doi:10.1016/j.jmb.2009.07.017 J. Mol. Biol. (2009) 392, 33–47

JMB

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

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Received 10 December 2008; received in revised form 26 June 2009; accepted 7 July 2009
Available online 14 July 2009

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, variants H2AX and H2AZ, and a canonical H2A1. 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 (where ϕ denotes a hydrophobic residue) known to be phosphorylated in response to DNA damage. We 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 found that expression of *h2ax*, but not *h2a1* or *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 expressed but *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 stage of the life cycle. 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.

Edited by J. Karn

Keywords: Toxoplasma gondii; histone; nucleosome; development; chromatin

Introduction

The protozoan parasite *Toxoplasma gondii* is an important human and veterinary pathogen.[1] 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.[2–4] In addition, *Toxoplasma* is listed as a Category B pathogen in the NIAID list of 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

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Abbreviations used: ChIP, chromatin immunoprecipitation; qPCR, quantitative real time PCR; qRT, quantitative reverse transcriptase; co-IP, co-immunoprecipitation.

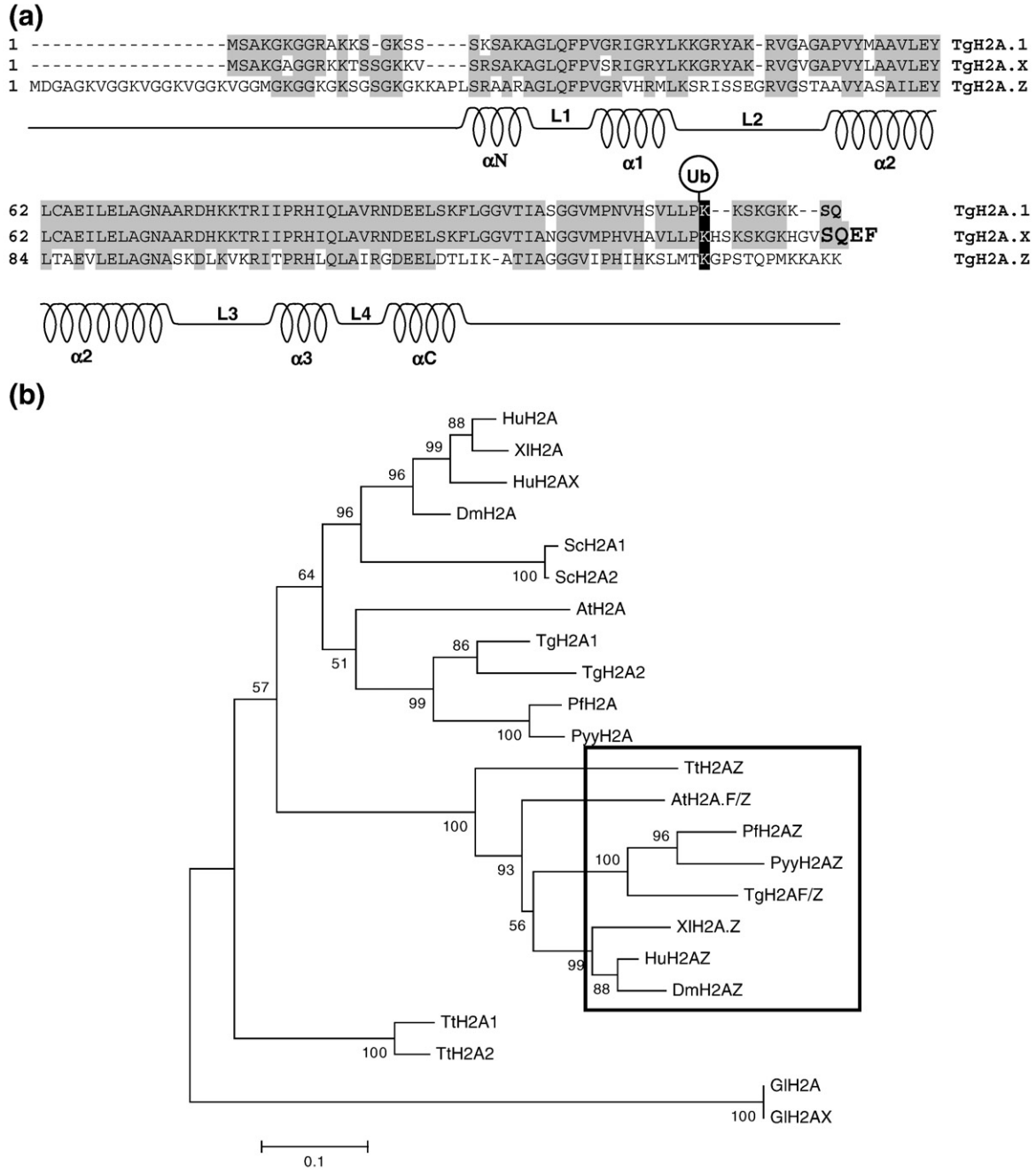


Fig. 1. Sequence alignment and phylogenetic analysis of histones H2A. (a) Alignment of *Toxoplasma* H2A1 (gene IDs at www.toxodb.org: ***55.m04926***; ***TGME49_061250***; ***TGGT1_chrVIIb 1811408-1811794***; and ***TGVEG_chrVIIb 1800384-1800743***), H2AX (***55.m04942***; ***TGME49_061580***; ***TGGT1_008650;*** and ***TGVEG_073130***) and H2AZ (***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* (***AAN11125*** and ***AAF56631***), Gl, *Giardia lamblia* (***AF139873***); Hu, human (***AAN59958***; ***P16104;*** and ***CAG33696***), Pf, *Plasmodium falciparum* (***P40282*** and ***CAB39069***); Pyy, *Plasmodium yoelii yoelii* (***CAQ40903*** and ***EAA15833***); Sc, *Saccharomyces cerevisiae* (***P04909***; ***P04910;*** and ***Q12692***), Tg, *Toxoplasma gondii*, Tt, *Tetrahymena thermophilus* (***AAC37292***; ***AAC37291***; and ***CAA33554***), Xl, *Xenopus laevis* (***AAA49769***; ***AAH74188***; and ***AAH74203***).

converting 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 has been 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

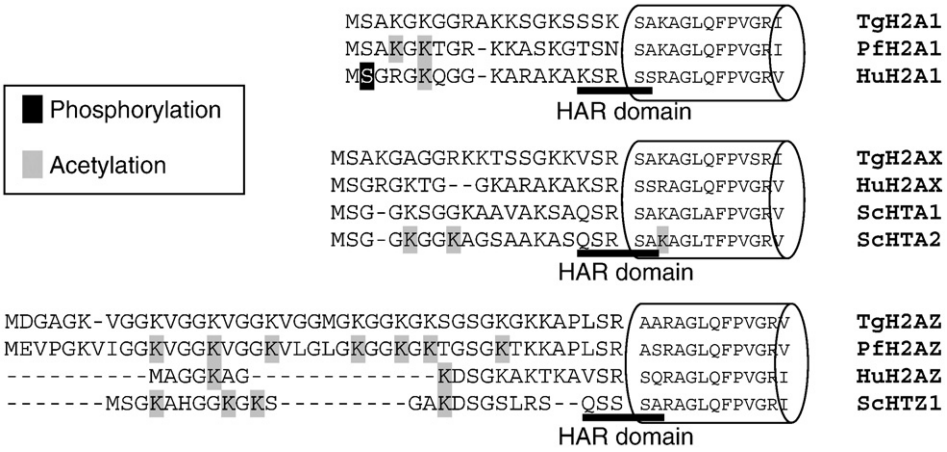


Fig. 2. Sequence analysis of histones H2A. (a) Comparison of the deduced amino acid sequences of histones H2A N-terminal region. Tg, *T. gondii* histones. Pf, *P. falciparum* histones; Hu, human histones (accession numbers ***AAN59958***; ***P16104***; and ***CAG33696***). Sc: *S. cerevisiae* histones (***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 by shaded letters. Cylinder indicates part of the histone core. Histone A repressive domain (HAR) is underlined.

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 have a significant role during parasite differentiation.

Nucleosome octamers are comprised of two copies each of the four core histone proteins H2A, H2B, H3, and H4.[7] H2A and H2B form dimers that pair with an H3-H4 tetramer to form the core nucleosome particle. Among the core histones, H2A has the most 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 H2AX.[8] 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 breaks.[9] 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 complex,[12] 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.[17-19] Expression analysis of *Toxoplasma h2b* genes showed that canonical *h2ba* is expressed mainly in the highly replicative tachyzoite, whereas the variant *h2bv* is expressed equally in tachyzoites and the dormant form, bradyzoites.[17] 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 roles.[20] 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 (ChIP) and quantitative real time PCR (qPCR). The expression profiles of H2A1, H2AX, and H2AZ were analyzed in tachyzoites and bradyzoites by quantitative reverse transcriptase (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‡ databases revealed three putative H2A histones, which we cloned and sequenced (Fig. 1a). Two, named H2A1 and H2AX, are highly similar (88% identity and 92% similarity) and are located on chromosome VIIb; the third, named H2AZ, is more divergent and is located on chromosome XII. These designations of the *Toxoplasma* H2A homologues were assigned on the basis of the 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

‡ http://toxodb.org

and H2AX cluster with canonical and H2AX from other species (Fig. 1b).[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 *Saccharomyces cerevisiae* H3 and H4. In contrast, *Toxoplasma* H2AX shows only 78–83% similarity (~69% identity) and H2AZ shows 80-89% similarity (67–81% identity) to human and *S. cerevisiae* counterparts, respectively. *Toxoplasma* H2A1 bears 86% similarity to 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 breaks.[9] We identified only one *Toxoplasma* H2A containing the entire C-terminal motif, and thus we designated it H2AX (Fig. 1a). Interestingly, the H2A we designated H2A1 has a truncated version of the H2AX signature (Fig. 1a); 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 (Fig. 1a).

Histone N-terminal sequences are subject to extensive post-translation modifications that 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 (Fig. 2a). Another notable feature is a conserved histone A repressive

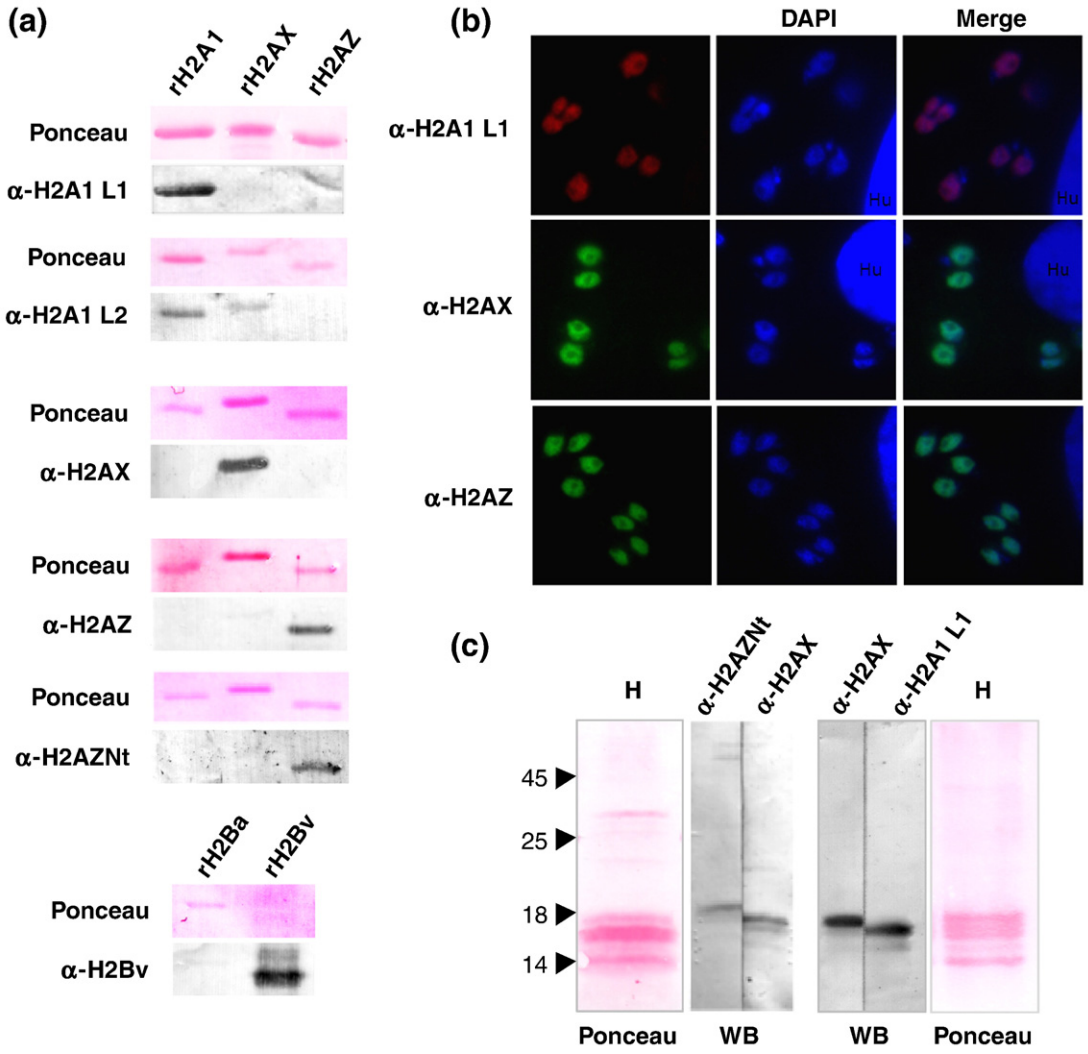


Fig. 3. Generation of recombinant histones and specific antibodies. (a) The specificity of the antibodies generated was tested on the basis of their ability to recognize the recombinant Toxoplasma histones by Western blot. rH2A1, rH2AX and rH2AZ were used to study α-H2A antibodies (α-H2A1 L1, α-H2A1 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, subjected to SDS-PAGE (15% (w/v) polyacrylamide gel) and blotted onto nitrocellulose. Filters were stained with Ponceau red and each one was cut into two pieces, which were analyzed by Western blot (WB). In both cases, the other H2As were compared to α-H2AX bands. α-H2AZ Nt is in the blot localized at the left, α-H2A1 L1 is at the right.

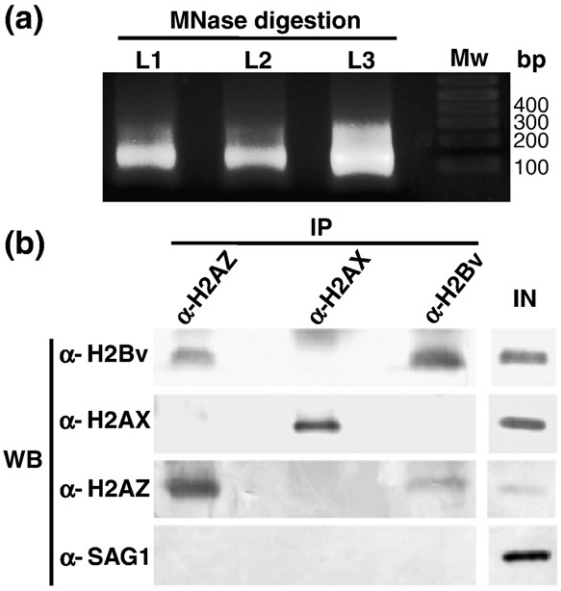


Fig. 4. Histone – histone interactions. (a) Tachyzoites were permeabilized with digitonin and treated with micrococcal endonuclease (MNase) to obtain a solution containing mononucleosomes form co-immunoprecipitations. The presence of mononucleosomes was evaluated by isolating DNA and examining it by electrophoresis in a 1.5% (w/v) agarose gel. (b) Lysates 1, 2, and 3 (L1, L2, 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).

(HAR) domain on *Toxoplasma* H2AX, which has been associated with transcriptional repression in other species (Fig. 2a).[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. Two lots of antibodies were obtained from mice immunized with rH2A1: α-H2A1 L1 recognizes specifically rH2A1 and α-H2A1 L2 and is cross-reactive with H2AX (Fig. 3a; Supplementary Data Fig. S1). α-H2AX was highly specific to rH2AX, presenting no reactivity against rH2A1 and rH2AZ proteins (Fig. 3a). In agreement with this result, α-H2AZ did not cross-react with rH2AX or rH2A1 (Fig. 3a). A mouse antibody to *Toxoplasma* H2AZ generated using the highly divergent N-terminal region (H2AZNt, residues 1 – 120) showed the same behavior as rabbit α-H2AZ (Fig. 3a). With respect to H2Bs, no reactive serum was obtained for rH2Ba (data not shown), whereas the α-H2Bv antibody[17] showed a very weak cross-reaction with rH2Ba (Fig. 3a). These antibody characterization results were confirmed by competition ELISAs (Supplementary Data Fig. S1). As expected, each α−H2A antibody stains the parasite nucleus (Fig. 3b).

When histones purified from *Toxoplasma* are separated by SDS-PAGE (15% (w/v) polyacrylamide 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, Western blot analysis was done with the different anti-H2A antibodies (Fig. 3c). Since the band recognized with the α-H2AZ is not observed with Ponceau red 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 red, suggesting a larger amount of protein. However, it is not possible to determine the relative amounts of H2AX and H2A1, because other histones (e.g. H3) or small proteins may 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

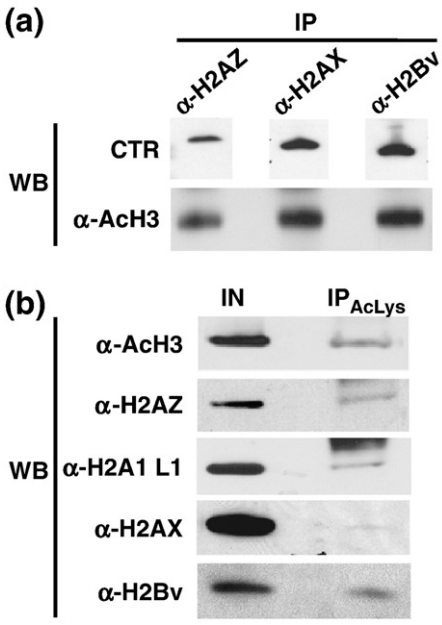


Fig. 5. Association of Toxoplasma H2A and H2Bs with active chromatin. Tachyzoite lysates generated by sonication were used for co-immunoprecipitations (co-IP). (a) Antibodies against Toxoplasma H2AZ, H2AX, and H2Bv were used for co-IP. They were analyzed by Western blots with the same antibodies to ensure 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 was done with α-AcH3 (positive control), α-H2A1 L1, α-H2AX, α-H2AZ, and α-H2Bv antibodies. IN, input (1% of tachyzoite whole extract).

H2Bv.[17] 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 (Fig. 4a). Subsequently, interactions among H2As and H2Bv were studied by co-immunoprecipitation (co-IP) on mononucleosomes followed by Western blot analysis. Pre-immune sera did not immunoprecipitate detectable protein (Supplementary Data Fig. S3). Antibody against the abundant surface antigen protein SAG1 was used as a control for the fidelity of the IP (Fig. 4b). We found that H2Bv interacts with H2AZ, but not with H2AX and *vice versa* (Fig. 4b). These data suggest that H2AZ and H2AX comprise different nucleosomes in tachyzoites.

The same interactions were observed when co-IPs were done using parasite lysate generated by sonication (Supplementary Data Fig. S4). The resulting genomic DNA (gDNA) fragments were 100 – 600 bp (Supplementary Data Fig. 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 (Supplementary Data Fig. S4(b)).

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

There is a clear relationship between active chromatin and acetylated histones.[24] We sought to determine if histone H2A and H2Bv variants are associated with active chromatin. Two independent approaches were used based on immunoprecipitation of one to several nucleosomes using sonicated lysates. We first immunoprecipitated H2AX, H2AZ and H2Bv with their respective polyclonal serum and performed Western blot analysis with anti-acetylated H3 antibody (α-AcH3), which is associated with active chromatin in *Toxoplasma*.[5,6] The three histone variants co-IP with the acetylated-H3 histone (Fig. 5a). Second, immunoprecipitation with anti-acetylated lysine (α-AcLys) was 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 (Fig. 5b). In contrast, H2AX was barely detectable in the acetylated lysine IP (Fig. 5b). By comparing band intensities, it could be observed that the percentage of H2AZ, H2A1 and H2Bv immunoprecipitated from input was lower than but similar to that observed for AcH3; however, the percentage of H2AX is more than 10-fold less (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 with active and inactive genes

Our data indicate that H2AX and H2AZ/H2Bv comprise different nucleosomes, and they could

Table 1. Primers used for qRT-PCR and qPCR

Name	Sequence	Description	Amplification product (bp from ATG)
qRT-H2A1 F	5′-AGGACGATGCAGCACACAAC	*h2a*1 3′UTR	622 to 715
qRT-H2A1 R	5′-CCATTAAAAGCCGCTTACGG		
qRT-H2AX F	5′-CCCGCCTTTTAGGCTTCACT	*h2aX* 3′UTR	615 to 708
qRT-H2AX R	5′-TGGTGAATTCGGCTCTTTGG		
qRT-H2AZ F	5′-TCTGGAGGGACAAACATGGG	*h2aZ* 3′UTR	581 to 674
qRT-H2AZ R	5′-AAAAAACACGCACCTGACCC		
qRT-TUB F	5′-ATGTTCCGTGGTCGCATGT	β-*tubulin* coding sequence	945 to 1039
qRT-TUB R	5′-TGGGAATCCACTGAACGAAGT		
qRT-BAG1 F	5′-TGAGCGAGTGTCCGGTTATTT	*bag*1 coding sequence	536 to 630
qRT-BAG1 R	5′-TAGAACGCCGTTGTCCATTG		
qRT-LDH2 F	5′-ACAATGGCCCAGGCATTCT	*ldh*2 coding sequence	750 to 844
qRT-LDH2 R	5′-CAATAAACATATCGTGAAGCCCATA		
qRT-SAG1 F	5′-TTAAGTGAGAACCCGTGGCAG	*sag1* coding sequence	696 to 789
qRT-SAG1 R	5′-GCTTTTTGACTCGGCTGGAA		
q-TUB F	5′-TCCTTTTCTTCATGCACTCGC	β-*tubulin* promoter	−627 to −526
q-TUB R	5′-GTCGTTCGAATTCTCTGCCC		
q-BAG1 F	5′-TCCTCCCCTGGATCTTCCTCC	*bag*1 promoter	−739 to −638
q-BAG1 R	5′-TTGCACAAAACTGGCAAAGG		
q-LDH2 F	5′-AAGTGTGCACGCTTTGCAAG	*ldh*2 promoter	−505 to −404
q-LDH2 R	5′-CATGCTCCGGGCAGTACCT		
q-TgIRE 100 F	5′-TTCTTCACGGTTCCGGACTT	*TgIRE* region at 100 bp	3 to 96
q-TgIRE 100 R	5′-CAGCAGTAGCCAACCACGT		
q-TgIRE 600 F	5′-GTTATGAGTCCACCACACGGG	*TgIRE* region at 600 bp	732 to 825
q-TgIRE 600 R	5′-CATGATGTGCATGAAACGTGC		
q-SAG1 F	5′-AACTTCCCACACGAGGCATT	*sag*1 promoter	−653 to −560
q-SAG1 R	5′-GATCGGCCCCTAATTCAGC		

q, Quantitative real time PCR; qRT, quantitative real time RT-PCR; F, forward; R, reverse.

have different acetylation status and/or proximity to acetylated histones, both a hallmark of active chromatin among other roles.[25] To address this further, ChIP experiments performed with α-H2AX, α-H2AZ and α-H2Bv antibodies were followed by qPCR. ChIP experiments with α-AcH3 and α-H4K20me1 (histone H4 monomethylated 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*: see 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 (Fig. 6a). These data suggest that H2AZ and H2AX 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 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 elements[26] retrieved poorly conserved and incomplete sequences (data not shown), but *TgIRE*[27] identified sequences in nine out of the 14

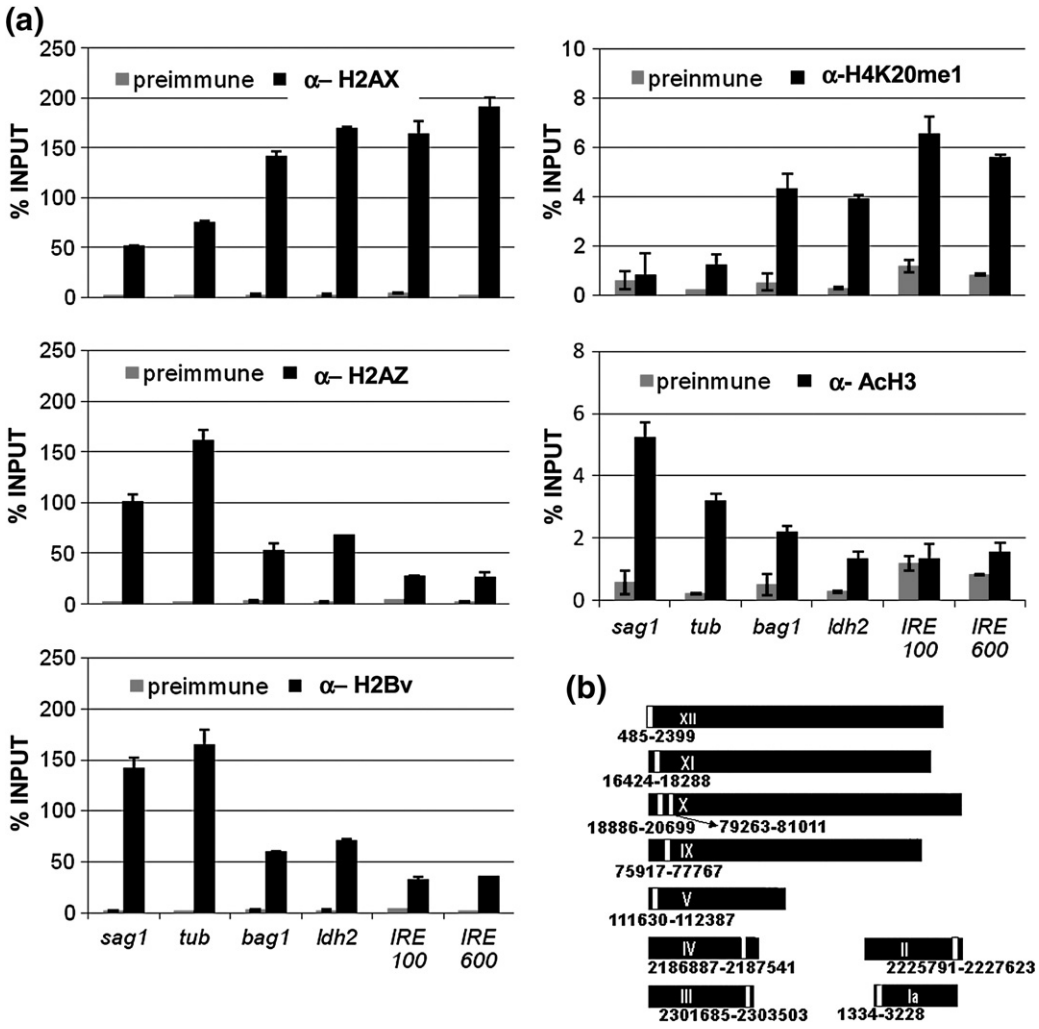


Fig. 6. Genomic distribution of H2A and H2B variants. (a) Chromatin immunoprecipitation (ChIP) was carried out using α-H2AX, α-H2AZ, and α-H2Bv. In addition, α-H4K20me1 and α-AcH3 antibodies were used as heterochromatin and euchromatin controls, respectively. Immunoprecipitated DNA was then analyzed 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.

chromosomes having high sequence homology among them (Fig. 6b). 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 for expressed products within *TgIRE* sequence retrieved just four ESTs that are evident in only 3 chromosomes (Supplementary Data Fig. S5)‡. Three EST (TIGR EST assemblies N61085, TC35464 and CK736714) are present in chromosomes Ia and XI, whereas the other one (TIGR EST assemblies TC39371) is in chromosome X (Supplementary Data Fig. S4). However, there is no evidence of annotated genes, mass spectrometry peptides or predicted proteins. Moreover, no annotated gene has 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 (Fig. 6a). In acccord with earlier observations, 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 motif (ϕ denotes a hydrophobic residue) following double strand breaks.[8,28] In order to determine whether this process is conserved in *Toxoplasma*, extracellular tachyzoites were incubated with 0 – 400 μM H_2O_2 for 1 h at 37°C. Samples were then subjected to Western blot analysis with α-H2AX and anti-phospho histone H2AX (α-γH2AX), the latter being a mouse monoclonal antibody that specifically recognizes the last eight amino acids, including the phosphorylated motif of human H2AX. Both antibodies recognize one band with the same migration rate under the conditions described in Materials and Methods. Anti-tubulin was used as a control for protein concentration. Band intensities were quantified and γH2AX/Tub and H2AX/Tub ratios were determined. While H2AX levels were the same under all conditions, γH2AX increased in a dose-dependent manner following exposure to DNA damage mediated by H_2O_2 exposure (Fig. 7a).

Although histone variants exhibit cell cycle-independent transcription,[29] no mechanism is described that could account for alterations in their expression patterns. We asked whether oxidant stress has an effect on H2A expression. qRT-PCR showed that transcription of *h2a1* and *h2ax* was stimulated by H_2O_2 in a dose-dependent manner, but this was not the case for *h2az* (Fig. 7b). Taken together, these data suggest that H2AX and H2A1 are likely to be associated with oxidant DNA damage stress.

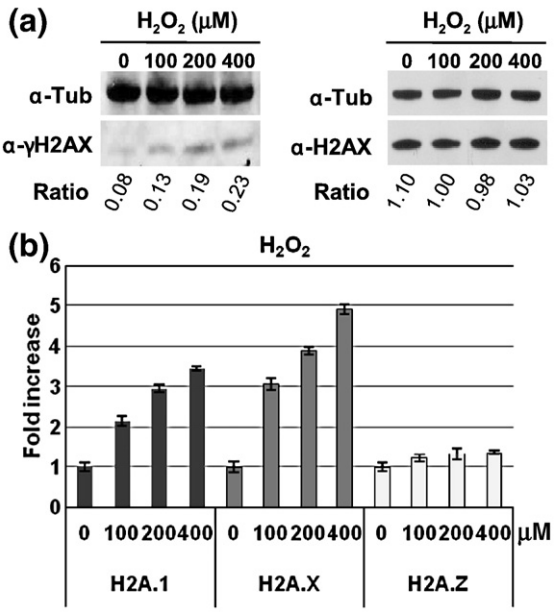
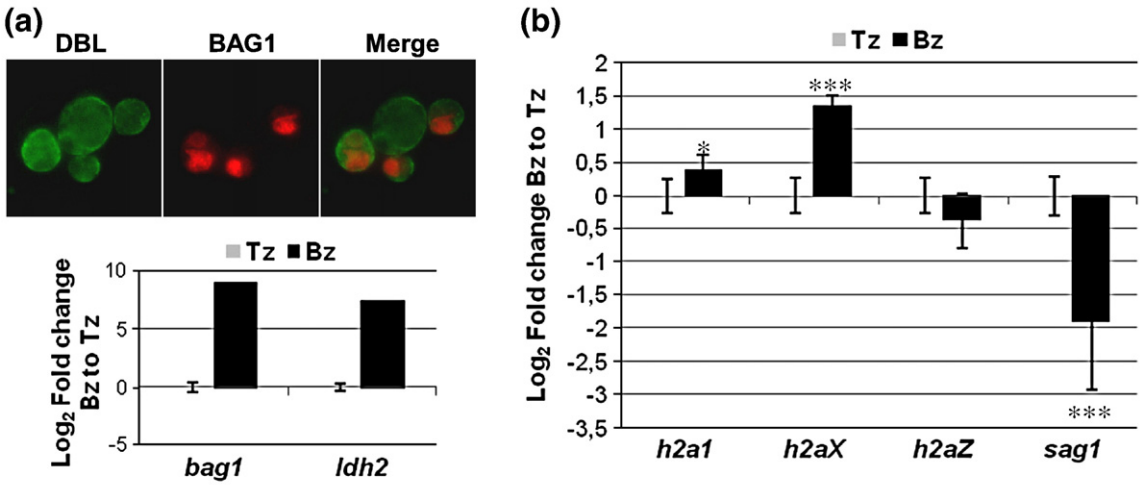


Fig. 7. Phosphorylation and expression analysis of *h2a* genes following DNA damage. Tachyzoites were treated with 0, 100, 200, or 400 μM H_2O_2 for 1 h at 37°C. (a) Western blots were performed to detect phospho H2AX (α-γH2AX) at the left and total H2AX (α-H2AX) at the right. Each one was also blotted 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.

H2A expression patterns during bradyzoite formation

The gene expression profiles of H2As were studied between tachyzoites and bradyzoites. Intracellular RHΔ*uprt* tachyzoites were incubated in bradyzoite conversion conditions (CO_2 deprivation and pH 8.1). The differentiation rate was analyzed by immunofluorescence assay (Fig. 8a) and by monitoring the expression of bradyzoite marker genes *bag1/hsp30* and *ldh2* and a tachyzoite marker gene *sag1* (Fig. 8a 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 (Fig. 8b). In contrast, *h2az* mRNA was not altered between stages (Fig. 8b). The qRT-PCRs were normalized to β-*tubulin*, which exhibits no significant change in expression during bradyzoite induction at pH 8.1.[30]

These results were corroborated by a microarray analysis of RH strain using the Affymetrix ToxoGeneChip,[31] in which parasites stressed for three days in pH 8.1 medium were compared to unstressed parasites (W.J.S. Jr, unpublished results). As shown in Fig. 8c, intracellular RH parasites exposed to alkaline pH, a known trigger of bradyzoite gene expression, increased the expression of



(c)

Gene ID	Fold Increase	P value	Description
55.m04926	2.16	0.000883	H2A1
55.m04942	2.08	0.007143	H2AX
145.m00002	1.03	0.48	H2AZ
541.m02039	1.77	0.000010	H2Ba
25.m00008	1.04	0.40	H2Bv

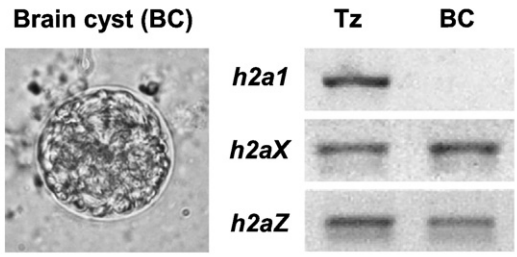


Fig. 8. *h2a* gene expression and bradyzoite induction. (a and b) RHΔ*uprt* parasites were induced to bradyzoites using pH 8.1 and low CO_2 for four days. Relative quantification of expression levels of several genes in tachyzoites (Tz) and *in vitro* bradyzoites (Bz) by qRT-PCR calibrated to tachyzoites using β-*tubulin* as the 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. Log_2 of fold change Bz to Tz are shown for each gene analyzed. Three independent experiments were performed and significant differences between Tz and Bz for each gene amplified were analyzed by Student's *t*-test (GraphPad Prism software) * $p<0.05$; *** $p<0.001$. Tachyzoite-specific gene *sag1* was used as a control. (c) A table summarizing the microarray data for select histone genes expressed in RH parasites stressed for 3 days with pH 8.1 relative to a 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.

h2ax, *h2a1* and *h2ba*, whereas *h2aZ* and *h2bv* were 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 with a specific cell cycle stage, 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 PCR was done using mRNA obtained from brain cysts of experimentally infected mice (30 days post infection). Fig. 8d shows that *h2a1* is not expressed in mature bradyzoites, whereas *h2ax* and *h2az* mRNAs were detected in this parasite stage. As canonical histones are known to be expressed exclusively during the S-phase of the cell cycle,[34] 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 H2AX.[16] The impact of these varied H2A subtypes among early-branching eukaryotes on cellular physiology is not known.

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. *Trypanosoma brucei* H2AZ dimerizes with H2Bv, but in this case H2AZ did not pull down with canonical H2A.[18] 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 an H2AX variant,[16] 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 are 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 positively and negatively regulate transcription. Genome-wide localization studies in yeast revealed that H2AZ is located preferentially at inactive promoters.[18,35-38] Raisner *et al.*[39] found no correlation between transcribed genes and the presence of H2AZ in their promoter regions. However, three recent studies of human T cells,[40] *Caenorhabditis elegans*[41] and *T. brucei*[42] found 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; 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 transcription start sites. 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 with 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 tail.[20] This study showed also 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 in response to DNA damage.[8] H2AX may function during the replication of facultative heterochromatin on the inactive chromosome X.[43,44] Our studies show that H2AX in tachyzoites 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 switching between the two processes. Interestingly, *Toxoplasma* H2AX has the typical SRS motif located at loop 1, resulting in a well conserved HAR pattern;[22] H2AZ, however, displays a low degree of similarity to the HAR domain relative to H2AX. It will be of interest to determine 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 H_2O_2-treated parasites in a dose-dependent manner, consistent with parasite H2AX being associated with the DNA damage response, as shown for other eukaryotes.[8] Since *Toxoplasma* has an H2A1 that contains a truncated C-terminal motif (SQ), it may be phosphorylated. Interestingly, *h2ax* and, to a lesser extent, *h2a1*, mRNA increase during H_2O_2 treatment, perhaps to replace the phosphorylated histones that are evacuated after DNA repair.[45] However, a similar *h2ax/h2a1* expression profile was observed during bradyzoite development, making it possible that the oxidative damage initiates bradyzoite differentiation. In contrast to the qRT-PCR results, increased expression of H2AX was not observed by Western blot. 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, canonical histones are the major histones and their expression is linked to the S-phase of the cell cycle. Our data show also that *h2a1* is expressed in tachyzoites and bradyzoites

generated *in vitro*, while the latter are still in a replicative state.[32] When mature bradyzoites were analyzed, only *h2aX* and *h2aZ* were 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 is 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 with a specific stage in the cell cycle. In contrast, *h2az* levels were stable during bradyzoite development, inferring that the effect on the *h2ax* gene is due to bradyzoite development signals. There are several genes that are upregulated during bradyzoite differentiation.[46] In general, however, 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, make 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 found also 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. On the basis of these data, it will be very important 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‡. We searched ToxoDB for putative H2A homologues using human, yeast and *Plasmodium* H2A sequences and blast.[19,47,48] Protein domains on the amino acid sequence were analyzed using the "conserved domain search§" and secondary structure was deduced‖. The neighbor-joining tree was constructed with Molecular Evolutionary Genetics Analysis Software version 4 (MEGA4) as described.[49] Internal support was measured using 1000 replicates of the heuristic search bootstrap option.

§ http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

‖ http://toolkit.tuebingen.mpg.de/sections/secstruct

Parasite sources, culture and manipulation

The RH*Δhxgprt* strain was used in all cases except in bradyzoite-related experiments. Bradyzoites were generated from RH*Δuprt* or PK parasites (a clone isolated from cystogenic *Toxoplasma* Me49 strain). These parasites were grown under standard tachyzoite conditions *in vitro*: human foreskin fibroblast monolayers were infected with tachyzoites and incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 1% (v/v) fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Parasites RH*Δuprt* (parasite/host cell ratio<1:10) were induced to differentiate into bradyzoites for four days in low CO_2 (0.03%) and pH 8.1 as described.[50] Briefly, a confluent monolayer of human foreskin fibroblast was infected with approximately 1×10^7 parasites in 10 cm diameter tissue culture dish. They were incubated for 10 min on ice and then incubated for 1 h under 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 was placed in a 37°C incubator (at ambient CO_2 0.03%). At the fourth day, cells were scraped and the cysts were disrupted by syringe passage. To determine the ratio of bradyzoites, we used *Dolichos biflorus* lectin (TRITC-labeled; Sigma) staining and antibody to BAG1.[51] Under all conditions, parasites were separated from the cell debris filtration through a 3 μm pore size. To obtain bradyzoites *in vivo*, PK parasites were used to infect C3H mice. After one month, the mousse brains were isolated and homogenized in PBS using a hand-held tissue grinder, centrifuged in 30% (w/v) dextran to sediment the cysts, washed and suspended 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′-*ggatcc*ATGTCGGCCAAAGGC
and antisense:
5′-*ggtacc*TTACTGAGACTTCTTGCCCTTG
5′-*ggatcc*ATGTCCGCCAAAGGTGCAGG
and antisense:
5′-*ggtacc*CACCAGACAGAATGGCTATCCT
For H2AZ, sense: 5′-*ggatcc*ATGGACGGAGCTGCAAAGT
and antisense:
5′-*aagctt*GAGCGACTTCTCGTGGAAAG

BamHI and KpnI/HindIII sites were included in the sense and antisense primers, respectively, (underlined sequence). These fragments were cloned in pGEM T easy vector (Promega) and sequenced using Sp6 and bacteriophage 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 wit accession numbers AY631392, AF502246 and AY573602).

Raising specific polyclonal antibodies

The H2AZ sequence was subcloned into 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).[17]

Recombinant proteins (rH2AZ, rH2AX, rH2A1, and rH2Ba) were expressed in *Escherichia coli* strain BL21pLys, under induction with 0.1 mM IPTG overnight at 30°C and purified through a Ni^{+}affinity column (Qiagen) under denaturing conditions following the manufacturer's instructions. In addition, the N-terminal sequence of H2AZ (first 40 amino acid residues) was cloned in the BamHI and SalI sites of the pGEX-4T-1 plasmid (Amersham) to generate a glutathione *S*-transferase fusion protein (GST)-H2AZNt. rH2AZNt was expressed and purified with glutathione-Sepharose 4B (Amersham). Rabbits were immunized with rH2AZ and rH2AX (300 μg) and mice were immunized with rH2A1, rH2AZNt and rH2Ba (10 μg). Three boosters of each antigen emulsified with incomplete Freund's adjuvant at intervals of 2 weeks followed a primary immunization performed with complete Freund's adjuvant. Samples of pre-immune serum were collected from each animal before antigenic stimulation. For anti-H2A1 (α-H2A1), two lots were obtained: lot 1, an aliquot obtained before finishing immunization plan; lot 2, an aliquot that corresponds to the final bleed (α-H2A1L1 and α-H2A1L2, respectively). The first was shown to be highly specific and the second 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 measurement of A_{280}. Histones were acid-purified as described.[17] In all cases, 1×10^7 parasites, 1.5 μg of recombinant protein or purified histones from a T-25 were loaded per well and resolved by SDS-PAGE (15% polyacrylamide gel). Proteins were transferred to cellulose acetate membrane for 1 h at 100 V and a Western blot was done as described.[50] The primary antibodies α-H2AZ, α-H2AX, α-H2AZNt, and α-H2Bv[17] were used at a dilution of 1/3000 for 1 h at room temperature, whereas α-H2A1 L1/L2 were used overnight 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 used with the ECL detection system (Amersham-GE).

Immunofluorescence assay (IFA)

Human foreskin fibroblast cells grown on coverslips were infected with tachyzoites. After 24 h they were fixed with 4% (v/v) paraformaldehyde and blocked with 1% BSA. Primary antibodies α-H2AX, α-H2AZ and α-H2AZNt diluted 1/500 and α-H2A1 L1 diluted 1/50 with 0.5% BSA were incubated at room temperature for 1 h. 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*Δuprt* and RH*Δhxgprt* tachyzoites washed twice with PBS were used for each immunoprecipitation. On one hand, parasites were disrupted by sonication: they were suspended in 1 ml of lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 4 mM EDTA, 1% (v/v) NP-40, and protease inhibitors), sonicated three times (15 s at 30% amplitude each) and centrifuged in a bench-top centrifuge at max speed for 10 min. A 1 % part of the supernatant (Input= IN) was phenol/chloroform-extracted and gDNA fragments were ethanol-precipitated overnight at 4 °C. Samples were visualized after electrophoresis in a 1.5% (w/v) agarose gel (Supplementary Data Fig. S3(a)). In some cases, tachyzoites were treated with micrococcal endonuclease in order to obtain mononucleosomes as described.[18] Briefly, parasites were suspended in 1 ml of permeabilization buffer (100 mM KCl, 10 mM Tris pH 8.0, 25 mM EDTA, 1 mM DTT) and permeabilized with 40 μM digitonin 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 $CaCl_2$, 5% (v/v) glycerol, 1 mM DTT, 1 mM PMSF). Six units of micrococcal nuclease (Sigma) were added and the cell suspension was incubated for 15 min at 28 °C. The reaction was stopped by adding EGTA to a final concentration of 10 mM. To improve chromatin solubility, NP-40 and NaCl were added to final concentrations of 0.5% and 200 mM, respectively. Following centrifugation at ~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 subjecting it to electrophoresis in a 1.5% agarose gel and then stained with ethidium bromide (Fig. 4a).

Sonicated parasites or mononucleosomes were incubated with 20 μl of the antibody of interest overnoght at 4 °C. On the following day, 40 μl of protein A/G (Santa Cruz) was added and incubated for 2 h. Immunocomplexes were washed six times with 50 mM Tris pH 8, 200 mM NaCl, 2 mM EDTA, 1 % NP-40, then suspended in 60 μl of SDS-PAGE loading buffer. Samples were boiled for 5 min and 20 μl was loaded into each well and subjected to SDS-PAGE (15% polyacrylamide gel) and Western blotting. The pre-immune serum of each antibody was used as a negative control. 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, centrifuged and suspended in cold lysis buffer (1% (w/v) SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1, 20 mM 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 for 15 s each time. 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% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 167 mM NaCl, 20 mM sodium butyrate) plus Protease Inhibitor Cocktail (Sigma p8340). A 10% portion of the lysate was used as input DNA (IN) for normalization, the rest was cleared with 80 μl of salmon sperm DNA/protein A agarose (Upstate) for 30 min with agitation. Following centrifugation at 1000 rpm for 1 min in a bench-top centrifuge, 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. A 60 μl sample of salmon sperm DNA/protein-A agarose was added and incubated for 1 h to collect the antibody–protein–DNA complex, which was washed consecutively with 1.0 ml of each of the following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl); high-salt wash buffer (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl); and LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1). Samples were washed six times with TE buffer before elution with 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature with agitation. A second elution step was done after a spin at 1000 rpm for 1 min. Crosslinks were reversed from the combined eluates and input samples with 20 μl of 5 M NaCl overnight at 65 °C. 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 the 5′UTR sequence of *sag1*, *ldh2*, β-*tubulin* (present on chromosome IX) and *bag1*, and two regions of *TgIRE* with a high degree of homology among all the copies present in the parasite. The primer sequences are given in Table 1. The calibration curve was constructed 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 constructed with each pair of primers detecting only one amplification product. The PCR products were also visualized following electrophoresis 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 $2^{-\Delta\Delta Ct}$. Each sample was normalized to β-*tubulin* and calibrated to the tachyzoite sample. Real-time PCRs were performed using the same reaction mix, the real-time PCR system, and the software described above. Primers were designed to amplify the 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***). Log_2 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 done with primers used to clone them using Taq polymerase (Invitrogen). Products were visualized following electrophoresis in a 1% agarose gel containing 0.5 mg/ml ethidium bromide.

Parasite treatment with H_2O_2

Freshly lysed, filter-purified tachyzoites were treated with 0, 100, 200 or 400 μM H_2O_2 for 1 h at 37 °C in a 5% CO_2 atmosphere. 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. The integrated optic density of histone proteins was related to the corresponding tubulin intensity to determine the ratio.

Acknowledgements

S.O.A. (Researcher) and M.C.D. (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 and W.J.S.), and National Institutes of Health grants AI077502 and AI073091 (to W.J.S.).

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.07.017

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