Published in final edited form as:

J Mol Biol. 2009 September 11; 392(1): 33–47. doi:10.1016/j.jmb.2009.07.017.

***Toxoplasma* H2A Variants Reveal Novel Insights into Nucleosome Composition and Functions for this Histone Family**

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Abstract

Toxoplasma gondii is an obligate intracellular parasite. Toxoplasmosis is incurable because of its ability to differentiate from the rapidly replicating tachyzoite stage into a latent cyst form (bradyzoite stage). Gene regulation pertinent to *Toxoplasma* differentiation involves histone modification, but very little is known about the histone proteins in this early branching eukaryote. Here we report the characterization of three H2A histones, a canonical H2A1 and variants H2AX and H2AZ. H2AZ is the minor parasite H2A member. H2A1 and H2AX both have an SQ motif, but only H2AX has a complete SQ(E/D)φ (φ denotes a hydrophobic residue) known to be phosphorylated in response to DNA damage. We also show that a novel H2B variant interacts with H2AZ and H2A1 but not with H2AX. Chromatin immunoprecipitation (ChIP) revealed that H2AZ and H2Bv are enriched at active genes while H2AX is enriched at repressed genes as well as the silent *TgIRE* repeat element. During DNA damage, we detected an increase in H2AX phosphorylation as well as increases in *h2a1* and *h2ax* transcription. We also found that *h2ax* expression, but not *h2a1* and *h2az*, increases in bradyzoites generated *in vitro*. Similar analysis performed on mature bradyzoites generated *in vivo*, which are arrested in G0, showed that *h2az* and *h2ax* are actively expressed and *h2a1* is not, consistent with the idea that *h2a1* is the canonical histone orthologue in the parasite. The increase of H2AX, which localizes to silenced areas during bradyzoite differentiation, is consistent with the quiescent nature of this life cycle stage. Our results indicate that the early-branching eukaryotic parasite *Toxoplasma* contains nucleosomes of novel composition, which is likely to impact multiple facets of parasite biology, including the clinically important process of bradyzoite differentiation.

Keywords

Toxoplasma gondii; histone; nucleosome; development; chromatin; epigenetics

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Introduction

The protozoan parasite *Toxoplasma gondii* is an important human and veterinary pathogen¹. Because of the late development of the cellular immune response during fetal maturation, *Toxoplasma* has long been associated with causing congenital birth defects. More recently, *Toxoplasma* has achieved additional notoriety as a cause of life-threatening opportunistic disease in immunocompromised individuals, including cancer chemotherapy patients, transplantation patients, and individuals with AIDS or other immunosuppressive disorders^{2; 3; 4}. In addition, *Toxoplasma* is listed as a Category B pathogen in NIAID's organisms of interest for biodefense research. Asexual replication of *Toxoplasma* parasites in humans and intermediate hosts is characterized by two stages: rapidly growing 'tachyzoites' and latent 'bradyzoite' tissue cysts. Tachyzoites are responsible for acute illness and congenital birth defects, while the more slowly dividing bradyzoite form can remain latent within the tissues for many years, but capable of reconvert to destructive tachyzoites if host immunity wanes. These two developmental stages are essential for disease propagation and causation.

The molecular mechanism driving *Toxoplasma* conversion from tachyzoite to bradyzoite is not understood. It was demonstrated, however, that covalent histone modifications influence gene expression relevant to the differentiation of *Toxoplasma*⁵. A number of histone acetylation and methylation modifications have been noted in the upstream regions of *Toxoplasma* genes that influence their expression^{5; 6}. These studies argue that epigenetic events involving the parasite's nucleosomes are likely to play a significant role during parasite differentiation.

Nucleosome octamers are comprised of four types of core histone proteins, two copies each of H2A, H2B, H3, and H4⁷. H2A and H2B form dimers that pair with a H3-H4 tetramer to form the core nucleosome particle. Among the core histones, H2A has the largest number of variants, and the variants found differ among species. The H2A class histones contribute to transcription regulation and DNA repair. DNA damage is associated with monoubiquitylation of H2A and phosphorylation of H2AX⁸. H2AX possesses a C-terminal motif, SQ(E/D) ϕ , where ϕ denotes a hydrophobic residue and S is the serine targeted for phosphorylation in response to double-stranded breaks⁹. Variant histone H2AZ contributes to transcriptional regulation, genome stability, and blocking the spread of heterochromatin^{10; 11}. H2AZ is incorporated into nucleosomes as a heterodimer with H2B by an ATP-dependent chromatin remodeling complex¹², and is an essential histone in most species^{13; 14}.

In contrast to H3 and H4, histones of the H2A and H2B class are remarkably different in protozoan parasites. For example, the H2A sequences are highly divergent compared to higher eukaryotes^{15; 16}, and protozoa possess novel variants of H2B^{17; 18; 19}. Expression analysis of *Toxoplasma h2b* genes showed that canonical *h2ba* is mainly expressed in the highly replicative tachyzoite whereas the variant *h2bv* is equally expressed in tachyzoites and the dormant form bradyzoites¹⁷. Similarly, *Plasmodium falciparum*, the malaria pathogen related to *T. gondii*, also has canonical H2Bs and the variant H2Bv^{19 17}. Recently, *Plasmodium* H2Bv was shown to be acetylated whereas canonical H2B did not exhibit this modification, suggesting these histones have different roles²⁰. Given the important role of histone modifications in parasite physiology, we sought to characterize the unusual *Toxoplasma* H2A histones. Here we describe the development and use of novel, specific antibodies against the H2A family histones to elucidate *Toxoplasma* nucleosome composition during the replicating tachyzoite form. The genomic positions of H2A and the H2B variants were characterized by chromatin immunoprecipitation and quantitative real time polymerase chain reaction (ChIP-qPCR). The expression profiles of H2A1, H2AX, and H2AZ were analyzed in tachyzoites and bradyzoites by quantitative reverse transcriptase -PCR (qRT-PCR) and *Toxoplasma* microarray analysis. The results obtained in this study provide significant insight into the chromatin structure and

gene regulation of early-branching eukaryotic cells such as *Toxoplasma*, and further illuminate the biological roles of H2AX and H2AZ variants.

Results

Identification of *Toxoplasma* H2A histones and sequence analysis

Searches of NCBI and ToxoDB (<http://toxodb.org>) databases revealed three putative H2A histones, which we cloned and sequenced (Figure 1(a)). Two, named H2A1 and H2AX, show high similarity to each other (88% identity and 92% similarity) and are located on chromosome VIIb; the third, named H2AZ, is more divergent and located on chromosome XII. These designations of the *Toxoplasma* H2A homologues were assigned based on sequence similarity to those found in other species. We have shown by neighbor joining analysis that *Toxoplasma* H2AZ clusters with members of the H2AZ group, whereas parasite H2A1 and H2AX cluster with canonical and H2AX from other species (Figure 1(b)) and Sullivan et al. 16.

Toxoplasma H2As show a high degree of divergence compared to H3 and H4, which are highly conserved among most eukaryotes^{16; 21}. *Toxoplasma* H3 and H4 exhibit 94–98% homology with human and *S. cerevisiae* H3 and H4. In contrast, *Toxoplasma* H2AX shows only 78–83% similarity (~69% identity) and H2AZ shows 80–89% similarity (67–81% identity) with human and *S. cerevisiae* counterparts, respectively. *Toxoplasma* H2A1 bears 86% similarity and 73% identity with canonical H2A from human.

In other eukaryotes, the H2AX variant has a trademark C-terminal motif, SQ(E/D) ϕ , where ϕ denotes a hydrophobic residue and “S” is the serine targeted for phosphorylation in response to DNA double-stranded breaks⁹. We identified only one *Toxoplasma* H2A containing the entire C-terminal motif, and thus we designated it H2AX (Figure 1(a)). Interestingly, the H2A we designated H2A1 has a truncated version of the H2AX signature (Figure 1(a)); therefore, we cannot rule out that H2A1 has H2AX-associated functions. The C-termini of all *Toxoplasma* H2As contain the conserved Lys120 shown to be ubiquitinated in other species (Figure 1(a)).

Histone N-terminal sequences are subject to extensive post-translation modifications, which are known to affect chromatin status. *Toxoplasma* H2As have several lysine, arginine, and serine residues in this region that could potentially be acetylated, methylated or phosphorylated (Figure 2(a)). Another notable feature is a conserved histone A repressive (HAR) domain on *Toxoplasma* H2AX, which has been associated with transcriptional repression in other species (Figure 2(a))^{22; 23}.

Expression levels of *Toxoplasma* H2A family members

To facilitate the study of the parasite H2A/H2B histone families, recombinant *Toxoplasma* H2Ba, H2AZ, H2AX and H2A1 (rH2Ba, rH2AZ, rH2AX and rH2A1, respectively) were purified and used to raise polyclonal antibodies. Their specificity was evaluated by Western blot. From mice immunized with rH2A1, two lots of antibodies were obtained: α -H2A1 L1, that recognizes specifically rH2A1 and α -H2A1 L2 that is cross-reactive with H2AX (Figure 3(a) and Figure S1). α -H2AX was highly specific to rH2AX, presenting no reactivity against rH2A1 and rH2AZ proteins (Figure 3(a)). In agreement with this result, α -H2AZ did not cross-react with rH2AX or rH2A1 (Figure 3(a)). A mouse antibody to *Toxoplasma* H2AZ generated using the highly divergent N-terminal region (H2AZNt, residues 1 to 120) showed the same behavior as rabbit α -H2AZ (Figure 3(a)). With respect to H2Bs, no reactive serum was obtained for rH2Ba (data not shown) whereas the α -H2Bv antibody¹⁷ showed a very weak cross-reaction to rH2Ba (Figure 3(a)). These antibody characterization results were confirmed

by competition ELISAs (Figure S1). As expected, each α -H2A antibody stains the parasite nucleus, (Figure 3(b)).

When histones purified from *Toxoplasma* are separated in a 15% SDS-PAGE gel, it is possible to distinguish each H2A histone by Western blot analysis using the specific antibodies we generated. This allows an analysis of the relative abundance of H2As in tachyzoites. Following visualization of histone proteins with Ponceau red, a Western blot analysis was performed with the different anti-H2A antibodies (Figure 3(c)). Since the band recognized with the α -H2AZ is not observed with Ponceau staining, it could be suggested that H2AZ is the minor *Toxoplasma* H2A. In contrast, the bands recognized by α -H2AX and α -H2A1 L1 showed abundant staining with Ponceau, suggesting higher amount of protein. However, it is not possible to determine the relative amount H2AX and H2A1, because other histones (e.g. H3) or small proteins may co-migrate with them.

Analysis of H2A-H2B interactions reveal that H2AZ and H2AX are contained in distinct nucleosomes in *Toxoplasma* tachyzoites

Unlike other eukaryotes, *Toxoplasma* possesses an H2B variant that seems to be the major H2B, called H2Bv¹⁷. Consequently, novel nucleosome arrangements are likely to exist in the parasite. To examine histone-histone interactions occurring within the same nucleosome, the chromatin was treated with micrococcal nuclease, resulting in material consisting of >95% mononucleosomes (Figure 4(a)). Subsequently, interactions among H2As and H2Bv were studied by co-immunoprecipitation (co-IP) on mononucleosomes followed by Western blot analyses. Pre-immune sera did not IP detectable protein (Figure S3). Antibody against the abundant surface antigen protein SAG1 was used as a control for the fidelity of the IP (Figure 4(b)). We found that H2Bv interacts with H2AZ, but not with H2AX and *vice versa* (Figure 4(b)). These data suggest that H2AZ and H2AX comprise different nucleosomes in tachyzoites.

The same interactions were observed when co-IPs were performed using parasite lysate generated by sonication (Figure S4). The resulting genomic DNA (gDNA) fragments were 100 to 600 bp (Figure S4(a)). Under these conditions, complexes including one to four nucleosomes could be immunoprecipitated, in accordance with the size of gDNA fragments generated. We propose that H2Bv- and H2AX-containing nucleosomes are not in close proximity since they do not co-immunoprecipitate (Figure S4(b)).

H2A variants and H2Bv: acetylation status and association with active chromatin

There exists a clear relationship between active chromatin and acetylated histones²⁴. We sought to determine if histone H2A and H2Bv variants are associated to active chromatin. Two independent approaches were performed based on immunoprecipitations of one to several nucleosomes using sonicates lysates. We first immunoprecipitated H2AX, H2AZ and H2Bv with their respective polyclonal serum and performed Western analysis with anti-acetylated H3 antibody (α -AcH3), which is associated with active chromatin in *Toxoplasma*^{5, 6}. The three histone variants co-IP the acetylated-H3 histone (Figure 5(a)). Second, immunoprecipitations with anti-acetylated lysine (α -AcLys) were performed and tested for the presence of H2As and H2Bv by Western blot. α -AcH3 was used as a positive control. Since more than one nucleosome could be pulled down, AcH3, H2AZ, H2A1 and H2Bv are acetylated, associated to acetylated histones, and/or are present in nucleosomes localized next to acetylated histone-containing nucleosomes (Figure 5(b)). In contrast, H2AX was barely detectable in the acetylated lysine IP (Fig 5(b)). By comparing band intensities, it could be observed that the percentage of H2AZ, H2A1 and H2Bv immunoprecipitated from input was lower but similar to that observed for AcH3; however, the percent of H2AX is more than ten times smaller (data not shown). In conclusion, while H2A variants and H2Bv may be associated with or close to AcH3, only

H2AZ and H2Bv show a clear association with acetylated histones and/or are acetylated themselves.

Differential association of H2A and H2B variants at active and inactive genes

Our data indicate that H2AX and H2AZ/H2Bv comprise different nucleosomes, and they could have different acetylation status and/or proximity to acetylated histones, both a hallmark of active chromatin among other roles²⁵. To address this further, ChIP experiments performed with α -H2AX, α -H2AZ and α -H2Bv antibodies followed by qPCR were performed. ChIP experiments with α -AcH3 and α -H4K20me1 (histone H4 mono methylated at K20) were performed at the same time as controls of euchromatin and heterochromatin, respectively^{5; 6}. We used primers that amplify upstream regions of a constitutively active gene (β -tubulin) and a tachyzoite-specific gene (*sag1*) as well as two bradyzoite-specific genes that are repressed during the tachyzoite stage (*ldh2* and *bag1*) (Table 1). Even though H2AX, H2AZ and H2Bv are associated with all genomic regions analyzed, H2AX and H4K20me1 are enriched upstream of repressed genes *ldh2* and *bag1* compared to the active genes, β -tubulin and *sag1*. On the contrary, H2AZ, H2Bv and AcH3 are enriched at active genes relative to the inactive genes (Figure 6 (a)). These data suggest that H2AZ and H2AX may have opposing roles in their regulation of chromatin.

H2AX is associated with TgIRE, a repeat element at the end of parasite chromosomes

The association of H2AX with inactive genes promoted the idea that this histone may also contribute to transcriptionally repressed genomic regions, i.e. silent DNA. In order to identify gene-free regions on *Toxoplasma* chromosomes as a hallmark of silent DNA, nucleotide sequence of different repeat elements (*sat350*, *sat680* and *TgIRE*) were used to search the database. Satellital (*sat350* and *sat680*) DNA repeat elements²⁶ retrieved poorly conserved and incomplete sequences (data not shown), but *TgIRE*²⁷ identified sequences in 9 out of the 14 chromosomes having high sequence homology among them (Figure 6(b)). This repetitive element length varies among chromosomes, but it is always approximately 1900 bp, except in chromosome IV and V, which have a deletion between base pairs 240/270 to 1402 (data not shown). A search analysis at www.toxodb.org for expressed products within *TgIRE* sequence retrieved just four ESTs that are evident in only 3 chromosomes (Figure S5). Three EST (TIGR EST Assemblies: N61085, TC35464 and CK736714) are present in chromosome Ia and XI, whereas the other one (TIGR EST Assemblies: TC39371) is in chromosome X (Figure S4). However, there is no evidence of annotated genes, mass-spectrometry peptides or predicted proteins. Moreover, no annotated genes have been described at *TgIRE* flanking regions. We propose, therefore, that *TgIRE* represents a generally silent DNA region. In support of this, H4K20me1 is enriched at these regions whereas AcH3 is completely absent when analyzed by ChIP-qPCR (Figure 6(a)). In correlation with that observed before, H2AX is highly enriched in *TgIRE* whereas H2AZ and H2Bv are not. This observation is consistent with the idea that H2AX is a histone variant that is associated with gene silencing in *Toxoplasma*.

H2AX and oxidative DNA damage in tachyzoites

H2AX is involved in the cellular DNA damage response, becoming phosphorylated at its SQ (E/D) ϕ (ϕ denotes a hydrophobic residue) motif following double strand breaks (DSB)^{8; 28}. In order to elucidate if this process is conserved in *Toxoplasma*, extracellular tachyzoites were incubated with 0 to 400 μ M H₂O₂ for 1 h at 37°C. Samples were then subjected to Western blot analysis with α -H2AX and anti-phospho histone H2AX (α - γ H2AX), the latter being a mouse monoclonal antibody that specifically recognizes the last 8 amino acids, including the phosphorylated motif of human H2AX. Both antibodies recognize one band with the same migration rate under conditions described in Materials and Methods. Anti-tubulin (α -Tub) was used as a control for protein concentration. Band intensities were quantified and γ H2AX/Tub

and H2AX/Tub ratios were determined. While H2AX levels were the same in all conditions, γ H2AX increased in a dose-dependent manner following exposure to DNA damage mediated by H₂O₂ exposure (Figure 7(a)).

Although histone variants exhibit cell cycle independent transcription²⁹, there are no mechanisms described that account for alterations in their expression patterns. We examined if oxidant stress has an effect on H2A expression by qRT-PCR. Results show that transcription of *h2a1* and *h2ax* was stimulated by H₂O₂ in a dose-dependent manner, but this was not the case for *h2az* (Figure 7(b)). Taken together, these data suggest that H2AX and H2A1 are likely to be associated with oxidant DNA damage stress.

H2A expression patterns during bradyzoite formation

The gene expression profiles of H2As were studied between tachyzoites and bradyzoites. Intracellular RH Δ *uprt* tachyzoites were incubated in bradyzoite conversion conditions (CO₂ deprivation and pH 8.1). The differentiation rate was analyzed by immunofluorescence assay (Figure 8(a)) and by monitoring expression of bradyzoite marker genes *bag1/hsp30* and *ldh2* and a tachyzoite marker gene *sag1* (Figure 8(a) and (b)). In all cases, bradyzoite induction showed a significant increase of *h2ax* ($p < 0.0001$) and *h2a1* ($p = 0.0272$) mRNA levels in comparison with tachyzoite when analyzed by qRT-PCR (Figure 8(b)). In contrast, *h2az* mRNA was not altered between stages (Figure 8(b)). The qRT-PCRs were normalized to β -*tubulin*, which exhibits no significant change in expression during bradyzoite induction at pH 8.1³⁰.

These results were corroborated by a microarray analysis of RH strain using the Affymetrix ToxoGeneChip³¹, in which parasites stressed for 3 days in pH 8.1 media were compared to unstressed parasites (Sullivan, to be published elsewhere). As shown in Figure 8(c), intracellular RH parasites exposed to alkaline pH, a known trigger of bradyzoite gene expression, increase expression of *h2ax*, *h2a1* and *h2ba*, whereas *h2az* and *h2bv* remain unchanged. Interestingly, H2Bv, which dimerized with H2AZ but not with H2AX, exhibits the same expression pattern as H2AZ. Moreover, H2Ba, the expected H2AX partner, increases in expression under bradyzoite conditions as well as H2AX and H2A1.

These results raise the possibilities that *h2ax* and *h2a1* are involved in bradyzoite development or are associated to a specific cell cycle state, or both. Since mature bradyzoites isolated from animals are in G1 or G0 growth arrest with uniform 1N DNA content^{32; 33}, we examined the expression profiles of H2As from cysts harvested from infected mouse brain. Reverse transcription followed by PCRs were performed using mRNA obtained from brain cysts of experimentally infected mice (30 days post-infection). Figure 8 (d) shows that *h2a1* is not expressed in mature bradyzoites, whereas *h2ax* and *h2az* mRNAs were detected in this parasite stage. As canonical histones are well known to be exclusively expressed during the S-phase of the cell cycle³⁴, it can be inferred that H2A1 is indeed the canonical H2A in *Toxoplasma*.

Discussion

Here we show that *Toxoplasma* possesses H2A1, H2AX, and H2AZ histones, with the latter seeming to be the minor H2A subtype in tachyzoites. Protozoan parasites like *Trypanosoma spp* and *Plasmodium spp*. do not have an H2AX. In contrast, *Giardia spp*. and *Cryptosporidium spp*. appear to have replaced the canonical H2A with H2AX¹⁶. The impact of these varied H2A subtypes among early-branching eukaryotes on cellular physiology is unknown.

An interesting finding from this study is that H2AX did not form a dimer with H2Bv whereas H2AZ did, and H2AX and H2AZ are not in the same nucleosome, indicating that H2AX and H2AZ/H2Bv histones could have different roles in chromatin dynamics. *T. brucei* H2AZ

dimerizes with H2Bv, but in this case H2AZ did not pull down with canonical H2A¹⁸. Moreover, both H2AZ and H2Bv localize within the nucleus in a pattern that is distinct from canonical H2A, suggesting that H2AZ and H2Bv function together within a single nucleosome. Since *T. brucei* does not have a H2AX variant¹⁶, it is possible that H2AZ and canonical H2A nucleosome deposition is analogous to H2AZ and H2AX in *T. gondii*. Since no H2B variant has been observed in higher eukaryotes, it is likely that protozoan parasites have a novel layer of chromatin regulation based on the incorporation of H2AZ and H2Bv in nucleosomes.

In agreement with the suggestion that *Toxoplasma* H2AZ and H2Bv histone variants could have unique features and differences in chromatin modulation, we observed that H2AX, H2AZ, and H2Bv can be found in upstream regions of active and inactive genes, but with different enrichments. A more refined analysis indicated that H2AX is enriched at inactive genes as well as silent genomic regions (*TgIRE*) whereas H2AZ and H2Bv are enriched at active genes. The precise role of H2AZ varies among the species that have been studied to date. It has been described to be positively and negatively regulate transcription. Genome-wide localization studies performed in yeast revealed that H2AZ is preferentially located at inactive promoters^{18; 35; 36; 37; 38}. Raisner *et al*³⁹ found no correlation between transcribed genes and the presence of H2AZ in their promoter regions. However, three recent studies in human T cells⁴⁰, *C. elegans*⁴¹ and *T. brucei*⁴² report a positive correlation between H2AZ occupancy at promoters and transcriptional activity. Notably, *T. brucei* H2AZ and H2Bv are contained in the same nucleosome and can be detected at probable RNAPol II transcription start sites (TSS); such nucleosomes were less stable than those containing the corresponding core histones, suggesting that both H2AZ and H2Bv contribute to a more open chromatin structure at the TSS. Our studies of *Toxoplasma* H2AZ support the idea that this histone subtype is correlated with active promoters. Once again H2AZ and H2Bv in Apicomplexas and Trypanosomatids seem to have a novel means of modulating active chromatin.

H2AZ and H2Bv could be immunoprecipitated with acetylated H3, which is a hallmark of active chromatin in eukaryotes including *Toxoplasma*^{5; 6}, or are acetylated or proximal to acetylated histones. This is in concordance with their enrichment on active promoters. In contrast, H2AX can co-IP AcH3, but seems to be poorly associated with acetylated histones and is clearly enriched at silent DNA regions. The AcH3 that is seen in our H2AX co-IPs may represent the portion of AcH3 that borders silent DNA regions or that co-localizes with the small fraction of H2AX detected in active regions by ChIP. Remarkably, when co-IPs were performed with only one antibody (anti-AcLys) able to pull-down any acetylated protein, H2AX was virtually undetectable whereas H2AZ, H2Bv and H2A1 were detected in a similar ratio to AcH3 compared with the input (1% of tachyzoite lysate). Recently, a proteomic analysis showed that *Plasmodium* H2AZ has a high level of acetylation at its N-terminal tail²⁰. This study also showed that H2Bv is acetylated at its N-terminal region whereas canonical H2B is not, implicating a novel differentiation of H2B function in *P. falciparum*. Future studies should be carried out to shed more light on the modification status of *Toxoplasma* H2As and H2Bs histones.

It is well established that H2AX becomes phosphorylated after double strand breaks (DSB) in response to DNA damage⁸. H2AX may also function during the replication of facultative heterochromatin on the inactive chromosome X^{45; 46}. Our studies show that in tachyzoites, H2AX is deposited preferentially at silent regions of DNA, either proximal to inactive genes or at probable non-transcribed repeat elements like *TgIRE*. These results link H2AX with a novel role associated with gene silencing in *Toxoplasma*. We have considered that the H2AZ/H2AX ratio is an important factor in the regulation of chromatin dynamics, including gene silencing, gene activation, and the switching between the two processes. Interestingly, *Toxoplasma* H2AX has the typical "SRS" motif located at loop 1, resulting in a well conserved HAR pattern²²; H2AZ, however, displays a low degree of similarity to the HAR domain

relative to H2AX. It will be of interest to analyze if these HAR domains are functional in *Toxoplasma* H2As and if they influence incorporation into silent or active DNA regions.

Further functional analysis of the H2A family in *Toxoplasma* revealed both conserved and novel functions. Phosphorylation of the SQ motif increased in H₂O₂-treated parasites in a dose-dependent manner, consistent with parasite H2AX being associated to DNA damage response as previously shown for other eukaryotes⁸. Since *Toxoplasma* has an H2A1 that contains a truncated C-terminal motif ("SQ"), it may also be phosphorylated. Interestingly, *h2ax*, and to a lesser extent, *h2a1*, mRNA increase during H₂O₂ treatment, perhaps to replace the phosphorylated histones that are evacuated after DNA repair⁴⁷. However, a similar *h2ax/h2a1* expression profile was observed during bradyzoite development, making it possible that the oxidative damage is also initiating bradyzoite differentiation. In contrast to that observed by qRT-PCR, the increase of H2AX expression by Western blot was not observed. It is possible that for the time of stress (30 min), the changes in transcription are more evident than changes at the protein level, or that extracellular tachyzoites are more transcriptionally active than translationally active.

In general, canonic histones are the major histones and their expression is linked to the S-phase of the cell cycle. Our data also shows that *h2a1* is expressed in tachyzoites and bradyzoites generated *in vitro*, the latter of which are still in a replicative state³². When mature bradyzoites were analyzed, only *h2aX* and *h2aZ* are expressed. These data indicate that *h2a1* expression is observed only in parasite populations that can undergo S-phase. This observation is consistent with H2A1 being the canonical H2A.

It has been established that histone modification is important for critical parasitic processes, such as differentiation, but it would also be important to investigate if nucleosome composition also changes during stage conversion. Our analysis of the bradyzoite stage gave a clear indication of *h2ax* expression, as well as a weak increase in *h2a1* expression associated to a specific state at the cell cycle. In contrast, *h2az* levels remained stable during bradyzoite development, inferring that the effect on the *h2ax* gene is due to bradyzoite development signals. Even though there are several genes that are upregulated during bradyzoite differentiation⁴⁸, in general gene expression would be expected to be decreased in bradyzoites since they are virtually dormant. It is tempting to speculate that the increase of H2AX is necessary to spread chromatin repression during the latent bradyzoite stage.

Understanding histones is essential to understand how transcription, replication and other cell cycle processes operate in the parasite. The presence of an H2B variant in *Toxoplasma*, and the highly divergent N-terminal tails of H2A/H2B histones and variants, makes this an intriguing field of study. In this regard, our results show that *Toxoplasma* has a novel nucleosome composition based on H2Bv dimerizing with H2AZ, but not with H2AX. We also found that H2AZ and H2Bv are enriched at active chromatin whereas H2AX is predominant in silent chromatin and over-expressed in the bradyzoite stage, suggesting an important role in parasite differentiation. Based on these data, it would be very important for future studies to determine the post-translational modification map of these histones and to define their genomic localization in different parasite stages.

Materials and methods

Database mining

In order to identify histones of *Toxoplasma*, we took advantage of the genome sequencing database available at www.toxodb.org. We searched ToxoDB for putative H2A homologues using human, yeast and *Plasmodium* H2A sequences and blast^{19; 44; 49}. Protein domains were analyzed on amino acid sequence by using the "conserved domain search" at

<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>. Secondary structure was deduced by using the tools at <http://toolkit.tuebingen.mpg.de/sections/secstruct>. Neighbor-joining tree was constructed with MEGA4 (Molecular Evolutionary Genetics Analysis Software version 4) as described Tamura *et al*⁵⁰. Internal support was measured using 1000 replicates of the heuristic search bootstrap option.

Parasite sources, culture and manipulation

RHΔ*hxgprt* strain was used in all cases except in bradyzoite-related experiments; bradyzoites were generated from RHΔ*uprt* or PK parasites (a clone isolated from cystogenic *Toxoplasma* Me49 strain). These parasites were grown in standard tachyzoites conditions *in vitro*: human foreskin fibroblast (HFF) monolayers were infected with tachyzoites and incubated with Dulbecco's modified Eagle medium (DMEM, Gibco BRL) supplemented with 1% fetal bovine serum at 37°C and 5% CO₂. Parasites RHΔ*uprt* (parasite/host cell ratio <1:10) were induced to differentiate into bradyzoites for 4 days in low CO₂ (0.03%) and pH 8.1 as described in Echeverria *et al*⁵¹. Briefly, a confluent monolayer of HFF was infected with approximately 1×10⁷ parasites in 10 cm diameter tissue culture dish. They were incubated for 10 min on ice and then incubated for 1 h in standard tachyzoite conditions, to permit invasion and initial growth. After this, the medium was removed and replaced with inducing medium (RPMI/HEPES, pH 8.1, 5% fetal bovine serum) and the culture placed in a 37°C incubator (at ambient CO₂ 0.03%). At the fourth day, cells were scraped and the cysts disrupted by syringe passage. To determine the ratio of bradyzoites, we used *Dolichos biflorus* lectin (TRITC-labeled from Sigma) staining and antibody to BAG1⁵². In all conditions, parasites were separated from the cells debris by 3 μm pore filtration. To obtain bradyzoites *in vivo*, PK parasites were used to infect C3H mice. After one month, brains were isolated and homogenized in PBS using a hand-held tissue grinder, centrifuged in 30% Dextran to sediment the cysts, washed and resuspended in PBS. Cysts were visualized and counted by phase contrast microscopy.

Source of the cDNA clone, sequencing and sequence analysis

Predicted H2A sequences were amplified from cDNA or genomic DNA by PCR using specific primers. For H2A1: sense 5'- ggatccATGTCGGCCAAAGGC and antisense 5'- ggatccTTACTGAGACTTCTTGCCCTTG. H2AX: sense 5'- ggatccATGTCCGCCAAAGGTGCAGG and antisense 5'- ggatccCACCAGACAGAATGGCTATCCT. H2AZ: sense 5'- ggatccATGGACGGAGCTGCAAAGT and antisense 5'- aagcttGAGCGACTTCTCGTGGAAG. BamHI and KpnI/HindIII sites were included in sense and antisense primers respectively (underlined sequence). These fragments were cloned in pGEM T easy vector (Promega) and sequenced using Sp6 and T7 primers in a sequencer Perkin Elmer Applied Biosystems ABI 377 and the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, USA). The sequences are available in GenBank (accession numbers: [AY631392](#), [AF502246](#) and [AY573602](#)).

Raising specific polyclonal antibodies

The H2AZ sequence was subcloned in the BamHI and HindIII sites of pQE30 plasmid (Qiagen). H2AX, H2A1 and H2Ba sequences were subcloned into the BamHI and KpnI sites of pRSET-A plasmid (Invitrogen). For H2Ba primers and sequence refer to¹⁷. Recombinant proteins (rH2AZ, rH2AX, rH2A1 and rH2Ba) were expressed in *E. coli* BL21pLys strain, under induction with 0.1mM IPTG overnight at 30°C and purified through a Ni²⁺ column (Qiagen) under denaturing conditions following the manufacturer's instructions. In addition, the N-terminal sequence of H2AZ (first 40 aa) was cloned in BamHI and SalI sites of the pGEX-4T-1 plasmid (Amersham) to generate a glutathion thiotransferase fusion protein (GST)-H2AZNt.

rH2AZNt was expressed and purified with glutathione-sepharose 4B (Amersham). Rabbits were immunized with rH2AZ and rH2AX (300 μ g) and mice with rH2A1, rH2AZNt and rH2Ba (10 μ g). Three boosters of each antigen emulsified with Incomplete Freund's Adjuvant at 2-week intervals followed a primary immunization performed with Complete Freund's Adjuvant. Before antigenic stimulation pre-immune serum was collected from each animal. For anti-H2A1 (α -H2A1) two lots were obtained: lot 1, an aliquot obtained before finishing immunization plan and lot 2 that corresponds to the final bleed (α -H2A1L1 and α -H2A1L2 respectively). The first one showed to be highly specific and the second one presented cross-reaction with rH2AX. No reactive serum anti-H2Ba was obtained.

Western blot (WB) analysis

Tachyzoites were collected, filtered and counted. Recombinant histones were quantified by Abs₂₈₀. Histones were acid purified as described before¹⁷. In all cases, 1×10^7 parasites, 1.5 μ g of recombinant protein or purified histones from a T-25 were loaded per well and resolved by 15% SDS-PAGE. Proteins were transferred to cellulose acetate membrane for 1h at 100V. Western blot was then performed as described⁵¹. The primary antibodies: α -H2AZ, α -H2AX, α -H2AZNt and α -H2Bv¹⁷ were used at 1/3000 for 1 h at room temperature, whereas α -H2A1 L1/L2 were used at 1/300 o.n. at 4°C. Appropriate secondary antibodies were used: phosphatase alkaline-conjugated goat anti-mouse or anti-rabbit (Sigma) along with the NBT and BCIP (Promega) detection system, or horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit employed along with the ECL detection system (Amersham-GE).

Immunofluorescence assay (IFA)

HFF cells grown on cover slips were infected with tachyzoites. After 24 h they were fixed with 4% paraformaldehyde and blocked with 1% BSA. Primary antibodies α -H2AX, α -H2AZ and α -H2AZNt diluted at 1/500 and α -H2A1 L1 diluted 1/50 with 0.5% BSA were incubated for 1 h at room temperature. After several washes with PBS they were incubated with secondary antibodies Alexa fluor goat anti-mouse 594 or Alexa fluor goat anti-rabbit 488 (Invitrogen).

Co-Immunoprecipitation (Co-IP)

Approximately 5×10^8 RH Δ uprt and RH Δ hxgprt tachyzoites washed twice with PBS were used for each immunoprecipitation. On one hand, parasites were disrupted by sonication: they were resuspended in 1 ml lysis buffer (50mM Tris-HCl pH 8, 150mM NaCl, 4mM EDTA, 1% NP-40, plus protease inhibitors), sonicated (15 s at 30% amplitude 3 $\times$) and centrifuged at max speed for 10 min. 1 % of supernatant (Input = IN) was phenol/chloroform extracted and gDNA fragments were ethanol precipitated at 4°C o.n. Samples were visualized in a 1.5% agarose gel electrophoresis (Figure S3(a)). In some cases, tachyzoites were treated with micrococcal endonuclease in order to obtain mononucleosomes as described¹⁸. Briefly, parasites were resuspended in 1 ml permeabilization buffer (100 mM KCl; 10 mM Tris, pH 8.0; 25 mM EDTA; 1 mM DTT) and permeabilized with digitonin (40 μ M) for 5 min at room temperature. After this treatment, parasites were washed and resuspended in cold isotonic buffer (100 mM KCl; 10 mM Tris pH 8; 10 mM CaCl₂; 5% glycerol; 1 mM DTT; 1 mM PMSF). Six units of micrococcal nuclease (Sigma) were added to the cell suspension and incubated for 15 minutes at 28°C. The reaction was stopped by adding EGTA (10 mM final concentration). To improve chromatin solubility, NP-40 and NaCl were added to a final concentration of 0.5% and 200 mM, respectively. Following centrifugation at $\sim 10,000$ g for 10 minutes at 4°C, the supernatant was analyzed for the presence of mononucleosomes by isolating DNA from an aliquot and examining it on a 1.5% agarose gel stained with ethidium bromide (Figure 4(a)).

Sonicated parasites or mononucleosomes were incubated with the antibody of interest (20 μ l) o.n. at 4°C. On the following day, 40 μ l of Protein A/G (Santa Cruz) was added and incubated for 2 h. Immunocomplexes were washed six times with buffer (50 mM Tris, pH8, 200 mM

NaCl, 2 mM EDTA and 1 % NP-40), then resuspended in 60 µl of SDS-PAGE loading buffer. Samples were boiled for 5 min and 20 µl was loaded per well in a 15% SDS-PAGE gel for Western blotting. Negative controls were performed using the pre-immune serum of each antibody. The absence of contaminating proteins was corroborated by Western blot with anti-SAG1 monoclonal antibody (a gift from J. F. Dubremetz, Universite de Montpellier II, France). IPs and immunoblots were also performed with anti-acetyl H3 (α -AcH3, Upstate 17–615) and anti-acetylated lysine (α -AcLys, ImmuneChem ICP0380).

Chromatin immunoprecipitation (ChIP)

Freshly lysed tachyzoites were cross-linked for 10 min with 1% formaldehyde at 37°C, spun and resuspended in cold lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 20mM sodium butyrate, plus protease inhibitors). Samples for ChIP consisted of $\sim 5 \times 10^7$ parasites that were subjected to sonication on ice three times, 15 s each. The following steps were carried out at 4°C unless indicated otherwise. Samples were diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, 167mM NaCl, pH 8.1 and 20mM sodium butyrate) plus protease inhibitor cocktail (Sigma p8340). Ten percent of the lysate was used as input DNA (IN) for normalization, the rest was pre-cleared with 80 µl salmon sperm DNA protein A agarose (Upstate) for 30 min with agitation. Following centrifugation at 1,000 rpm for 1 min, antibody – 10 µl of the rabbit serums (α -H2AX, α -H2AZ or α -H2Bv) or 5 µl of commercial antibodies (α -AcH3 (rabbit polyclonal, Upstate 17–615) or α -H4K20me1 (rabbit polyclonal, Abcam ab9051)) – was added for overnight incubation. Sixty µl of salmon sperm DNA/protein-A agarose was added and incubated for 1 h to collect the antibody-protein-DNA complex. The resulting complex was washed 3× with 1.0 ml of each of the following buffers: Low Salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, 150mM NaCl, pH 8.1); High Salt wash buffer (0.1% SDS, 1% Triton-X 100, 2mM EDTA, 20mM Tris-HCl, 500mM NaCl, pH 8.1); LiCl wash buffer- 0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1. Samples were washed 6× in TE prior to elution in 250 µl elution buffer (1% SDS, 0.1M NaHCO₃) for 15 min at room temperature with agitation. After a spin at 1,000 rpm for 1 min, a second elution step was performed. Crosslinks were reversed from the combined eluates and input samples with 20 µl 5M NaCl and 65°C o.n. Samples were then treated with proteinase K and DNA was purified through PCR Purification Kit columns (Qiagen). DNA samples (1.0 µl) were used as a template for qPCR to detect specific targets. Amplification was performed in a 25 µl final volume containing SYBR Green PCR Master Mix (Applied Biosystems, CA) and 0.5 µM of each forward and reverse primer designed to amplify 5'UTR sequence of *sag1*, *ldh2*, β -*tubulin* (present in chromosome IX) and *bag1* and two regions of *TgIRE* that bear high homology among all the copies present in the parasite. Primer sequences are listed in Table 1. Calibration curve was performed with serial dilutions of the input DNA sample. All reactions were performed in triplicate using the 7500 Real-time PCR system and analyzed with SDS software version number 1.2.1 (Applied Biosystems, CA). A dissociation curve was performed with each pair of primers detecting only one amplification product. PCR products were also visualized in a 3% agarose gel to confirm a single amplicon.

Expression analysis of H2A histones

RNA from tachyzoites and bradyzoites was extracted with the RNAeasy Mini Kit (Qiagen). Reverse transcription was performed with Omniscript RT Kit (Qiagen) using 1 – 2 µg of RNA. Levels of mRNA for designated H2A analyzed by qRT-PCR were given by $2^{-\Delta\Delta C_t}$. Each sample was normalized to β -*tubulin* and calibrated to the tachyzoite sample. Real-time PCRs were performed using the same reaction mix, Real-time PCR system and software described above. Primers were designed to amplify 3'UTRs of each H2A in order to guarantee specificity (Table 1). qRT Tub primers amplify a region in the CDS of β -*tubulin* (ToxoDB gene ID: 57.m00003), showing 84% homology with the other β -*tubulin* genes ((ToxoDB gene ID:

41m00036 and 38m00301). Log₂ of fold change Bradyzoites to Tachyzoites were graphed for each gene analyzed.

The presence of *h2a1*, *h2ax* and *h2az* mRNAs in brain cyst bradyzoites was examined by standard PCRs carried out with primers used to clone them using Taq polymerase (Invitrogen). Products were visualized on a 1% agarose gel containing 0.5 mg/ml ethidium bromide.

Parasite treatment with H₂O₂

Freshly lysed, filter-purified tachyzoites were treated with 0, 100, 200 or 400 μM of hydrogen peroxide (H₂O₂) for 1 h in incubator at 37°C with 5% CO₂. Parasite RNA was extracted, reverse transcribed, and analyzed for histone gene expression using qRT-PCR as described above. For immunoblots, parasites were lysed by sonication and extracts separated in 4–12 % NuPAGE minigels (Invitrogen) run with MES buffer (Invitrogen) for subsequent transfer to nitrocellulose membrane. Membranes were probed with α-H2AX (1/3000), anti-phospho H2AX (α-γH2AX, 1/4000) a mouse monoclonal antibody from Upstate (05–636) and anti-tubulin antibody (1/3000) as protein charge control. Anti-tubulin antibody was a gift generously supplied by Dr. David Sibley (Washington University, MO). Band intensities were quantified using Gel Pro Analyzer version 4.0 (Media Cybernetics) using 1D gel analysis and determining integrated optic density (IOD). The IOD of histone proteins was related to the corresponding tubulin intensity to determine the ratio.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SO Angel (Researcher) and MC Dalmasso (Fellow) are members of National Research Council of Argentina (CONICET). This work was supported by: ANPCyT grant BID1728 OC-ARPICT 05-34415 (to S.O.A.), CONICET-NSF collaborative grant (to S.O.A.-W.J.S.), and National Institutes of Health grants AI077502 and AI073091 (to W.J.S.).

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Figure 1. Sequence alignment and phylogenetic analysis of histones H2A

(a) Alignment of *Toxoplasma* H2A1 (Gene IDs at www.toxodb.org: 55.m04926; TGME49_061250; TGGT1_chrVIIb 1811408–1811794 and TGVEG_chrVIIb 1800384–1800743), H2AX (Gene IDs at www.toxodb.org: 55.m04942, TGME49_061580, TGGT1_008650 and TGVEG_073130) and H2AZ (Gene IDs at www.toxodb.org: 145.m00002, TGME49_100200, TGGT1_095100 and TGVEG_007580) amino acid sequences. Predicted sites of ubiquitylation (Ub), α -helix structure and loops L1 and L2 are indicated. (b) Neighbor joining (NJ) tree of H2A amino acid sequences. Box: H2AZ subfamily. At, *Arabidopsis thaliana* (Accession numbers: AAL85051 and BAF00012), Dm, *Drosophila melanogaster* (Accession numbers: AAN11125 and AAF56631), Gl, *Giardia lamblia*

(Accession number: [AF139873](#)); Hu, human (Accession numbers: [AAN59958](#), [P16104](#) and [CAG33696](#)), Pf, *Plasmodium falciparum* (Accession numbers: [P40282](#) and [CAB39069](#)); Pyy, *Plasmodium yoelii yoelii* (Accession numbers: [CAQ40903](#) and [EAA15833](#)); Sc, *Saccharomyces cerevisiae* ([P04909](#), [P04910](#) and [Q12692](#)), Tg, *Toxoplasma gondii*, Tt, *Tetrahymena thermophilus* ([AAC37292](#), [AAC37291](#) and [CAA33554](#)), Xl, *Xenopus laevis*. Accession numbers ([AAA49769](#), [AAH74188](#) and [AAH74203](#))

Figure 2. Sequence analysis of histones H2A

(a) Comparison of the deduced amino acid sequences of histones H2A N-terminal region. Tg: *T. gondii* histones. Pf: *P. falciparum* histones Hu: human histones (Accession numbers: AAN59958, P16104 and CAG33696). Sc: *S. cerevisiae* histones (P04909, P04910 and Q12692). HTA1 and HTA2 are H2AX histones; HTAZ1 is H2AZ variant. The panel shows sites of post-translational modifications identified in human, yeast and *Plasmodium* H2A. Post-translationally modified residues are indicated as shaded letters. Cylinder indicates part of the histone core. Histone A repressive domain (HAR) is underlined.

Figure 3. Generation of recombinant histones and specific antibodies

(a) The specificity of the antibodies generated was tested based on their ability to recognize the recombinant *Toxoplasma* histones by Western blot. rH2A1, rH2AX and rH2AZ were used to study α -H2A antibodies (α -H2A1 L1, α -H2A1 L2, α -H2AX, α -H2AZ and α -H2AZNt); rH2Ba and rH2Bv were used to detect cross-reaction of α -H2Bv antibody. (b) IFA of intracellular RH tachyzoites performed with α -H2A1 L1, α -H2AX, α -H2AZ and antibodies, and DAPI (nuclear labeling). hN = host cell nucleus. (c) *Toxoplasma* histones analyzed by Western blot with α -H2AZNt, α -H2AX and α -H2A1 L1 antibodies. Histones (H) were purified by acid extraction from RH tachyzoites, electrophoresed in 15% SDS-PAGE, and blotted onto nitrocellulose. Filters were stained with Ponceau red and each one was cut in two pieces. They were then analyzed by Western blot (WB). In both cases, the other H2As were compared to α -H2AX bands. In the blot localized at the left is α -H2AZ Nt whereas in the one at the right side is α -H2A1 L1.

Figure 4. Histone – histone interactions

(a) Tachyzoites were permeabilized with digitonin and treated with micrococcal endonuclease (MNase) in order to obtain mononucleosomes-containing solution to perform co-immunoprecipitations. The presence of mononucleosomes was evaluated by isolating DNA from an aliquot and examining it on a 1.5% agarose gel. **(b)** Lysates 1, 2 and 3 (L1, L2 and L3) were used to perform immunoprecipitations (IP) with α -H2AX, α -H2AZ and α -H2Bv, respectively. The input (IN) corresponds to 1 % of each lysate. Immunoprecipitated material was then examined by Western blotting (WB) for the presence of H2AZ, H2AX, H2Bv and SAG1 (as a contamination control).

Figure 5. Association of *Toxoplasma* H2A and H2Bs with active chromatin

Tachyzoite lysates generated by sonication were used for co-IP analysis. **(a)** Antibodies against *Toxoplasma* H2AZ, H2AX, H2Bv were used to perform co-IP. They were analyzed by Western blots with the same antibodies to ensure the IP worked correctly (CTR). Additionally, anti-acetylated H3 (α -AcH3) antibody was used for Western blot. **(b)** IP was performed with anti-acetylated lysine (α -AcLys) antibody and Western blot analysis were performed with α -AcH3 (positive control), α -H2A1 L1, α -H2AX, α -H2AZ, and α -H2Bv antibodies. IN: input (1% of tachyzoite whole extract).

Figure 6. Genomic distribution of H2A and H2B variants

(a) Chromatin immunoprecipitation (ChIP) was carried out using α -H2AX, α -H2AZ and α -H2Bv. In addition, α -H4K20me1 and α -AcH3 antibodies were used as heterochromatin and euchromatin controls, respectively. Immunoprecipitated DNA was then analyzed by qPCR with primers of *sag1*, β -tubulin (*tub*), *bag1* and *ldh2* promoters and two *TgIRE* regions. **(b)** Localization of *TgIRE* sequences on *Toxoplasma* chromosomes as detected by *in silico* analysis (white boxes). Numbers below chromosomes indicate the position of *TgIRE* in each chromosome, identified by roman numerals.

Figure 7. Phosphorylation and expression analysis of *h2a* genes following DNA damage

Tachyzoites were treated with 0, 100, 200, or 400 μM H_2O_2 for 1 h at 37°C. **(a)** Western blots were performed to detect phospho H2AX (α - γ H2AX) at the left and total H2AX (α -H2AX) at the right. Each one was also blotted for tubulin (α -Tub) as a loading control. Band intensities were quantified and histones were related to tubulin intensities (Ratio). **(b)** Expression of *h2ax*, *h2a1* and *h2az* was analyzed by qRT-PCR normalized to β -tubulin and calibrated to the control without H_2O_2 . This graphic represents one of three independent experiments.

Figure 8. *h2a* gene expression and bradyzoite induction

(a) and **(b)**. RH Δ *uprt* parasites were induced to bradyzoites using pH 8.1 and low CO₂ for 4 days. Relative quantification of expression levels of several genes in tachyzoites (Tz) and *in vitro* bradyzoites (Bz) by qRT-PCR calibrated to tachyzoites using β -tubulin as endogenous control. **(a)** Efficiency of bradyzoite induction (higher than 80%) was analyzed by qRT-PCR of bradyzoite-specific genes *bag1* and *ldh2* and by IFA using *Dolichos biflorus* lectin (DBL) staining (cyst wall labeling) and anti-BAG1 antibody (labels the bradyzoite cytosol). **(b)** Expression of histones H2A. Log₂ of fold change Bz to Tz are graphed for each gene analyzed. Three independent experiments were performed and significant differences between Tz and Bz for each gene amplified were analyzed by Student *T* test (GraphPad Prism software) * $p < 0.05$; *** $p < 0.001$. Tachyzoite-specific gene *sag1* was used as a control. **(c)** Table summarizing microarray data for select histone genes expressed in RH parasites stressed for 3 days with pH 8.1 relative to parallel culture maintained in normal pH. 1.0 indicates no change between stressed and unstressed parasites. **(d)** Brain cysts (BC) were purified from mice brains and visualized by phase contrast microscopy. They were processed to obtain mRNA of mature bradyzoites used to analyze H2A expression by reverse transcription and PCR assay.

Table 1

Primers used for qRT-PCR and qPCR

Name	Sequence	Description	Amplification product (bp from ATG)
qRT-H2A1 F qRT-H2A1 R	5'-AGGACGATGCAGCACACAAC 5'-CCATTAAAAGCCGCTTACGG	<i>h2a1</i> 3'UTR	622 to 715
qRT-H2AX F qRT-H2AX R	5'-CCCGCCTTTTAGGCTTCACT 5'-TGGTGAATTCGGCTCTTTGG	<i>h2aX</i> 3'UTR	615 to 708
qRT-H2AZ F qRT-H2AZ R	5'-TCTGGAGGGACAAACATGGG 5'-AAAAACACGCACCTGACCC	<i>h2aZ</i> 3'UTR	581 to 674
qRT-TUB F qRT-TUB R	5'-ATGTTCCGTGGTCGCATGT 5'-TGGGAATCCACTGAACGAAGT	β -tubulin coding sequence	945 to 1039
qRT-BAG1 F qRT-BAG1 R	5'-TGAGCGAGTGTCGGTTATTT 5'-TAGAACGCCGTTGTCCATTG	<i>bag1</i> coding sequence	536 to 630
qRT-LDH2 F qRT-LDH2 R	5'-ACAATGGCCCAGGCATTCT 5'-CAATAAACATATCGTGAAGCCCAT	<i>ldh2</i> coding sequence	750 to 844
qRT-SAG1 F qRT-SAG1 R	5'-TTAAGTGAGAACCCGTGGCAG 5'-GCTTTTGTACTCGGCTGGAA	<i>sag1</i> coding sequence	696 to 789
q-TUB F q-TUB R	5'-TCCTTTTCTTCATGCACTCGC 5'-GTCGTTCGAATTCTCTGCCC	β -tubulin promoter	-627 to -526
q-BAG1 F q-BAG1 R	5'-TCCTCCCCTGGATCTTCTCTCC 5'-TTGCACAAAACCTGGCAAAGG	<i>bag1</i> promoter	-739 to -638
q-LDH2 F q-LDH2 R	5'-AAGTGTGCACGCTTTGCAAG 5'-CATGCTCCGGGCAGTACCT	<i>ldh2</i> promoter	-505 to -404
q-TgIRE 100 F q-TgIRE 100 R	5'-TTCTTCACGGTTCGGACTT 5'-CAGCAGTAGCCAACCACGT	<i>TgIRE</i> region at 100bp	3 to 96
q-TgIRE 600 F q-TgIRE 600 R	5'-GTTATGAGTCCACCACACGGG 5'-CATGATGTGCATGAAACGTGC	<i>TgIRE</i> region at 600bp	732 to 825
q-SAG1 F q-SAG1 R	5'-AACTTCCCACACGAGGCATT 5'-GATCGGCCCTAATTCAGC	<i>sag1</i> promoter	-653 to -560

q: quantitative real time PCR. qRT: quantitative real time RT-PCR. F: forward. R: reverse