Cellular differentiation leading to formation of the bradyzoite tissue cyst stage is the underlying cause of chronic toxoplasmosis. Consequently, mechanisms responsible for controlling development in the Toxoplasma intermediate life cycle have long been sought. Here, we identified 15 Toxoplasma mRNAs induced in early bradyzoite development that encode proteins with apicomplexan AP2 (ApiAP2) DNA binding domains. Of these 15 mRNAs, the AP2IX-9 mRNA demonstrated the largest expression increase during alkaline-induced differentiation. At the protein level, we found that AP2IX-9 was restricted to the early bradyzoite nucleus and is repressed in tachyzoites and in mature bradyzoites from 30-d infected animals. Conditional overexpression of AP2IX-9 significantly reduced tissue cyst formation and conferred alkaline pH-resistant growth, whereas disruption of the AP2IX-9 gene increased tissue cyst formation, indicating AP2IX-9 operates as a repressor of bradyzoite development. Consistent with a role as a repressor, AP2IX-9 specifically inhibited the expression of bradyzoite mRNAs, including the canonical bradyzoite marker, bradyzoite antigen 1 (BAG1). Using protein binding microarrays, we established the AP2 domain of AP2IX-9 binds a CAGTGT DNA sequence motif and is capable of binding cis-regulatory elements controlling the BAG1 and bradyzoite-specific nucleoside triphosphatase (B-NTPase) promoters. The effect of AP2IX-9 on BAG1 expression was direct because this factor inhibits expression of a firefly luciferase reporter under the control of the BAG1 promoter in vivo, and epitope-tagged AP2IX-9 can be immunoprecipitated with the BAG1 promoter in parasite chromatin. Altogether, these results indicate AP2IX-9 restricts Toxoplasma commitment to develop the mature bradyzoite tissue cyst.

gene regulation | gene expression | Apicomplexa

The apicomplexan Toxoplasma gondii has an exceptional range of animals that may serve as host for its intermediate life cycle, whereas the definitive life cycle occurs in a feline host (1). Together, oocysts shed by cats into the environment and tissue cysts in food products contribute to rates of human exposure that are estimated at one in three by age 50 in the US (25% for >20 y of age) (2, 3) and nearly 100% by the end of childhood in other parts of the world (4). Toxoplasma infections are thought to be lifelong because of the development of the tissue cyst, which is effectively invisible to the immune system and clinically untreatable. The bradyzoite tissue cyst is an essential part of the life cycle of Toxoplasma, and recrudescence of the tissue cyst leading to chronic cycles of toxoplasmosis is a major cause of mortality in AIDS patients. Experimental data indicates Toxoplasma sporozoite or bradyzoite infections follow a similar developmental course through the tachyzoite stage that is characterized by changes in growth and stage-specific gene expression, which leads ultimately to the mature tissue cyst (5–7). Determining how gene expression is regulated in these critical developmental transitions will be important to unraveling the underlying mechanisms responsible for chronic Toxoplasma infections.

The mechanisms of gene-specific regulation in Apicomplexa have remained elusive. The expression profiles of large portions of the cell cycle transcriptome of Plasmodium falciparum and Toxoplasma asexual stages are progressive with little understanding yet as to how these serial patterns are controlled (8, 9). Toxoplasma, conversion between developmental stages is also linked to significant changes in gene expression (5). Data mining of genome sequences has recently identified a family of plant-related AP2 domain (DNA binding) containing proteins in the Apicomplexa [apicomplexan AP2 (ApiAP2) proteins] (10). Based on genome sequence annotation, 68 of these factors are found in Toxoplasma and 27 in P. falciparum (8, 11, 12). In Toxoplasma, 24 ApiAP2 factors are cell cycle regulated in tachyzoites (8), whereas 18 are expressed in a cascading fashion during the P. falciparum intraerythrocytic cycle (12). Relatively few ApiAP2 proteins are characterized; however, stage-specific gene activation (13–16) as well as chromatin biology and genome maintenance (17) are functions identified so far. Like their plant counterparts, ApiAP2 factors can bind promoter elements of distinct coregulated gene clusters (12) and, therefore, it is likely these proteins will be major regulators of apicomplexan transcription.

Here, we report the characterization and functional validation of an ApiAP2 transcription factor in Toxoplasma. AP2IX-9 is a nuclear-restricted protein expressed transiently during tachyzoite to bradyzoite development. AP2IX-9 specifically binds cis-regulatory elements (CRE) functionally mapped to bradyzoite promoters (18) including the classic bradyzoite marker, small heat shock protein bradyzoite antigen 1 (BAG1) (19). Using gene disruption and conditional expression, we demonstrate AP2IX-9 is the first Apicomplexa transcriptional repressor that operates to regulate developmental gene expression. By preventing bradyzoite gene expression and promoting parasite growth, AP2IX-9 regulates the balance between parasite expansion and forming end-stage bradyzoites required for transmission to the definitive host.

Results

Toxoplasma ApiAP2 Factors Are Induced During Early Bradyzoite Differentiation. Of the 68 ApiAP2-encoding genes predicted in the Toxoplasma genome (11), there is evidence for expression by AP2IX-9.
microarrays for 44 of these genes (>40th percentile) in the tachyzoite (constitutive and stage specific) (8). To identify ApiAP2 genes regulated during early bradyzoite differentiation in Toxoplasma, we queried developmental transcriptome data representing the three genetic lineages (type I-GT1, type II-ME49, and type III-CTG) that dominate parasite populations of North America and Europe (18). From these microarray data, we classified 15 ApiAP2 mRNAs as increased (9 down-regulated) during in vitro bradyzoite induction in at least one strain (heatmap; SI Appendix, Fig. S1A). This collection of developmentally regulated ApiAP2 genes is predicted to encode a diverse group of proteins ranging in size from 286 to 3,256 amino acids containing one to three ApiAP2 domains (protein maps: SI Appendix, Fig. S1A). Interestingly, more than half of the ApiAP2 genes showing regulated mRNA expression (up and down) in the bradyzoite (14 of 24 total) also had cycllical mRNA profiles in the tachyzoite cell cycle.

One of the largest expression changes of any ApiAP2 mRNA was AP2IX-9, which was consistently increased during early bradyzoite induction in all three Toxoplasma lineages. To verify that the mRNA profile was predictive of AP2IX-9 protein expression, we endogenously tagged the AP2IX-9 locus in a type II Prugniaud strain carrying a disruption of the KU80 gene that enhances gene targeting (strain designated PruQ) (20). PruQ-AP2IX-9HA parasites induced to differentiate by pH 8.2 media were positive for tissue cyst wall formation by Dolichos biflorus agglutinin (DBA) lectin staining, and they coexpressed AP2IX-9HA protein exclusively in the nucleus (Fig. 1) similar to other ApiAP2 factors (8). The number of vacuoles positive for AP2IX-9HA reached 79% by 2 d after shift into pH 8.2 media (%HA positive), and this induction profile was confirmed by Western blot analysis and semiquantitative RT-PCR (SI Appendix, Fig. S1B).

By contrast, microarray analysis (see ToxoDB for data summary) indicates AP2IX-9 mRNA is minimally expressed in tachyzoites and in mature bradyzoites in tissue cysts (isolated from 21 d infected mice) (21), indicating AP2IX-9 mRNA expression is transient. AP2IX-9HA protein largely followed the native mRNA profile with minimal expression in tachyzoites of the PruQ-AP2IX-9HA transgenic strain (Fig. 1 and SI Appendix, Fig. S1B), and AP2IX-9HA protein was not detected in mature bradyzoites from PruQ-AP2IX-9HA-infected mice (30 d after infection; SI Appendix, Fig. S1C). Parasites from in vivo tissue cysts are capable of expressing other ApiAP2 proteins, such as the cell cycle factor AP2V1-1HA (SI Appendix, Fig. S1C) (8). Altogether, these results indicate AP2IX-9 is only expressed during early bradyzoite differentiation and, consistent with this conclusion, we found AP2IX-9HA-positive vacuoles decreased after peaking at 2 d in alkaline-shifted cultures (Fig. 1). The decrease in AP2IX-9HA nuclear fluorescence was not associated with a loss of tissue cysts because the number of DBA-positive vacuoles continued to increase throughout the experiment (Fig. 1, % DBA); by 4 d after alkaline shift, nearly half the DBA-positive vacuoles were negative for AP2IX-9HA protein.

AP2IX-9 binds cis-Regulatory Elements of the Bradyzoite NTPase Promoter. To determine the DNA binding motif recognized by AP2IX-9, we expressed the single ApiAP2 domain of AP2IX-9 as a glutathione-S-transferase (GST) fusion protein (GST-IX-9). Affinity-purified GST-IX-9 recombinant protein was assayed by using a protein binding microarray (12), and the top three motifs from this analysis revealed a conserved 6-bp core DNA sequence motif 5′-CAGTGT-3′/5′-GTCACA-3′ (enrichment scores >0.45; SI Appendix, Fig. S2A). GST-IX-9 binding to this motif was validated by using electrophoretic mobility shift assays (EMSAs) (SI Appendix, Fig. S2B; Dataset S1 for all sequence designs). Few functional CRE sequences are known in Toxoplasma; however, the promoter CRE controlling a bradyzoite NTPase isoform (B-NTPase) shares the GT/CA repeats (18) found in the AP2IX-9 binding motif. In EMSAs, GST-IX-9 protein bound the B-nucleoside triphosphatase (NTPase) CRE sequence in a manner consistent with the results of previous promoter mutagenesis (Fig. 2) (18). GST-IX-9 binding to the B-NTPase CRE was effectively competed by a mutant sequence (Fig. 2, mut 4) that retains 51% of promoter function, whereas binding to the B-NTPase probe was not affected by a mutant sequence that fully disrupts promoter function (Fig. 2, mut 5) (18). These results suggest AP2IX-9 could regulate the transcription of B-NTPase and, perhaps, other bradyzoite genes.

AP2IX-9 Regulates Bradyzoite Differentiation in Multiple Strains. The induction of AP2IX-9 during bradyzoite differentiation suggests this factor may serve a role in promoting parasite development and, by extension, deleting this factor might block development. Several attempts to verify this hypothesis by gene knockout in type II Prugniaud parasites failed despite successful epitope tagging by recombination of the AP2IX-9 locus in this strain (Fig. 1). We also failed to disrupt the AP2IX-9 locus in low-passage isolates of GT1, ME49, and CTG (types I–III, respectively). By contrast, disruption of the AP2IX-9 gene was readily achieved in the PLK clone of type II ME49 (PLKΔap2IX-9) and in the laboratory strain RHΔhxgprtΔuprt developed previously as a model for bradyzoite differentiation by using CO2 starvation (22). These genetic results suggest that AP2IX-9 may be essential to developmentally competent Toxoplasma strains, whereas dispensable in parasites adapted to cell culture (Dataset S1; knockout frequencies). AP2IX-9 is inducible in the parental parasites of both RHΔhxgprtΔuprt and PLK strains, and these strains are able to form tissue cysts, although they have lost the capacity to complete the definitive life cycle in the feline host (23). Therefore, we examined in vitro tissue cyst formation of PLKΔap2IX-9 clones as well as RH strain clones carrying a AP2IX-9 knockout in addition to the deletion of the hypoxanthine-xanthine-guanine
phosphoribosyl transferase (HXGPR) and uracil phosphoribosyl transferase (UPRT) genes (triple knockout strain designated RHΔap2IX-9) (SI Appendix, Fig. S3). Contrary to expectations, under CO₂-starvation culture conditions (22), RHΔap2IX-9 clones differentiated much better (49% tissue cysts; SI Appendix, Fig. S3, gray bars) than the double knockout parent (15% cysts in RHΔhxgprtΔuprt strain). Tissue cyst numbers were also increased in PLKΔap2IX-9 clones compared with the PLK parental strain following pH 8.2 induction (SI Appendix, Fig. S3, black bars).

The increased formation of tissue cysts in PLK and RHΔhxgprtΔuprt parasites lacking the AP2IX-9 protein suggested this factor might operate as a repressor rather than an activator of bradyzoite development. To test whether AP2IX-9 is a repressor, a conditional expression allele of AP2IX-9 was engineered in the developmentally competent Prugniaud strain (Pru) by creating a fusion of the FKBP destabilization domain (DD) (24) with the N terminus of AP2IX-9 (DDΔAP2IX-9; see design SI Appendix, Fig. S4A). In this ectopic expression design, DDΔAP2IX-9 transcription was controlled by the constitutive α-tubulin promoter and, as expected, steady-state levels of DDΔAP2IX-9 mRNA were independent of Shield-1 addition or the media pH (SI Appendix, Fig. S4A, mRNA). Importantly, regulation of the DDΔAP2IX-9 fusion protein was achieved by stabilization of the DD domain by using the small molecule Shield-1 (SI Appendix, Fig. S4A and B).

Endogenous posttranscriptional mechanisms also contributed to DDΔAP2IX-9 protein levels with the highest expression resulting from the combination of pH 8.2 media plus Shield-1, whereas in tachyzoites (pH 7.0 media), DDΔAP2IX-9 expression was lower because of native degradation that could be partially overcome with Shield-1 (SI Appendix, Fig. S4A and B).

Using this conditional model, we explored how extended stabilization of DDΔAP2IX-9 under alkaline conditions influenced tissue cyst formation. In the Pru-parental strain, tissue cyst formation in pH 8.2 media was robust and independent of Shield-1 (>75% cysts formed by 72 h; SI Appendix, Fig. S4C). By contrast, Pru-DDΔAP2IX-9 parasites pretreated with Shield-1 (250 nM Shield-1, 6 h before alkaline shift) produced half the number of tissue cysts (32%) as parent controls (75%) (SI Appendix, Fig. S4C). The addition of Shield-1 at the time of pH 8.2 shift inhibited cyst numbers in Pru-DDΔAP2IX-9 parasites by 60%. To confirm AP2IX-9 inhibition of tissue cyst formation was not strain specific, we obtained a DDΔAP2IX-9-expressing clone in a low-passage type III CTG strain (Fig. 3). CTG-DDΔAP2IX-9 parasites shifted into pH 8.2 media plus Shield-1 were severely restricted in tissue cyst formation (12% tissue cysts; Fig. 3A), and this inhibition was substantially reversed if DDΔAP2IX-9 levels were lowered by omitting Shield-1. Thus, AP2IX-9 acts to repress bradyzoite differentiation in developmentally competent type II and III strains.

It is generally accepted that bradyzoite differentiation requires a slowing of parasite growth (25) and, ultimately, this rate change leads to growth arrest in the tissue cyst as mature bradyzoites with nearly 100% haploid DNA content (7). The tight association between bradyzoite development and the parasite cell cycle led us
to explore the growth rate of parental CTG and CTG-DDAP2IX-9 parasites. Parental CTG showed the expected slowing of growth in pH 8.2 media, reaching an average vacuole size of ~22 in 96 h (Fig. 3B), and these populations fully growth arrested before lysing the host cell monolayer. By contrast, CTG-DDAP2IX-9 parasites grew faster in pH 8.2 media than parental controls and, unlike parental CTG cultures, CTG-DDAP2IX-9-infected monolayers were lysed by 96 h (Fig. 3B, arrows). Resistance to the growth inhibition of alkaline media was independent of Shield-1 in CTG-DDAP2IX-9 parasites. The low levels of DDAP2IX-9 in pH 8.2 media (Fig. 3B, Inset) appeared to be sufficient to confer pH-resistant growth on CTG-DDAP2IX-9 parasites, and they also prevented a full reversal of the AP2IX-9 block to tissue cyst formation (Fig. 3A, pH 8.2 minus Shield-1 = 55% cysts compared with >75% cysts in the induced CTG parent). These results suggest AP2IX-9 may act to forestall the growth changes that ultimately lead to the dormant bradyzoite in mature tissue cysts (6, 7). In such a growth promoting role, deletion of AP2IX-9 could cause developmentally competent strains to readily leave the cell cycle, perhaps explaining why knockouts of APIX-9 were not recovered in low-passage strains. To support this idea, we easily disrupted the AP2IX-9 locus in CTG-DDAP2IX-9 parasites (17% of stable transgenics were knockouts), whereas repeated knockout attempts had failed in the developmentally competent CTG parent strain. The phenotype of CTGΔap2IX-9-DDAP2IX-9 parasites with respect to tissue cyst formation was unchanged from the CTG-DDAP2IX-9 transgenic strains, indicating the DDAP2IX-9 fusion protein had fully replaced the function of the native protein. [Tissue cyst results: CTG-Δap2IX-9-DDAP2IX-9 in pH 8.2 minus Shield-1 produced 74% DBA-positive vacuoles, whereas DBA-positive vacuoles were counted in only 28% of cultures where Shield-1 was added.]

**AP2IX-9 Represses Major Elements of the Bradyzoite Transcriptome.** To identify the genes potentially controlled by AP2IX-9, duplicate total RNA samples from parental CTG and CTG-DDAP2IX-9 parasites induced by pH 8.2 media plus Shield-1 were isolated and analyzed on a custom Toxoplasma Affymetrix GeneChip (26). A total of 116 mRNAs were altered (5% FDR) in this comparison (full gene lists in Dataset S1) with the largest change shown) in CTG-DDAP2IX-9 parasites compared with CTG parental controls under pH 8.2 media plus Shield-1 induction conditions. BAG1 mRNA was the most repressed along with other known bradyzoite mRNAs: LDH2, #4 mRNA; ENO1, #5; SAG2D, #8; SAG4.2, #23; BK1, #24; B-NTPase, #29 (see Dataset S1 for full gene list). Colored boxes indicate the number of AP2IX-9 motifs present in each promoter (2 kb upstream of ATG start). (B) DDAP2IX-9 expression represses induction of a firefly luciferase transgene controlled by the BAG1 promoter (~1,195-bp region) (18). Luciferase activity in whole-cell lysates from PruIC2 parent and PruIC2-DDAP2IX-9 parasites was determined at 72 h after shift to pH 8.2 media ± 200 nM Shield-1 and compared with the appropriate pH 7.0 controls (data presented as the ratio pH 8.2/7.0, see SI Appendix, Methods for average light unit values). Gray bars, PruIC2-DDAP2IX-9 parasites; black bars, PruIC2 parent (**P < 0.001) (C) An EMSA was performed with 0, 70, 200, and 600 ng (lanes 1–4) purified GST-IX-9 and 20 fmol of 59-5'biotin-labeled probe containing the BAG1 CRE sequence (5'-TACTGG-3'3'/ATGACC-5') (18). A large complex (arrow) above the two probe-associated bands was competed with an unlabeled probe (300x, lane 5). See Dataset SI for all oligonucleotide sequences. (D) DDAP2IX-9 occupies the BAG1 promoter in parasite chromatin. Specific binding was observed in all six target regions (1–6) tiled across the BAG1 promoter (~950 bp flanking the ATG indicated by arrow in diagram) with the central three regions showing the highest enrichment (see SI Appendix, Fig. S5 for full experimental details). Ten repeat motifs matching 5 of 6 bases in the AP2IX-9 DNA binding motif are indicated by black triangles above the diagram, whereas the BAG1 promoter CRE previously mapped by luciferase assay lies within region 4 and is marked by a triangle below the diagram (18).

**EMSAs and chromatin immunoprecipitation followed by quantitative (qPCR) of DDAP2IX-9—bound genomic DNA showed specific enrichment across the BAG1 promoter (Fig. 4D and SI Appendix, Fig. S5B).** Altogether, these results support the view that ApiAP2 factors may have greater flexibility in DNA binding than plant AP2 factors (9, 11). AP2IX-9 was able to bind the B-NTPase functional CRE (Fig. 2) through the GT/CA repeat motif (APIX-9 binding motif) (18). Second, the direct regulation of BAG1 by AP2IX-9 is supported by repression of luciferase activity in a transgenic clone where the BAG1 promoter controls the firefly luciferase gene (PruIC2; Fig. 4B) (18). As expected, the introduction and stabilization of DDAP2IX-9 expression by Shield-1 in the PruIC2 strain strongly inhibited luciferase induction under alkaline stress (Fig. 4B), and this repression was partially reversed by omitting Shield-1 (Fig. 4B). Finally, the direct interaction of AP2IX-9 with the BAG1 promoter was supported by GST-IX-9 binding to the BAG1 CRE (18) in the introduction and stabilization of DDAP2IX-9 expression by Shield-1 in the PruIC2 strain strongly inhibited luciferase induction under alkaline stress (Fig. 4B), and this repression was partially reversed by omitting Shield-1 (Fig. 4B). Finally, the direct interaction of AP2IX-9 with the BAG1 promoter was supported by GST-IX-9 binding to the BAG1 CRE (18) in
bradyzoite transcriptome. We demonstrate AP2IX-9 is a nuclear protein induced in response to alkaline stress in the early bradyzoite, whereas it is minimally expressed in tachyzoites and end-stage bradyzoites in murine brain tissue cysts. AP2IX-9 binds DNA in a sequence-specific manner, and the AP2IX-9 binding motif is repeated in many bradyzoite promoters where direct binding of this factor to the BAG1 promoter in chromatin was established. ApiAP2 factors have roles in activating developmental gene expression (13–16), and there is evidence an ApiAP2 factor may assist the silencing of var gene expression in P. falciparum (17). The AP2IX-9 factor is the first ApiAP2 transcriptional repressor to be characterized in the Apicomplexa, and this mechanism begins to explain how the early steps in tachyzoite to bradyzoite development are regulated in Toxoplasma. The extent of transcriptional repressors among the ApiAP2 family is unknown; however, this type of mechanism is commonly associated with related AP2 factors of plants. Multiple AP2 family members in plants are repressors of stage-specific gene regulation, especially in flowering (27) and stress responses (28).

In its natural life cycle, Toxoplasma is a heteroxenous parasite. Its sexual reproductive cycle is exclusive in the feline host with a second clonal reproductive phase that is possible in virtually any endothermic animal. The traditional developmental course widely assumed to unfold in the intermediate host involves three sequential stage switches (e.g., sporozoite to tachyzoite to bradyzoite), although this scheme is likely oversimplified (see model in Fig. 5). The development and cell cycle transitions of the intermediate life cycle are accessible in host cells infected with sporozoites or bradyzoites (6, 7), where these end stages first differentiate into the tachyzoite stage (<2 d) followed by limited replication of the emergent tachyzoite (Fig. 5, day 3–6). Spontaneously switching to a longer division cycle (day 7) signals formation of a prebradyzoite, which is primed to develop into the mature bradyzoite (equivalent to D3-6; Fig. 5) (37), and supporting this finding is the pattern of AP2IX-9 expression in these strains (25), enrichment for S and mitotic parasites characterizes the course of bradyzoite development (7), and peak expression of bradyzoite transcripts coincides with the S/M periods of the tachyzoite cell cycle (8). Thus, replicating forms in Toxoplasma can be envisioned as reprogramming states required for changing one transmissible end stage (G0/G1 phase) into another to ensure survival in multiple hosts (e.g., sporozoite switch to bradyzoite). A precise relationship between chromosome replication and transcriptome remodeling is not established; however, a surprisingly short timeframe (1–4 d) produces infectious tissue cysts following bradyzoite inoculation of mice (32).

The prebradyzoite is an important, if also a poorly understood transition of the intermediate life cycle. Low passage strains readily form this developmental stage that is characterized by a spontaneous switch to slower growth, while retaining tachyzoite proteins on the parasite surface (7). Here, the activity of AP2IX-9 provides new insight into the molecular mechanisms regulating Toxoplasma development. We propose that the prebradyzoite stage produced by native parasite proliferation is held in the poised state by ApiAP2 factors like AP2IX-9 (see model in Fig. 5). As a transcriptional repressor, the role for AP2IX-9 could be to prevent premature commitment of the prebradyzoite in a less optimal host tissue or balance the parasite response to different immune system reactions. There is little evidence for quorum sensing mechanisms in apicomplexa infections, although if they occur, the AP2IX-9 mechanism could play a role here as well. Importantly, AP2IX-9 expression may be the first marker for this specific developmental transition. In providing a transient adaptive response, the AP2IX-9 is similar to the AP2 factor, abscisic acid-insensitive 4 (ABI4) of plants that is induced by stress (high sugar) where it reduces critical gene expression to protect these sensitive plants during early stages of development (35). In later stages of seedling development, ABI4 does not appear to be required and is no longer induced by stress (36) as much as AP2IX-9 is dispensable for the mature bradyzoite. Dividing tachyzoites appear to actively degrade AP2IX-9, which could reflect a protective response, or, alternatively, stabilization of AP2IX-9 may be tied to mechanisms active only during the prebradyzoite developmental transition. The latter explanation has some support because conditional overexpression of AP2IX-9 in the Shield-1 model was tolerated by tachyzoites (Fig. 3 and SI Appendix, Fig. S4A), and induction of this protein is tightly associated with strain developmental competency. In Prugniaud and CTG strains, AP2IX-9 is inducible by stress, whereas in the developmentally resistant RH strain, AP2IX-9 does not accumulate under any pH condition although the encoded mRNA is inducible. Based on transcriptome profile, RH strain is trapped upstream of the prebradyzoite (equivalent to D3-6; Fig. 5) (37), and supporting this assignment, the chromatin signature of bradyzoite promoters in RH indicates they are transcriptionally inactive (18). Therefore, we interpret the difference in AP2IX-9 expression in these strains to indicate that when the AP2IX-9 repressor complex is bound to chromatin it is stabilized and, therefore, the native transient pattern of AP2IX-9 expression is strongly influenced by histone modification at specific promoters. The changing epigenetic landscape during bradyzoite differentiation may also explain the decline of AP2IX-9 as development progresses to the mature tissue cyst. Here, other ApiAP2 factors likely replace AP2IX-9 to activate the bradyzoite transcriptome such as the newly described bradyzoite activator AP2XI-4 that binds GT/CA DNA repeats (14). We suspect the principle of ApiAP2 posttranscriptional control orchestrated by dynamic chromatin states will apply to many other family members including those in the large group of cycling ApiAP2 factors that act in the tachyzoite cell cycle (8). In conclusion, evidence presented here indicates AP2IX-9 is a major regulator of the intermediate life cycle of Toxoplasma where it may help control the balance between parasite expansion and transmission and, through the prevention of premature commitment.

Fig. 5. Model for control of bradyzoite development by the AP2IX-9 transcriptional repressor. Four major transitions are thought to occur in the intermediate life cycle: following infections with sporozoites (or bradyzoites) parasites rapidly differentiate into replicating tachyzoites (A), limited tachyzoite proliferation leads to a slower growing prebradyzoite state (B), and (C) the timing of commitment to the dormant bradyzoite in the mature tissue cyst is asynchronous (C and D*). AP2IX-9 balances the competing interests of parasite expansion and efficient transmission through direct inhibition of commitment to the mature bradyzoite. This model is based on studies here and published results with the VEG strain (6, 7) that has been maintained in the Jitender P. Dubey laboratory (US Department of Agriculture, Beltsville, MD) by exclusive mouse-cat serial passage to preserve the native Toxoplasma developmental pathway (32).
to the bradyzoite end-stage, could help steer tissue cyst formation to a favorable host cell environment.

Materials and Methods

Information about parental and transgenic strains; culture conditions; plasmid construction; and more detailed descriptions of the assays used for IFA, Western blot analysis, and EMSAs, and luciferase activity, can be found in SI Appendix. T. gondii genomic sequences were accessed via http://ToxoDB.org.

Development and Conditional Expression Models. To induce bradyzoite differentiation in vitro, infected cultures were subjected to alkaline media (pH 8.2) for indicated time periods according to ref. 22 with media replaced every 24 h to maintain pH. Conditional expression of the AP2IX-9 protein fused to HA epitope tag was performed by published methods (39) with the modifications fully described in SI Appendix.

Protein and RNA Microarray Analysis. The AP2IX-9 DNA binding domain (amino acids 1142–1204) was cloned in pGE4X4T, expressed as a GST-fusion in BL21DE3 cells (Novagen), and purified by glutathione affinity column. Protein binding microarrays were performed as described (12). All RNA samples were collected as independent biological replicates. RNA quality was determined by using the Agilent Bioanalyzer 2100. Total parasite RNA was collected and prepared for hybridization on the ToxoGeneChip as described in ref. B. Data were analyzed in Genespring GX software (version 11.5, Agilent) and in Bioconductor (38). Microarray data are available in the Gene Expression Omnibus repository under accession no. GSE16037.

Chromatin Immunoprecipitation and qPCR. Parasites were inoculated at 3:1 multiplicity of infection in T715cm² flask, allowed to invade for 3 h, rinsed three times with HBSS to remove free floating parasites, and media was replaced with pH 8.2 media supplemented with 200 nM Shield-1 (Clontech). ChIP-qPCR was performed by published methods (39) with the modifications fully described in SI Appendix.

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**Supplement Information (SI) Appendix**

**Material and Methods:**

**Cell culture and transgenic strains.** Parasite cultures were maintained in primary human foreskin fibroblasts (HFF) as previously described (1). To determine growth rates for CTG and CTG-\(^{DD}\)AP2IX-9 parasites, HFF monolayers were inoculated at a 4:1 MOI, the parasites allowed to invade for 3 h, and then shifted to pH 8.2 media plus or minus 250nM Shield-1. The parasite population in each flask was monitored in real time every 24 h for 4 days with 50 randomly selected vacuoles counted in triplicate at each time point and the values averaged.

A summary of the parent strains used and transgenic clones produced for this study is provided in Dataset S1 along with the oligonucleotide primers used for cloning. **Genomic locus epitope fusions with hemagglutinin (HA) tags.** *Toxoplasma* ApiAP2 factors, AP2IX-9 (TGME49_306620) and AP2VI-1 (TGME49_240460), were tagged at the endogenous locus with a triple copy of the HA epitope in the Type II Prugniaud strain lacking the *KU80* gene (designated in this study as the PruQ strain) by genetic knock-in using the pLIC-HA3x plasmid as described in (2). **Conditional expression model.** For conditional expression, the AP2IX-9 single exon coding region was PCR amplified from genomic DNA to incorporate in frame Mfe1/Sbf1 sites, which were used to clone the purified PCR fragment into the pCTDDHA3x plasmid provided by Dr. Striepen, University of Georgia (3). This cloning results in the fusion of the FKBP peptide (11.2 kDa) and 3 copies of the HA epitope for detection (4.4 kDa) in frame with the N-terminal end of AP2IX-9 protein with a final fusion protein mass of ~128kDa (designated \(^{DD}\)AP2IX-9). The plasmid pCTDDHA3x-AP2IX-9 was introduced by electroporation into the Prugniaud strain (wild type for *KU80*), and also into a Prugniaud transgenic clone, PruIC2, expressing firefly luciferase under the BAG1 promoter (see details of the PruIC2 strain in Appendix ref 4). Transgenic parasites were selected using chloramphenicol (20µM) and clones were isolated by limiting dilution. To produce Type III CTG parasites expressing the conditional DDHA3x-AP2IX-9 allele (\(^{DD}\)AP2IX-9), the pCTDDHA3x-AP2IX-9 plasmid was digested with SpeI/NotI to remove the sagCAT selectable marker and this was switched with the DHFR-TS selectable marker from the pT7S4HA plasmid. The resulting pDHFR/DDHA3x-AP2IX-9 plasmid was introduced by electroporation into low passage CTG strain parasites, selected with pyrimethamine (1µM) and resistant clones isolated by limited dilution. **AP2IX-9 gene knockouts.** Transgenic parasites carrying a deletion of the AP2IX-9 gene were obtained in the PLK, CTG-\(^{DD}\)AP2IX-9 transgenic, and the RH\(\Delta hxgprt\Delta uprt\) strains as follows. To generate the PLK-\(\Delta ap2ix-9\) transgenic strain the pTCY plasmid (a kind gift from Dr. Striepen, University of Georgia) was first modified to expand the multiple cloning sites surrounding the sagCAT selectable marker (5’ KpnI-Apal-Xhol-Sall-ClaI-HindIII--sagCAT--3’AatII-Smal-AvrII-Nhel-BglII-SpeI) to generate the pTCY-exMCS vector. The plasmid pTCY-AP2IX-9KO was then constructed in this new vector by cloning the 5’ and 3’ flanking regions of the single AP2IX-9 coding exon. A 3,067bp PCR product (primer sequences are listed in Dataset S1) amplified from Prugniaud Type II strain genomic DNA supplied the left arm using KpnI/HindIII to clone and a 1960bp PCR fragment down stream of the single AP2IX-9 exon was cloned by AatII/SpeI sites in order to supply the right arm. The resulting pTCY-AP2IX-
9KO plasmid was electroporated into PLK strain, transgenic parasites selected using chloramphenicol (20µM), and cloned by limiting dilution. To produce CTG-Dap2IX-9-[DDAP2IX-9] parasites the sagCAT selectable marker in pTCY-AP2IX-9KO was swapped with a phleomycin (Ble) expression cassette using PCR using HindIII/AvrII sites to generate the plasmid pT(ble)Y-AP2IX-9KO. This Ble knockout plasmid was electroporated into CTG-DDAP2IX-9 parasites, subjected to two rounds of extracellular phleomycin (50µg/ml) selection and resistant parasites cloned by limiting dilution. Finally, to generate an AP2IX-9 knockout in the RHΔhxgprtΔuprt strain, cosmid TOXP727 containing the AP2IX-9 locus was modified by recombineering to delete the single AP2IX-9 exon as previously described (5). The resulting TOXP727-AP2IX-9KO cosmid was electroporated into RHΔhxgprtΔuprt strain, selected in chloramphenicol and resistant clones isolated by limiting dilution.

**Immunofluorescence assays (IFA).** Parasites were inoculated on confluent HFF coverslips for indicated time and IFA assays performed as previously described (6) using the following primary reagents: anti-HA antibody (Roche; rat mAb 3F10; 1:500), biotin-labeled *Dolichos biflorus* agglutinin (1:3000, Vector labs, CA), and DAPI (0.5mg/ml). All Alexa (Molecular Probes, CA) and streptavidin (Vector Labs, CA) conjugated secondary antibodies were used at 1:1000. Image acquisition was performed on a Zeiss Axiovert microscope equipped with 100x objective.

For IFA analysis of *in vivo* tissue cysts from murine brain, glass cover slips were treated with 1% polylysine for 20 min and then washed with PBS. Tissue cysts were placed on the cover slips and left at room temperature (RT) for 15 min followed by incubation with trypsin solution (4µl of a 1mg/ml solution, Sigma-Aldrich) for 4 min at RT. Cover slips were then fixed 4% paraformaldehyde for 30 min, washed with PBS and permeabilized with 0.2% Triton X-100 for 20 min. Lastly, cover slips were blocked with 3% BSA and 5% goat serum overnight at 4°C. IFA was preformed using the following antibodies at 37°C for 90 min: HA (Roche; rat mAb 3F10, 1:500) and articulin 4 (polyclonal mAb, Dr Louis Weiss, 1:500) and visualized using Alexa-conjugated secondary antibodies at 1:1000 (Molecular Probes).

**Production and purification of cysts:** Mice (Bab/cDM1) were infected by i.p. with 1x10^4 parasites and 2 days post-inoculation Sulfamerazine (30mg/l, Sigma-Aldrich) was added to the drinking water for the duration of the experiment. Four weeks after infection, mice were sacrificed and the cysts were purified from brain tissues as follows: Five mouse brains were washed with 10 ml PBS, placed in 10 ml 0.9% NaCl and homogenized with a potter glass homogenizer. The homogenized tissue was layered on top of 25 ml Percoll buffer (45 ml Percoll solution and 55 ml PBS ) in a 50 ml centrifugation tube and separated by centrifugation (20 min at 1,500 rpm, 4°C). After centrifugation, 20 ml of the middle layer solution containing the cysts was transferred to a new 50 ml tube. Cysts were washed with 30 ml of PBS by centrifugation (10 min, 750 rpm, 4°C). The resulting pellet was resuspended in 1 ml PBS and cyst number quantified by light microscopy.

**Western analysis.** Freshly needle passed and filtered parasites were collected by centrifugation and total lysates made by boiling for 10 min at 95°C in Laemmli buffer (6). Each lane on a SDS-PAGE gel was loaded with 25x10^6 parasites, separated by electrophoresis and transferred to a nitrocellulose membrane.
The blots were probed with anti-HA (Roche, 3F10, 1:500) for AP2IX-9HA or DDAP2IX-9 stripped with Ponceau S (0.1%) and re-probed with anti-TgPCNA1 (1:1000)(7) to monitor nuclear TgPCNA1 as a loading control. Proteins were detected with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, PA) and visualized using enhanced chemiluminescence reaction.

**Electrophoretic mobility shift assays (EMSA).** Complementary single stranded DNA oligonucleotides that included a 5’-biotinylated nucleotide were annealed to produce DNA probes from 35-65bp in length. Standard binding reactions were carried out at RT for 20 min using 20fmol of biotinylated DNA probe and varying concentrations of GST-AP2IX-9 protein (70-900ng) according to manufacturer’s protocol (Pierce, IL). Non-biotinylated competitor DNA probes were added at 100-300x concentrations as indicated. Complexes were resolved on 6% PAGE gel, transferred to nylon membrane and detected according to manufacturer’s protocol (Pierce, IL). All EMSA oligonucleotides are listed in Dataset S1.

**Luciferase assays.** Luciferase assays were performed according to manufacturers protocols (Promega, Madison, WI) with modifications as previously described (4). Briefly, HFF cells in T25cm² flasks were inoculated with PruI2 parent or PruI2-DDAP2IX-9 transgenic parasites at 3:1 MOI, parasites were allowed to invade for 3 h, and the culture media changed to either pH 8.2 or 7.0 media plus or minus 200nM Shield-1 (Clontech). The alkaline-shifted cultures were grown in non-CO₂ conditions for 72 h and then harvested for whole cell lysates according to manufacturers protocols. Each experimental condition was assessed in three independent cultures with average statistical significance for parental PruI2 compared to the PruI2-DDAP2IX-9 transgenic strain determined by the unpaired t-test. Average luminometer values for the results shown in Figure 4B: PruI2 parent grown as tachyzoites=674 light units; pH 8.2 media alone=284,476 units; pH 8.2 plus Shield-1=327,870 units. PruI2-DDAP2IX-9 parasites grown as tachyzoites=216 units; in pH 8.2 media alone=56,574 units; in pH 8.2 media plus Shield-1=11,839.

**Chromatin immunoprecipitation and quantitative PCR.** Chromatin-immunoprecipitation (ChIP) was performed by published methods (8) with the following modifications. Following intracellular formaldehyde crosslinking and purification of the nuclear fraction, lysed nuclear material was subjected to sonication (Misonix S-4000, cuphorn probe, 30min at 80% amplitude, 30sec ON/OFF pulse) to produce 200-1000bp fragments. The soluble fraction was then isolated by centrifugation at 4°C and pre-cleared with protein-G dynabeads (Dynabeads, Invitrogen) before overnight incubation with rabbit anti-HA antibody (5µg, ab9110; Abcam). Anti-HA-DNA complexes were purified using protein-G coupled magnetic beads (Dynabeads, Invitrogen) for 1 h at 4°C, subjected to salt and lithium chloride washes and incubated overnight at 65°C in 1%SDS/TE to reverse cross-links. Samples were treated with RNase cocktail (Ambion) prior to standard phenol-chloroform extraction and DNA precipitation with ethanol. Whole genome amplification (Sigma-Aldrich) was performed on ChIP-DNA and purified using Qiagen Mini-Elute PCR Kit. Quantitative PCR (qPCR) was performed on amplified ChIP-DNA (20ng/rxn) using Fast SYBR® green master mix on an ABI 7900 according to manufacturers protocols. All qPCR DNA probes were designed using Primer3 software (9) and amplified 75-364bp fragments. The primer sequences can be found in Dataset S1. Here, ChIP was performed DDAP2IX-9 (specific chromatin) or parent strains (non-specific chromatin). Relative enrichment
values were determined using the equation $2^{\Delta \Delta Ct \text{ target} - \Delta \Delta Ct \text{ non-target}}$ where the change in Ct value of specific versus nonspecific chromatin at target and non-target loci was calculated.

**SI Appendix reference list.**

Figure S1. The expression of 24 ApiAP2 mRNAs regulated (up and down) during *in vitro* tachyzoite to bradyzoite development.

(A) Heatmap showing mRNA levels for ApiAP2 mRNAs that have >2 fold change in alkaline media (pH 8.2) and/or compound 1 (3 µM) bradyzoite induction conditions in at least one of three major genetic lineages (Type I=GT1, Type II=ME49, Type III=CTG; for microarray dataset see ref 4). Relative mRNA levels obtained from bradyzoite-induced cultures compared to the mRNA level in the matching tachyzoite strain are shown. Fold change color scale is indicated: green=downregulated; red=upregulated; black=no mRNA change compared to tachyzoites. Fourteen of these AP2s were previously identified as periodic in the tachyzoite cell cycle (10). Note that 13 of 14 cell cycle AP2s with modulated expression (up or down) during bradyzoite differentiation had maximum mRNA levels in late S to mitotic periods of tachyzoite replication (cell cycle peak times indicated on left, see also ref 10). The predicted protein sizes with the position of the ApiAP2 domain(s) indicated by black boxes for all included proteins are indicated (11).

(B) mRNA panel: AP2IX-9\(^{\text{HA}}\) mRNA could be detected in alkaline-induced PruQ-AP2IX-9\(^{\text{HA}}\) clones (48 h in pH 8.2 media). AP2IX-9 primers are designed from a single exon, while GAPDH mRNA primers span a 432bp intron (TGME49_089690) (See Dataset S1 for primer designs). PCR fragments generated for GAPDH were included as loading and template quality controls. The presence of a larger GAPDH PCR fragment would indicate possible genomic DNA contamination (g lane indicates the genomic DNA fragment size), as the smaller GAPDH fragment is produced only from mRNA sequence. The absence of bands in the no reverse transcriptase controls (lane -) also indicate the RNA templates were DNA-free. Protein panel: AP2IX-9\(^{\text{HA}}\) tagged protein (endogenous promoter control) was highly expressed only in alkaline-stressed PruQ-AP2IX-9\(^{\text{HA}}\) transgenic parasites (lane Bz=72h post-infection in pH 8.2 media) and not in PruQ-AP2IX-9\(^{\text{HA}}\) tachyzoites (lane Tz=24 h post-infection in pH 7.0 media). Staining for *Toxoplasma* nuclear protein TgPCNA1 is included as a loading control. Molecular mass standards (kDa) are indicated to the right. Note AP2IX-9\(^{\text{HA}}\) is a larger mass than currently annotated (ToxoDB.org; 112 kDa prediction plus 4.4kDa 3xHA tag) due to mis-prediction of an intron, which was confirmed by sequencing of AP2IX-9 cDNA fragments (not shown).

(C) PruQ transgenic clones epitope tagged with 3xHA by genetic knock-in at the endogenous AP2IX-9 or the AP2VI-1 locus were used to individually infect mice. At four weeks post-infection murine brain cysts were purified for immunofluorescent analysis of expression (see SI Appendix Methods for full details). No expression of AP2IX-9\(^{\text{HA}}\) was observed in any tissue cyst, while all cysts stained positive for AP2VI-1\(^{\text{HA}}\). Antibodies against mouse α-articulin 4 included here allow visualization of the parasite tissue cyst and was used as a co-stain in AP2IX-9\(^{\text{HA}}\) IFAs.
Figure S2. AP2IX-9 binds to DNA with sequence specificity.

(A) Sequence logo representation of the position weight matrices generated by the Seed and Wobble algorithm from probing an Agilent DNA microarray with purified GST-IX-9 protein (see SI Appendix Methods). The three related DNA motifs identified a 6 bp consensus sequence 5’-CAGTGT-3’ (3’-GTCACA-5’ opposite strand sequence). (B) An electrophoretic mobility shift assay was performed with 0, 0.5 and 2.5 µg of purified GST-IX-9 protein and 20 fmol of 59bp 5’-biotin-labeled probe containing the CAGTGT motif or a scrambled probe (Scram; see Dataset S1 for all sequence designs). The arrow denotes a single band with slower electrophoretic migration only observed when GST-IX-9 is incubated with the CAGTGT but not the scrambled probe.
Figure S3: Targeted disruption of the AP2IX-9 gene in laboratory strains increases tissue cyst formation in alkaline media.

*Dolichos biflorus* agglutinin (DBA) positive vacuoles in RHΔhxgrtΔuprt,Δap2IX-9 and PLKΔap2IX-9 transgenic parasites after *in vitro* bradyzoite induction were counted in triplicate. Tissue cyst number was determined after 4 days of CO2 starvation for the RHΔhxgrtΔuprt strain background or 3 days in alkaline media (pH 8.2) media for the PLK strains. Results reflect the average of two independent clones in each knockout strain. Tissue cyst counts for the parent strains RHΔhxgrtΔuprt (12) and PLK (13) are included. The legend indicates strain genetic background; RHΔhxgrtΔuprt=grey bars, PLK=black bars. Statistical significance compared to parental strain was determined using the unpaired two-tailed Student’s t-test (***: p-value =0.006).
Figure S4. The development of a conditional expression model for AP2IX-9 in the Prugniaud strain.

(A) Schematic of the AP2IX-9 conditional expression design. The DDHA3x sequence was N-terminally fused to the single coding exon of AP2IX-9 (designated \( \text{DD}\text{AP2IX-9} \)). The construct was driven by the Toxoplasma \( \alpha \)-tubulin promoter and terminated with the DHFR-TS 3'-untranslated region. The \( \text{DD}\text{AP2IX-9} \) expression construct was transfected into the Prugniaud strain (Pru; wild type for the \( KU80 \) gene) and stable clones obtained under chloramphenicol selection in the absence of Shield-1. (mRNA panel) Total RNA purified from Pru-\( \text{DD}\text{AP2IX-9} \) parasites was reverse transcribed and relative levels of mRNA compared following limited PCR with primers specific for the \( \text{DD}\text{AP2IX-9} \) transgene. Expression was assessed in parasites cultured under tachyzoite (pH 7.0 for 24 h) and alkaline induction conditions (pH 8.2 for 48 h) with or without 100nM of Shield-1. Constitutive GAPDH mRNA was included as a control and was evaluated as in Figure S1. (protein panel) Following a limited invasion (3 h), Pru-\( \text{DD}\text{AP2IX-9} \) parasites were shifted into alkaline media (pH 8.2 lanes) for 72h plus or minus 250nM Shield-1 and compared to parasites grown in standard pH 7.0 media (24 h growth +/- Shield-1). Note that \( \text{DD}\text{AP2IX-9} \) expression was detected in parasites grown in either media condition when Shield-1 was added especially in pH 8.2 media, which combines natural and Shield-1 influences leading to sustained high levels of \( \text{DD}\text{AP2IX-9} \). Each gel lane was loaded with 25x10^6 purified parasites lysed in SDS-PAGE buffer. Following transfer to nitrocellulose the membrane was probed with anti-HA antibody, stripped and re-probed with a rabbit anti-PCNA1 antibody as a loading control. (B) Pru-\( \text{DD}\text{AP2IX-9} \) parasites were cultured in tachyzoite (pH 7.0 media for 24 h) or bradyzoite-induction conditions (pH 8.2 for 72 h) plus or minus Shield-1 (0, 100nM, 500nM) and then examined by immunofluorescence for \( \text{DD}\text{AP2IX-9} \) expression (red). Red stain=anti-HA antibody and blue=DAPI staining. DIC images are shown for reference with scale bar=5 \( \mu \)m. (C) Expression of
DDAP2IX-9 reduces the occurrence of tissue cysts in vitro. HFF cells were grown on glass coverslips and parasites allowed to invade for 3 h (1:1 MOI). Pru-DDAP2IX-9 parasites were pretreated for 6 h with 250 nM Shield-1 prior to shifting the cultures into pH 8.2 media with (black bars) or without (grey bars) 250nM Shield-1 for 72h. Induction of tissue cysts by pH 8.2 media in the Pru-parental strain was robust and unaffected by Shield-1 addition. All experimental conditions were run in triplicate with 50 vacuoles/experiment counted and scored for a full DBA staining. The average results from two independent transgenic clones (A6 and B4) are shown. A significant 2-fold difference in tissue cysts number was observed between the Pru-parent and Pru-DDAP2IX-9 parasites (Student's two-tailed t-test, *** p-val <0.001).

A. RT-PCR of BAG1, LDH2 and GAPDH mRNAs

<table>
<thead>
<tr>
<th>pH 8.2 media</th>
<th>- Shield-1 + Shield-1</th>
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<tbody>
<tr>
<td>S1 S2 S1 S2 Tz g</td>
<td>BAG1</td>
</tr>
<tr>
<td>CTG parent</td>
<td>LDH2</td>
</tr>
<tr>
<td>Tz=tachyzoite RNA sample</td>
<td>GAPDH</td>
</tr>
<tr>
<td>S1=first RNA sample</td>
<td>S2= second RNA sample</td>
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B. AP2IX-9 ChIP-qPCR; enrichment of BAG1 promoter DNA compared to three off target sites
Figure S5. AP2IX-9 overexpression inhibits bradyzoite gene expression through direct promoter interaction.

(A) To validate the microarray results in Figure 4A, duplicate total RNA samples (S1 and S2) from the CTG or CTG-DDAP2IX-9 parasites were purified and analyzed for BAG1 and LDH2 mRNAs as well as control GAPDH mRNA by semi quantitative RT-PCR. Following stimulation with pH 8.2 media (plus or minus Shield-1) BAG1 and LDH2 mRNAs were detected in CTG parent RNA, but not from CTG-DDAP2IX-9 RNA. Consistent with induced expression only during bradyzoite differentiation, BAG1 and LDH2 mRNAs were also not detected in tachyzoite RNA samples (Tz), while the constitutively expression GAPDH mRNA was detected in all RNA samples. The lack of BAG1 and LDH2 mRNA expression detected in CTG-DDAP2IX-9 parasites by the RT-PCR here is comparable to the low RMA values observed for the CTG-DDAP2IX-9 samples in the microarray analysis (see Dataset S1 for RMA values). Generally, fold change determined from microarray analysis when RMA values fall near or below the fluorescent background of the GeneChip (50-100 RMA) poorly estimate the actual mRNA change as occurs for the repression of the BAG1 and LDH2 mRNAs in these experiments. A genomic DNA reference (g) was included here as control. Note that intron containing fragments are larger for GAPDH and BAG1, while LDH2 primers did not span an intron (see Dataset S1 for primer designs). The lower diffuse bands in the DDAP2IX-9 transgenic PCR reactions are primer-dimers that form in the absence of real product. Shield-1 (200nM) additions are indicated above each lane. (B) DDAP2IX-9 is enriched at the BAG1 promoter. Chromatin immunoprecipitation was performed using anti-HA antibodies on DDAP2IX-9 (specific chromatin) and parent strains (non-specific chromatin). Equal amounts of specific or nonspecific chromatin were subjected to quantitative-PCR at six target regions (1-6) tiled across the BAG1 promoter and compared to three non-target loci within the genome (Non-target 1=TgME49_chrVI, 1,281,571-1,281,849bp, non-target 2=TgME49_chrIa: 31,895-32,259bp, non-target 3=TgME49_chrVI: 1,595,703-1,596,052bp). Relative enrichment values for each BAG1 promoter region was determined as the change in Ct value of specific versus nonspecific chromatin at target and non-target loci, using the equation $2^{\Delta\Delta Ct}$ for each individual non-target region and all showed similar DNA enrichment patterns. The average of BAG1 enrichment against all three non-target chromosome sites is presented in Figure 4D. The diagram below shows the arrangement of the six BAG1 promoter regions (-950bp to translation start indicated by arrow) with triangles above showing placement of AP2IX-9 motifs in the promoter. The results of published promoter mutagenesis that localizes the BAG1 CRE to region 4 and the specific mutations in the BAG1 promoter with reference to the results of luciferase reporter activity (*in vivo, promoter mutations are underlined)(4).