5-2018

Investigation of bradyzoite differentiation initiation in Toxoplasma gondii

Harim I. Won
University of Nebraska at Omaha, hwon@unomaha.edu

Paul H. Davis Ph.D.
University of Nebraska at Omaha, pdavis@unomaha.edu

Investigation of bradyzoite differentiation initiation in *Toxoplasma gondii*

Honors Program Senior Thesis

University of Nebraska at Omaha

Submitted by:

Harim I. Won

April 2018

Paul H. Davis, Ph.D.

Faculty Mentor

Department of Biology

Table of Contents

Abstract

Like other eukaryotic organisms, *Toxoplasma gondii* promoters feature both constitutive and life-stage regulated *cis*-elements. Using a transcriptomic microarray approach, a cluster of transcripts upregulated early during bradyzoite differentiation was identified. Computational analysis of the promoter regions of these “up-early” transcripts identified a shared upstream consensus motif, a putative transcription factor binding site. Using a dual luciferase assay adapted for recombinational cloning and reporter gene quantification by qPCR, we demonstrate developmental stage-specific expression of the luciferase reporter gene inserted downstream of the transcription factor binding site. The shared consensus motif was found to be an autonomous *cis*-element by conversion of a constitutive promoter into a bradyzoite growth condition-inducible promoter. A gel-shift experiment showed the *cis*-element’s binding to bradyzoite nuclear proteins. Mass spectrometry of the shifted gel band identified a protein possessing an apicomplexan AP2 transcription factor domain. Much detail concerning the mechanism of differentiation is yet to be discovered and this work highlights the mediation of life cycle progression by bradyzoite differential gene expression. Taken together, these data demonstrate the control an early bradyzoite promoter element exercises on stage differentiation.

Key Words: bradyzoite differentiation, gene regulation, gene expression, *Toxoplasma gondii*

Introduction

Toxoplasma gondii is a single-celled, obligate intracellular parasite with a worldwide distribution, chronically infecting one third of the world's population [1,2]. This apicomplexan pathogen is capable of infecting all nucleated mammalian cells, and is the etiologic agent of toxoplasmosis in humans [3]. Recrudescence of the chronic infection in immunocompromised patients (e.g. AIDS, cancer, and organ transplant patients) can cause fatal encephalitis [4,5]. Additionally, primary infection in otherwise healthy pregnant women can cause severe birth defects or spontaneous abortion of the fetus [6,7]. The wide distribution and significant public health impact of *T. gondii* highlight the critical need to further characterize the basic biology of this important parasite to discover novel biomedical solutions to it.

The *T. gondii* developmental cycle includes the conversion from tachyzoite to bradyzoite – stages which give rise to acute and chronic infection, respectively [8]. While prophylaxis for acute infection with tachyzoites is available [9] and many promising experimental compounds are in development [10,11], the cyst-forming bradyzoite stage has no treatment or cure. Many apicomplexan transcriptome analyses have focused on developmental gene expression, as shown in the variety of microarray and RNA-Seq datasets investigating developmental stages found in the ToxoDB, PlasmoDB, and CryptoDB genomics resources [12–14]. Analyses of apicomplexan transcriptomes have shown differential expression of transcripts accompanying the progression of the parasite through life-cycle stages [15–17]. This is particularly true of *Plasmodium*, as staged transcription is observed in a large portion of mRNAs with limited shared expression among the life stages [18]. In *T. gondii*, serial analysis of gene expression

(SAGE) tags have been used to characterize the pool of transcripts expressed through development; it was shown that mRNAs which are stage-specific accounted for 18% of all SAGE tags identified [17]. The stage-specific nature of large subsets of apicomplexan transcripts as shown by microarray, RNA-Seq, and SAGE analyses lends support to the idea that apicomplexan development is, in part, driven by the ordered activation of the appropriate transcript subsets which correspond to the life stage being transitioned to. It appears that regulation at transcription initiation is a major mechanism by which gene expression is controlled in apicomplexans.

In this work, we utilize a bradyzoite differentiation time series microarray dataset to identify a cluster of transcripts upregulated early during bradyzoite differentiation and use a computational analysis to extract a shared, consensus DNA motif from their promoter regions – a putative transcription factor binding site. An apicomplexan Apetala 2 (AP2)-domain transcription factor is predicted to bind the identified motif by mass spectrometry analysis followed by peptide mass fingerprinting. It is further shown by a dual luciferase model adapted for recombinational cloning and quantitative PCR that this motif acts as an autonomous *cis*-element which regulates the expression of bradyzoite-specific transcripts and can convert a constitutive promoter into one which is chronic stage-specific.

Materials and Methods

Cell culture. The Type II Prugniaud (Pru) strain shows robust bradyzoite differentiation and Pru*Δku80Δhxgprt* was used to generate the transient transgenic strains. This strain was maintained by serial passage into confluent human foreskin fibroblast (HFF) monolayers cultured in D10 media containing Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) fetal bovine serum, 2 mM L-alanyl-L-glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 20 µg/mL Gentamicin at 37 °C with 5% CO_2. Bradyzoite induction was accomplished by growth in capped flasks with CO_2-depleted alkaline media containing Eagle's minimum essential medium (MEM), 1% (v/v) dialyzed fetal bovine serum, 25 mM HEPES solution, 2 mM L-alanyl-L-glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 25 µg/mL Gentamicin adjusted to pH 8.2 with NaOH.

Promoter constructs. The Dest-p-firefly destination vector which carries the cassette B fragment (containing the *ccd*B toxic and chloramphenicol resistance markers) upstream of the firefly luciferase coding region was used for Gateway™ recombinational cloning. The Dest-p-renilla-α-tubulin was used as a transfection efficiency control, and both described plasmids were generous gifts from Michael White [19]. Promoter fragments of the well-documented, constitutive dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene [20] were designed to include the 5'-upstream genomic sequence, flanking attB sites, and the translational start site, designed such that the attB2 sequence would be translated in frame with the firefly luciferase CDS. These fragments were ordered as double-stranded gBlocks® Gene Fragments (Invitrogen). Promoter fragments were first cloned into the pDONR221 entry vector (Invitrogen) by an 18-hr incubation with the Gateway® BP Clonase™ II Enzyme (Invitrogen) to produce modified pDONR221 entry

clones. Following verification by Sanger sequencing, the promoter fragment from each entry clone was cloned into the Dest-p-firefly destination vector by an 18-hr incubation with the Gateway® LR Clonase™ II Enzyme (Invitrogen) to yield destination clones. The destination clones were also verified by Sanger sequencing to ensure successful recombination and correct orientation. Plasmids featuring the *ccd*B toxic marker (Dest-p-firefly and pDONR221) were maintained in One Shot® *ccd*B Survival™ 2 $T1^R$ *Escherichia coli* cells (Invitrogen), and all other vectors were maintained in 10-beta *E. coli* cells (NEB).

Dual Luciferase Assay by Quantitative PCR. Pru*Δku80Δhxgprt* strain parasites were electroporated according to an amended protocol described previously [3,21] with 50 µg each of a promoter construct destination clone and the control Dest-p-renilla-α-tubulin plasmid. Transfections were performed in duplicate and each sample was transferred into one T25 cm^2 flask each (one each for tachyzoite and bradyzoite growth conditions) and allowed to recover for 12 h at 37 °C with 5% CO_2. After ~24 h, one flask was selected for bradyzoite induction by shifting to alkaline (pH 8.2), CO_2-depleted media. To assay the level of transcription early in bradyzoite differentiation, infected monolayers were Dounce homogenized, filtered, and parasite RNA was isolated 16 h-post induction from both tachyzoite and bradyzoite flasks with RNeasy Plus Mini reagents (Qiagen) and cDNA was produced from mRNAs with Maxima reverse transcriptase (Thermo Scientific) and a poly(T) primer according to manufacturers' instructions. Quantitative PCR was used to measure transcript abundance of the dual luciferases and a constitutively-expressed housekeeping gene (histone H2B variant TGME49_009910) for each transfected life stage, using the primers listed in

Supplemental Table 1. Synthesized cDNA was mixed with the Luminaris HiGreen qPCR Master Mix (ThermoFisher Scientific) and the qPCR reactions were performed in triplicate for each promoter construct at 50 ˚C for 2 min, 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 sec, 60 ˚C for 30 sec, and 72 ˚C for 30 sec. Reaction specificity was assayed by melt curve analysis.

Relative quantification of transcript abundance was calculated using the ΔΔCt method [22], experiments were conducted on a Rotor-Gene Q 5plex HRM Platform (Qiagen), and data was processed using the Rotor-Gene Q Series software v2.3.1 (Qiagen). Fold expression of both firefly and renilla luciferase in both tachyzoites and bradyzoites were normalized to histone H2B variant expression; fold expression of firefly luciferase was then normalized to renilla levels by comparing the ratio of the firefly promoter construct expression to that of the α-tubulin-renilla promoter construct.

Results

Induction of Bradyzoite-specific Expression by the DNA Motif.

Previous work included microarray analysis of *T. gondii* expression in a 48-hour time course identified subsets of transcripts expressed at distinct timepoints during bradyzoite induction. A consensus DNA motif was also identified computationally in the promoters of 71 early-expression genes. The DNA motif was examined to assess whether it was an autonomous-acting *cis*-element by inserting the motif into a weak promoter upstream of the firefly luciferase reporter gene. DHFR-TS is known to be a constitutive, weakly-expressed transcript; the constitutive expression of this gene regardless of growth stage has been confirmed by microarray both by the dataset detailed in this study and by others (M.S. Behnke and M.W. White, ToxoDB). The 1000 bp genomic fragment 5' of the DHFR-TS gene [23] was selected for this experiment. The native DHFR-TS promoter and the promoter with three copies of the pyrimidine-rich DNA motif inserted 100 bp upstream of the major transcriptional start site at -369 bp [23] were designed and ordered as described (sequences are given in Supplemental Table 2). A promoter with three scrambled copies of the DNA motif was also designed to confirm that any observed impact on expression during bradyzoite induction was not sequence-specific and not simply an artefact of adding a pyrimidine-rich sequence or disrupting a repressor site. A diagram of the three promoter constructs cloned into Dest-p-firefly destination vectors is shown in Figure 1. The introduction of the DNA motif *cis*-element, but not the native or scrambled *cis*-elements, led to upregulation of the target gene in bradyzoite conditions. (Figure 2). This conversion was also seen to be specific to the period of early induction; following 7 days of induction, parasites transfected with

the construct with three copies of the DNA motif showed no significant difference in luciferase expression between tachyzoite and bradyzoite populations (data not shown). This data indicates that the identified DNA motif is autonomous, i.e., is sufficient to effect an increase in gene expression during bradyzoite induction.

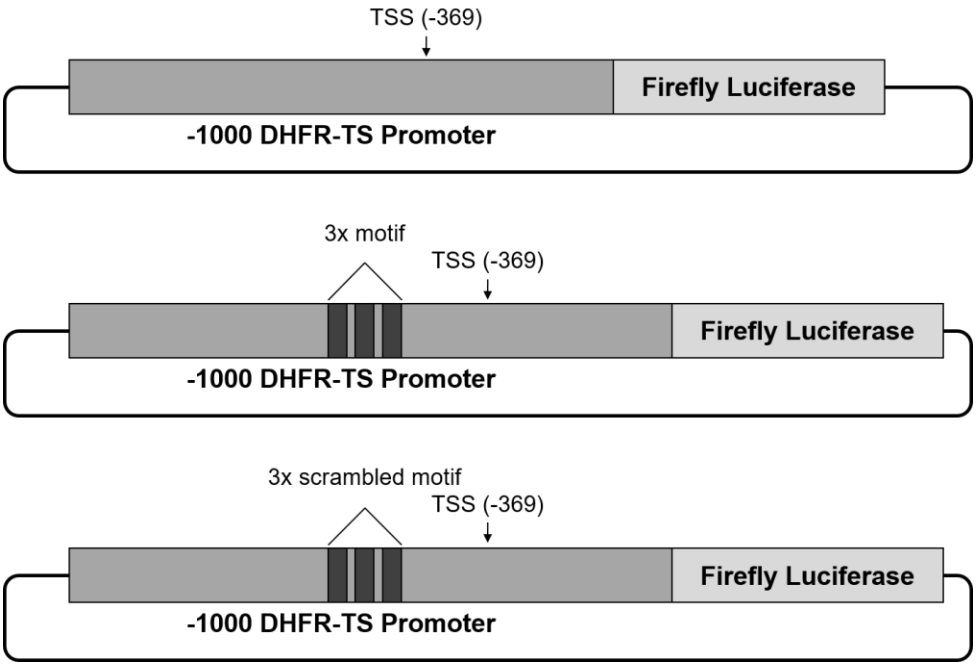

Figure 1. Diagram of Promoter Constructs. The three promoter constructs used in this study were designed and ordered as gBlocks™ from Invitrogen with attB1 and attB2 sites flanking them, corresponding to the attP1 and attP2 sites on the Dest-p-firefly destination vector.

The DHFR-TS promoter (-1000 bp) was modified to contain three copies of either the putative motif or scrambled versions of the motif, placed 100 bp 5' of the major transcriptional start site (TSS). Each promoter construct was inserted into the destination vector by recombinational cloning, immediately upstream of the firefly luciferase coding region.

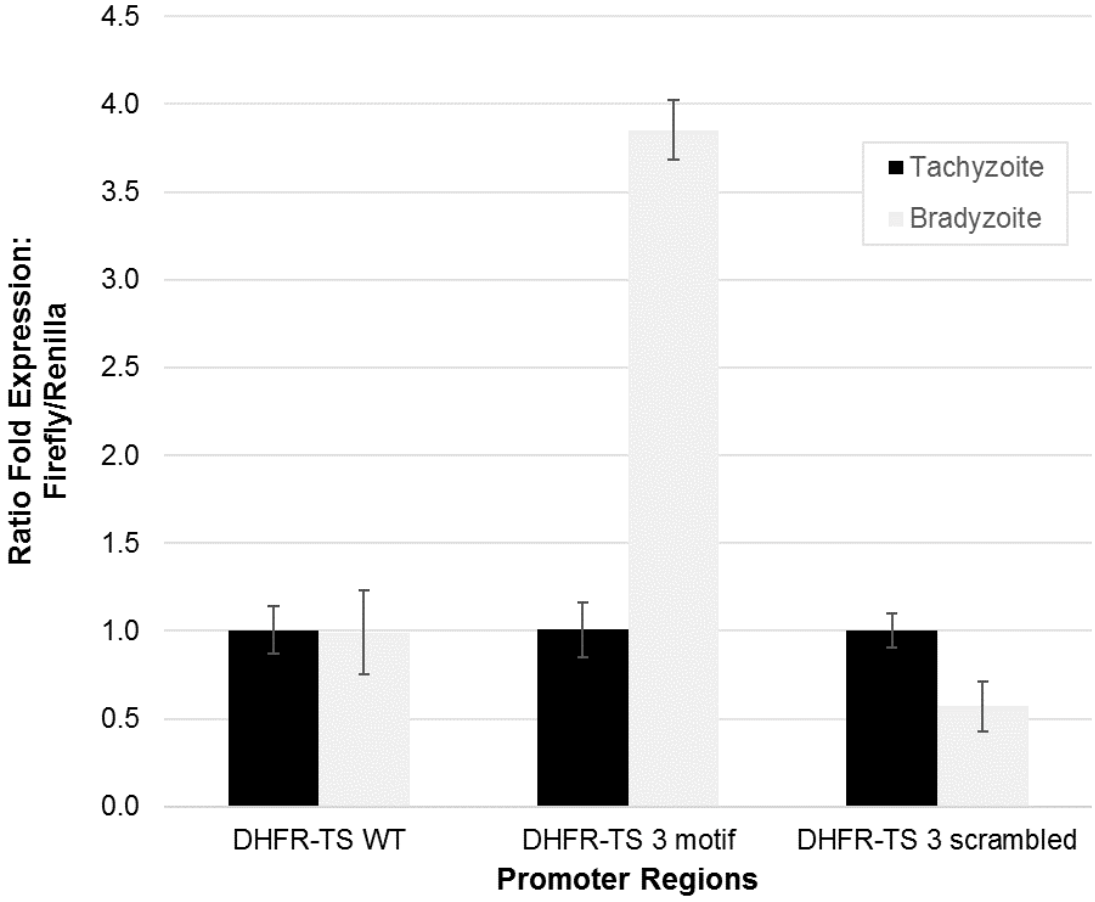

Figure 2. Induction of Expression During Bradyzoite Differentiation by the DNA Motif. The Pru*Δku80Δhxgprt* strain was transiently transfected in duplicate and bradyzoite-condition flasks were induced for 16 h by pH 8.2, CO_2-depleted media. Parasites were separated by homogenization and filtration, RNA was isolated, and quantitative PCR was performed in triplicate to assay the transcription of the dual luciferases and a housekeeping gene. Results of both luciferases were normalized to histone H2B variant and are reported as the fold expression of firefly luciferase relative to that of renilla luciferase driven by the α-tubulin promoter. No change in luciferase expression was observed in the tachyzoite stage for any of the constructs. In contrast,

induced parasites showed a marked increase in luciferase transcription in the construct with three copies of the DNA motif *cis*-element compared to the wild-type DHFR-TS promoter.

Discussion

Utilizing the dual luciferase assay and alkaline bradyzoite induction models, we show that a putative transcription factor binding site identified in the 5' promoter regions of a cluster of transcripts upregulated early during *T. gondii* bradyzoite differentiation is able to induce luciferase transcription under alkaline bradyzoite induction. This *cis*-element was identified as autonomous by its ability to induce greater expression during bradyzoite differentiation following insertion into the constitutive DHFR-TS promoter (Figure 2). A protein binding assay demonstrated the site-specific binding of nuclear proteins to this *cis*-element (previous data not shown), and mass spectrometry followed by computational analysis identified a small group of proteins predicted to bind the consensus DNA motif. These results confirm the concept that a major control mechanism of apicomplexan development is transcriptional initiation and this study is the first to examine the control of transcriptionally-active mRNAs which are specifically upregulated early in bradyzoite differentiation.

The transcription factor which bound to the DNA motif was identified as the apicomplexan AP2-domain transcription factor, AP2III-3. The AP2 transcription factors are a well-documented family of transcription factors which have been implicated in stage-specific transcription [24–28]. While AP2III-3 has been identified in the literature as being upregulated ~200-fold during bradyzoite differentiation [27], it has otherwise not been characterized in the literature. It is conceivable that because this transcription factor activates the transcription of such a large subset of bradyzoite-initiating proteins, it is necessary for bradyzoite differentiation and cyst development. This expression analysis work will continue with a knock-out of AP2III-3 to examine whether the gene

knock-out impacts *T. gondii*'s ability to differentiate into the bradyzoite stage. If it is shown that deletion of this gene removes or severely inhibits *T. gondii* bradyzoite formation, thus reducing risk of chronic infection, an excellent live vaccine candidate strain will have been identified. Considering the serious public health issues that accompany lifelong chronic infection in immunocompromised individuals, the production of an efficacious vaccine is paramount.

Acknowledgements

This work was supported by NIH grant P20GM103427 (HIW, PHD). Additional financial support was provided by a University of Nebraska at Omaha Fund for Undergraduate Scholarly Experiences (FUSE) grant. HIW was also partially supported by the Barry Goldwater Scholarship. The University of Nebraska Medical Center Genomics Core Facility provided DNA sequencing and receives partial support from the Nebraska Research Network in Functional Genomics, The Molecular Biology of Neurosensory Systems CoBRE P30GM110768, The Fred & Pamela Buffett Cancer Center P30CA036727, The Center for Root and Rhizobiome Innovation (CRRI) 36-5150-2085-20, and the Nebraska Research Initiative. This work was also completed utilizing the Holland Computing Center of the University of Nebraska which receives support from the Nebraska Research Initiative. Genomic and/or cDNA sequence data were accessed via http://ToxoDB.org. This report's contents are the sole responsibility of the author and do not necessarily represent the official views of the funding agencies.

References

1. Lehmann T, Marcet PL, Graham DH, Dahl ER, Dubey JP. Globalization and the population structure of *Toxoplasma gondii. Proc Natl Acad Sci U S A*. 2006;103: 11423–11428. doi:10.1073/pnas.0601438103

2. Dubey JP. Toxoplasmosis of Animals and Humans, Second Edition. CRC Press; 2016.

3. Weiss LM, Kim K. *Toxoplasma Gondii*: The Model Apicomplexan. Perspectives and Methods. Academic Press; 2011.

4. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet Lond Engl*. 2004;363: 1965–1976. doi:10.1016/S0140-6736(04)16412-X

5. McFarland MM, Bartlett ML, Davis PH. Toxoplasmic Encephalitis. Avid Science; 2016.

6. Montoya JG, Remington JS. Management of *Toxoplasma gondii* infection during pregnancy. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2008;47: 554–566. doi:10.1086/590149

7. Giakoumelou S, Wheelhouse N, Cuschieri K, Entrican G, Howie SEM, Horne AW. The role of infection in miscarriage. *Hum Reprod Update*. 2016;22: 116–133. doi:10.1093/humupd/dmv041

8. Dubey JP. Advances in the life cycle of *Toxoplasma gondii. Int J Parasitol.* 1998;28: 1019–1024.

9. Neville AJ, Zach SJ, Wang X, Larson JJ, Judge AK, Davis LA, et al. Clinically Available Medicines Demonstrating Anti-*Toxoplasma* Activity. *Antimicrob Agents Chemother*. 2015;59: 7161–7169. doi:10.1128/AAC.02009-15

10. McFarland MM, Zach SJ, Wang X, Potluri L-P, Neville AJ, Vennerstrom JL, et al. Review of Experimental Compounds Demonstrating Anti-*Toxoplasma* Activity. *Antimicrob Agents Chemother*. 2016;60: 7017–7034. doi:10.1128/AAC.01176-16

11. Sanford AG, Schulze TT, Potluri LP, Hemsley RM, Larson JJ, Judge AK, et al. Novel *Toxoplasma gondii* inhibitor chemotypes. *Parasitol Int.* 2017;67: 107–111. doi:10.1016/j.parint.2017.10.010

12. Kissinger JC, Gajria B, Li L, Paulsen IT, Roos DS. ToxoDB: accessing the *Toxoplasma gondii* genome. *Nucleic Acids Res*. 2003;31: 234–236.

13. Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, et al. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res.* 2009;37: D539-543. doi:10.1093/nar/gkn814

14. Puiu D, Enomoto S, Buck GA, Abrahamsen MS, Kissinger JC. CryptoDB: the *Cryptosporidium* genome resource. *Nucleic Acids Res*. 2004;32: D329–D331. doi:10.1093/nar/gkh050

15. Cleary MD, Singh U, Blader IJ, Brewer JL, Boothroyd JC. *Toxoplasma gondii* asexual development: identification of developmentally regulated genes and distinct patterns of gene expression. *Eukaryot Cell*. 2002;1: 329–340.

16. Singh U, Brewer JL, Boothroyd JC. Genetic analysis of tachyzoite to bradyzoite differentiation mutants in *Toxoplasma gondii* reveals a hierarchy of gene induction. *Mol Microbiol.* 2002;44: 721–733.

17. Radke JR, Behnke MS, Mackey AJ, Radke JB, Roos DS, White MW. The transcriptome of *Toxoplasma gondii. BMC Biol.* 2005;3: 26. doi:10.1186/1741-7007-3-26

18. Llinás M, DeRisi JL. Pernicious plans revealed: *Plasmodium falciparum* genome wide expression analysis. *Curr Opin Microbiol*. 2004;7: 382–387. doi:10.1016/j.mib.2004.06.014

19. Matrajt M, Nishi M, Fraunholz MJ, Peter O, Roos DS. Amino-terminal control of transgenic protein expression levels in *Toxoplasma gondii. Mol Biochem Parasitol.* 2002;120: 285–289.

20. Behnke MS, Radke JB, Smith AT, Sullivan WJ, White MW. The transcription of bradyzoite genes in *Toxoplasma gondii* is controlled by autonomous promoter elements. *Mol Microbiol.* 2008;68: 1502–1518. doi:10.1111/j.1365-2958.2008.06249.x

21. Donald RG, Roos DS. Stable molecular transformation of *Toxoplasma gondii:* a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. *Proc Natl Acad Sci U S A.* 1993;90: 11703–11707.

22. Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat Bioinforma Biomath*. 2013;3: 71–85.

23. Matrajt M, Platt CD, Sagar AD, Lindsay A, Moulton C, Roos DS. Transcript initiation, polyadenylation, and functional promoter mapping for the dihydrofolate reductase-thymidylate synthase gene of *Toxoplasma gondii. Mol Biochem Parasitol*. 2004;137: 229–238. doi:10.1016/j.molbiopara.2003.12.015

24. Huang S, Holmes MJ, Radke JB, Hong D-P, Liu T-K, White MW, et al. *Toxoplasma gondii* AP2IX-4 Regulates Gene Expression during Bradyzoite Development. *mSphere.* 2017;2. doi:10.1128/mSphere.00054-17

25. Lai B-S, Witola WH, El Bissati K, Zhou Y, Mui E, Fomovska A, et al. Molecular target validation, antimicrobial delivery, and potential treatment of *Toxoplasma*

gondii infections. *Proc Natl Acad Sci U S A*. 2012;109: 14182–14187. doi:10.1073/pnas.1208775109

26. Radke JB, Lucas O, De Silva EK, Ma Y, Sullivan WJ, Weiss LM, et al. ApiAP2 transcription factor restricts development of the *Toxoplasma* tissue cyst. *Proc Natl Acad Sci U S A*. 2013;110: 6871–6876. doi:10.1073/pnas.1300059110

27. Walker R, Gissot M, Croken MM, Huot L, Hot D, Kim K, et al. The *Toxoplasma* nuclear factor TgAP2XI-4 controls bradyzoite gene expression and cyst formation. *Mol Microbiol.* 2013;87: 641–655. doi:10.1111/mmi.12121

28. Walker R, Gissot M, Huot L, Alayi TD, Hot D, Marot G, et al. *Toxoplasma* transcription factor TgAP2XI-5 regulates the expression of genes involved in parasite virulence and host invasion. *J Biol Chem.* 2013;288: 31127–31138. doi:10.1074/jbc.M113.486589

Supplemental Data

Primer #	Primer Name	Amplicon and Direction	Sequence (5' to 3')
BB09	qRen2-F	Renilla luciferase forward	GGGTGCTTGTTTGGCATTTCA
BB10	qRen2-R	Renilla luciferase reverse	GGCAACATGGTTTCCACGAAG
BB19	qLuc4-F	Firefly luciferase forward	CGTTCGGTTGGCAGAAGCTA
BB20	qLuc4-R	Firefly luciferase reverse	CACTACGGTAGGCTGCGAAA
BB33	qHis2b-F	Histone H2B variant forward	GCTTGGCTGATGAAGCAGTTCGTT
BB34	qHis2b-R	Histone H2B variant reverse	AGTCGTGTACTTGGTCACTGCCTT

Table S1. Oligonucleotides used for quantitative PCR. The qPCR reactions were performed using the described primers. To ensure specificity of the luciferase primer pairs, that is, having no *T. gondii* or human host cell targets, qPCR was also run on a mock transfected strain and the absence of either luciferase amplicon was confirmed.

Promoter Construct	**Sequence (5’ to 3’)**
Native DHFR-TS -1000 bp	GGGG**ACAAGTTTGTACAAAAAAGCAGGCT**TAGTGCCACGACTTCTAAATCCGGCGACAGGC TGGTCTTTTGTCTTACCACGTATTAGCCCGCGTGCGGATTTCTCGGAGCGCACCTGTTCAACA CTAGAAAACGGAGTTTCCTGATCGAGAAGCCACCACCTTTCCAGAAGTTGAACGCTAGCATGT CATTCGATTTTCACCCCCCGCGTAGTTCCTGTGTGTCATTCGTTGTCGAGACAACTCTGTCCC GCCCCGGTGCTGTTCCATATGCGTGACTTTCCCGCAATTTTTTCAGACTTTCAGGAAAGACAG GCTCCGGAACGATCTCGTCCATGACTGGTAAATCCACGACACCGCAATGGCCCCCAGCACCT CTATCTCTCGTGCCAGGGGACTAACGTTGTATGCGTCTGCGTCTTGTCTTTTTGCATTCGCTTT CCAAAAAAGAGAGCCATCCGTTCCCCCGCACATTCAACGCCGCGAGTGCGGTTTTTGTCTTTT TTGAGTGGTAGGACGCTTTTCATGCGCGAACTACGTGGACATTAAGTTCCATTCTCTTTTTCGA CAGCACGAAACCTTGCATTCAAACCCGCCCGGGGAAGATCCGATCTTGCTGCTGTTCGCAGT CCCAGTAGCGTCCTGTCGGCCGCGCCGTCTCTGTTGGTGGGCAGCCGCTACACCTGTTATCT GACTGCCGTGCGCGAAAATGACGCCATTTTTGGGAAAATCGGGGAACTTCATTCTTTAAAAGT ATGCGGAGGTTTCCTTTTTCTTCTGTTCGTTTCTTTTTCTCGGGTTTGATAACCGTGTTCGATG TAAGCACTTTCCGTCTCTCCTCCGTGCTTTGTTCGACATCGAGACCAGGTGTGCAGATCCTTC GCTTGTTGATCCGGAGACGCGTGTCTCGTAGAACCTTTTCATTTTACCACACGGCAGTGCTGA GCACTGCTCTGAGTGCAGCAGGGACGGGTGAAGTTTCGCTTTAGTAGTGCGTTTCTGCTCTA CGGGGCGTTGTCGTGTCTGGGAAG**ATG**GCGG**ACCCAGCTTTCTTGTACAAAGTGGT**GGGG
DHFR-TS -1000 bp with DNA Motif 3x	GGGG**ACAAGTTTGTACAAAAAAGCAGGCT**TAGTGCCACGACTTCTAAATCCGGCGACAGGC TGGTCTTTTGTCTTACCACGTATTAGCCCGCGTGCGGATTTCTCGGAGCGCACCTGTTCAACA CTAGAAAACGGAGTTTCCTGATCGAGAAGCCACCACCTTTCCAGAAGTTGAACGCTAGCATGT CATTCGATTTTCACCCCCCGCGTAGTTCCTGTGTGTCATTCGTTGTCGAGACAACTCTGTCCC GCCCCGGTGCTGTTCCATATGCGTGACTTTCCCGCAATTTTTTCAGACTTTCAGGAAAGACAG GCTCCGGAACGATCTCGTCCATGACTGGTAAATCCACGACACCGCAATGGCCCCCAGCACCT CTATCTCTCGTGCCAGGGGACTAACGTTGTATGCGTCTGCGTCTTGTCTTTTTGCATTCGCTTT CCAAAAAAGAGAGCCATCCGTTCCCCCGCACATTCAACGCCGCGAGTGCGGTTTTTGTCTTTT TTGAGTGGTAGGACGCTTTTCATGCGCGAACTACGTGGACATTAAGTTCCATTCTCTTCTCTCT TCTTCTCTCTTCCTTCTTTACTAGTCCTACTCTCTTCTTCTCTCTTCCTTCTTTTCGATGAGACG CTCTCTTCTTCTCTCTTCCTTCTTTTTTCGACAGCACGAAACCTTGCATTCAAACCCGCCCGGG GAAGATCCGATCTTGCTGCTGTTCGCAGTCCCAGTAGCGTCCTGTCGGCCGCGCCGTCTCTG

	TTGGTGGGCAGCCGCTACACCTGTTATCTGACTGCCGTGCGCGAAAATGACGCCATTTTTGG GAAAATCGGGGAACTTCATTCTTTAAAAGTATGCGGAGGTTTCCTTTTTCTTCTGTTCGTTTCT TTTTCTCGGGTTTGATAACCGTGTTCGATGTAAGCACTTTCCGTCTCTCCTCCGTGCTTTGTTC GACATCGAGACCAGGTGTGCAGATCCTTCGCTTGTTGATCCGGAGACGCGTGTCTCGTAGAA CCTTTTCATTTTACCACACGGCAGTGCTGAGCACTGCTCTGAGTGCAGCAGGGACGGGTGAA GTTTCGCTTTAGTAGTGCGTTTCTGCTCTACGGGGCGTTGTCGTGTCTGGGAAG**ATG**GCGG**A** **CCCAGCTTTCTTGTACAAAGTGGT**GGGG
DHFR-TS -1000 bp with Scrambled Motif 3x	GGGG**ACAAGTTTGTACAAAAAAGCAGGCT**TAGTGCCACGACTTCTAAATCCGGCGACAGGC TGGTCTTTTGTCTTACCACGTATTAGCCCGCGTGCGGATTTCTCGGAGCGCACCTGTTCAACA CTAGAAAACGGAGTTTCCTGATCGAGAAGCCACCACCTTTCCAGAAGTTGAACGCTAGCATGT CATTCGATTTTCACCCCCCGCGTAGTTCCTGTGTGTCATTCGTTGTCGAGACAACTCTGTCCC GCCCCGGTGCTGTTCCATATGCGTGACTTTCCCGCAATTTTTTCAGACTTTCAGGAAAGACAG GCTCCGGAACGATCTCGTCCATGACTGGTAAATCCACGACACCGCAATGGCCCCCAGCACCT CTATCTCTCGTGCCAGGGGACTAACGTTGTATGCGTCTGCGTCTTGTCTTTTTGCATTCGCTTT CCAAAAAAGAGAGCCATCCGTTCCCCCGCACATTCAACGCCGCGAGTGCGGTTTTTGTCTTTT TTGAGTGGTAGGACGCTTTTCATGCGCGAACTACGTGGACATTAAGTTCCATTCTCTTTTCCTC TCCTCTTTCTTCTCTTCTTACTAGTCCTATCCTTTTCCTTCCTTTCTCTCTTCTTCGATGAGACG TCCTTTCTCCTTTCTTCTCTCTTCTTTTCGACAGCACGAAACCTTGCATTCAAACCCGCCCGGG GAAGATCCGATCTTGCTGCTGTTCGCAGTCCCAGTAGCGTCCTGTCGGCCGCGCCGTCTCTG TTGGTGGGCAGCCGCTACACCTGTTATCTGACTGCCGTGCGCGAAAATGACGCCATTTTTGG GAAAATCGGGGAACTTCATTCTTTAAAAGTATGCGGAGGTTTCCTTTTTCTTCTGTTCGTTTCT TTTTCTCGGGTTTGATAACCGTGTTCGATGTAAGCACTTTCCGTCTCTCCTCCGTGCTTTGTTC GACATCGAGACCAGGTGTGCAGATCCTTCGCTTGTTGATCCGGAGACGCGTGTCTCGTAGAA CCTTTTCATTTTACCACACGGCAGTGCTGAGCACTGCTCTGAGTGCAGCAGGGACGGGTGAA GTTTCGCTTTAGTAGTGCGTTTCTGCTCTACGGGGCGTTGTCGTGTCTGGGAAG**ATG**GCGG**A** **CCCAGCTTTCTTGTACAAAGTGGT**GGGG

Table S2. Promoter Constructs. Promoter constructs were designed and ordered as gBlocks™ Gene Fragments (Invitrogen). The bolded nucleotides (25 bp on each end) are the attB1 and attB2 sites used for recombination. In the second and third constructs,

the underlined nucleotides indicate either motif or scrambled motif sequences. The ATG start codon is bolded and underlined in each construct; translation starts at that location in the transcribed mRNA, and the attB2 site is translated in frame with the firefly luciferase coding region.

Appendix: Glossary of Terms

AP2 = Apetala 2

AP2III-3 = indicates the third (3) Apetala 2 transcription factor identified on *T. gondii* chromosome III

DHFR-TS = dihydrofolate reductase-thymidylate synthase

Pru = Prugniaud strain of *T. gondii*

qPCR = quantitative PCR

SAGE = serial analysis of gene expression

TSS = transcriptional start site