Histone H2AZ dimerizes with a novel variant H2B and is enriched at repetitive DNA in *Trypanosoma brucei*

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Summary

H2AZ is a widely conserved histone variant that is implicated in protecting euchromatin from the spread of heterochromatin. H2AZ is incorporated into nucleosomes as a heterodimer with H2B, by the SWR1 ATP-dependent chromatin-remodeling complex. We have identified a homolog of H2AZ in the protozoan parasite *Trypanosoma brucei*, along with a novel variant of histone H2B (H2BV) that shares ~38% sequence identity with major H2B. Both *H2AZ* and *H2BV* are essential for viability. H2AZ localizes within the nucleus in a pattern that is distinct from canonical H2A and is largely absent from sites of transcription visualized by incorporation of 5-bromo-UTP (BrUTP). H2AZ and H2BV colocalize throughout the cell cycle and exhibit nearly identical genomic distribution patterns, as assessed by chromatin immunoprecipitation. H2AZ co-immunoprecipitates with H2BV but not with histones H2B or H2A nor with the variant H3V. These data strongly suggest that H2AZ and H2BV function together within a single nucleosome, marking the first time an H2AZ has been shown to associate with a non-canonical histone H2B.

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Introduction

The basic chromatin unit – the nucleosome – is composed of ~146 bp of DNA wrapped around two histone H2A/H2B dimers and an H3/H4 tetramer. One way in which nucleosome structure and function can be altered is by exchanging a major histone with a specialized variant histone. Many variant histones have been identified, including some that are unique to vertebrates and others that are highly conserved among all eukaryotes (Malik and Henikoff, 2003). The variant H2AZ (also known as H2A.F/Z) falls into this latter category, having been described in organisms ranging from *Tetrahymena* to humans, and sharing as much as ~90% sequence identity among organisms (Dryhurst et al., 2004; Iouzalen et al., 1996). H2AZ split from the H2A lineage early in eukaryotic evolution (Thatcher and Gorovsky, 1994) and its function, although not fully elucidated, is clearly distinct from H2A (Jackson and Gorovsky, 2000). H2AZ has been linked to transcriptional activation (Adam et al., 2001; Santisteban et al., 2000; Stargell et al., 1993), gene silencing (Dhillon and Kamakaka, 2000), and to the protection of euchromatin from the spread of heterochromatin (Meneghini et al., 2003). H2AZ is also involved in chromosome segregation (Carr et al., 1994; Rangasamy et al., 2004), with a role in the formation of pericentric heterochromatin (Rangasamy et al., 2003).

Structural and biochemical studies indicate that H2AZ-containing nucleosomes exhibit important differences from nucleosomes composed entirely of major histones, and these differences could facilitate transcription (Abbott et al., 2001; Fan et al., 2002; Placek et al., 2005; Suto et al., 2000). H2AZ-containing nucleosomes are less stable than H2A-containing nucleosomes, owing to changes in the interface between the H2AZ/H2B dimer and the H3/H4 tetramer (Suto et al., 2000). Furthermore, the L1 loop region, through which H2AZ self-interacts, differs significantly from the L1 loop in H2A, and thereby dictates that H2AZ and H2A cannot be present in the same nucleosome (Suto et al., 2000). In vitro, H2AZ apparently promotes the folding of nucleosomal arrays but impedes their ability to oligomerize (Fan et al., 2002). This finding suggests that H2AZ-containing chromatin might be resistant to condensation and therefore primed for transcription (Fan et al., 2002).

The major histones are deposited onto newly synthesized DNA at replication forks. By contrast, many histone variants – including H2AZ – are incorporated into nucleosomes outside of S phase of the cell cycle (Dryhurst et al., 2004; Malik and Henikoff, 2003). Recently, several groups have made major inroads into understanding this process (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). The SWR1 multi-protein chromatin-remodeling complex specifically exchanges H2AZ/H2B dimers for H2A/H2B dimers on nucleosome arrays in a reaction that requires ATP (Mizuguchi et al., 2004). How the SWR1 complex is targeted to appropriate sites is unknown, but might involve the acetylation of major histones (Kobor et al., 2004).

It has been suggested that only histones that are self-interacting within a nucleosome (H2A and H3) have variants (Suto et al., 2000). In fact, variant forms of H2B do exist, but might function outside the context of a nucleosome (Aul and

Oko, 2001). Those that have been described are not widely conserved (Malik and Henikoff, 2003) and are primarily involved either in packaging DNA in pollen (Ueda et al., 2000) and sperm (Churikov et al., 2004; Gineitis et al., 2000; Zalensky et al., 2002) or in other aspects of sperm development (Aul and Oko, 2001).

Trypanosoma brucei is an extracellular protozoan parasite that is responsible for sleeping sickness in Africa. In its bloodstream form, *T. brucei* escapes elimination by the immune system of its host by periodically switching its variant surface glycoprotein (VSG) coat, a process known as antigenic variation (Pays et al., 2004). Although there are hundreds of *VSGs* scattered about the genome, only a *VSG* positioned at a sub-telomeric 'expression site', of which there are ~20, can be transcribed. However, *VSG* expression is monoallelic, as only one expression site is active at a time. Switches in *VSG* expression occur by recombination or the in situ activation of an inactive expression site. When ingested by its vector, the tsetse fly, the parasite differentiates to the procyclic form, and its VSG coat is replaced by one or more members of the procyclin glycoprotein family (Roditi et al., 1998).

In *T. brucei*, nearly all transcription is polycistronic and transcription initiation is unlikely to be an important place for regulating mRNA expression (Clayton, 2002). The RNA polymerase I-driven polycistronic *VSG* expression site contains ~11 *ESAGs* (for 'expression-site associated genes') and a *VSG* transcribed from a single promoter located ~50 kb upstream of the *VSG*. All expression sites are bounded at one end (proximal to the *VSG*) by a telomere and at the other, immediately upstream of the promoter, by an array of ~10-50 kb of a 50 bp repeat sequence. The function of the 50 bp repeats is unknown, but they have been speculated to act as a boundary element, serving to prevent transcriptional machinery from centromere-proximal regions from extending into the expression site (Sheader et al., 2003).

T. brucei histones are extremely divergent from those found in model organisms (Alsford and Horn, 2004). Consequently, the relationship between chromatin and transcription in *T. brucei* has largely been unexplored. Since it has been established that the histone variant H2AZ plays key roles in transcriptional activation (Adam et al., 2001; Santisteban et al., 2000; Stargell et al., 1993), and in preventing the spread of heterochromatin (Meneghini et al., 2003), we sought to identify its homolog in *T. brucei*. Here, we present the identification of *T. brucei* H2AZ and a novel variant of histone H2B (H2BV). Unexpectedly, H2AZ and H2BV colocalize, share nearly identical chromatin immunoprecipitation profiles and co-immunoprecipitate, strongly suggesting that they heterodimerize within an individual nucleosome. Our results mark the first time an H2AZ has been shown to associate with a variant form of H2B – a surprising finding given the evolutionary conservation of H2AZ.

Materials and Methods

Trypanosome culture

The 'single marker' cell line, a derivative of *T. brucei* Lister 427, antigenic-type MITat 1.2, clone 221a (Doyle et al., 1980), expresses T7 RNA polymerase and the *tet* repressor, allowing inducible expression of ectopic genes under control of the T7 promoter and *tet* operator (Wirtz et al., 1999). All cell lines used in this study were derived from the 'single marker' cell line. Cells were grown in HMI-9 medium (Hirumi and Hirumi, 1989) and stable transfections were performed as described (Wirtz et al., 1994).

Cloning histone genes

To map the start codons of *H2AZ* and *H2BV*, both genes were amplified by PCR from cDNA using primers that were homologous to the 3′ end of the relevant gene and the spliced leader sequence. They were then cloned into pBluescript II SK+, generating pPY1 and pJEL24, respectively, and sequenced. The *H2AZ* open reading frame (ORF) (Tb927.7.6360), flanked by ~300-500 bp of adjacent sequence, was amplified by PCR from genomic DNA and cloned into pBluescript II SK+, generating pFK1. The *H2BV* gene and flanking sequence was cloned using the pJEL24 insert to probe a bacterial artificial chromosome (BAC) library derived from *T. brucei* Lister 427 (Zeng et al., 2001). A BAC containing *H2BV* was digested with *Bam*HI and *Hind*III, and the resulting DNA fragments were cloned into pBluescript II SK+ and screened by colony blotting. pJEL35 contains the H2BV ORF flanked by 500-2100 bp of adjacent DNA. The *H2BV* sequence was submitted to GenBank (accession number AY179218). ORFs corresponding to H2A (*HTA*) and H2B (*HTB*) were amplified by PCR using genomic DNA as a template and cloned into pBluescript II SK+, generating pJEL1 and pJEL3, respectively.

Cell lines expressing tagged and fusion proteins

To generate the cell line BFpFK8, which is capable of inducible ectopic expression of TY1-tagged H2A, the *HTA* gene was PCR amplified from pJEL1 such that the DNA corresponding to the TY1 epitope [EVHTNQDPLD (Bastin et al., 1996)] was introduced at the 5′ end of the ORF. This PCR fragment was cloned into the tetracycline-inducible pLEW82 (Wirtz et al., 1999), to produce pFK8, which was linearized with *Not*I and stably transfected into the 'single marker' cell line. Inducible expression of TY1-H2A was confirmed by western blot using αTY BB2 mAb.

To generate the cell line BFJEL8, which contains an inducible, ectopic, FLAG-tagged H2B, the *HTB* gene was PCR amplified from pJEL3 such that DNA encoding the FLAG epitope (DYKDDDDK) was introduced into the 5′ end of the *HTB* ORF. The PCR fragment was cloned into pLEW82, to generate pJEL52, which was subsequently linearized with *Not*I and stably transfected into the 'single marker' cell line. Inducible expression of FLAG-H2B was confirmed by western blot using αFLAG mAb (Sigma).

To generate the cell line BFJEL28, which constitutively expresses TY1-tagged H3V from its endogenous locus, a strategy identical to that described before (Lowell and Cross, 2004) was used.

To generate the cell lines BFpFK3.4.5 and BFJEL41, in which a single ectopic tagged version of *H2AZ* replaces the endogenous *H2AZ* alleles, the *H2AZ* ORF was amplified by PCR from pFK1 such that DNA encoding the TY1 epitope was introduced at the 5′ end of the ORF and cloned into pLEW82, producing pFK3. Similarly, to generate a plasmid capable of expressing an inducible fusion protein between GFP and H2AZ, *H2AZ* was again PCR amplified from pFK1 and cloned into pCO55 (a derivative of pLEW82 containing *GFP*), generating pJEL95. pFK3 and pJEL95 were linearized with *Not*I, and stably transfected into the 'single marker' cell line, to produce the cell lines BFpFK3 and BFJEL30, respectively. Inducible expression of TY1-H2AZ and GFP-H2AZ was confirmed by western blot. Drug resistance cassettes corresponding to puromycin *N*-acetyltransferase [derived from pHD309-*PUR* (Wirtz et al., 1994)] or hygromycin [derived from pLEW90 (Wirtz et al., 1999)] were cloned such that they were flanked by a few hundred bp of DNA adjacent to the *H2AZ* ORF. The resulting plasmids, pFK4 (*h2az::PUR*) and pFK5 (*h2az::HYG*), were released from the vector backbone by restriction digestion, and used sequentially to transfect BFpFK3 and BFJEL30. The elimination of endogenous *H2AZ* ORFs was confirmed by Southern blotting.

BFJEL43, a cell line in which a single ectopic TY1-tagged copy of *H2BV* replaced both endogenous *H2BV* alleles, was prepared following a similar approach. Using pJEL24 as a template, the *H2BV* ORF was amplified by PCR such that it contained the TY1 epitope in the region of the ORF corresponding to the N-terminus and was cloned into pFLAN, a derivative of pLEW82 containing the resistance marker blasticidin in place of bleomycin. The resulting plasmid, pJEL92, was linearized with *Not*I and stably transfected into the 'single marker' cell line to generate the cell line BFJEL29. Inducible expression of TY1-H2BV was confirmed by western blotting. To generate the deletion plasmids pJEL74 and pJEL75 (*h2bv::PUR* and *h2bv::HYG*, respectively), pJEL35 was PCR amplified such that the *H2BV* ORF was deleted. This PCR product was then ligated to the drug resistance cassettes corresponding to puromycin *N*-acetyltransferase or hygromycin. pJEL74 and pJEL75 were digested with appropriate restriction enzymes to release the knock-out cassettes and were used sequentially to transfect BFJEL29. Elimination of both *H2BV* ORFs was confirmed by Southern blotting.

Antibody generation

Polyclonal antibodies specific for H2AZ and di- or trimethylated histone H3 lysine 76 were raised by immunizing rabbits (Sigma) with the KLH-conjugated peptides: LTGDDAVPQAPLVGC, VSGAQK[Me_2]EGLRFC or VSGAQK[Me_3]EGLRFC. All antisera were affinity purified using the corresponding peptides immobilized to SulfoLink coupling gel (Pierce) as described (Harlow and Lane, 1999).

Fluorescence microscopy

The cell lines BFpFK8 or BFJEL43 were subject to immunofluorescence microscopy as described (Lowell and Cross, 2004) except cells were fixed with 2% formaldehyde in trypanosome dilution buffer (5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 20 mM glucose, pH 7.7). Following permeablization, cells were incubated for $\geqslant$1 hour with either the αTY1 BB2 mAb or αH2AZ. Following incubation with secondary antibody, cells were stained with DAPI, mounted in antifade solution, and examined using DeltaVision deconvolution microscopy (Applied Precision). BrUTP incorporation in the presence or absence of 100 μg/ml α-amanitin was carried out on the permeabilized 'single marker' cell line as described (Navarro and Gull, 2001), except that cells were fixed in formaldehyde.

Chromatin immunoprecipitation and co-immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on the cell lines 'single marker', BFpFK8, BFpFK3.4.5 and BFJEL43 as described (Lowell and Cross, 2004), except cells were fixed with formaldehyde in HMI-9 medium for 5 minutes and lysates were immunoprecipitated with either αFLAG mAb (Sigma) or αTY1 BB2 mAb. Each slot blot includes an input control that corresponds to 10% of total input chromatin. Blots were hybridized with the following probes: telomeric repeat [TEL (de Lange et al., 1990)]; mini-chromosome 177 bp repeat [MC177 (Ersfeld and Gull, 1997)]; 50 bp repeat (Chaves et al., 1998); 5SDNA (Ersfeld and Gull, 1997); rDNA spacer, a ~318 bp *Not*I/*Sac*II fragment from pLEW82 (Wirtz et al., 1999); α-tubulin (*αTUB*) ORF from pZJM, a 650 bp fragment, corresponding to amino acids 1-216 (Wang et al., 2000); β-tubulin (*βTUB*) ORF, a ~464 bp *Pvu*II/*Not*I fragment of pSgl42 corresponding to amino acids 163-318; histone H3 (*HHT*), a ~400 bp complete ORF from pJEL19 (Lowell and Cross, 2004); procyclin (*EP1*), a ~400 bp complete ORF; *VSG221* ORF, 1-1364 bp; *VSG224* ORF, 513-1210 bp; *VSGVO2* ORF, 1-1103 bp; INGI retrotransposon-like element (*INGI*), a ~4900 bp fragment amplified by PCR from genomic DNA; ES promoter, 1580 bp fragment of pLF16 (GenBank accession number AL671259, sequence range 70323-71898). Each ChIP was performed 2-4 times and the results quantified by PhosphorImager using ImageQuaNT software (Molecular Dynamics).

Co-immunoprecipitations were carried out on the cell lines BFJEL43, BFJEL8, BFpFK8 and BFJEL28 as follows: 2×10^8 bloodstream-form cells were washed and resuspended in permeabilization buffer (100 mM KCl; 10 mM Tris, pH 8.0; 25 mM EDTA; 1 mM DTT), incubated with digitonin (40 μM final concentration) for 5 minutes, then washed and resuspended in isotonic buffer (100 mM KCl; 10 mM Tris, pH 8.0; 10 mM $CaCl_2$; 5% glycerol; 1 mM DTT) as described (Navarro and Cross, 1998), all in the presence of 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM *N*-tosyl-L-lysine-chloromethyl ketone (TLCK) and a commercial protease inhibitor cocktail (Sigma, P8340). Four units of micrococcal nuclease (Sigma) were added to the cell suspension and incubated for 15 minutes at room temperature. 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 0.05% 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 examining it on an 1.5% agarose gel stained with ethidium bromide. Typically, the population was >95% mononucleosomes as assessed by ImageJ (available at http://rsb.info.nih.gov/ij/).

Mononucleosome-containing solutions, precleared by incubation for 1 hour with protein-G agarose beads, were combined with either 4 μg αTY1 BB2 or αFLAG mAb and incubated at 4°C with rotation for ~1.5 hours. Subsequently, protein-G beads were added to immunoprecipitations and incubated at 4°C with rotation for 1 hour. Bound material was then washed for 15-30 minutes with wash buffer 1 (200 mM NaCl; 50 mM Tris; 0.05% NP40), wash buffer 2 (300 mM NaCl; 50 mM Tris, pH 8.0; 0.05% NP-40) and TE. Immunoprecipitated material was eluted from the beads by boiling for 10 minutes in sample buffer. To analyze each immunoprecipitation, western blotting was carried out using BB2, αFLAG, αH2AZ, or a combination of the αH3 antibodies.

Results

Identification of *T. brucei* H2AZ

A search of the *T. brucei* genome database revealed the presence of an unannotated ORF predicted to encode a polypeptide sharing 51-58% sequence identity with H2AZ from a variety of organisms (Fig. 1A). By contrast, it shared only 43% sequence identity with major H2A from *T. brucei* (data not shown). We cloned this ORF from cDNA and further confirmed by northern blotting that it is transcribed in both the mammalian bloodstream and procyclic (tsetse midgut) stages of the parasite's life cycle (data not shown). On the basis of its sequence conservation, we named this gene *H2AZ* and the protein it encodes H2AZ. Owing to an N-terminal extension, *T. brucei* H2AZ is considerably larger than H2AZ from other organisms (Fig. 1A; see also Fig. S1, supplementary material). Putative H2AZ orthologs in *Trypanosoma cruzi* and *Leishmania major* have similar extensions (data not shown), suggesting that this feature of H2AZ is conserved among kinetoplastid protozoa.

As a tool to investigate H2AZ function, we generated a polyclonal antibody to a peptide contained within the unique N-terminal region (Fig. 1A, bar; see also Materials and Methods). To test the specificity of the αH2AZ antibody, we performed western analysis on a variety of cell lines (Fig. 1B). In wild-type procyclic and bloodstream-form cells, H2AZ was present as a single band running at an apparent molecular

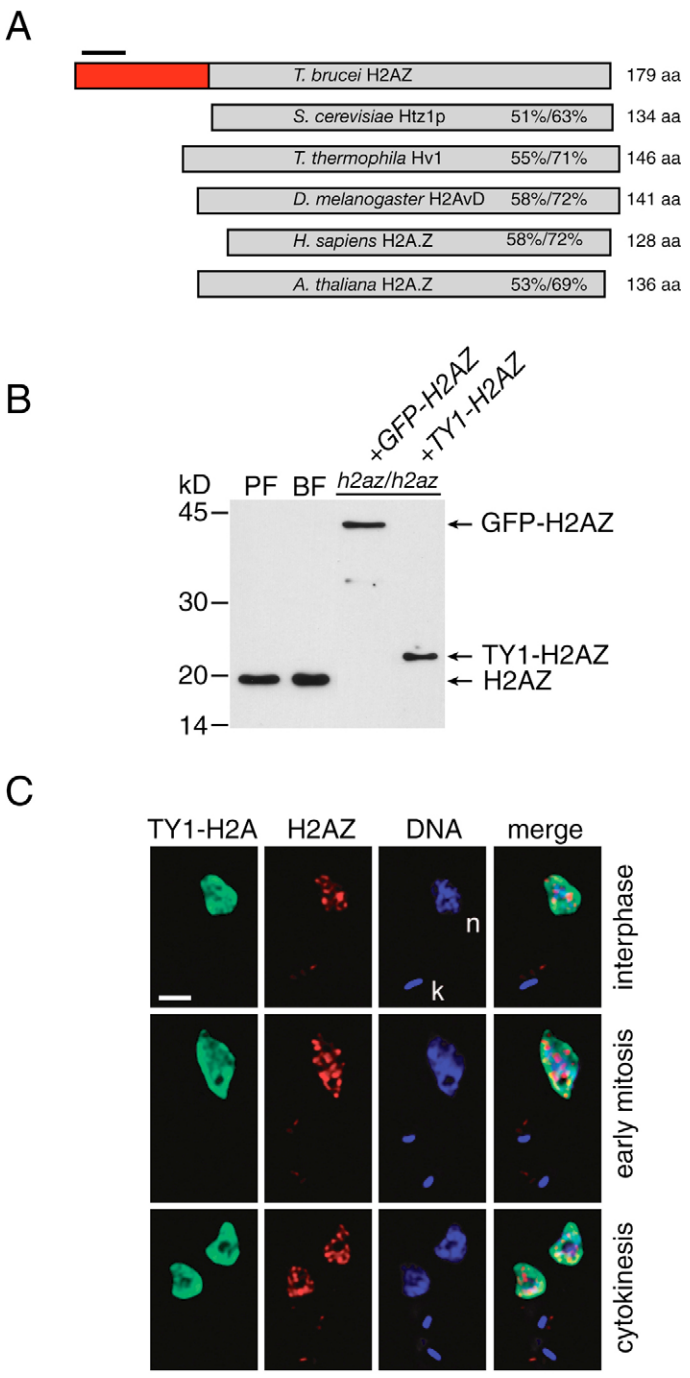

Fig. 1. *T. brucei* H2AZ localizes to distinct foci within the nucleus. (A) Sequence conservation of H2AZ. For each H2AZ, the polypeptide length is denoted as well as the percent sequence identity/similarity relative to *T. brucei* H2AZ. The amino terminal extension of *T. brucei* H2AZ (red) is unique and was excluded from these calculations. (B) A polyclonal antibody to a 14 amino-acid region of the amino-terminal tail of H2AZ (marked by a black bar in A) recognizes endogenous H2AZ in wild-type procyclic (PF) and bloodstream-form (BF) cell lines as well as those in which ectopically expressed GFP- or TY1-tagged H2AZ has replaced the endogenous H2AZ. (C) A comparison of the localization of H2AZ (red) and TY1-H2A (green) by indirect immunofluorescence in a bloodstream-form cell line over the course of the cell cycle reveals that H2AZ is not uniformly distributed. Both nuclear (n) and mitochondrial kinetoplast (k) DNA (blue) were detected with DAPI. Owing to its compact state, the kinetoplast DNA signal appears stronger than the nuclear DNA at this focal plane. Top, an interphase cell (1n 1k); middle, an early mitotic cell (1n 2k); bottom, cytokinesis (2n 2k). Bar, 2 μm.

weight of less than 20 kDa, close to its predicted size of 18.7 kDa. This result confirms that H2AZ is expressed in both life stages of the parasite. We created two cell lines that exclusively expressed tagged ectopic versions of H2AZ, by deleting both alleles of the *H2AZ* gene from cell lines engineered to express either an H2AZ fusion with the TY1 epitope (Bastin et al., 1996) (TY1-H2AZ) or with GFP (GFP-H2AZ). By western blot analysis, endogenous H2AZ could no longer be detected in either cell line, as expected. Instead, the αH2AZ antibody detected bands of ~22 kDa or ~44 kDa, which matched the expected sizes of TY1-H2AZ and GFP-H2AZ, respectively. We confirmed that these bands corresponded to the tagged versions of H2AZ, by demonstrating that they crossreacted with TY1- or GFP-specific antibodies (data not shown).

H2A and H2AZ have distinct localization patterns in *Drosophila* polytene chromosomes (Leach et al., 2000) and in early mouse embryos (Rangasamy et al., 2003). To compare the localization of H2A and H2AZ in *T. brucei*, we introduced a TY1-tagged copy of H2A into a bloodstream-form cell line and performed indirect immunofluorescence using antibodies to the TY1 epitope and to H2AZ (Fig. 1C). In *T. brucei*, the kinetoplast (mitochondrial DNA) completes its replication and division prior to nuclear DNA (Woodward and Gull, 1990). Thus, by counting the kinetoplasts and nuclei within a cell, its cell-cycle stage can be determined (see legend, Fig. 1C). As expected, TY1-H2A was distributed throughout the nucleoplasm over the course of the cell cycle. By contrast, H2AZ was detected as bright, punctate spots within the nucleus at all stages of the cell cycle. Proximal to the kinetoplasts, weak cytoplasmic staining was also observed. However, in cell lines that expressed TY-H2AZ or GFP-H2AZ, the H2AZ localization was entirely punctate nuclear (data not shown), strongly suggesting the cytoplasmic staining observed with the αH2AZ antibody was a result of crossreactivity. The H2AZ localization pattern was unchanged in procyclic cells (data not shown), indicating its distribution is not radically altered at this stage of the life cycle.

A histone H2B variant colocalizes with H2AZ

Of the few variant forms of H2B identified, most are involved in packaging chromatin in pollen and sperm (Malik and Henikoff, 2003). Thus, it was surprising that a search of the *T. brucei* genome database led to the discovery of an ORF predicted to encode a polypeptide with sequence similarity to H2B, yet clearly distinct from the canonical H2B (Fig. 2A). We cloned the gene from a BAC library (Zeng et al., 2001), mapped the start ATG codon by cloning and sequencing its cDNA (the protein does not initiate at the first in-frame genomic ATG), and confirmed by northern blot analysis that it is transcribed in both bloodstream and procyclic cells (data not shown). The predicted protein shares ~38% sequence identity with histone H2B but is 31 amino acids longer, owing to the presence of both N- and C-terminal extensions. Because of its

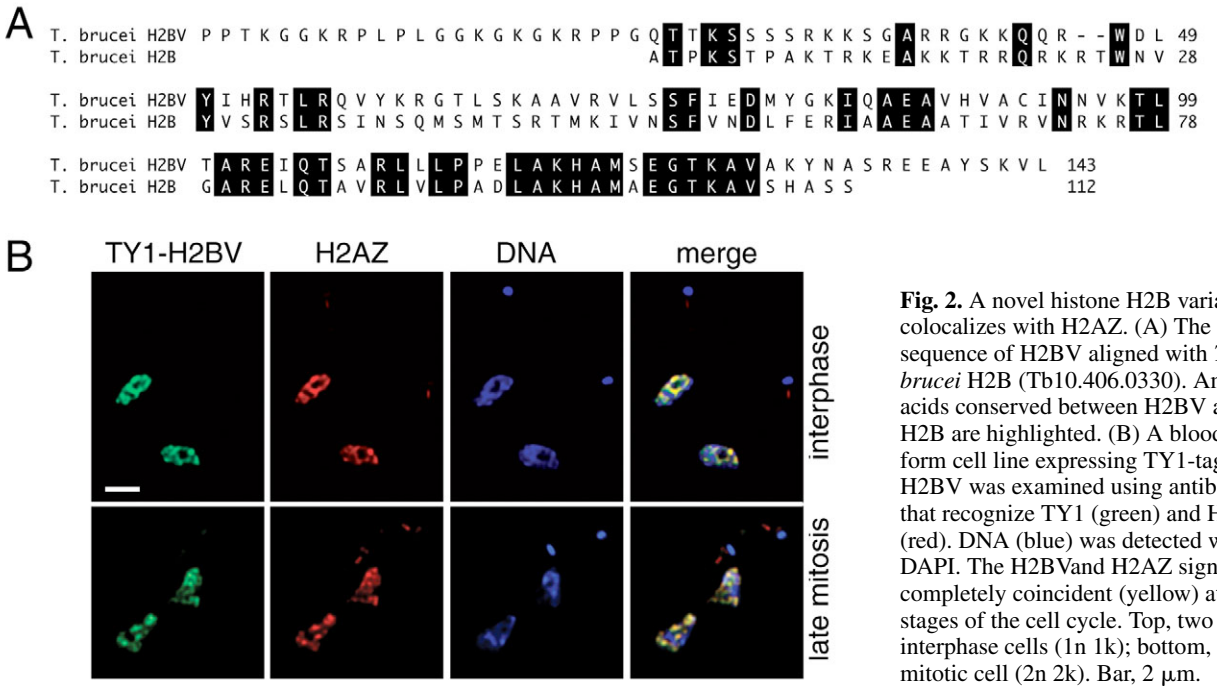

Fig. 2. A novel histone H2B variant colocalizes with H2AZ. (A) The sequence of H2BV aligned with *T. brucei* H2B (Tb10.406.0330). Amino acids conserved between H2BV and H2B are highlighted. (B) A bloodstream-form cell line expressing TY1-tagged H2BV was examined using antibodies that recognize TY1 (green) and H2AZ (red). DNA (blue) was detected with DAPI. The H2BVand H2AZ signals are completely coincident (yellow) at all stages of the cell cycle. Top, two interphase cells (1n 1k); bottom, a late mitotic cell (2n 2k). Bar, 2 μm.

similarity to H2B, we named this gene *H2BV* (for *H2B V*ariant) and the protein it encodes H2BV. Apparent orthologs of H2BV are present in *T. cruzi* and *L. major* (data not shown), suggesting that H2BV is conserved among kinetoplastid protozoa.

Like histone H2A, immunofluorescence microscopy revealed that H2B is distributed throughout the nucleoplasm at all points during the cell cycle (data not shown). To examine the localization of H2BV by indirect immunofluorescence, we introduced a TY1-tagged ectopic copy of *H2BV* (TY1-H2BV) into a bloodstream-form cell line and subsequently deleted both endogenous alleles of *H2BV*. H2BV formed numerous foci within the nucleus in a pattern that was remarkably similar to that observed for H2AZ (Fig. 2B). To test if H2BV and H2AZ colocalized, we simultaneously visualized both proteins. The signals corresponding to the two histones were completely coincident at all stages of the cell cycle (Fig. 2B, merge). Comparable results were observed in procyclic cells (data not shown), indicating that the colocalization is not dependent on the life-cycle stage of the parasite.

H2AZ and H2BV are encoded by essential genes

H2AZ is required for viability in mice, *Drosophila* and *Tetrahymena* (Faast et al., 2001; Liu et al., 1996; van Daal and Elgin, 1992), whereas both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* can survive its absence but exhibit growth phenotypes (Carr et al., 1994; Jackson and Gorovsky, 2000; Santisteban et al., 2000). To test if *H2AZ* is essential in *T. brucei*, we generated 'knock-out' cassettes conferring resistance to hygromycin or puromycin. When transfected into wild-type bloodstream-form cells, either cassette could replace one of the two endogenous *H2AZ* alleles, but multiple attempts at replacing both alleles were unsuccessful unless an ectopic copy of *H2AZ* was first introduced into the cell line (data not shown).

Of the variant forms of H2B described to date (Aul and Oko, 2001; Churikov et al., 2004; Gineitis et al., 2000; Ueda et al., 2000; Zalensky et al., 2002), whether any are required for viability or – when deleted – produce mutant phenotypes, has never been examined. To test if *H2BV* is essential, we employed a strategy similar to that used for *H2AZ* and achieved comparable results: all attempts to exchange the second endogenous *H2BV* allele with a 'knock-out' cassette failed except when an ectopic copy of *H2BV* was present (data not shown). On the basis of these combined results, we conclude that both *H2AZ* and *H2BV* are essential for viability.

Relative to their canonical histone counterparts, both H2AZ and H2BV have N-terminal extensions (Figs 1A and 2B; see also Fig. S1 in supplementary material). To test if these extensions are required for viability or influence localization, we created mutant alleles of *H2AZ* and *H2BV* in which the regions corresponding to the first 33 and 23 amino acids respectively were deleted and replaced by the TY1 epitope. Both TY1-H2AZΔ1-33 and TY1-H2BVΔ1-23 had localization patterns similar to their full-length counterparts (data not shown). Cell lines in which either mutant allele supplied the sole source of H2AZ or H2BV were readily generated, indicating that neither N-terminal extension is required for viability. For H2AZ, this result is consistent with previous studies, which demonstrated that the essential function of H2AZ maps to its C-terminal domain (Adam et al., 2001; Clarkson et al., 1999).

H2AZ and H2BV exhibit similar genomic distributions and are absent from sites of BrUTP incorporation

On the basis of the findings that H2AZ and H2BV colocalize and are required for viability, we proposed that they might function at the same genomic loci, possibly as components of the same nucleosome. If true, the ChIP profiles of H2AZ and H2BV should be identical. Formaldehyde-crosslinked

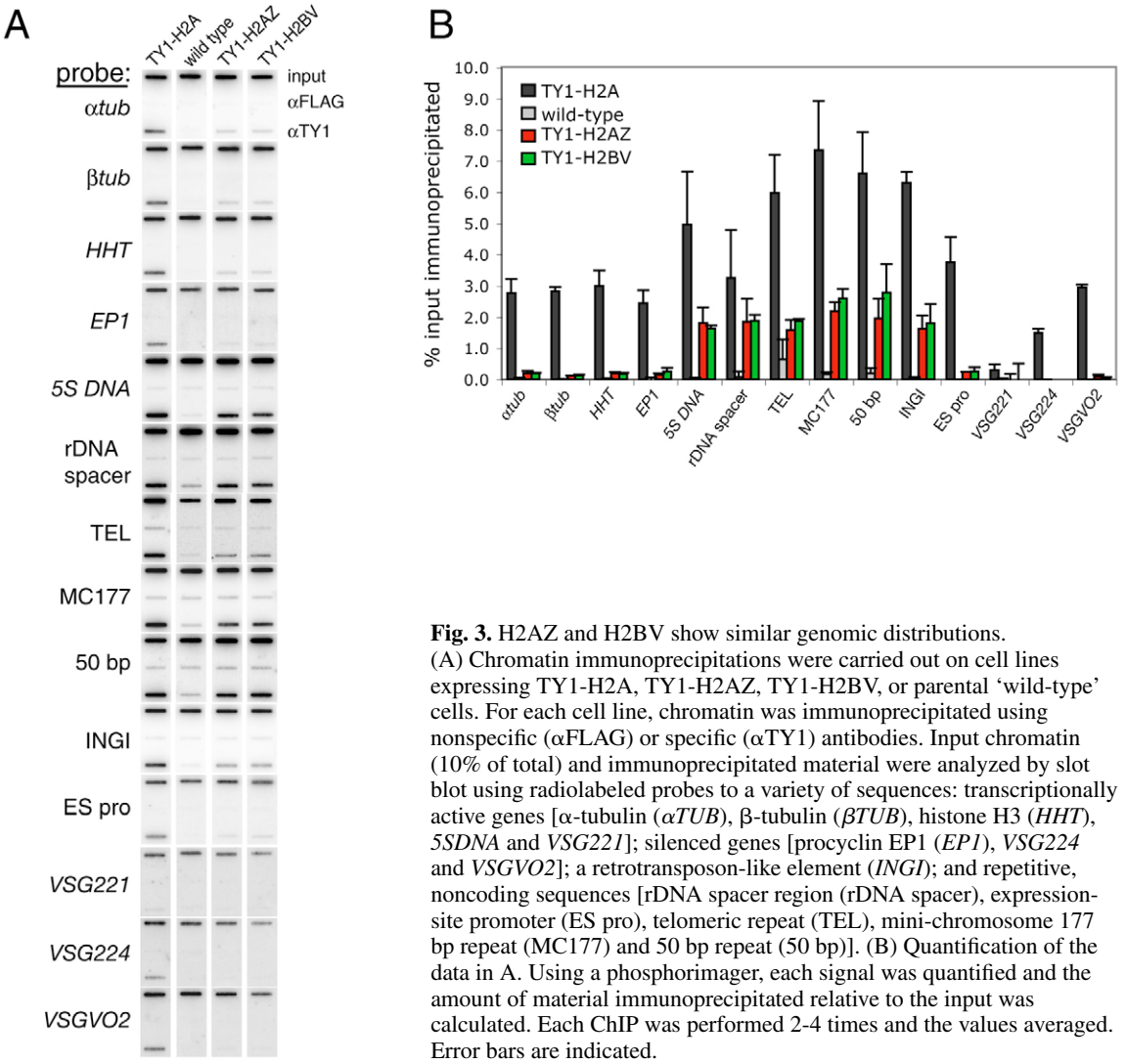

Fig. 3. H2AZ and H2BV show similar genomic distributions. (A) Chromatin immunoprecipitations were carried out on cell lines expressing TY1-H2A, TY1-H2AZ, TY1-H2BV, or parental 'wild-type' cells. For each cell line, chromatin was immunoprecipitated using nonspecific (αFLAG) or specific (αTY1) antibodies. Input chromatin (10% of total) and immunoprecipitated material were analyzed by slot blot using radiolabeled probes to a variety of sequences: transcriptionally active genes [α-tubulin (*αTUB*), β-tubulin (*βTUB*), histone H3 (*HHT*), *5SDNA* and *VSG221*]; silenced genes [procyclin EP1 (*EP1*), *VSG224* and *VSGVO2*]; a retrotransposon-like element (*INGI*); and repetitive, noncoding sequences [rDNA spacer region (rDNA spacer), expression-site promoter (ES pro), telomeric repeat (TEL), mini-chromosome 177 bp repeat (MC177) and 50 bp repeat (50 bp)]. (B) Quantification of the data in A. Using a phosphorimager, each signal was quantified and the amount of material immunoprecipitated relative to the input was calculated. Each ChIP was performed 2-4 times and the values averaged. Error bars are indicated.

chromatin from cells expressing either TY1-H2AZ or TY1-H2BV was sheared and immunoprecipitated using αTY1 antibody or, as a nonspecific control, αFLAG antibody. As additional controls, ChIP was carried out on cells expressing TY1-H2A and on wild-type cells. Since we lacked genetic clues about H2AZ and H2BV function, immunoprecipitated DNA was assessed by slot blot for the presence of a variety of sequences (see Materials and Methods for detailed probe information) including: actively transcribed genes (*αTUB*, *βTUB*, *HHT*, *5SDNA* and *VSG221*); silenced genes (*EP1*, *VSG224* and *VSGVO2*); a retrotransposon-like element (*INGI*); and noncoding sequences including the rDNA spacer region (rDNA spacer), expression-site (ES) promoters, telomere repeats (TEL), mini-chromosomal 177 bp repeats (MC177), and 50 bp repeats (50 bp) (Fig. 3A). With two exceptions (*VSG221* and *VSG224*), all loci tested are present in multiple copies.

All loci were detected in TY1-H2A-expressing cells when immunoprecipitated by αTY1 antibody, whereas no material was immunoprecipitated in wild-type cells or with αFLAG antibody. Because we used dCTP-labeled probes to detect DNA, the percentage of immunoprecipitated material relative to total material could be quantified (Fig. 3B). For TY1-H2A, with one exception (see below), ~1.5-7% of input material could be immunoprecipitated, with the highest percentages generally corresponding to the repetitive DNA tracts. These values underestimate the association of H2A with these loci because TY1-H2A was in competition with endogenous H2A. Both TY1-H2AZ and TY1-H2BV were detected at repetitive DNA tracts, *INGI* elements, *5SDNA* and rDNA spacer regions, where the percentage of immunoprecipitated material ranged from ~1.5-3.0%. By contrast, TY1-H2AZ and TY1-H2BV were barely detectable or undetectable at *αTUB*, *βTUB*, *HHT*, *EP1*, *VSG224*, *VSGVO2* or the ES promoter. Likewise, neither TY1-H2AZ nor TY1-H2BV appeared to associate with *VSG221* (the active *VSG* in these cells). However, the significance of this last result is unclear because *VSG221* was nearly undetectable in immunoprecipitates from TY1-H2A-expressing cells, suggesting the limits of detection might have been reached. Overall, the genomic distribution patterns of H2AZ and H2BV were very similar, consistent with the hypothesis that they function together.

To examine globally if H2AZ localized to transcriptionally active regions of the genome, we labeled nascent RNA

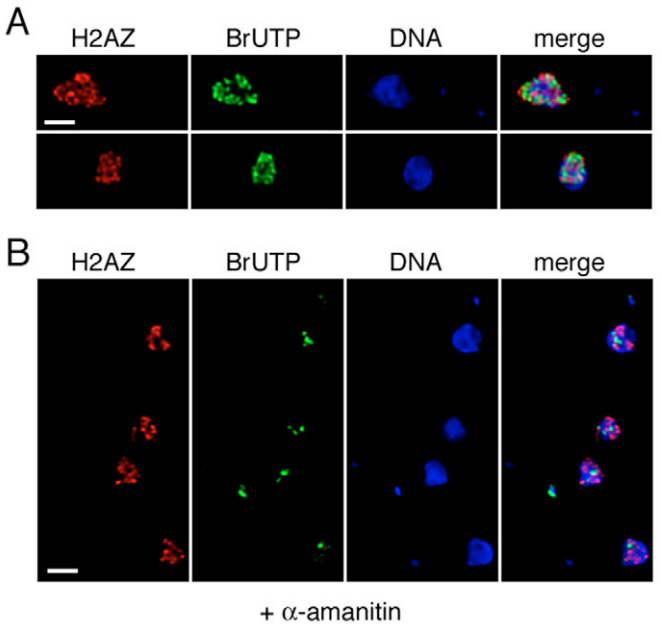

Fig. 4. H2AZ does not colocalize with sites of transcription. (A) Nascent RNA transcripts from bloodstream-form cells were labeled with BrUTP and examined by indirect immunofluorescence. H2AZ (red) and BrUTP (green) occupy distinct sites within the nucleus. (B) In the presence of 100 μg/ml α-amanitin, incorporation of BrUTP (green) is limited to sites of RNA polymerase I transcription, which do not overlap with H2AZ (red). For both A and B, DNA (blue) was detected with DAPI. Bar, 2 μm. BrUTP incorporation into the kinetoplasts is detected in the presence and, less obviously, in the absence of α-amanitin, probably reflecting the massive uridine incorporation that occurs during RNA editing, including in the bloodstream form (Schnaufer et al., 2001).

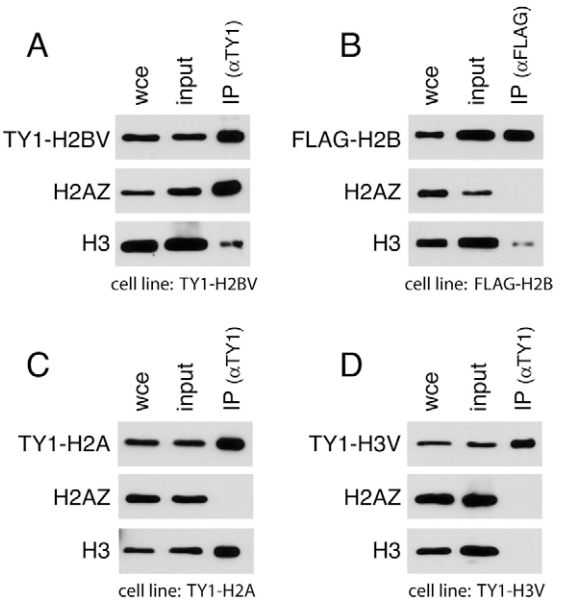

Fig. 5. H2AZ and H2BV co-immunoprecipitate. Mononucleosomes were prepared from cell lines expressing TY1-H2BV, FLAG-H2B, TY1-H2A or TY1-H3V, and were subjected to immunoprecipitations using antibodies to TY1 or FLAG. Immunoprecipitated material was then examined by western blotting for the presence of the TY1- or FLAG-tagged histone along with H2AZ and H3. For each experiment, a lane corresponding to whole-cell extract (wce), input material (input) and immunoprecipitated material (IP) is shown. (A) Both H2AZ and histone H3 co-immunoprecipitate with TY1-H2BV. (B) Histone H3, but not H2AZ, co-immunoprecipitates with FLAG-H2B. (C) Histone H3, but not H2AZ, co-immunoprecipitates with TY-H2A. (D) Neither H2AZ nor histone H3 co-immunoprecipitate with TY1-H3V.

transcripts with BrUTP and compared their localization with H2AZ (Fig. 4A). Although many BrUTP-containing foci were detected, few overlapped with H2AZ, suggesting that H2AZ is absent or is physically displaced from most sites of transcription. Similar results were observed for H2BV (data not shown). In the presence of α-amanitin, which inhibits transcription by RNA polymerase II and III, but not RNA polymerase I, the few BrUTP-containing foci that can be detected correspond to the nucleolus and the presumptive expression-site body, the RNA polymerase I-containing extranucleolar structure at which the active *VSG* is transcribed (Navarro and Gull, 2001). No dramatic reorganization of H2AZ occurred in the presence of α-amanitin, and H2AZ did not colocalize with sites of RNA polymerase I transcription (Fig. 4B), which is in agreement with our observation that H2AZ did not immunoprecipitate *VSG221* or any ES promoters.

H2AZ and H2BV co-immunoprecipitate

The colocalization and ChIP data were consistent with the intriguing possibility that H2AZ and H2BV heterodimerize in the same nucleosome. We therefore performed a series of experiments to test the ability of TY-H2BV and other epitope-tagged histones to co-immunoprecipitate with H2AZ. To ensure that any interaction occurred between histones within the same nucleosome, the chromatin input of each immunoprecipitation was treated with micrococcal nuclease, resulting in material consisting of >95% mononucleosomes. When TY1-H2BV was immunoprecipitated with αTY1 antibody, a robust signal corresponding to H2AZ was present in the immunoprecipitate (Fig. 5A). By contrast, H2AZ did not co-immunoprecipitate with FLAG-H2B (Fig. 5B), suggesting that in *T. brucei* H2AZ does not heterodimerize with H2B. Similarly, H2AZ did not co-immunoprecipitate with TY-H2A (Fig. 5C), consistent with previous experiments indicating that structural constraints would prevent a nucleosome from containing one molecule of H2AZ and another of H2A (Suto et al., 2000). Finally, as an additional specificity control, we demonstrated that H2AZ does not co-immunoprecipitate with TY1-H3V (Fig. 5D), a variant histone H3 enriched at telomeres in *T. brucei* (Lowell and Cross, 2004).

Failure to detect an interaction between H2B and H2AZ could be because mononucleosomes did not remain intact during immunoprecipitation. To rule out this possibility, we tested whether histone H3 co-immunoprecipitated with FLAG-H2B as well as with TY1-H2BV and TY1-H2A. In each instance, we were able to detect H3 in the immunoprecipitate (Fig. 5A-C). We note that the efficiencies of H3 co-immunoprecipitation varied, and less H3 was generally detected in the immunoprecipitate than in the input, presumably because of differences in the expression levels of

the tagged histones and because much of the H3 was packaged with endogenous (untagged) histones. We also showed that H3 did not co-immunoprecipitate with TY1-H3V (Fig. 5D), which implies that H3V does not interact with H3, as is the case in nucleosomes containing the histone H3 variants Cenp-A and H3.3 (Black et al., 2004; Tagami et al., 2004). Taken together, these data strongly suggest that *T. brucei* nucleosomes containing H2AZ also contain H2BV but not canonical H2B.

Discussion

We have identified a *T. brucei* homolog of the variant histone H2AZ and demonstrated that it associates with H2BV, a novel variant of histone H2B. Three lines of evidence support this claim. First, H2AZ and H2BV were observed to colocalize over the course of the cell cycle. Second, H2AZ and H2BV exhibited nearly identical ChIP profiles, indicating their association with the same genomic sites. Third, H2AZ and H2BV co-immunoprecipitate. Since H2AZ is highly conserved whereas variant forms of H2B are not, it has been assumed that in all organisms H2AZ would dimerize with H2B. Thus, our results are both surprising and significant because H2AZ has never before been shown to associate with a non-canonical form of H2B.

Our results provide the first direct evidence that an H2B variant functions in a nucleosomal context. On the basis of the structure of the nucleosome, we presume that the core unit of an H2AZ/H2BV-containing nucleosome is composed of two H2AZ/H2BV dimers and an H3/H4 tetramer. Steric constraints make it theoretically unlikely that H2AZ and H2A can exist within the same nucleosome (Suto et al., 2000). Consistent with this prediction, we were unable to co-immunoprecipitate H2AZ and H2A in *T. brucei*. Likewise, we were unable to detect a physical interaction between H2AZ and the telomere-enriched histone variant H3V (Lowell and Cross, 2004). Might there be a subpopulation of H2AZ-containing nucleosomes in *T. brucei* in which H2AZ dimerizes with H2B as it clearly does in many species? We were never able to detect such an interaction by co-immunoprecipitation, suggesting the H2AZ and H2BV dimerize exclusively. However, because our co-immunoprecipitation attempts were carried out on an epitope-tagged H2B that was in competition with endogenous H2B, if the input quantity of H2AZ was sufficiently low, a minor interaction may have been missed.

The machinery by which H2AZ/H2B dimers are deposited into chromatin has recently been identified in *S. cerevisiae* (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). The SWR1 chromatin-remodeling complex consists of Swr1, a Swi2/Snf2-related ATPase, Swc2-7, Bdf1, and subsets of both the Ino80 chromatin-remodeling complex and the NuA4 histone acetyltransferase complex. Orthologs of Swr1 are present in *Drosophila* and humans, strongly suggesting that the SWR1 complex is evolutionarily conserved (Kobor et al., 2004). Whether similar machinery could load H2AZ/H2BV dimers into chromatin in *T. brucei* is unknown and, at present, the SWR1 complex has not been identified. Inspection of the *T. brucei* genome reveals the presence of a putative ATP-dependent Snf2-family DNA helicase, but few clear candidate orthologs for other members of the complex.

Much progress has recently been made in understanding the functions of H2AZ in a range of organisms. In *S. cerevisiae*, the molecular mechanisms by which H2AZ (named Htz1) influences transcription have begun to emerge. Htz1 is relatively enriched at promoters and within ORFs of active genes, many of which are near telomeres and would ordinarily be subject to telomeric silencing (Meneghini et al., 2003). By acting synergistically with boundary elements, Htz1 disrupts Sir-dependent silencing and heterochromatin formation. Early in mammalian development, H2AZ is enriched at pericentric heterochromatin (Rangasamy et al., 2003), where it apparently assists in chromatin folding by promoting HP1 binding (Fan et al., 2004). When H2AZ is depleted by RNA interference, HP1 association with chromatin is disrupted and chromosome segregation becomes unstable (Rangasamy et al., 2004).

In light of these observations, a straightforward hypothesis is that H2AZ and its partner H2BV might be involved in similar processes in *T. brucei*. Since *H2AZ* and *H2BV* are essential, we were unable to look for phenotypes in either null mutant, which might have provided us with information about gene function. Instead, a close inspection of the localization and ChIP data might reveal clues. H2AZ and H2BV are widely distributed over the nucleus, but their pattern of localization clearly differs from H2A. By ChIP, all three proteins are associated with highly repetitive DNA, including the mini-chromosomal 177 bp repeats, the ES-proximal 50 bp repeats, and telomeric repeats. Because the ChIP readout was by slot-blot and the probes were often large in size, resolution was limited, and we could not infer whether specific sites within the repeats were occupied exclusively by H2A or H2AZ. However, only H2A was detected within the ORFs of arrays of RNA polymerase II-transcribed genes such as those encoding α- and β-tubulin and histone H3. Consistent with this observation, H2AZ and H2BV do not colocalize with sites of nascent RNA transcription visualized by BrUTP incorporation. Given these data, it is tempting to speculate that H2AZ and H2BV are primarily enriched at transcriptionally inactive regions of the genome, where they might function to block the spread of heterochromatin in a manner similar to Htz1. Alternatively, they might bind within ORFs and/or within promoter regions and be displaced during transcription through the actions of RNA polymerase II. In this case, the failure to observe colocalization with BrUTP-labeled nascent RNA would not be surprising. Clearly, a more thorough inventory of the sequences with which H2AZ and H2BV are associated must be achieved to establish if these limited ChIP data are representative of a larger trend; however, the necessary tools for such studies are not yet available in trypanosomes.

If H2AZ and H2BV do play roles in transcriptional control in *T. brucei*, it is important to remember that they must function in the context of an organism in which most transcription is polycistronic. Moreover, regulation of gene expression primarily occurs not at transcriptional initiation, but at the level of transcriptional elongation and RNA stability (Clayton, 2002). However, several lines of evidence point to the importance of chromatin structure in regulating transcription in *T. brucei*. For example, developmental expression-site repression correlates with a decrease of transcriptional accessibility of chromatin, suggesting that it is mediated by chromatin remodeling (Navarro et al., 1999). Furthermore, homologs of known chromatin-modifying enzymes exist (Ingram and Horn, 2002). Additionally, we have now demonstrated that *H2AZ* and *H2BV* are required for viability.

Although the sequencing of the *T. brucei* genome has been completed, the beginning and end of RNA polymerase II polycistronic transcription units have yet to be defined. Accordingly, the regions between polycistronic units, which presumably contain both termination sequences and promoters (and possibly enhancer and boundary elements), are uncharacterized. Perhaps H2AZ and H2BV bind to these interpolycistronic regions and promote transcription directly by altering the chromatin composition such that RNA polymerase is more stably bound to its template and transcription elongation occurs more efficiently. Alternatively, H2AZ and H2BV might function by blocking the spread of transcription from one polycistronic unit to another.

T. brucei represents an anciently diverged eukaryotic lineage (Stevens et al., 2001). Our observation that H2AZ and H2BV are within the same nucleosome in *T. brucei* opens up the exciting possibility that similar interactions between H2AZ and variant forms of H2B might occur in other organisms. Potential homologs of H2BV exist in other kinetoplastids and a variant form of H2B is present in the malarial parasite *Plasmodium falciparum* (accession number Q8IBV7). Inspection of the human genome reveals that it too contains (non-testis-specific) putative variant forms of H2B (accession numbers XP_210048, XP_373359, XM_498379). Whether any of these might be specifically associated with H2AZ must be evaluated, but their presence is an important reminder of the dynamic nature of the nucleosome.

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