of doxycycline (Dox) before luciferase and LacZ (as an internal standard) expression was determined. A summary of three independent experiments is shown.

Fig. 2. Functional expression of TATI-1 and TATI-2 in *P. falciparum* analyzed by transient transfection. (A) Schematic diagram of the expression vectors used to generate drug-resistant *P. falciparum* (D10 line) transfectants expressing either TATI-1 or TATI-2. (B) Western blot analysis of parasites expressing TATI-1 or TATI-2 and, as control, the TetR protein. (C) Quantification of CAT activity of parasites transiently transfected with reporter constructs containing minimal CAM promoters of different lengths flanked upstream by seven TetO sequences or the full-length CAM promoter (pCAM5/3; ref. 8). Black boxes represent previously defined promoter elements (8). D10-TATI-2 parasites were incubated for 48 h in the presence or absence of ATc after transient transfection with reporter plasmids. Data are from three independent experiments. (D) Measurement of promoter activity in the presence of different ATc concentrations analyzed under transient conditions as above. In this instance the pCAM5.1/3 plasmid was used as a positive control (8).

T. gondii genetic screen are capable of interacting with factors

conserved in higher eukaryotic transcription machinery, TATi-1 and TATi-2 were tested for their ability to transactivate the Tet-responsive minimal promoter, P_{hCMV*}-1 (7) in HeLa cells. Under transient transfection conditions, no stimulation of promoter activity was detectable with either TATi-1 or TATi-2, whereas the tTA allowed gene regulation of >5 orders of magnitude (Fig. 1D). This result suggests that TATi-1 and TATi-2 do not interact with transcription factors present in higher eukaryotes. Similarly, the transactivating domain of TATi-3, which was originally identified in yeast, is not active in mammalian cells (18).

TATi-1 and TATi-2 Act as Functional Transactivators in *P. falciparum*.

To determine whether the *T. gondii* transactivators functioned in *P. falciparum*, we generated constructs in which either TATi-1 or TATi-2 was placed under the control of the *P. falciparum* HSP86 promoter (Fig. 2A). After selection for episomal maintenance of the plasmid we obtained transgenic parasite lines expressing either TATi-1 or TATi-2 (Fig. 2B). The strong expression of

Fig. 3. Transactivation and regulation of promoter activity in stably transfected *P. falciparum*. (A) Stable induction of CAT under the control of inducible minimal promoters. Parasites stably transfected with plasmid constructs designed to express both TATi transactivators and CAT under the control of particular regulatable CAM minimal promoters were assayed for CAT activity. The elements controlling transcription of TATi proteins and hDHFR in these plasmids were the same as those in Fig. 2A. Results are expressed relative to CAT activity in a previously described CAT-expressing line, D10-HC1/CAT (20), analyzed in parallel. (B) Regulation of stable transgenic *P. falciparum* lines with ATc. On day 0, identical cultures were split into two groups, termed "sample A" and "sample B." Sample A cultures were maintained in the absence of ATc for 4 days (two growth cycles), whereas sample B parasites were grown in the presence of 0.5 μ g/ml ATc for this period. Results are expressed as a percentage of the ratio of CAT activity of sample B over sample A. (C) D10-HC1/CAT and D10-TC5.6/TATi-2 parasites were subjected to ATc regulation as described above, except here sample B was maintained in the presence of ATc until day 6, after which time ATc was removed. Each data point is the mean ($\pm$ SD) of all values in the linear range from two to six independent experiments.

these transactivators was not surprising because we have achieved similar stable expression in *P. falciparum* of TetR (Fig. 2B) and of a number of TetR mutants using similar approaches (data not shown). Moreover, despite the extreme AT bias of *P. falciparum*, the codons of these transactivators were not optimized for *P. falciparum*, because this optimization has not proven to be necessary for strong expression of GC-rich transgenes in this organism (20–22).

To monitor promoter activation by TATi-1 or TATi-2, we generated ATc-responsive promoters based on previously described CAM-minimal promoters fused to seven TetO sequences (Fig. 2C). CAT activity was monitored 48 h after transient transfection of these constructs into parental parasites or parasites expressing TATi-1 or TATi-2. In a preliminary experiment, we observed that, whereas transfection into wild-type control parasites yielded little expression from the minimal promoters, there was substantial activation of CAT expression in parasites expressing TATi-2 and a small degree of activation in parasites expressing TATi-1 (data not shown). We noticed that expression of TATi-1 was diminished after persistent maintenance of the parasites under selection (data not shown). Hence, the discrepancy in activation between TATi-1 and TATi-2 may be explained by the reduced expression level of TATi-1. In view of the loss of expression of TATi-1, only transient transfections with TATi-2-expressing parasites were repeated, and the cumulative results are presented in Fig. 2C. All three minimal

promoter constructs were activated to some degree by the TATi-2 in these transient conditions.

Establishment of an ATc-Regulated-Expression System in *P. falciparum*. Because Tet and some of its derivatives are toxic for *P. falciparum*, we used ATc to regulate TATi-induced transactivation. As evidenced throughout this study (see below), ATc is not toxic for *P. falciparum* at levels of 0.5–1.0 μ g/ml, even over longer-term continuous culture ($\approx$ 1 month). Under transient conditions, CAT expression in parasites transfected with a control construct encoding CAT under transcriptional control of the full length CAM promoter (pCAM5/3) was not affected by the presence of ATc. In contrast, the ATc-responsive minimal promoter plasmids showed a clear difference in CAT activity between the noninduced and induced states (Fig. 2C). We established that ATc concentrations as low as 0.1 μ g/ml achieved similar levels of regulation (Fig. 2D).

To test the efficiency of transactivation in parasites stably transfected with the ATc-responsive promoters, we generated constructs containing an expression cassette for TATi-1 or TATi-2 as well as CAT as the reporter gene under the control of the ATc-responsive promoters from pTOCAM5.4/3 or pTOCAM5.6/3 (Fig. 3A). Substantial promoter activation was observed in parasites transfected with pTC5.6/TATi-2, reaching up to 30% CAT activity when compared with parasites transfected with the positive control plasmid pHC1/CAT (20). Rel-

atively weaker promoter activation was seen with other combinations of transactivator and minimal promoter (Fig. 3A). The high degree of variation among different reporter constructs may be explained by different levels of TATi-1 or TATi-2 expression in the different lines (data not shown).

We compared CAT activities after growth for two parasite cycles in the presence or absence of ATc in parasite lines D10-TC5.4/TATi-1, D10-TC5.6/TATi-2, and D10-HC1/CAT. In both regulatable parasite lines, significant reduction (10- to 20-fold) of CAT activity was evident in the presence of ATc relative to identical cultures grown in parallel in the absence of ATc, whereas no difference in CAT activity was observed in positive control parasite D10-HC1/CAT in the presence or absence of ATc (Fig. 3B).

Time course experiments were performed by using the D10-TC5.6/TATi-2 line, which had consistently demonstrated the best regulation (Fig. 3C). After 2 days in the presence of ATc, CAT activity was significantly reduced, reaching baseline activity after 4 days of incubation. On day 6, ATc was removed, resulting in reactivation of the regulatable promoter up to maximal expression level after a further 6 days of incubation. The absence of an ATc effect on the expression of CAT in the control D10-HC1/CAT line shows that ATc has no toxic effect on *P. falciparum* at this concentration (Fig. 3C).

To better examine regulation and further validate the system, we inserted GFP under the control of the ATc-regulatable promoter from pTOCAM5.6/3. In an attempt to maximize the prospects of rapid turnover of the reporter, the GFP gene was fused to sequence encoding an N-terminal signal peptide and a C-terminal GPI addition sequence. The resulting construct allows ATc-regulated expression of GFP and stage-specific expression of TATi-2 under the control of the MSP-2 promoter (9) (Fig. 4A). Because we have observed that some transgenes encoding GPI-anchored proteins seem to be toxic to *P. falciparum* (R. A. O'Donnell and B.S.C., unpublished data), this line was maintained in the presence of 0.5 μ g/ml ATc for the duration of the selection period after transfection (3–4 weeks). After a drug-resistant line was established, ATc was removed from the medium, resulting in the presence of fluorescent parasites in $\approx$ 20% of the population. The activated (i.e., without ATc) line was synchronized and separated into two identical cultures. ATc was added to one of the cultures, and samples were taken from both cultures with and without ATc over a 48-h period. Using both Western blotting and fluorescence microscopic analysis, we confirmed that GFP is effectively and specifically down-regulated after addition of ATc to undetectable levels within a single parasite cycle (Fig. 4B and C). Using a limiting-dilution Western blot approach, we estimated this to be $>$ 50-fold regulation. No difference in expression level was observed for endogenous protein MSP-2 or the heat shock protein HSP70 (Fig. 4C). Importantly, the timing of the induced GFP throughout the life cycle seemed to be very similar to the schizont-stage protein MSP-2, the promoter of which drives the transactivator TATi-2 in this line.

Discussion

Methods to modulate gene expression in *P. falciparum* blood stages are required for the analysis of gene function in this organism. Our own group, for example, has focused on developing a direct TetR-regulated system whereby endogenously expressed TetR is intended to reversibly inactivate full-length parasite promoters by binding to Tet operators placed in close proximity to transcriptional start sites. Despite achieving strong stable expression of wild-type and mutant TetR under two different *P. falciparum* promoters, we did not observe repression in any of six different TetO-containing HSP86 or MSP-2 promoter constructs when using a transient transfection approach (T.F.d.K.-W. and B.S.C., unpublished data). It is possible that

Fig. 4. Inducible and stage-specific expression of a GFP fusion protein in *P. falciparum*. (A) Diagram of the pTGPI-GFP plasmid containing all necessary elements including the selectable marker (hDHFR) under the full-length CAM promoter, transactivator (TATi-2), and GPI-GFP cassettes, the latter controlled by a Tet-responsive promoter. TATi-2 is under the control of the MSP-2 promoter (M5'; ref. 9) and a Rep-20 element (3). This plasmid was stably transformed in D10 parasites in the continual presence of ATc. (B) Assessment of ATc regulation. A culture of transformed parasites maintained in the absence of ATc was split into two identical cultures and maintained in the absence or presence of ATc over one growth cycle. Lanes 1–6 represent 8-h time points from synchronized ring-stages analyzed by Western blotting and probing with antibodies against HSP70, MSP-2, and GFP. (C) GFP fluorescence of schizont-stage parasites. Parallel cultures of the parasites were maintained in the presence or absence of ATc and were isolated from uninfected red blood cells before microscopy. Phase, phase contrast.

such a system may function when all elements are stably integrated in the genome. Nevertheless, our lack of success to date with this approach, together with the uncertainty surrounding the potential of RNA interference (RNAi) in this organism, led us to explore the possibility that ATc-regulated transactivators that are functional in *T. gondii* may also function in *P. falciparum*.

TATi-1 and TATi-2 were obtained by a random insertion-based genetic screen in *T. gondii*. It eventuated that the insertion into this *T. gondii* line was not random: rather, integration into preexisting plasmid backbone (in two different reading frames) was favored. Hence, the transactivating domains of TATi-1 and TATi-2 do not correspond to parasite proteins but are instead essentially adventitiously derived polypeptides that have in common a predominantly hydrophobic amino acid composition and an absence of charged amino acids. It is presumably these general characteristics that provide transactivating capacity to the TetR-fusion protein. In contrast, the transactivating domain of tTA, the strong transactivator used for Tet-regulated expression in higher eukaryotes, is highly acidic. In HeLa cells, both TATi-1 and TATi-2 are inactive, suggesting fundamental differences in the transcription machinery between *T. gondii* and its host. Consistent with this hypothesis, a bioinformatics approach revealed that the basic transcription machinery in Apicomplexan parasites contains only a minimal set of general transcription

factors including TBP, TFB (a homologue to TFIIB), and TFE (a homologue to TFIIE- α) and resembles the RNA polymerase system of Archaea (M.M. and D.S., unpublished observations).

TATi-1 and TATi-2 function as ATc-responsive transactivators within a range of ATc that shows no toxicity effects on the blood stages of *P. falciparum*. TATi-2 was well tolerated when expressed in *P. falciparum* and was capable of regulating a CAT reporter by 10- to 20-fold and a GFP reporter by a factor of >50-fold in stable lines. Using GFP, we demonstrated that inactivation of gene expression is rapid and occurs within a single cycle upon addition of ATc. Such rapid regulation was not observed with CAT where two growth cycles were required to maximally suppress expression. It is likely that the primary reason for the slower regulation and higher background in the presence of ATc seen with bacterial CAT is related to the stability of this enzyme. The stability of CAT would result both in carryover of preexisting enzyme into the next growth cycle (resulting in slower regulation) and in the accumulation of small amounts of CAT in parasites maintained in the presence of ATc as a result of the expected minimal promoter activity in this circumstance (resulting in higher background). In contrast, the GFP reporter protein, which is localized in the endoplasmic reticulum (data not shown), is turned over rapidly, as evidenced by its absence in ring- and early-trophozoite stage parasites in the absence of ATc (Fig. 4B, lanes 1–3).

In addition, it was evident that only $\approx 20\%$ of the GFP-expressing parasites in the population were brightly fluorescent. There are a number of potential reasons for this, including the fact that GFP expression is controlled by TATi-2, which is itself regulated by the MSP-2 promoter. This means that GFP will only be maximally expressed at a specific time in the schizont stage. Because parasites in the population are not tightly synchronous, at any given time only a subpopulation will be at this optimal stage. Furthermore, the GFP plasmid is maintained episomally and hence is unlikely to be present in equal copy numbers per cell (3, 23). Refinement of this system will involve integrated forms of the plasmid and the cloning of transactivator-expressing lines

that should eliminate heterogeneity due to the latter. The fact that the expression profile of GFP perfectly mimics the steady-state level of MSP-2 across the entire erythrocytic cycle suggests that TATi-2 transcript and protein are rapidly turned over by the parasites. Therefore, the regulation of TATi-2 expression by a stage-specific promoter imposes a second level of control for the activity of the regulatable promoter in addition to modulation by ATc. This is of considerable interest in a parasite in which the expression of each individual gene seems to be carefully induced only when it is required.

As it stands, the system has considerable potential for the analysis of blood-stage gene function in *P. falciparum*. Because of the inability to observe transgene expression transiently (except when using very sensitive reporters) and the long time period between initial transfection and analysis in the case of stable transformants (usually 1–2 months), the strong expression of transgenes is particularly problematic in this organism, especially when such a transgene impacts negatively on growth. Hence, the ATc-regulation system described here should have immediate practical value for stage-specific expression of transgenes of interest. In particular, the expression of proteins that possess mutations conferring dominant-negative phenotypes could be highly diagnostic of gene function. Furthermore, we anticipate that the system could be adapted to generate conditional knockout lines in a manner similar to that in *T. gondii* (6).

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