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Cloning and analysis of a *Toxoplasma gondii* histone acetyltransferase: a novel chromatin remodelling factor in Apicomplexan parasites

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ABSTRACT

The yeast transcriptional adaptor GCN5 functions as a histone acetyltransferase, directly linking chromatin modification to transcriptional regulation. Homologues of yeast GCN5 have been found in *Tetrahymena*, *Drosophila*, *Arabidopsis* and human, suggesting that this pathway of chromatin remodelling is evolutionarily conserved. Consistent with this view, we have identified the *Toxoplasma gondii* homologue, referred to here as TgGCN5. The gene codes for a protein of 474 amino acids with an estimated molecular mass of 53 kDa. The protein reveals two regions of close similarity with the GCN5 family members, the HAT domain and the bromodomain. Tg*GCN5* occurs in a single copy in the *T.gondii* genome. The introduction of a second copy of Tg*GCN5* in *T.gondii* tachyzoites is toxic unless the HAT activity is disrupted by a single point mutation. Full Tg*GCN5* does not complement the growth defect in a yeast *gcn5*$^-$ mutant strain, but a chimera comprising the *T.gondii* HAT domain fused to the remainder of y*GCN5* does. These data show that *T.gondii* GNC5 is a histone acetyltransferase attesting to the significance of chromatin remodelling in gene regulation of Apicomplexa.

INTRODUCTION

Transcription is a complex process requiring the coordinate action of multiple basal and transactivating proteins (1). In eukaryotic cells, the packing of DNA into chromatin complicates this process. Nucleosomes are the fundamental repeating unit of chromatin with 146 bp of DNA wrapped around a histone octamer, which is itself composed of two copies of each of the four histones: H2A, H2B, H3 and H4. Nucleosomes have the potential to inhibit transcription strongly by blocking binding of transcription factors to their cognate DNA sites. Accordingly, chromatin structures are remodelled prior to or during transcriptional activation (2,3). Mechanisms that are involved in chromatin remodelling include histone acetylation and deacetylation and ATP-dependant nucleosome remodelling (4–6).

A major advance in understanding the connection between histone acetylation and gene transcription came with the discovery that the yeast transcriptional adaptor GCN5 serves as the catalytic subunit of a histone acetyltransferase type A activity (7). The GCN5 protein is associated with at least two multisubunit complexes in yeast, which include Ada proteins (ADA complex) and/or certain Spt proteins (SAGA complex) (8–13). These complexes are required for transcriptional activation in combination with a particular set of transactivators *in vivo* (14–16). They bridge the interaction between the specific activation domains and the general transcriptional machinery, thus providing an adaptor or co-activator function in addition to histone acetyltransferase activity (17).

GCN5 is responsible for the acetylation of the ε amino groups of lysine residues in the histone H3 (K14) and H4 (K8 and K16) N-terminal tails (18). The GCN5 protein is composed of a histone acetyltransferase (HAT) domain, spanning residues 95–261, required for *in vivo* full catalytic activity (8), an ADA2 interaction domain (residues 253–280) necessary for *in vitro* activity, and a C-terminal bromodomain (residues 148–440), which contributes to highly specific histone acetylation by tethering transcriptional HATs to specific chromosomal sites by interaction with acetyl-lysine residues (AcK) (19,20). To date, two human homologues (hGCN5 and hPCAF) (21,22), a *Drosophila* homologue (dGCN5) (23), and a *Tetrahymena* homologue of yGCN5 (Tetp55) (7), have been cloned and shown to possess HAT activity.

The phylum Apicomplexa comprises numerous pathogens of medical and veterinary significance. Among them, *Plasmodium falciparum* is the most virulent of the *Plasmodium* species responsible for malaria in human. The opportunistic pathogen *Toxoplasma gondii* causes diseases in immune-compromised patients and in congenitally infected infants. The protozoan parasite *T.gondii* has a complex life cycle that involves two morphologically distinct stages in the intermediate host: the rapidly dividing tachyzoite and the dormant encysted bradyzoite stage. Development and differentiation of the parasite are coincident with expression of different patterns of genes. However, transcriptional regulation mechanisms of these genes in the parasite remain largely unknown. Histone-modifying enzymes have not been identified in *T.gondii* so far and the involvement of histone acetylation in the modulation of gene expression has not been documented. However, a recent study

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illustrates the importance of HATs and deacetylases in Apicomplexan parasites (24). Agents such as apicidin that are known to block histone deacetylases, resulting in histone hyperacetylation, have potent antiparasitic activities.

In an attempt to understand the role and impact of histone acetylation in *T.gondii* regulation of gene expression, we have characterised the *T.gondii* homologue of the HAT and transcriptional co-activator GCN5, referred to here as TgGCN5. The protein sequence of TgGCN5 shares significant homology with other eukaryotic GCN5 proteins. We have shown that the HAT domain of *T.gondii* GCN5 is able to complement a *gcn5*$^-$ mutation in yeast, when fused to the remainder of yeast GCN5. We failed to stably introduce a second copy of the *GCN5* gene in *T.gondii* tachyzoites unless the HAT activity of the gene was disrupted by a point mutation.

MATERIALS AND METHODS

Nucleotide sequence accession numbers

The cDNA sequence of *T.gondii GCN5* has been deposited in GenBank under accession no. AF155929. The accession nos of other related sequences referred to here are: yGCN5, Q03330; Tetp55/HATA1, U47321; PCAF, U57317; hGCN5-S, U57316; *Arabidopsis* GCN5, AF031958; dGCN5, AF029776; *P.falciparum* partial sequence, AA550570.

Strains and reagents

The bacterial strains used for recombinant DNA techniques were *Escherichia coli* XL1-Blue and XLOLR. The helper phage ExAssist from Stratagene was used for *in vivo* excision of the phagemid vectors from the λZAPII clones. Restriction enzymes were purchased from New England Biolabs.

Growth of parasites and isolation of DNA

Toxoplasma gondii tachyzoites (RH wild-type strain and RH*hxgprt*$^-$) were grown in human foreskin fibroblasts (HFF) or in Vero cells (African green monkey kidney cells) maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 25 μg/ml gentamicin. Parasites were harvested after complete lysis of the host cells and purified by passage through 3.0 μm filters and centrifugation in PBS. Genomic DNA was isolated from purified parasites by SDS/proteinase K lysis followed by phenol/chloroform and chloroform extractions and ethanol precipitation (25).

Cloning of Tg*GCN5* and Southern analysis

The *T.gondii* RH (EP) cDNA expression library in λZAPII was obtained from NIH AIDS reagents (kindly provided by D.S. Roos). The library was screened by plaque hybridisation using the digoxigenin system (Boehringer Mannheim) for non-radioactive labelling and detection of nucleic acids. As probe, we used a DIG-dUTP-labelled fragment of 250 bp generated by PCR with primers designed on the basis of the *T.gondii* EST sequence found in GenBank (accession no. N61067): sense, 5′-CACGTCAAGGCGCAGATTG-3′; antisense, 5′-AAGTAGTAAATTGTGTCGGG-3′. The detection of positive clones was achieved by chemiluminescence with CSPD according to the manufacturer. A total of 15×10^4 plaques were screened and two positive clones were identified in both duplicates. After two additional cycles of hybridisation, positive clones were excised *in vivo* and the insert sizes determined by restriction digestion. The cosmid library used the SuperCos vector modified with the SAG1/ble *T.gondii* selection cassette inserted into a *Hin*dIII site. The library was prepared from a *Sau*3AI partial digest of RH genomic DNA ligated into the *Bam*HI cloning site and was kindly provided by D. Sibley and D. Howe.

Total genomic DNA was prepared as described above. An aliquot of 10 μg genomic DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, *Xho*I and *Bam*HI, separated on a 0.9% agarose gel, and blotted to a positively charged Nylon membrane (Boehringer) according to standard protocols. The Southern blot was hybridised with the DIG-labelled probe used for screening the cDNA library according to Engler-Blum *et al.* (26). To visualise the bound probe a CSPD Star chemiluminescent substrate (Boehringer) was used.

Construction of *T.gondii* and yeast expression vectors

The coding sequence of Tg*GCN5* was amplified by PCR to introduce *Nsi*I and *Bam*HI sites at the start and stop codons, respectively. The oligonucleotide 5′-CGGGATCCGATCATAAAGGCGCTCCACAGGTCT-3′ was combined with the antisense oligonucleotide primer 5′-GCGGATCCTCAGAAACTCCCGAGAGCCTCGAC-3′ for PCR amplification. The PCR product was cloned into the bacterial expression vector pET-19b (pET19bHisTgGCN5) and analysed for expression of the recombinant protein in BL21 (DE31, expressing T7 polymerase) after IPTG induction. Before subcloning of Tg*GCN5* into a *T.gondii* expression vector, we introduced a c-myc epitope tag at the N-terminus of GCN5 by cloning double-stranded oligonucleotides into the *Nsi*I site of the pET19bHisTgGCN5 construct. The oligonucleotide pair 5′-TGAGCAGAAGCTCATCTCCGAGGAGGACCTGCTGCA-3′ and 5′-GCAGGTCCTCCTCGGAGATGAGCTTCTGCTCATGCA-3′ codes for the myc epitope. The *T.gondii* expression vector (pS) used in this work contains a chimeric promoter composed of the five repeat elements present in the promoter of *SAG1* fused to a minimal *SAG4* promoter and carries the 3′-flanking sequence of the *SAG1* gene (Soete, Hettmann and Soldati, unpublished results). pSTgGCN5 was generated by subcloning c-mycTgGCN5 from pET19bHismycTgGCN5 into pS between the *Nsi*I and *Bam*HI sites. To introduce the point mutation F224A into the pSTgGCN5 vector (pSTggcn5F224A), we used the Quick Change Site-Directed Mutagenesis Kit from Stratagene according to the instructions of the manufacturer. The mutation was confirmed by sequencing.

The yeast episomal high copy number plasmid containing the wild-type y*GCN5* gene (pRS423ADHyGCN5) and the construct expressing the hhyy chimera were kindly provided by Dr Shelly Berger (27). For construction of the yeast pRS423TgGCN5 plasmid, pET19bHismycTgGCN5 (described above) was digested with *Bam*HI and blunt end ligated with *Eco*RI + *Sal*I-digested pRS423yGCN5 to replace the y*GCN5* coding region with the *Tg*GCN5 coding region.

To clone the ttyy chimera, the Tg*GCN5* cDNA from amino acid 1 to 264 was amplified by PCR using the oligonucleotides 5′-CGGGATCCGCGGCCGCAATGAAAGGCGCTCCAACAGGTC-3′ and 5′-CGGGATCCGCGGCCGCGTAATTTATTCGGGTGGTGAGAC-3′ to introduce *Not*I sites and the hh was replaced by tt in the hhyy vector using the *Eag*I sites. The Quick Change Site-Directed Mutagenesis Kit from Stratagene was used to

introduce the point mutation F224A into the ttyy chimera, creating ttyyF224A.

Parasite transfection and selection

Toxoplasma gondii tachyzoites (RH*hxgprt*$^-$) were transfected by electroporation as previously described (28) using 10^7 freshly lysed-out tachyzoites, 80 µg plasmid DNA and 100 U of *Bam*HI for restriction enzyme-mediated integration (29). After electroporation parasites were inoculated into HFF cells grown on glass coverslips (for immunofluorescence microscopy) or in 25 cm^2 T-flasks for selection. Stable transformants containing the transfected vectors were selected using medium containing 25 µg/ml mycophenolic acid and 50 µg/ml xanthine and cloned by limited dilution in 96-well plates (30).

Indirect immunofluorescence microscopy

All manipulations were carried out at room temperature. Intracellular parasites grown for 24 h in HFF on glass slides were fixed with 4% paraformaldehyde and 0.005% glutaraldehyde for 15 min. Following fixation, slides were briefly rinsed in PBS containing 0.1 M glycine. Cells were then permeabilised in PBS containing 0.2% Triton X-100 for 20 min and blocked in the same buffer with 2% FCS. Slides were incubated for 60 min with primary antibodies diluted in PBS containing 1% FCS, washed and incubated for a further 60 min in FITC-labelled goat anti-mouse IgG diluted in PBS containing 1% FCS. Slides were mounted in Vectashield and kept at 4°C in the dark. The anti-myc mAb was an ascites preparation of 9E10 used at a dilution 1:2000. The images were examined with a Zeiss Axiophot microscope equiped with a camera (Photometriecs Type CH-250). Adobe PhotoShop (Adobe Systems, Mountain View, CA) was used for processing of images.

Western blotting analysis

SDS–PAGE was performed using standard methods (31). Crude extracts from *T.gondii* tachyzoites or recombinant bacteria were separated by SDS–PAGE and transferred to nitrocellulose. Western blot analyses were carried out essentially as described (32) using 8–10% polyacrylamide gels run under reducing conditions with 144 mM β-mercaptoethanol in the loading samples. After electrophoresis, proteins were transferred to Hybond ECL nitrocellulose. For detection, the membranes were incubated with the mAb 9E10 (mouse ascites fluid diluted 1:1000 in PBS, 0.5% Tween 20) or with an anti-His mAb (Sigma) and then with horseradish peroxidase-conjugated purified goat anti-mouse IgG (1:2000) and bound antibodies visualised using the ECL system (Amersham Corp.).

Complementation in yeast

The yeast wild-type strain (the *trp1* derivative of PSY316) and the *gcn5*$^-$ mutant used in the complementation experiments were described previously (21). Yeast transformation was carried out by the lithium acetate protocol (33). The yeast strains were provided by Dr Shelly Berger (27). To test complementation the transformed yeasts were plated in fully supplemented SD medium. After 3 days, single colonies were inoculated into fully supplemented liquid SD medium and rotated at 30°C overnight. Ten-fold serial dilutions of the transformants were transferred to plates with SD minimal medium.

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  1 MHKGAPTGLG LASFFGKSFI FHTLHAALPA LLEELANTVV GTELRRFVLA LAAAVGLSSS
 61 HAEELLHRAV AVRSSRLESI LPSETGLGFL HRDAGGAREE ELGIISFCCV TNDRQPLHMR
121 HLVTVKNIFS RQLPKMPREY IVRLVFDRAH FTFCLCKQGR VIGGVCFRPY FREKFAEIAF
181 LAVTSTEQVK GYGTRLMNHL KEHVKKSGIE YFLTYADNFA VGYFRKQGFS SKITMPRDRW
241 LGYIKDYDGG TLMECRLSTR INYLKLSQLL ALQKLAVKRR IEQSAPSVVC PSLSFWKENP
301 GQLLMPSAIP GLAELNKNGE LSLLLSSGRV GAAPQGSGAL PGGRTGALGS KKGPFGRAGF
361 AKGEKGLRAA SLKAQIAALL STLEKHSSSW PFRRPVSVSE APDYYEVVRR PIDISTMKKR
421 NRNGDYRTKE AFQEDLLLMF DNCRVYNSPD TIYYKYADEL QAFIWPKVEA LGSF
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Figure 1. Coding sequence of *T.gondii GCN5* gene. The predicted protein translated from Tg*GCN5* is shown with the catalytic domain underlined and the bromodomain double underlined (GenBank accession no. AF155929).

Sequence alignment

Published sequences were obtained by searching the GenBank, PIR-Protein and SWISS Prot databases. Sequence alignment was carried out with the Lasergene DNASTAR MegAligne program using the Clustal method.

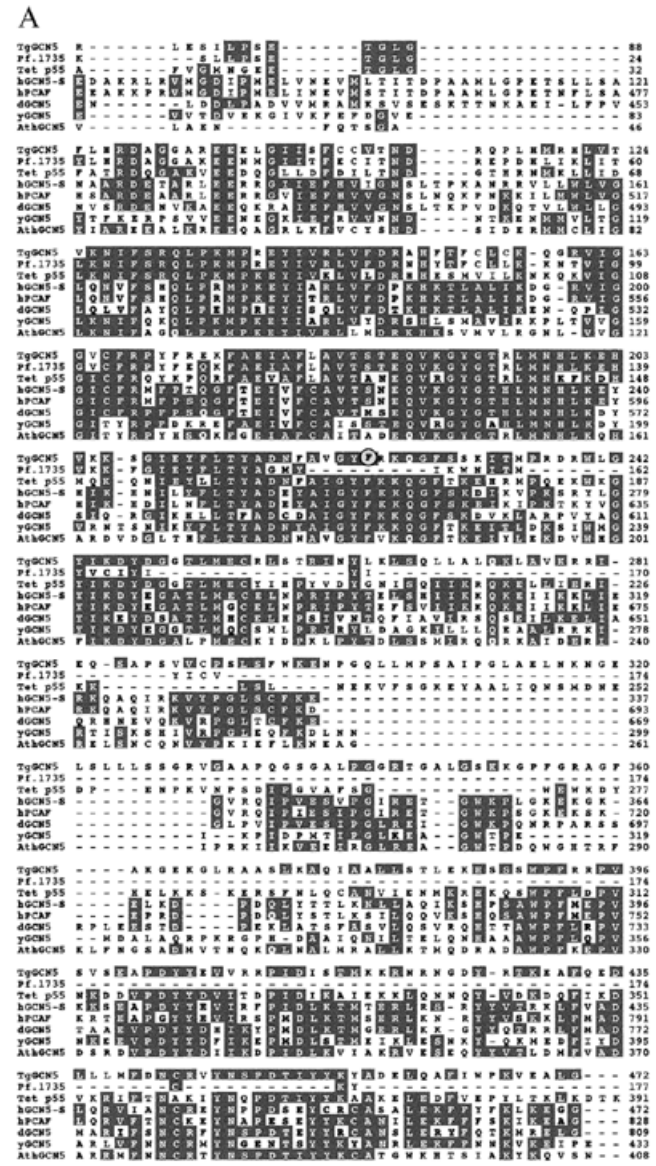
RESULTS

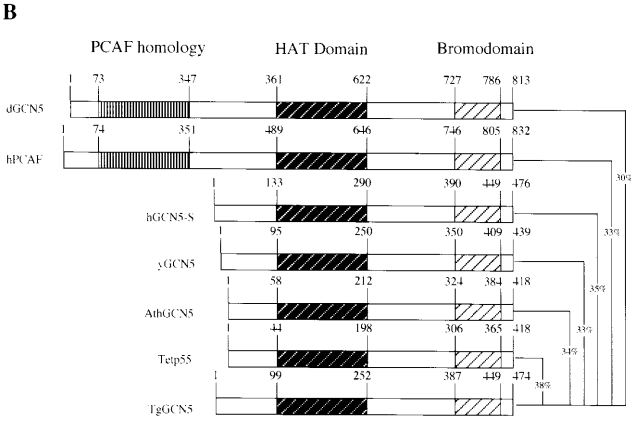
Cloning and characterisation of the *T.gondii GCN5* gene

In an attempt to identify a *T.gondii* factor involved in gene expression we searched the *T.gondii* expressed sequence tag (EST) database. This search revealed the presence of a single cDNA sequence of 294 bp with homology to *Saccharomyces cerevisiae GCN5* and a *Tetrahymena* homologue of *GCN5*, *Tetp55*. To isolate *T.gondii* cDNA and genomic clones corresponding to this EST, we designed primers within the 294 bp region of homology. The PCR amplification product on cDNA showed the expected size of 250 bp, whereas amplification from genomic DNA exposed an intron of ~550 bp. The cDNA fragment was used as probe to screen a λZAPII cDNA library from *T.gondii* strain RH. Two overlapping positive recombinant phage clones were obtained. The two clones were analysed by restriction mapping and sequenced on both strands.

One clone contained a full-length cDNA of 2.3 kb encompassing an open reading frame (ORF) encoding a predicted 474 amino acid protein (GenBank accession no. AF155929). The end of the ORF is marked by a stop codon followed by a 3′-untranslated region (UTR) ending with a polyadenylated tract. The putative start methionine is in-frame with an upstream stop codon. The second clone contained a cDNA of 1.2 kb exhibiting the same ORF but incomplete at the N-terminus. The deduced translated protein TgGCN5 is shown in Figure 1. Protein database searches (BLAST) retrieved Tetp55 (HATA1) as the strongest match, while hGCN5, an *Arabidopsis thaliana* GCN5-related protein, yGCN5, hPCAF and *Drosophila* dGCN5 are the next highest matches. Other significant matches are restricted to the bromodomain (34), which is a motif found in GCN5 family members and in a wide variety of co-activators and other transcription factors, including the HATs TAFII250 (35) and CBP (36).

Sequence comparison analysis of the predicted TgGCN5 protein with all GCN5-related proteins reveals two regions of close similarity, the HAT domain and the bromodomain, presented in Figure 2. The alignment of all seven proteins reveals the most significant homology in the HAT domain: 56% identity between *T.gondii* GCN5 and the Tetp55 HAT domain; 57% identity to hGCN5; 53% to hPCAF; 50% to





Arabidopsis GCN5-related protein; 49% to yGCN5; 45% identity to dGCN5. The similarity in the bromodomain is slightly lower (ranging between 36 and 47% identity). Nevertheless, residues previously noted to be conserved between the bromodomain of yGCN5 and other transcriptional factors (37) are also conserved in *T.gondii* GCN5 (Fig. 2).

Southern hybridisation analysis using the PCR-amplified cDNA Tg*GCN5* as probe on *T.gondii* genomic DNA cut with different restriction enzymes indicates that Tg*GCN5* is a single copy gene (Fig. 3). The presence of some of the introns interrupting the Tg*GCN5* gene was determined by sequencing or restriction mapping of genomic cosmid clones encompassing the Tg*GCN5* locus. The genomic library screening was carried out with the same probe used in the λZAPII screening in collaboration with Dr Jim Ajioka (Department of Pathology, Cambridge, UK). The pattern of bands obtained in Southern blot analysis of the cosmids match those predicted from the restriction map of the genomic locus.

Identification of a putative *GCN5* homologue in *P.falciparum*

An EST showing a high degree of homology with the amino acid sequence of TgGNC5 is present in the database of the *P.falciparum* sequencing project. The EST corresponds to a cDNA sequence of 748 bp (GenBank accession no. AA550570, clone 1735m). This partial *P.falciparum* cDNA clone contains an ORF of 178 amino acids showing high homology to the HAT domain of TgGCN5. BLAST searches with the 1735m *P.falciparum* clone retrieved an *A.thaliana* GCN5-related protein as the strongest match, while Tetp55 (HATA1), yGCN5 and hGCN5 are the next highest matches. The 1735m clone sequence reveals 74% identity to the *T.gondii* HAT domain and between 56 and 45% identity with the HAT domains of other GCN5 proteins (Fig. 2). The striking similarities of the 1735m clone with the HAT regions of all the known representatives of the GCN5 family members suggests that this incomplete *P.falciparum* cDNA corresponds to a true GCN5 homologue rather than to a member of the GCN5-related *N*-acetyltransferase superfamily (GNAT) (38).

Introduction of an additional copy of Tg*GCN5* appears to be toxic for *T.gondii* tachyzoites

In order to determine the subcellular localisation of TgGCN5, we used an epitope tagging strategy, taking advantage of the accessibility of *T.gondii* to genetic manipulation. At first, full-length Tg*GCN5* was amplified by PCR and cloned into a bacterial expression plasmid (pET19bHismycTgGCN5), in-frame with six histidines residues and a c-myc epitope tag at the N-terminus of the protein, immediately downstream of the ATG. After induction, bacterial lysate was analysed by SDS–PAGE

Figure 2. Comparison of the predicted *T.gondii* GCN5 protein with the other GCN5 protein family members. (**A**) Partial alignment of the conserved domains of the known GCN5-related sequences. Amino acids are shaded if three proteins have identical (black) residues at that position. Amino acid deletions are indicated with a dotted line. The F224A mutation is circled. (**B**) A schematic representation of the GCN5 family is shown, along with the percentage of identical amino acid residues shared between TgGCN5 and the GCN5 and P/CAF proteins from other species. Positions of the catalytic HAT domains, the bromodomains and the P/CAF homology are indicated above the diagram.

followed by Coomassie blue staining (data not shown). The TgGCN5 protein migrates to a position of ~65 kDa, although the predicted molecular mass of TgGCN5 is 53 kDa. Western blot analysis using monoclonal antibodies recognising the His tag confirmed the identification of the polypeptide expressed by the pET19bHismycTgGCN5 construct detected in the Coomassie blue staining (Fig. 4). The lower molecular weight band is due to the presence of a cross-reaction of anti-His antibody with a bacterial protein, since the band is also detectable in bacteria lacking pET19bHismycTgGCN5 (data not shown). The pET19bHismycTgGCN5 construct was used to construct the pSTgGCN5 *T.gondii* expression vector carrying the positive selectable marker gene *HXGPRT*. pSTgGCN5 was introduced into tachyzoites by electroporation. Recombinant parasites were selected for resistance to mycophenolic acid in the presence of xanthine (30). Unfortunately, no stable recombinant parasites could be obtained after selection, even after several independent trials suggesting that an additional copy of *GCN5* is not tolerated by the tachyzoites. Only transient recombinant parasites expressing the c-myc-tagged version of TgGCN5 could be detected by indirect immunofluorescence assay. Transfected parasites were able to undergo at least two rounds of replication before dying. No significant alteration in their morphological appearance was noticeable. The mAb 9E10 recognising the myc epitope revealed that TgGCN5 transgenic protein was distributed homogeneously in the cytoplasm of the parasite (Fig. 5A). To confirm that the recombinant parasites express a complete version of TgGCN5, transiently transfected parasites expressing TgGCN5 were analysed by western blot using mAb 9E10 as illustrated in Figure 5B. The migration of transiently expressed mycGCN5, as observed on SDS–PAGE, was in agreement with its predicted size of 53 kDa.

Disrupting the HAT activity allows overexpression of TgGCN5 in *T.gondii* tachyzoites

To determine whether the inability to express a second copy of the *GCN5* gene was due to the presence of excessive HAT activity or abundance of the bromodomain titrating out unknown factors, we have introduced a point mutation in the HAT domain of TgGCN5 to reduce its HAT activity (15). In a previous study, a single mutation (F221A) in the catalytic domain of the yGCN5 protein has been shown to inactivate the HAT activity *in vivo* and *in vitro*. Due to the high conservation of the amino acids in the HAT domains of all GCN5-related proteins we introduced a point mutation, F224A, at the same position in the TgGCN5 protein by site-directed mutagenesis. The resulting construct, pSTggcn5F224A, could be successfully integrated into tachyzoites, again using *HXGPRT* as the positive selectable marker gene. Transgenic parasites stably expressing pSTggcn5F224A were cloned and IFA analysis using mAb 9E10 revealed that the protein is faithfully expressed in the parasites (Fig. 5A). The Tggcn5F224A mutant showed the same homogeneous expression in *T.gondii* tachyzoites as the wild-type mycGCN5 (Fig. 5A). Western blot analysis with 9E10 showed that the transgenic protein migrates on SDS–PAGE as predicted by the amino acid sequence deduced from the Tg*GCN5* gene (Fig. 5B).

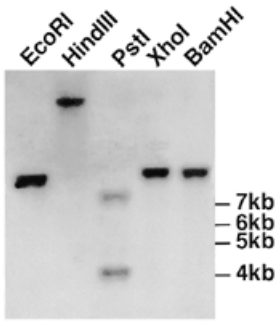


Figure 3. Southern blot analysis of Tg*GCN5*. Southern hybridisation of *T.gondii* genomic DNA digested with different restriction enzymes. The probe of 250 bp spans the Tg*GCN5* bromodomain.

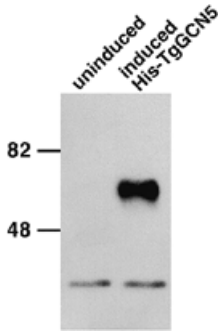


Figure 4. Bacterial expression of TgGCN5 after IPTG induction. Western blot analysis of recombinant TgGCN5. Crude bacterial extract was loaded on SDS–PAGE gels and analysed by staining with His mAb. A protein size standard is shown on the right.

The HAT of *T.gondii* GCN5 complements the *gcn5*$^-$ growth defect in yeast

In order to determine whether TgGCN5 can functionally replace yeast GCN5 *in vivo*, we tested the ability of the *T.gondii* gene to complement the *gcn5*$^-$ mutant of *S.cerevisiae*. The characteristic phenotype of *gcn5*$^-$ is poor growth on minimal medium (10). The yeast *gcn5*$^-$ mutant strain (21) was transformed with the multicopy expression vector pRS423 bearing either complete *S.cerevisiae GCN5* (pRS423yGCN5) or the *T.gondii GCN5* gene (pRS423TgGCN5). The mutant strain (*gcn5*$^-$) as well as the wild-type strain (GCN5) (21) were transformed with the empty pPRS423 vector to serve as a control for the *gcn5*$^-$ phenotype and wild-type growth on minimal medium. The growth of each transformed strain on minimal medium was then examined (Fig. 6A). On minimal medium only the wild-type strain transformed with the empty pPRS423 plasmid and the *gcn5*$^-$ mutant strain transformed with the plasmid bearing the yeast *GCN5* gene could grow. The construct bearing the Tg*GCN5* gene failed to complement the *gcn5*$^-$ phenotype (Fig. 6A). In conclusion, Tg*GCN5* is not capable of complementing the *gcn5*$^-$ mutant phenotype in *S.cerevisiae*.

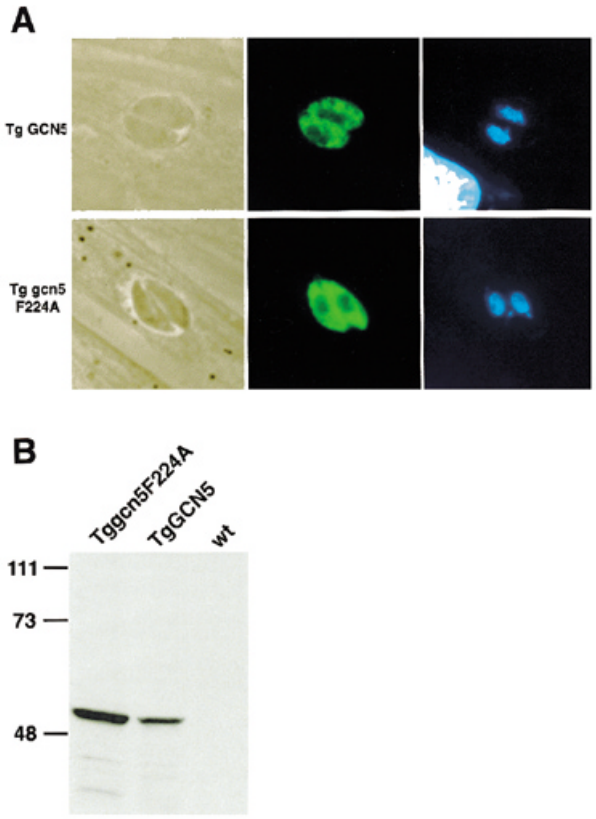


Figure 5. Localisation and expression of c-myc-tagged TgGCN5 and the mutated protein in *T.gondii* tachyzoites. (**A**) Parasites transiently transfected with pSTgGCN5 or stably with the pSTggcn5F224A construct were fixed after 24 h, permeabilised and incubated with mAb 9E10 followed by FITC-labelled secondary antibody for immunofluorescence microscopy. TgGCN5 as well as the point mutated Tggcn5F224A protein show the same distribution throughout the cytoplasm. (**B**) Western blot analysis of parasites transformed transiently with pSTgGCN5 or stably with pSTggcn5F224A. Parasites were transfected with 80 µg of each plasmid. Cell lysate corresponding to 10^7 parasites was loaded in each lane. After blotting the membrane was probed with an anti-myc Ab. A band of M_r ~50 kDa was detected in both transient and stable parasites. As a control the same amount of wild-type RH parasites were loaded.

The chimera hhyy, comprising the first half of hGCN5 (including the HAT domain) fused to the second half of yGCN5, has been shown to partially complement *gcn5*$^-$ (27). This demonstrates that the HAT function is sufficiently conserved to exhibit complementation for growth.

To test whether the *T.gondii* HAT domain also provides the same conserved function for growth complementation, we constructed the ttyy chimera, comprising the HAT domain of TgGCN5 (amino acids 1–264) fused to the remainder of yGCN5, and transformed this chimera into the yeast *gcn5*$^-$ background. As a control the *gcn5*$^-$ strain was transformed with the hhyy chimera and the empty plasmid as negative control. The wild-type strain was transformed with the empty plasmid to show normal growth on minimal medium. The growth abilities of the transformed strains were examined again on minimal medium (Fig. 6B). In agreement with the results obtained for the hhyy chimera, the ttyy chimera showed partial complementation (Fig. 6B). This result demonstrates that TgGCN5 protein possesses a HAT activity and that this activity is interchangeable between the members of the GCN5-related proteins.

To confirm that point mutation F224A introduced into the HAT domain of TgGCN5 has reduced HAT activity, we constructed the chimera ttyyF224A and tested for phenotypic complementation of *gcn5*$^-$ as described above. As predicted and as shown in Figure 6B, the ttyyF224A chimera grew as slowly as the *gcn5* mutant strain on minimal medium, indicating that HAT activity is compromised in the F224A point mutant.

DISCUSSION

The protozoan parasite *T.gondii* has a complex life cycle alternating between the rapidly growing tachyzoites and the slow growing encysted bradyzoites. A large number of genes are differentially regulated during the life cycle of the parasite (39–43). Very little is known about the regulation of gene expression in protozoan parasites in general and in Apicomplexa in particular. In the past few years, the role of chromatin has emerged at the forefront of transcription research (44,45). Discovery and characterisation of the chromatin modifying machinery suggest that it drastically influences transcriptional activity in other systems and it is likely to be important in protozoan parasites as well. Two components of the chromatin remodelling pathway have recently been identified in apicomlexan parasites: the histone deacetylase in *P.falciparum* (PfHDAC1) (46) and a *P.falciparum* homologue of the Snf-2 family (SNF2L), which belongs to the Swi/Snf complex of ATP-dependant nucleosome remodelling factors (47). PfHDAC1 was recently suggested to be a target of apicidin, a fungus-derived antiprotozoal agent exhibiting structural similarity to known HDAC inhibitors (24). Apicidin irreversibly prevents the *in vitro* development of intracellular apicomplexan parasites at low nanogram per millilitre levels by inducing hyperacetylation of parasite histones, suggesting that continuous acetylation/deacetylation of histone tails plays a key role in transcriptional regulation also in apicomplexan parasites. This implies that pathways of similar importance and probable complexity exist in protozoa. The enzymatic machinery involved in gene expression in the Apicomplexa therefore represents a novel target for antiprotozoal therapy. Investigation of the effect of chromatin on promoter activity in apicomplexan parasites is relevant, given the putative implications of chromatin conformation in antigenic variation of the *var* genes in *P.falciparum* (48) and in transcriptional control of the variant surface glycoprotein gene expression sites in the protozoan parasite *Trypanosoma brucei* (49).

We have characterised a HAT in *T.gondii* by virtue of its homology to the yeast *GCN5* gene. The identity ranges between 30 and 38%, when compared to the GCN5 family members. The predicted 53 kDa TgGCN5 protein shows the two typical domains found in all members of the GCN5 family, the N-terminal HAT domain and the C-terminal bromodomain. These high similarities classify TgGCN5 as a member of the GCN5 family. The presence of a *P.falciparum* clone (*P.falciparum* clone 1735m) in the EST database suggests the existence of a homologue of GCN5, although the sequence is only partial and lacks the bromodomain region. Comparison of the putative HAT domain of this clone with the HAT domain of the superfamily of GCN5-related *N*-acetyltransferases emphasises that it belongs to the GCN5 family and not to a different class of putative

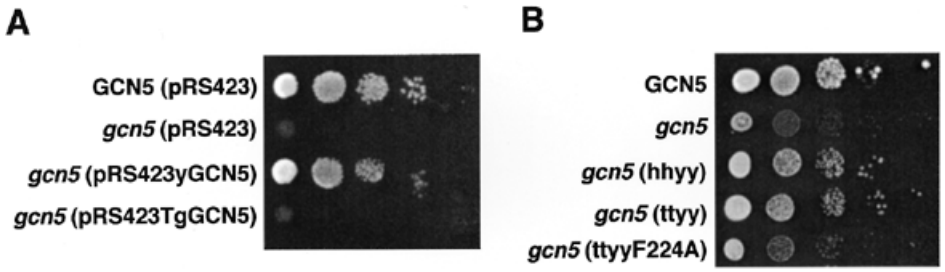


Figure 6. The ttyy chimera partially complements the *gcn5*⁻ growth defect, but not full TgGCN5 protein. (**A**) Growth complementation assay of *gcn5*⁻ transformed with either pRS423yGCN5 or pRS423TgGCN5. The wild-type strain (GCN5) and *gcn5*⁻ transformed with the empty plasmid pRS423 were used as controls. Ten-fold serial dilutions of the transformants were transferred to plates with minimal medium. The plates were incubated for 4 days at 30°C and photographed. (**B**) Growth complementation of TgGCN5–yGCN5 chimeras in the *gcn5*⁻ strain. The *gcn5*⁻ strain was transformed with hhyy, ttyy and ttyyF224A vectors. The wild-type strain (GCN5) and the *gcn5*⁻ strain were used as controls. Ten-fold serial dilutions of the transformants were transferred to plates with minimal medium. The plates were incubated for 4 days at 30°C and photographed.

acetyltransferases (Fig. 2A). The GNAT superfamily includes protein *N*-acetyltransferases, metabolic enzymes, detoxification and drug resistance enzymes and other proteins whose function is unknown (38,50,51). Among the HAT enzymes included in this superfamily are the cytosolic HATs and their homologues, the GCN5 family of proteins and the Esa 1 family, which includes yeast SAS, human MOZ and Tip60, and fly MOF (38,52–57). Neuwald and Landsman (38) have described four conserved sequence motifs (A–D) to characterise the GNAT superfamily. Of these, motif A is the longest and most highly conserved. The cytosolic HATs lack motif C, and the Esa 1 family appears to contain motifs A and B in reverse order. The *P.falciparum* HAT domain of clone 1735m contains all of the four conserved regions in the right order. The presence of *P.falciparum* and *T.gondii* homologues of GCN5 reinforce the notion that, as in higher eukaryotes, chromatin remodelling is also a key player in the regulation of gene expression in lower eukaryotes.

We failed to stably introduce an additional copy of *GCN5* in *T.gondii* tachyzoites. This result suggests that a fine balance between histone acetylation and deacetylation is likely to be essential for survival. This notion is confirmed by the fact that the histone deacetylase inhibitor apicidin, which is a potent novel antiprotozoal agent, causes hyperacetylation of histones in treated parasites (24). We postulated that overexpression of GCN5 in tachyzoites would induce hyperacetylation of histones and thus cause deleterious effects. To test this hypothesis directly, we disrupted the HAT activity of TgGCN5 by introducing a point mutation. The choice of the point mutation was based on a recent study reporting that a single point mutation, F221A, in the minimal catalytic domain of the yeast GCN5 protein significantly reduced HAT activity *in vivo* and *in vitro* (15). Due to the high conservation of the amino acid sequence of the HAT domain in all GCN5 family members, we introduced the point mutation (F224A) at the same position in *T.gondii* GCN5. As expected, this mutated version of TgGCN5 could be stably expressed in tachyzoites. This result suggests that hyperacetylation, due to overexpression of TgGCN5 protein, is likely to be responsible for the toxic effect observed. It stresses the fact that likely key genes are also controlled by HATs and deacetylases in apicomlexan parasites.

GCN5 is a HAT enzyme using nucleosomal histones as substrate and therefore is expected to localise in the nucleus. The *Tetrahymena* enzyme was purified from isolated macronuclei (7), but the enzyme has never been immunolocalised. We used an epitope tagging-based approach to determine localisation of the Tg*GCN5* gene product in *T.gondii* tachyzoites. TgGCN5 transgenic protein as well as the mutated version of TgGCN5 are faithfully and homogeneously expressed in the parasite (Fig. 5A). It was surprising to find c-myc-tagged GCN5 evenly distributed in the cell rather than accumulated in the nucleus, but we cannot exclude possible overexpression which might consequently cause mislocalisation by saturation of localisation and binding partners. The *S.cerevisiae* GCN5 protein apparently has no nuclear localisation signal (NLS) but it is the catalytic subunit of two high molecular mass native HAT complexes, the ADA and the SAGA (Spt-Ada-Gcn5 acetyltransferase) complexes (58). In these complexes GCN5 has been shown to interact directly or indirectly through ADA2 with a number of transactivators, for example VP16 (59), GCN4 (17) and Adr1 (60). Therefore, the catalytic activity of GCN5 is likely to be targeted to a promoter through interaction with one of these complexes and might contribute to entry into the nucleus.

TgGCN5 protein shows high similarity to yeast GCN5. Despite the common structural characteristics, the Tg*GCN5* gene is unable to complement the growth defect in the *gcn5*⁻ *S.cerevisiae* mutant. This was not unexpected, since the full human GCN5 also failed to complement this mutant even when it was expressed at levels that far exceeded normal endogenous yGCN5 levels (27).

The highly conserved middle region of yGCN5 (amino acids 116–260, the HAT domain) shares features with other acetyltransferases (7). This region encompasses the HAT activity (14,61), which was confirmed by analysing deletion mutants of yGCN5. Recently the crystal structures of the HAT domain of yGCN5 as well as of *Tetrahymena* GCN5 and P/CAF were determined (62–65). Deletion of this region in yGCN5 resulted in failure to complement growth and complete loss of HAT activity (14,27). When the N-terminal half of TgGCN5 (amino acids 1–264), including the HAT region, was fused to the functionally inert C-terminal half of yGCN5 (amino acids 262–439), the resulting chimera (ttyy) partially complemented

function in the *gcn5*⁻ mutant strain in a growth assay. Our results confirm that TgGCN5 protein possesses a HAT activity and that this activity is functionally homologous to the HAT domain of yGCN5 protein. The ttyy chimera (ttyyF224A), harbouring a point mutation in the HAT domain, is reduced in its capacity to complement the growth defect in the yeast *gcn5*⁻ mutant strain. Thus, the interspecies chimeras demonstrate that the HAT domain is functionally conserved and important for cell growth, however, for complementation the two other domains in the y*GCN5* gene are necessary. The level of sequence conservation in the C-terminal domain clearly identifies a bromodomain in the Tg*GCN5* gene (37), which is found in the GCN5 family as well as in other transcriptional regulatory factors (35,36). The role of the bromodomain is still unclear, though it is known that the bromodomain is indispensable for the function of GCN5 in yeast (19,66,67). The yGCN5 protein requires, besides the HAT domain and the bromodomain, its ADA2 interaction domain for *in vivo* function (14). Since Tg*GCN5* does not complement the corresponding yeast mutants, the complex must differ in a significant way, which might imply that gene regulation by this remodelling factor is different from that in yeast. *GCN5* is a non-essential gene in yeast (37) whose product participates in the expression of only a small number of genes (68). Overproduction of functional yGCN5 leads to hyperacetylation of histones in the promoter region and transcriptional activation of target genes (15). Overexpression of TgGCN5 in tachyzoites seems to be toxic for the parasite. The impossibility of expressing a second wild-type copy of *GCN5* in tachyzoites suggests that fine tuning between histone acetylation and deacetylation is important for survival of the parasites and that key genes are controlled by TgGCN5 activity. Disruption of the *GCN5* gene in *T.gondii* might assist us in the identification of the pathway controlled by this transcriptional regulator.

In *P.falciparum* and *T.brucei* there are several hints at the participation of chromatin remodelling in antigenic variation of the *var* genes and regulation of the *VSG* genes. Antigenic variation has not been reported in *T.gondii* but intracellular growth or differentiation are subject to remarkable changes in gene expression which might be controlled at the level of chromatin structure. The importance of chromatin remodelling in the control of gene expression in eukaryotic pathogens has not been investigated yet, but the clear parasiticidal effect of the histone deacetylase inhibitor apicidin demonstrates the importance of acetylation and deacetylation of histones in Apicomplexa.

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