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MYST Family Histone Acetyltransferases in the Protozoan Parasite *Toxoplasma gondii*

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The restructuring of chromatin precedes tightly regulated events such as DNA transcription, replication, and repair. One type of chromatin remodeling involves the covalent modification of nucleosomes by histone acetyltransferase (HAT) complexes. The observation that apicidin exerts antiprotozoal activity by targeting a histone deacetyltransferase has prompted our search for more components of the histone modifying machinery in parasitic protozoa. We have previously identified GNAT family HATs in the opportunistic pathogen *Toxoplasma gondii* and now describe the first MYST (named for members MOZ, Ybf2/Sas3, Sas2, and Tip60) family HATs in apicomplexa (TgMYST-A and -B). The TgMYST-A genomic locus is singular and generates a ~3.5-kb transcript that can encode two proteins of 411 or 471 amino acids. TgMYST-B mRNA is ~7.0 kb and encodes a second MYST homologue. In addition to the canonical MYST HAT catalytic domain, both TgMYST-A and -B possess an atypical C2HC zinc finger and a chromodomain. Recombinant TgMYST-A exhibits a predilection to acetylate histone H4 in vitro at lysines 5, 8, 12, and 16. Antibody generated to TgMYST-A reveals that both the long and short (predominant) versions are present in the nucleus and are also plentiful in the cytoplasm. Moreover, both TgMYST-A forms are far more abundant in rapidly replicating parasites (tachyzoites) than encysted parasites (bradyzoites). A bioinformatics survey of the *Toxoplasma* genome reveals numerous homologues known to operate in native MYST complexes. The characterization of TgMYST HATs represents another important step toward understanding the regulation of gene expression in pathogenic protozoa and provides evolutionary insight into how these processes operate in eukaryotic cells in general.

The phylum Apicomplexa includes an assortment of parasitic protozoa responsible for significant medical and economic burdens. *Plasmodium* spp., the causative agents of malaria, kill ~1 million people a year in Africa, with children representing 75% of the fatalities (38). *Cryptosporidium parvum* has gained notoriety as a potential waterborne menace for which no treatment currently exists (17, 29). Major economic losses are associated with *Eimeria* spp., which cause intestinal coccidiosis in livestock (36). *Toxoplasma gondii* causes 400 to 4,000 cases of congenital toxoplasmosis each year in the United States alone (15) and is a life-threatening complication in immunocompromised (AIDS) and heart transplant patients (47, 48). Recent reports linking *Toxoplasma* to first-episode schizophrenia and cryptogenic epilepsy are drawing even more attention to the study of this parasite's pathology, fueling speculation that long-term effects of infection are currently underestimated (41, 52).

Critical to pathogenesis and transmission is the conversion of the acute form of *Toxoplasma* (tachyzoite) into an encysted form (bradyzoite). Neither the immune response nor our current arsenal of pharmacological agents can eradicate the cysts from the host. Moreover, the toxicity associated with the common therapy administered to fight *Toxoplasma* infection (pyrimethamine plus sulfonamides) underscores the urgency for novel drug target research and development. The discovery that the antiprotozoal agent apicidin targets a histone deacetyltransferase (10) suggests that the chromatin remodeling machinery may be a new source of targets, but very little is known about the regulation of gene expression in apicomplexan parasites.

Once thought to serve little more than a structural function, the primary constituents of chromatin are now considered to play key roles in the regulation of DNA transcription, replication, and repair (7). The histone proteins that form nucleosomal DNA are covalently modified to attenuate their interaction with DNA (49) or to generate epigenetic markers for gene expression (42). Histones are subject to an ever-increasing variety of posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitinylation, glycosylation, ADP ribosylation, and sumoylation (35).

A direct link between gene activation and histone acetylation was made by the discovery that the *Saccharomyces cerevisiae* transcriptional coactivator GCN5 was an enzyme capable of mediating this modification (6). Many other proteins possessing histone acetyltransferase (HAT) activity have been identified (40), falling into one of two superfamilies based on the architecture of the catalytic domain: GNAT, GCN5-related *N*-acetyltransferases (28), and MYST, named for the founding members MOZ, Ybf2/Sas3, Sas2, and Tip60 (4, 22, 31). In addition to the founding members, other MYST HATs include yeast ESA1 (37), human HBO1 (18) and MORF (8), mouse Querkopf (45), and *Drosophila* and human MOF (16, 27).

We have recently shown that acetylation of histones H3 and H4 accompanies stage-specific gene activation in *Toxoplasma* (33), emphasizing the importance of characterizing the enzyme complexes mediating these activities. GNAT family HATs that

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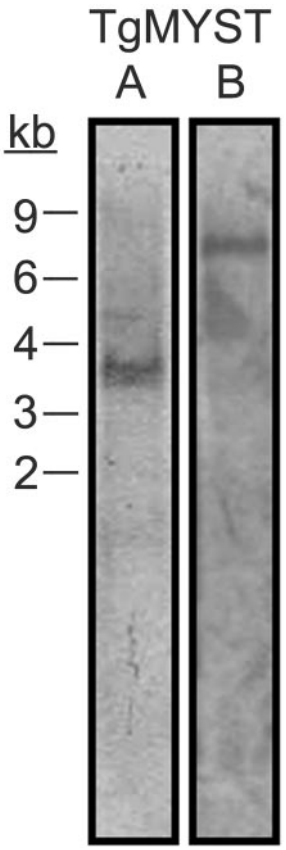

FIG. 1. Northern analysis of two independent *Toxoplasma* MYST HAT homologues. The mRNA from RH tachyzoites was purified, blotted, and probed with cDNA-derived fragments corresponding to the two putative TgMYST sequences (A and B). Sizes shown are in kilobases.

target H3 have been identified in apicomplexan parasites (14, 44). Now we report for the first time the discovery that MYST family HATs, which have a predilection to acetylate H4, also exist in apicomplexa. *Toxoplasma* contains two independent loci that encode MYST HATs (TgMYST-A and -B). Further characterization of TgMYST-A reveals that its transcript gives rise to a long and short version of the HAT protein, both of which are more abundant in the tachyzoite stage than in the bradyzoite stage. We expressed and purified TgMYST-A transiently in *Toxoplasma*, verified its preference for H4, and determined that it is capable of specifically acetylating lysines 5, 8, 12, and 16. Attempts to knock out or stably overexpress TgMYST-A have failed, suggesting that the expression levels of this HAT require precise regulation. This work represents another important step toward a better understanding of the mechanics of gene regulation in apicomplexa.

MATERIALS AND METHODS

Parasite culture and methods. *Toxoplasma* (RH and ME49 strains) was maintained in primary human foreskin fibroblast (HFF) cells as previously described (32). ME49 tachyzoites were induced to differentiate into bradyzoites in vitro by application of alkaline pH (8.1) medium for 3 days (39). Genomic DNA for Southern analysis was isolated from freshly lysed filter-purified RH tachyzoites using sodium dodecyl sulfate (SDS)-proteinase K lysis, phenol-chloroform extraction, and ethanol precipitation. Parasite mRNA was purified from freshly lysed filter-purified RH tachyzoites using the Poly(A)Pure system (Ambion), followed by treatment with DNase. Blotting and probe hybridizations conformed to conventional methods (34). *Toxoplasma* nuclear and cytoplasmic fractions were isolated using a Nuclear Extraction Kit according to the manufacturer's instructions (Chemicon). Protein concentrations of each fraction were determined by bicinchoninic acid protein assay (Pierce).

Antibodies and Western blot analysis. An antibody was generated in rabbit to the peptide sequence of TgMYST-A, LERNEGRISVQDLSRMTC (Biosource), called anti-TgMYST-A (used at a dilution of 1:10,000), and affinity purified. Anti-AcH3 (Upstate) was used at 1:1,000. Antibodies used to examine specific lysine residues included acetylated (Ac) H4 (K5) (1:600; Abcam), AcH4 (K8) (1:600; Upstate), AcH4 (K12) (1:1,000; Upstate), or AcH4 (K16) (1:1,000; Upstate,). Antibody to *Toxoplasma* tubulin and MIC2 was kindly provided by David Sibley (Washington University, St. Louis, MO) and used at a dilution of 1:1,000 and 1:10,000, respectively. Appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies were employed along with the ECL detection system to visualize results (Amersham-GE). Western blotting for all applications was performed using NuPAGE 4 to 12% or 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels using MOPS (morpholinepropanesulfonic acid) or MES (morpholineethanesulfonic acid) running buffer (Invitrogen).

Cloning, expression, and purification of TgMYST-A. RNA ligase-mediated rapid amplification of cDNA ends (RACE) was performed using GeneRacer (Invitrogen), and mRNA was harvested from *Toxoplasma* tachyzoites. Amplified products were gel purified, subcloned into TA-TOPO vectors (Invitrogen), and sequenced. All nucleotide sequencing was performed on both strands at the Indiana University Biochemistry Biotechnology Facility (Indianapolis, IN).

To express and purify recombinant epitope-tagged TgMYST-A from *Toxoplasma*, we followed a strategy analogous to a method previously employed (13). TgMYST-A short [S] and long [L] forms were each cloned into a *Toxoplasma* expression vector (2) containing a tubulin promoter to make *p*tub$_{FLAG}$MYSTA(S)::HX and *p*tub$_{FLAG}$MYSTA(L)::HX. The TgMYST-A coding sequences were amplified from *Toxoplasma* cDNA using *Pfu*Ultra (Stratagene) and primers containing NdeI and SpeI restriction enzyme sites for cloning purposes (italicized below). The 5′ primer also consisted of sequence encoding the FLAG epitope tag (underlined). Primers for TgMYST-A(S) were the following: sense, 5′-ATCG*CATATG*AAAATGGACTACAAGGACGACGACGACAAGAGCGATTCCTACGTCGTCTCGTTTCC; antisense, 5′-ATCG*ACTAGT*TTAGGCTTGAGGACTGTACTCAAAAGGC. The sense primer for TgMYST-A(L) was 5′-ATCG*CATATG*AAAATGGACTACAAGGACGACGACGACAAGAAGAGAGTCTCGGGAGCGGCTGCTCC. RH tachyzoites were transiently transfected by electroporating 100 μg of the expression vector into ~3.0 × 10^7 parasites in triplicate to be pooled 48 h postinfection. Transfection methods were as previously described (32). FLAG-tagged protein was purified by sonicating parasite pellets in 1.0 ml of 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with a protease inhibitor cocktail (Sigma P8340). The parasite lysate was cleared by centrifuging at 13,000 rpm for 10 min at 4°C prior to mixing with 40 μl of equilibrated anti-FLAG M2-agarose affinity resin (Sigma). The resin and lysate were mixed overnight at 4°C and spun at 8,200 × *g* for 30 s. The supernatant was removed, and the resin was washed three times in 500 μl of 1× wash buffer (0.5 M Tris HCl [pH 7.4], 1.5 M NaCl, and 0.01 M EDTA) and two times in 100 μl of 1× HAT assay buffer (50 mM Tris HCl [pH 8.0], 5% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate) at 4°C. FLAG-tagged protein bound to the resin was used directly in HAT assays as described below.

HAT assay and acid-urea-Triton (AUT) gels. Fifty-microliter reaction mixtures containing 1× HAT assay buffer (above), 4 μg of chicken erythrocyte histones (Upstate), 0.25 μCi of [^{3}H]acetyl-coenzyme A (CoA) (Amersham/GE), and purified enzyme or control were incubated at 30°C for 1 h. Subsequently, 35 μl of each sample was separated by SDS-PAGE using a 10% Bis-Tris MES gel (Invitrogen). For autoradiography, gels were soaked in protein fix solution (5% acetic acid and 5% isopropanol), washed in distilled water, soaked in Autofluor (National Diagnostics), dried, and placed on film for several days. To determine the residue substrate specificity, HAT assays were performed as described above, substituting 1 mM nonradioactive acetyl-CoA (Sigma) and 1.0 μg of recombinant H4 (Upstate). The reaction was resolved on SDS-PAGE for immunoblotting with antibodies specific for acetylated H4 (K5, K8, K12, or K16). FLAG-tagged TgMYST-A(L) and TgMYST-A(S) were purified as described above; 1.0 μg of recombinant glutathione transferase (GST)-tagged Esa1 from *S. cerevisiae* was used as a positive control (produced in *Escherichia coli* and purified over glutathione).

AUT gel assays were performed essentially as described previously (25). Parasite histones were extracted from ~10^8 filter-purified tachyzoites lysed in 20 mM HEPES (pH 7.2), 1% Triton X-100, 1% sodium deoxycholate, 100 mM

FIG. 2. The 5′ UTR of TgMYST-A. Two possible transcription start sites were determined using RNA ligase-mediated 5′ RACE, designated by arrowheads labeled 1 and 2, respectively. Numbering of the 5′ UTR nucleotide sequence is based on the consensus ATG shown in bold italics. A second, nonconsensus ATG in frame to the one previously mentioned is in bold (−177). An upstream stop codon in frame with both ATGs is underscored. A triple-repeat region of 18 nucleotides is highlighted in gray, with T representing the beginning of each repeating unit. Underscored twice is a *cis*-acting enhancer element identified previously in promoters of *Toxoplasma* (26).

NaCl, 5 mM EDTA, 50 mM NaF, and protease inhibitors. After incubation on ice for 10 min, lysates were centrifuged and pellets were resuspended in 1.0 ml of distilled water. Incubation on ice continued for at least 30 more min after the addition of 22 μl of concentrated H_2SO_4. Cleared lysate was mixed with 10 volumes of cold acetone overnight at −20°C. Samples were spun at 9,000 rpm for 15 min to pellet extracted histones.

Sequence data and analysis tools. DNA and protein sequences were analyzed for homologues using BLAST programs at http://www.ncbi.nlm.nih.gov. Protein mapping and motif searches were performed with Pfam (http://www.sanger.ac.uk/Software/Pfam/) and SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignments were compiled using Vector NTI 9.0 (Informax). Preliminary genomic sequence data were accessed via http://ToxoDB.org.

Nucleotide sequence accession numbers. Sequence data described in this report are available in the GenBank, EMBL, and DDBJ databases under the accession numbers AY578183 for TgMYST-A and DQ104220 for TgMYST-B.

RESULTS

Identification of MYST family HATs in *Toxoplasma*. We have previously identified GNAT family HATs in *Toxoplasma* (44) (accession no. AAW72884). The *Toxoplasma* genome sequencing database (ToxoDB.org) was surveyed for potential members of the MYST family of HATs. Two entries, TGG_994607 (TgMYST-A, chromosome IV) and TGG_994619 (TgMYST-B, chromosome X), were predicted to encode proteins possessing unequivocal homology to HAT catalytic domains of the MYST family type. Reverse transcription-PCR (RT-PCR) was used to generate cDNA-derived fragments from each gene to probe Northern blots containing tachyzoite mRNA, revealing that the transcripts for TgMYST-A and -B are ~3.5 and ~7 kb, respectively (Fig. 1). The fainter band at virtually the same size seen on the Northern blot probed with TgMYST-A is probably background from large-subunit rRNA (we detected no alternatively spliced forms while cloning the gene).

We acquired full-length sequence for TgMYST-A using 5′ and 3′ RACE. A single band from a nested 5′ RACE reaction was obtained and subcloned. Sequencing of three clones revealed the transcription initiation site to be −1496 upstream of the putative ATG, and one was observed that began 14 nucleotides further upstream (Fig. 2). An interesting feature of the 5′ untranslated region (UTR) is the repeating motif TCTTCT TCTCCAGCTTCG (−876 to −822), whose functional significance (if any) has not been determined. We verified the continuity and nucleotide sequence in an RT-PCR designed to amplify the entire presumed open reading frame of 1,236 bp. The 5′ and 3′ UTRs are ~1.5 and ~0.7 kb, respectively, yielding a transcript size consistent with that observed on the Northern blot. An in-frame stop codon is located 719 nucleotides upstream of the proposed start ATG, which conforms well to Kozak's consensus site rules (23). An additional in-frame ATG is found 177 nucleotides upstream of the consensus ATG, but it does not conform to consensus rules for translational start sites. A partial fragment of the sequence representing TgMYST-B was cloned by RT-PCR, encompassing the region encoding the chromodomain (CHD) through the end of the protein.

The 8.6-kb TgMYST-A genomic locus is comprised of 10 exons (Fig. 3A). A schematic diagram of the predicted protein

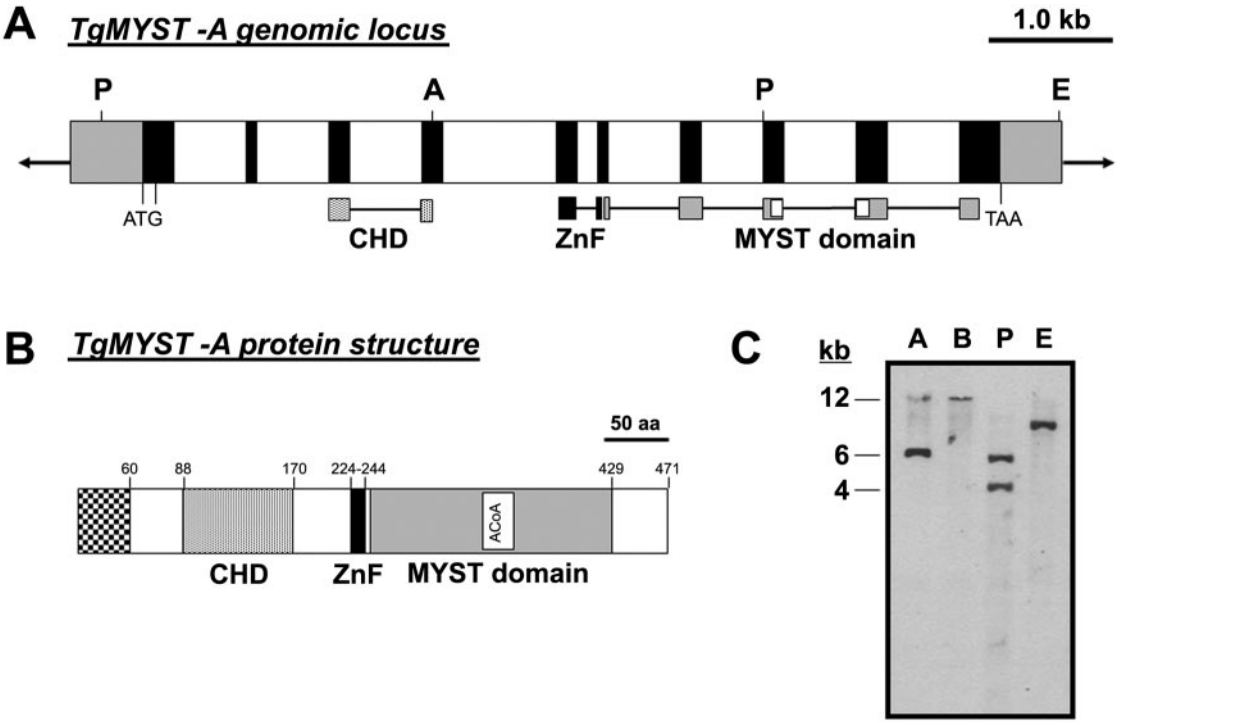

FIG. 3. TgMYST-A genomic locus and protein structure. Schematic diagram of the genomic locus (A) and predicted protein (B). Exons are represented by black boxes, introns by white boxes, and UTRs by gray boxes. ZnF, C2HC zinc finger. The acetyl-CoA binding site within the MYST HAT domain is represented by a white box. The checkered box indicates a region that is not present on TgMYST-A(S) form, reducing protein length to 411 amino acids. (C) Southern analysis of *Toxoplasma* genomic DNA cut with the following restriction enzymes and probed with TgMYST-A cDNA: A, AvrII; B, BamHI (not present in locus); P, PstI; E, EcoRI.

with key domains highlighted is shown in Fig. 3B, along with the 60 amino acids that would be added to the N terminus if the nonconsensus start site were used. These additional residues do not have homology to any protein in the databases and are devoid of known protein motifs and signal sequences. To distinguish the two possible proteins, we chose to call the longer one TgMYST-A(L) and the shorter one TgMYST-A(S). A cDNA-derived probe spanning the entire open reading frame was hybridized to a Southern blot containing *Toxoplasma* genomic DNA digested with various restriction enzymes. Results agree with our genomic map and demonstrate that the locus is present as a single copy in the parasite genome (Fig. 3C).

TgMYST-A and -B exhibit unequivocal similarity to the MYST family of HATs. Top BLASTp hits using TgMYST-A include hypothetical MYST proteins in fellow apicomplexans *Plasmodium* spp. (e^{-131}) and *Cryptosporidium* spp. ($8e^{-93}$), followed by numerous homologues from plants: *Solanum chacoense* ($9e^{-90}$), *Arabidopsis thaliana* (e^{-90}), *Zea mays* ($4e^{-88}$), and *Oryza sativa* ($2e^{-87}$). The closest metazoan homologues are human and mouse MOF ($\sim e^{-77}$). BLASTp searches using the predicted TgMYST-B protein sequence yielded nearly identical results. Closer analysis of the genomes of fellow apicomplexan parasites *Plasmodium* and *Cryptosporidium* shows that they possess 1 and 2 MYST HATs, respectively. An alignment of the protein sequences comparing these apicomplexan MYST homologues to other representative species is shown in Fig. 4.

Highlighted in Fig. 4 is the MYST-type HAT domain, including the two strictly conserved residues (Cys and Glu) postulated to be critical in forming the self-acetylated intermediate required for activity (50). Apicomplexan MYST HATs also possess the conserved cysteine-rich zinc finger region CXX CX_{12}HXXXC that is essential for HAT activity and interacts with the globular region of the nucleosome core (1). In addition, they contain a CHD sequence N-terminal to the HAT domain. Some metazoan MYST family HATs possess PHD fingers and H15 domains, neither of which is evident in any of the apicomplexan MYST family HATs. Thus, based on homology alignments and the structural features present, the MYST homologues identified in apicomplexan parasites more closely resemble the "MYST+CHD" subset, which includes yeast Esa1, human Tip60, and MOF (46).

Two forms of TgMYST-A are predominantly expressed in tachyzoites. An antibody was raised to a peptide of TgMYST-A (noted on Fig. 4) that is capable of recognizing recombinant TgMYST-A produced in *E. coli* (see below) (data not shown). When used in Western blotting of *Toxoplasma* tachyzoite whole-cell extract (type I RH strain), a predominant band of ~49 kDa is observed, consistent with the size expected for TgMYST-A(S) (Fig. 5A). A slightly larger band with less intensity is also apparent, consistent with the 53-kDa expected size of TgMYST-A(L). This suggests that TgMYST-A(L) is expressed in vivo regardless of the nonconsensus ATG start site, albeit at lower levels. Type II ME49 strain tachyzoites exhibited the same pattern of expression (Fig. 5B). Interest-

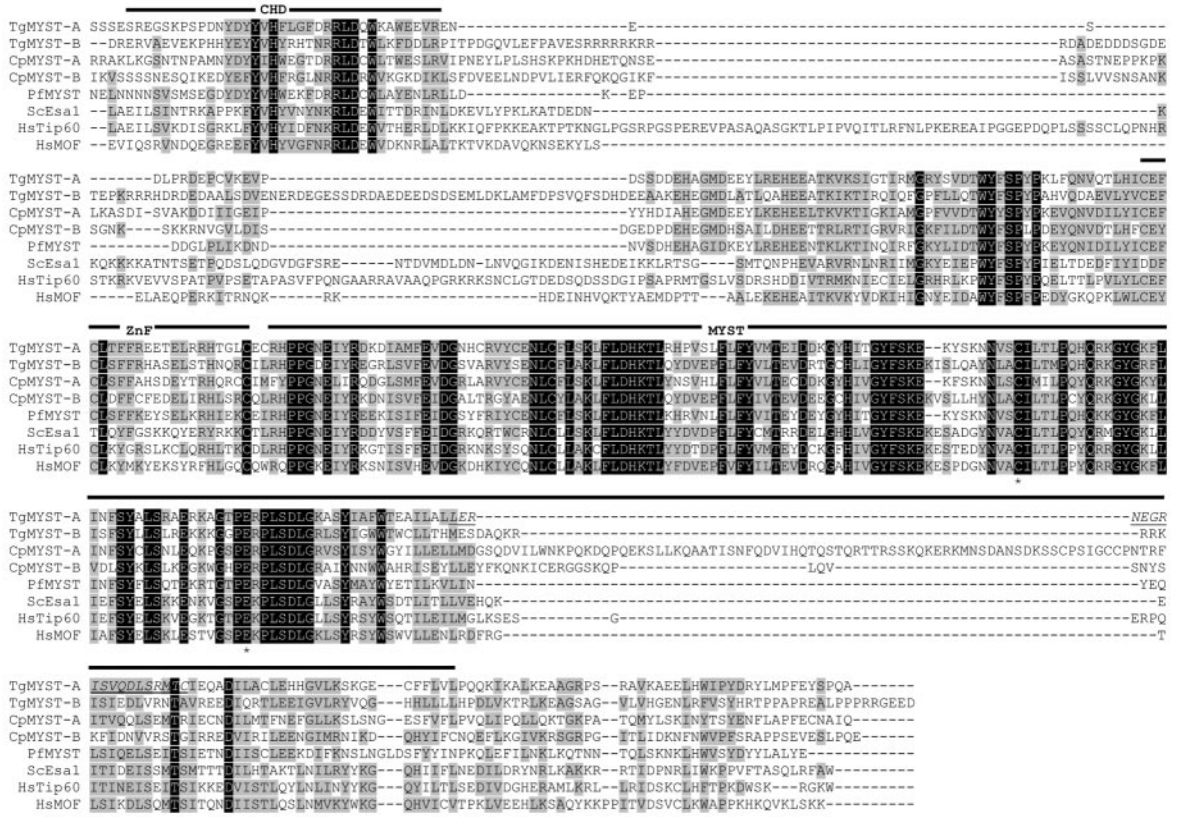

FIG. 4. Protein alignments of MYST family HATs. The TgMYST amino acid sequences were aligned with representatives of the MYST HAT family from other organisms using the Vector NTI AlignX tool. The N-terminal sequence varies considerably in length and composition between family members; thus, only the C-terminal portion beginning with the CHD is shown. Letters in black boxes are residues identical in every species, while shaded letters are chemically similar. Locations of key domains are labeled and denoted above with a solid bar. The two strictly conserved residues (C and E) modeled to be crucial for catalytic activity are denoted with asterisks. Peptide antigen used to generate antibody to TgMYST-A is italicized and underscored. ZnF, zinc finger; Cp, *C. parvum* (CpMYST-A, accession no. CpIOWA_EAK87476; CpMYST-B, accession no. CpIOWA_EAK89901); Pf, *Plasmodium falciparum* (accession no. PF11_0192); Hs, *Homo sapiens* MOF (accession no. NM_032188) and Tip60 (accession no. NP_874369); Sc, *S. cerevisiae* Esa1 (Q08649).

ingly, a decrease of both forms of TgMYST-A is seen when ME49 parasites are cultured in bradyzoite-induction conditions (alkaline pH): TgMYST-A(S) is diminished, and TgMYST-A(L) virtually disappears (Fig. 5B). Fidelity of the in vitro conversion was tested by RT-PCR for the stage-specific genes SAG1 (tachyzoite) and BAG1 (bradyzoite). As shown in Fig. 5C, BAG1 is not present in the ME49 tachyzoites but is present in the converted population. The persistence of some SAG1 is likely due to the inefficiency of in vitro stage conversion. The efficiency was ~61%, as determined by immunofluorescence using *Dolichos* lectin staining of the cyst wall (data not shown). Thus, it is likely that the TgMYST-A(S) detected in the bradyzoite sample is from undifferentiated parasites, suggesting that this HAT is predominantly expressed in tachyzoites. Also, the additional bands appearing in the ME49 bradyzoite lane probed with anti-TgMYST-A correspond to bands of identical size in an uninfected host cell (HFF) lysate (Fig. 5B), arguing that these minor bands are not of parasite origin.

Recombinant TgMYST-A acetylates histones. To test if TgMYST-A is a HAT exhibiting the familial proclivity to target H4, we attempted to express recombinant enzyme in *E. coli* for purification. The generation and purification of recombinant protein were unproblematic, but no HAT activity could be detected despite various attempts to refold the enzyme. Attempts were made to express recombinant $_{\text{FLAG}}$TgMYST-A (L and S forms) stably in *Toxoplasma* RHΔHX strain using *p*tub$_{\text{FLAG}}$MYSTA(L or S)::HX (see Materials and Methods) and the HXGPRT selection system (12). No viable clones were obtained, which suggests that overexpression is detrimental to the parasite. Thus, we harvested protein by virtue of the FLAG epitope tag from transiently transfected populations of *Toxoplasma* for use in HAT assays. Figure 6A shows that both long and short forms of FLAG-tagged TgMYST-A have a preference to acetylate H4 in a mix of free histones. Some MYST+CHD HATs such as MOF exclusively target H4 (K16) while others, such as Esa1, target all four lysines (46). Western blots of HAT reactions performed for TgMYST-A(S) show this HAT to be capable of acetylating any of the four target lysines in the H4 tail (K5, K8, K12, and K16) (Fig. 6B).

Relevance of histone H4 acetylation in vivo. While TgMYST-A clearly acetylates histone H4 in vitro, we wished to ascertain that *Toxoplasma* histones are posttranslationally modified with acetyl groups in vivo. The AUT gels in Fig. 6C show that *Toxoplasma* H4 exists in mono-, di-, tri-, and tetra-acetylated

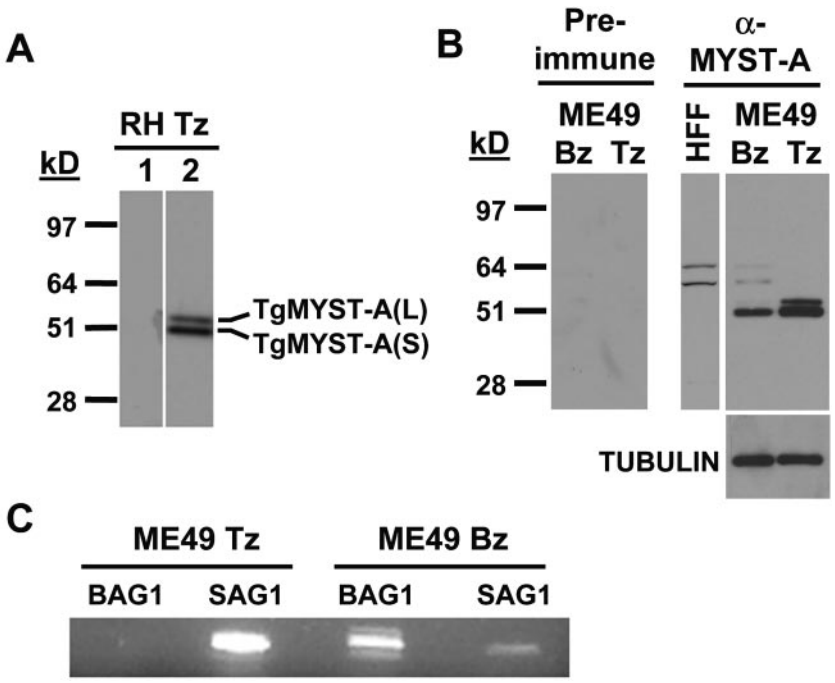

FIG. 5. Western blot of TgMYST-A. (A) Lysate from RH tachyzoites (Tz) was probed with preimmune sera (lane 1) or anti-TgMYST-A (lane 2). (B) Equivalent concentrations of extracts from ME49 tachyzoites cultivated under normal conditions (Tz) or bradyzoite-inducing conditions (Bz) were probed with preimmune sera or anti-TgMYST-A. Western probing for constitutively expressed tubulin was used as a normalizing control. Lysate from uninfected host cells (HFF) was also probed with anti-TgMYST-A. Molecular masses are given in kilodaltons (kDa). (C) RT-PCR for stage-specific genes, showing the fidelity of the bradyzoite differentiation process.

forms, correlating with the four lysine residues in H4 N-terminal tails known to be susceptible to this modification. In order to better define the role of TgMYST-A in this process, several attempts were made to disrupt the genomic locus in the haploid tachyzoites by homologous recombination. However, allelic replacement of the TgMYST-A locus was not possible, implying that it may encode an essential gene product. As mentioned above, no stable transgenic parasites overexpressing TgMYST-A were obtainable either, suggesting that levels of this protein may be tightly controlled. The inability of *Toxoplasma* to tolerate a second copy is due to the HAT activity of TgMYST-A. A point mutation of the conserved glutamic acid (E279 of the short form) to glycine abolished MYST HAT activity (16, 37) and allowed for the selection of stable *Toxoplasma* clones overexpressing $_{\mathrm{FLAG}}$TgMYST-A(S), as determined by immunofluorescence with anti-FLAG (data not shown).

We have also recently shown that H4 acetylation correlates with stage-specific gene activation in vivo (33), making it likely that the TgMYST proteins identified here participate in parasite differentiation.

Subcellular localization of TgMYST-A. Based on its role in the cell, we expected that TgMYST-A would localize to the parasite nucleus like the HAT TgGCN5 (2). However, no conventional nuclear localization signal is evident in the predicted TgMYST-A(L) or TgMYST-A(S) protein sequences. To check the subcellular compartmentalization of TgMYST-A, nuclear and cytosolic fractions were examined by immunoblotting with anti-TgMYST-A. As shown in Fig. 7, while both forms of TgMYST-A are capable of translocating to the parasite nucleus, there is abundant protein in the cytoplasm as well. The TgMYST-A observed in the nucleus is not likely due to impurities, as the cytoplasmic protein MIC2 is not detectable in this fraction.

MYST complex homologues in *Toxoplasma*. We also surveyed the *Toxoplasma* genomic resource (ToxoDB.org) for potential homologues of proteins known to associate with MYST+CHD family HATs in other species. Table 1 lists numerous potential orthologues present in the *Toxoplasma* genome that are similar to proteins previously identified in the MYST+CHD complexes NuA4 (yeast), MSL (fruit fly), and Tip60 (human).

Toxoplasma appears to share more homologues with the human Tip60 complex than NuA4 or MSL, possessing every subunit identified to date, with the possible exception of Enhancer of Polycomb (EPC/Epl1) and the SWI2/SNF2-related ATPase p400. However, TgSRCAP (Snf2-related CBP activator protein) is very similar to p400 and could serve as a functional equivalent (43). *Toxoplasma* also has possible components related to proteins found in the yeast NuA4 complex (Tra1, Arp4, and Yaf9) but lacks clear Eaf, Yng2, and Epl1 homologues. Similarly, while there are homologues to the fruit fly helicase called maleless (MLE) and the H3 phosphorylase JIL-1, *Toxoplasma* appears to lack MSL-1, MSL-2, and MSL-3, complicating the argument that an MSL complex exists in the parasite.

DISCUSSION

Once thought to serve little more than a structural function, chromatin is now receiving substantially more attention be-

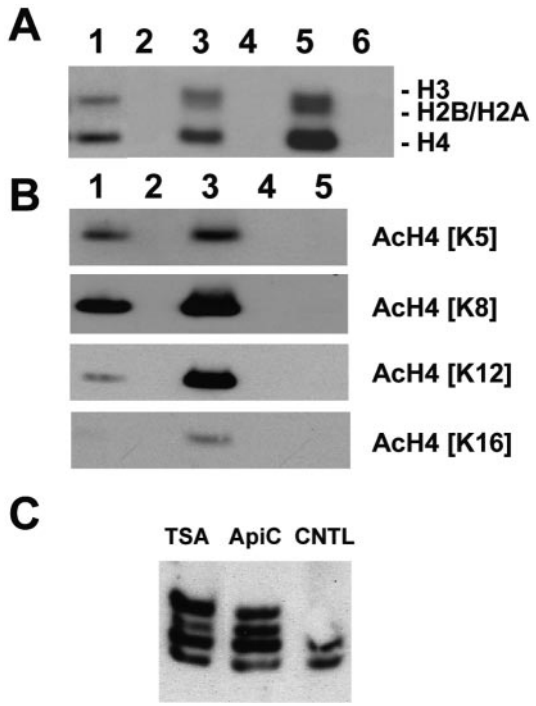

FIG. 6. TgMYST-A mediated histone H4 acetylation. (A) Recombinant TgMYST-A(L) and TgMYST-A(S) preferentially acetylate histone H4. FLAG-tagged TgMYST proteins were transiently expressed in *Toxoplasma* and purified by virtue of anti-FLAG affinity resin for use in HAT assays [lane 3 is $_{FLAG}$TgMYST-A(S); lane 5 is $_{FLAG}$TgMYST-A(L)]. Recombinant GST-Esa1 was used as a positive control (lane 1). Negative controls included anti-FLAG resin with no lysate (lane 2), histones alone (lane 4), and untransfected parasite lysate processed on anti-FLAG resin (lane 6). (B) Substrate specificity was further delineated for TgMYST-A(S) by performing Western blotting of the HAT reaction using antibodies to each acetylated lysine shown. Lane 1, GST-Esa1 control; lane 2, anti-FLAG resin with no lysate; lane 3, $_{FLAG}$TgMYST-A(S); lane 4, histones alone; lane 5, untransfected parasite lysate processed on anti-FLAG resin. Esa1 has been reported to weakly acetylate H4 (K16) (37); our failure to detect this may be related to the expression/purification of recombinant GST-Esa1 from *E. coli*. (C) AUT gel shows that acetylated forms of *Toxoplasma* H4 exist in vivo. TSA, trichostatin A; ApiC, apicidin; CNTL, control (no HDAC inhibitor).

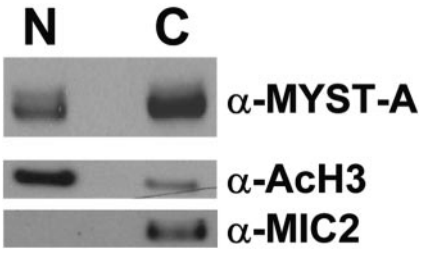

FIG. 7. Localization of TgMYST-A(L) and TgMYST-A(S). *Toxoplasma* tachyzoites were fractionated into nuclear (N) and cytosolic (C) fractions for Western blotting with anti-MYST-A. Two micrograms of each fraction was loaded onto gel for analysis. Anti-AcH3 and anti-MIC2, proteins that are enriched in the nucleus and cytoplasm, respectively, were used to examine the purity of each fraction.

cause of its new-found regulatory power. Vital to epigenetics and the modulation of DNA transcription, repair, and replication, chromatin remodeling machines are at the forefront of development and gene expression research. Investigation of chromatin remodeling and the regulation of gene expression in medically relevant pathogens like *Toxoplasma* is a significant area of research. Of particular concern is how chromatin remodeling complexes orchestrate important pathological processes such as parasite differentiation.

Like other eukaryotic organisms, apicomplexans possess the machinery required to carry out noncovalent and covalent modifications of nucleosomes. The former class is represented by the identification of SWI2/SNF2 homologues resembling p400 and SRCAP in apicomplexans, as well as an ISWI homologue in *Plasmodium* (20, 43). Histone deacetylases and HATs of the GNAT class have also been cloned in these parasites (21, 33, 44), yet no information is documented describing any MYST family HATs in apicomplexan parasites.

HATs of the MYST class in apicomplexa. Here we report the identification of MYST family HATs in *Toxoplasma*. BLASTp analysis of predicted proteins for *Plasmodium* (PlasmoDB.org) and *Cryptosporidium* (CryptoDB.org) was performed, using protein sequences of TgMYST-A and the partial TgMYST-B. While *Toxoplasma* and *Cryptosporidium* ssp. each harbor two MYST HATs, *Plasmodium* ssp. appear to have only one. This is not likely to be an artifact of gene prediction since all *Plasmodium* species in the database show only one MYST HAT homologue. Why *Plasmodium* evidently lost a MYST HAT is an intriguing question, but the fact is consistent with the idea that transcriptional regulation has been marginalized in this organism and that protein levels are primarily determined by posttranscriptional mechanisms (9).

All of the apicomplexan MYST HATs are of the MYST+ CHD subfamily, as commonly found in early eukaryotes. In other words, some metazoan MYST HATs such as human MOZ and MORF that contain additional domains (e.g., PHD zinc fingers and H15 domains) do not appear to have homologues in apicomplexa. PHD finger and H15 (named for homology to histones H1 and H5) domains may be protein-interaction modules, but their functional significance is unclear. Higher eukaryotes have five to six different MYST HAT family members and may have evolved these domains to maintain tighter regulation of the possible MYST complexes that could form.

Both TgMYST HATs are encoded by independent loci, each present in the parasite genome as a single copy. However, TgMYST-A encodes a transcript that gives rise to two versions of TgMYST-A, which we have termed long (L) and short (S). TgMYST-A(S) is predominant, presumably because its start codon fits Kozak consensus rules (23). The alternative start codon, found 177 nucleotides upstream, does not conform to a consensus translational start site but, nevertheless, appears to be used to synthesize modest levels of a longer form of TgMYST (Fig. 5). The additional 60 amino acids on TgMYST-A(L) do not appear to alter subcellular localization or substrate preference for H4. Thus, the differential roles (if any) of the long and short forms of TgMYST-A remain unresolved. Another curious feature is that neither TgMYST-A(L) nor TgMYST-A(S) harbors a classical nuclear localization signal. It would be of interest to investigate how TgMYST-A acquires

TABLE 1. Potential MYST + CHD-associating protein homologues in *Toxoplasma*

Protein (source)	Database entry[a]	Homologue
NuA4 (yeast)		
Esa1	TGG_994607-2-139325-139981 ($1.0e^{-12}$)	TgMYST-A
	TGG_994619-3-45819-46301 ($6.9e^{-17}$)	TgMYST-B
Tra1	TGG_994537-2-162320-166291 ($1.3e^{-10}$)	
Arp4	TGG_994254-5-202165-201701 ($3.4e^{-18}$)	
Yaf9	TGG_994577-1-679138-679536 ($7.9e^{-05}$)	
Tip60 (human)		
Tip60	TGG_994607-2-139325-139981 ($9.2e^{-11}$)	TgMYST-A
	TGG_994619-3-45819-46301 ($1.9e^{-14}$)	TgMYST-B
TRRAP	TGG_994537-2-162320-166291 ($3.2e^{-13}$)	
BAF53	TGG_994254-5-203548-202565 ($1.0e^{-29}$)	
p400	TGG_994324-6-26109-24721 ($3.6e^{-45}$)	TgSRCAP
Tip49	TGG_994290-5-416906-416523 ($1.8e^{-18}$)	
MSL (fruit fly)		
MOF	TGG_994607-1-137410-137670 ($3.1e^{-10}$)	TgMYST-A
	TGG_994619-3-45819-46301 ($7.3e^{-17}$)	TgMYST-B
MLE	TGG_994305-3-403584-404870 ($2.3e^{-38}$)	
JIL-1	TGG_994300-4-44315-43476 ($5.2e^{-40}$)	

[a] ToxoDB entry format: TGG_accession number-reading frame-nucleotide numbers (negative logarithm of expect value).

access to the parasite nucleus and, further, if an interaction with *Toxoplasma* importin alpha is observed, as seen in the case of TgGCN5 (2).

TgMYST-A acetylates multiple lysines in H4. While GCN5 family members show bias toward acetylating H3, MYST family HATs display a preference for H4. Recombinant TgMYST-A exhibits this substrate bias in in vitro HAT assays. Given the high conservation of the TgMYST-B catalytic domain (Fig. 4), it would be surprising if it was not capable of the same function. Of greater interest to resolve is the delineation of lysine(s) acetylated in H4 by each MYST. Our enzymatic data support the idea that TgMYST-A may be an orthologue of Esa1/Tip60 since it can target every lysine in the H4 tail (MOF has exquisite preference for H4 [K16]). It is tempting to speculate that TgMYST-B, like *Drosophila* MOF, may exclusively target H4 (K16), but experimental data are required to confirm this. It is likely that TgMYST-A and -B operate in two distinct multiprotein complexes analogous to those in other species. Our bioinformatics survey revealed that *Toxoplasma* possesses some homologues to proteins found in both Esa1/Tip60 and MOF complexes (Table 1). It will be of interest to purify the native complexes from *Toxoplasma* in order to assess which TgMYST is associated with the various MYST complex homologues.

TgMYST-A levels may be critical for parasite survival. Despite multiple attempts, neither the long nor the short form of TgMYST-A could be stably overexpressed in *Toxoplasma*. In addition, repeated attempts to generate a TgMYST-A knockout clone by homologous recombination have failed. Collectively, these observations speak to a delicate balance of MYST-mediated acetylation and deacetylation in the parasites. Tellingly, Esa1 is an essential gene in yeast, with its loss resulting in cell cycle arrest (37). Western analysis argues that TgMYST-A is predominantly expressed in the rapidly dividing tachyzoite stage and clearly decreases during the virtually quiescent bradyzoite stage (Fig. 5). It is thus possible that TgMYST-A may play a role in cell cycle progression, but no direct data exist to validate that hypothesis. The observation that TgMYST-A localizes to the cytoplasm as well as the nucleus suggests that it may acetylate H4 prior to nuclear import; however, it remains possible that TgMYST-A is also vital for the acetylation of critical nonhistone substrates, as shown for a variety of HATs (51). If TgMYST-A proves to be an essential gene, as our studies imply, it may be a useful target for drug development. A complemented knockout strategy would resolve this question.

To date, a limited amount of information is available on what genes each MYST family complex regulates or if, in fact, they operate as global regulators of gene expression. In contrast to GNAT family HAT complexes like SAGA, which contain proteins known to be associated with transcription, the MYST family HAT complexes contain subunits related more to epigenetics and cell proliferation control (46). Esa1 has been linked to ribosomal protein and heat shock genes (30). Tip60 was discovered by virtue of its association with human immunodeficiency virus type 1 Tat (22) and has been implicated as a coactivator of class I nuclear hormone receptors and NF-κB (5, 11). Both Esa1 and Tip60 complexes have been linked to participating in DNA repair (3, 19). MOF controls dosage compensation and is male-specific lethal in fruit flies (16). The development of conditional knockout strategies (24) and oligonucleotide microarrays for *Toxoplasma* will greatly assist the efforts to delineate the subset of genes that TgMYST HATs may control.

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