A Novel Chromatin Protein, Distantly Related to Histone H2A, Is Largely Excluded from the Inactive X Chromosome

Brian P. Chadwick and Huntington F. Willard

Department of Genetics, Case Western Reserve University School of Medicine and Center for Human Genetics and Research Institute, University Hospitals of Cleveland, Cleveland, Ohio 44106-4955

Abstract. Chromatin on the mammalian inactive X chromosome differs in a number of ways from that on the active X. One protein, macroH2A, whose amino terminus is closely related to histone H2A, is enriched on the heterochromatic inactive X chromosome in female cells. Here, we report the identification and localization of a novel and more distant histone variant, designated H2A-Bbd, that is only 48% identical to histone H2A. In both interphase and metaphase female cells, using either a myc epitope–tagged or green fluorescent protein–tagged H2A-Bbd construct, the inactive X chromosome is markedly deficient in H2A-Bbd staining, while the active X and the autosomes stain throughout. In double-labeling experiments, antibodies to acetylated histone H4 show a pattern of staining indistinguishable from H2A-Bbd in interphase nuclei and on metaphase chromosomes. Chromatin fractionation demonstrates association of H2A-Bbd with the histone proteins. Separation of micrococcal nuclease–digested chromatin by sucrose gradient ultracentrifugation shows cofractionation of H2A-Bbd with nucleosomes, supporting the idea that H2A-Bbd is incorporated into nucleosomes as a substitute for the core histone H2A. This finding, in combination with the overlap with acetylated forms of H4, raises the possibility that H2A-Bbd is enriched in nucleosomes associated with transcriptionally active regions of the genome. The distribution of H2A-Bbd thus distinguishes chromatin on the active and inactive X chromosomes.

Key words: histones • X chromosome inactivation • euchromatin • histone H4 acetylation • macroH2A

Introduction

To achieve comparable levels of gene expression for X-linked loci in mammals, one of the X chromosomes in female cells is subject to inactivation early in development and is transmitted clonally through successive somatic divisions (Lyon, 1961; Heard et al., 1997; Willard, 2000). The inactive X chromosome (Xi)[1] can be observed in interphase nuclei as a densely staining body at the periphery of the nucleus, which is referred to as the Barr body (Barr and Bertram, 1949). Most X-linked genes are transcriptionally silent on the Xi (Disteche, 1995), ensuring correct embryonic development and adult viability. Genes from the pseudoautosomal regions (PARs) of the X chromosome escape X inactivation, as expected, since they have functional homologues on the Y chromosome (Lyon, 1962; Lahn and Page, 1997). More recently, a number of other human genes outside of the PARs have also been found to escape gene silencing on the Xi, with a majority of these mapping to the short arm of the chromosome (Brown et al., 1997; Carrel et al., 1999).

Several distinguishing features of the Xi have been identified when compared with the active X chromosome (Xa). These include hypoacetylation of histones H3 and H4 (Jeppesen and Turner, 1993; Boggs et al., 1996; Gilbert and Sharp, 1999), hypermethylation of CpG islands (Mohandas et al., 1981; Pfeifer et al., 1990), late replication in S phase (Gilbert et al., 1962; Morishma et al., 1962), and association of the chromosome with a large untranslated RNA, the Xi-specific transcript (Xist) (Brown et al., 1991, 1992; Brockdorff et al., 1992). In addition, the chromatin of the Xi is enriched for a novel core histone variant, macroH2A (Costanzi and Pehrson, 1998).

MacroH2A is an unusual histone variant: one third of the protein (at the amino terminus) shares >60% amino acid sequence identity to conventional H2A, while the remaining two thirds of macroH2A have no sequence homology to known proteins (Pehrson and Fried, 1992). Immunolocalization studies demonstrated that macroH2A

Address correspondence to Dr. H.F. Willard, Department of Genetics, Case Western Reserve University School of Medicine, BRB 731, 2109 Adelbert Rd., Cleveland, OH 44106-4955. Tel.: (216) 368-1617. Fax: (216) 368-3030. E-mail: hfw@po.cwru.edu

[1]*Abbreviations used in this paper:* Cy5, Cyanine 5.18; DAPI, 4,6-diamidino-2-phenylindole; EST, expressed sequence tag; GFP, green fluorescent protein; H4Ac12, histone H4 acetylated at lysine 12; MCB macrochromatic body; TR, Texas red; Xa, active X chromosome; Xi, inactive X chromosome; Xist, X inactive-specific transcript.

appears as a dense staining region in female interphase cells, termed a "macrochromatin body" (MCB), which is coincident with the Xi and remains associated with the chromosome at metaphase (Costanzi and Pehrson, 1998). MacroH2A first becomes associated with the inactive X chromosome after initiation and propagation of X inactivation in differentiated female mouse embryonic stem cells, which suggests that macroH2A is not necessary for the early stages of the inactivation process (Mermoud et al., 1999; Rasmussen et al., 2000). MacroH2A is associated with Xist RNA (Gilbert et al., 2000), and its localization is disrupted when the Xist gene is deleted, but this does not appear to affect the maintenance of X inactivation (Csankovszki et al., 1999). The exact role macroH2A plays in the X inactivation process is thus unclear.

The enrichment of a histone variant on the Xi suggests that there may be additional differences between the chromatin of the Xa and Xi. Using the nucleic acid sequence of histone H2A and the genome databases, we have now identified a second H2A-related molecule and have investigated its chromosomal distribution in relation to the Xi.

Materials and Methods

Isolation of Human cDNA Clones Related to H2A

The nucleotide sequences of members of the human H2A gene family were used in BLASTX searches against entries in the human expressed sequence tag (EST) database at GenBank using the NIH BLAST server (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). cDNA clones for two distantly related H2A IMAGE EST entries (IMAGE No. 782067 and 1409797) were obtained from Research Genetics. DNA was prepared with the Wizard plus miniprep DNA purification system (Promega), and the cDNAs were sequenced with a fluorescence labeled dye-terminator cycle sequencing kit according to the manufacturer's instructions (PRISM Ready DyeDeoxy Terminator Premix; Applied Biosystems) and electrophoresed on an ABI 373 (PerkinElmer).

Expression Analysis of the H2A-related Transcript

To prepare a probe for the H2A-Bbd gene, the full coding sequence from clone 782067 was PCR-amplified (Fwd 5′-CCA GCA TGC CGA GGA GGA G-3′; Rev 5′-CCG GGT GTC AGA AGC TAG TC-3′) and gel purified using standard techniques (Sambrook et al., 1989). The PCR product was radiolabeled and hybridized to a Northern blot according to the manufacturer's instructions (human multiple tissue Northern blot, No. 7759-1; CLONTECH Laboratories, Inc.).

Poly(A)$^+$ RNA was prepared from cell lines T-3352, 293 and a female, 46,XX EBV-transformed lymphoblast cell line using the Fast-Track 2.0 system (Invitrogen) and reverse transcribed using standard techniques (Sambrook et al., 1989). The H2A-Bbd transcript was detected by PCR using 100 ng of each primer described above, and 1 U *Taq* polymerase in a 25-μl 1.5-mM Mg^{2+} reaction. Amplification was carried out using an initial denaturation of 94°C (2 min), followed by 40 cycles of 94°C (20 s), 60°C (20 s), and 72°C (30 s), and a final extension cycle at 72°C (10 min). PCR products were separated by electrophoresis through a 2% agarose gel.

Mammalian Expression Constructs

The full coding sequence from clone 782067 was PCR amplified with primers incorporating BamHI and EcoRI restriction enzyme recognition sites (Fwd 5′-GGA ATT CTG ATG CCG AGG AGG AGG AG-3′; Rev 5′-GGA ATT CGT CCT CGC CAG GGG C-3′) and subcloned using standard techniques (Sambrook et al., 1989). Both a myc epitope-tagged and green fluorescent protein (GFP) fusion were generated by cloning into pcDNA3.1-CT-myc-His and pcDNA3.1-CT-GFP (Invitrogen), and subclones were verified for sequence integrity as above.

A full-length human macroH2A cDNA was assembled from fragments from two overlapping IMAGE cDNA clones (2212400 and 1086835). The coding sequence was PCR amplified with primers incorporating EcoRI restriction sites (Fwd 5′-GGA ATT CTC ATG TCG AGC CGC GGT GG-3′; Rev 5′-GGA ATT CGT TGG CGT CCA GCT TGG C-3′) and cloned in-frame into pcDNA3.1-CT-myc-His vector. Subclones were sequenced to ensure sequence integrity and orientation as above.

A myc epitope-tagged histone H2B was derived by subcloning the KpnI–BamHI fragment from pEGFP-H2B (Kanda et al., 1998) into pcDNA3.1-CT-myc-His. pEGFP-H2B was a generous gift of Drs. Teru Kanda and Geoffrey Wahl (Salk Institute, La Jolla, CA).

Cell Culture and Transfection

Human cell lines used in this study were T-3352, a 46,XX primary fibroblast strain (provided by Dr. Stuart Schwartz, Case Western Reserve University, Cleveland, OH); 293, a female fetal kidney tumor cell line; GM04626 and GM00254, both 47,XXX primary fibroblast strains (National Institute of General Medical Sciences Cell Repository, Camden, NJ); and Y87, a 46,XY primary fibroblast strain.

Cell lines were maintained in culture as monolayers in alpha-MEM supplemented with 20% FBS, 100 μg/ml antibiotics (penicillin and streptomycin), and 2 mM L-glutamine (GIBCO BRL) at 37°C in a 5% CO_2 atmosphere. Stably transformed 293 cell lines (see below) were cultured in the presence of 300 μg/ml Neomycin (G418; GIBCO BRL). For immunofluorescence experiments, 2–4 × 10^4 cells were seeded on microscope slides 24–48 h after transfection.

Transfections were performed in serum-free media using Superfect reagent according to the manufacturer's recommendations (QIAGEN). To establish a stably transformed 293 cell line, 5 μg of clone 782067 expression construct (see Mammalian Expression Constructs) was linearized with 5 U of PvuI (GIBCO BRL) for 1 h at 37°C before transfection with Superfect. Cells were grown for 48 h before selection with G418.

Immunofluorescence and Fluorescence In Situ Hybridization

Transfected cells were grown directly on microscope slides and were permeabilized with 0.1% Triton X-100 in PBS for 10 min before incubations with a 1:100 dilution of anti–myc mAb (Invitrogen) in PBS-tween and 1% BSA for 1 h at 37°C. Detection was achieved using a 1:200 dilution of goat anti–mouse IgG conjugated with either FITC or Texas red (TR) in PBS-tween and 1% BSA at 37°C for 30 min (Jackson ImmunoResearch Laboratories). Sequential fluorescence in situ hybridization (FISH) was performed after fixation in 3.7% formaldehyde in PBS for 10 min. Slides were denatured in 70% formamide, 2× SSC for 14 min at 72°C before dehydration through a 70–80–100% ethanol series, and then air dried. A direct-labeled human X chromosome alpha satellite probe was obtained from Vysis (Spectrum Orange). A direct-labeled Cyanine 5.18 (Cy5) human X alpha satellite probe was generated by labeling pBamX7 DNA (Waye and Willard, 1985) by nick translation in the presence of Cy5-dUTP (Amersham Pharmacia Biotech). The probes were denatured for 5 min at 42°C before placing on ice, and hybridization was carried out overnight in a humid chamber at 37°C. Slides were washed twice in 50% formamide, 2× SSC for 10 min at 42°C and once in 2× SSC at 42°C before application of 200 ng/ml 4,6-diaminidino-2-phenylindole in diazabicyclol-2-2-2-octane (DABCO; Sigma-Aldrich). Images were collected with a Vysis imaging system equipped with a cooled CCD camera (Photometrics) controlled via the Quips M-FISH™ software (Vysis).

Cells transfected with GFP-tagged proteins were detected with a FITC excitation filter (Chromatech) using a Vysis Quips™ imaging system. Slides were fixed with 3.7% formaldehyde, 0.1% Triton X-100 for 10 min, before washing with PBS, and counterstaining nuclei with 4,6-diamidino-2-phenylindole (DAPI).

Metaphase Analysis

Metaphase chromosomes were obtained by cytospinning in a Shandon Cytospin-3 essentially as described (Sullivan and Warburton, 1999). Unfixed chromosomes were extracted in 0.1% Triton X-100 in PBS for 10 min before incubation with a 1:100 dilution of anti–myc monoclonal antibody for 1 h at 37°C. Detection of histone H4 acetylated at lysine 12 (H4Ac12) was achieved using a 1:90 dilution of anti–H4Ac12 rabbit polyclonal antisera (pAb; 06-761; Upstate Biotechnology), according to the manufacturer's instructions. Coincubations of both anti–myc mAb and anti–H4Ac12 pAb were carried out using conditions favored by anti–H4Ac12. H4Ac12 pAb was detected using a 1:200 dilution of a goat anti–rabbit IgG conjugated with FITC and a 1:200 dilution of a goat anti–mouse IgG conjugated with Texas red (Jackson ImmunoResearch Laboratories). After

incubation with the secondary antibody, chromosomes were fixed in 3.7% formaldehyde in PBS for 10 min, followed by 15 min in 3:1 methanol:acetic acid. Sequential FISH was carried out as described above.

Chromatin Extraction and Immunoblotting

Chromatin preparation and histone extraction was performed by a modified version of previously described methods (Holt et al., 1989; Lennox and Cohen, 1989). Nuclei were isolated from stably transfected 293 cells and from untransfected 293 cells as a control. 10^8 cells were homogenized in resuspension buffer at 4°C (80 mM NaCl, 20 mM EDTA and 1% Triton X-100) centrifuged at 1,500 *g* and washed twice more in the same buffer. The nuclei pellet was then homogenized twice in wash buffer (150 mM NaCl, 10 mM Tris, pH 8.0) before resuspending in water and lysing for 30 min on ice. Chromatin was pelleted by centrifugation at 20,000 *g* and resuspended in 10 mM Tris, pH 8.0. Histones were extracted with 2.2 N H_2SO_4 on ice for 30 min. The extraction was centrifuged at 20,000 *g* and the acid-soluble fraction was precipitated overnight at 4°C in 5 vol of 100% ethanol, before centrifugation at 20,000 *g*. Both the acid soluble and insoluble fractions were resuspended in 10 mM Tris, pH 8.0.

Proteins were separated by gel electrophoresis on an 18% SDS PAGE gel before transfer to polyvinyldifluoride membrane (Bio-Rad Laboratories) as previously described (Harlow and Lane, 1989). The blot was probed with a 1:5,000 dilution of anti–His mAb in 5% nonfat milk, PBS, 0.1% Tween-20, followed by incubation with a horseradish peroxidase conjugated goat anti–mouse secondary antibody. Detection was performed using the ECL chemiluminescence substrate (Amersham Pharmacia Biotech).

Fractionation of Micrococcal Nuclease-digested Chromatin and Immunoblotting

Nuclei were isolated from cell lines as described above. Nuclei were resuspended in digestion buffer (10 mM NaCl, 10 mM Nabutyrate, 10 mM Tris, pH 7.2, 2 mM $MgCl_2$, and 1 mM $CaCl_2$), and digested with 15 U of micrococcal nuclease (Worthington) and incubated at 37°C for 8 min. Digestion was stopped by addition of EDTA to 10 mM final and chilling suspension on ice. Nuclei were centrifuged at 10,000 *g* for 4 min and supernatant S1 retained. The pellet was resuspended in lysis buffer (10 mM Tris, pH 7.2, 10 mM Nabutyrate, and 250 mM EDTA) and incubated on ice for 30 min before centrifugation at 10,000 *g* for 4 min. The supernatant S2 was retained and pooled with S1. Nucleosome oligomers were separated by ultracentrifugation through 5–30% sucrose gradient containing 10 mM Tris, pH 7.2, 10 mM Nabutyrate, and 250 mM EDTA at 37,000 rpm for 16 h at 4°C in a SW41 rotor (Beckman Coulter).

Gradient fractions were collected and nucleosome containing fractions were identified by gel electrophoresis on an 18% PAGE gel and coomassie staining. Nucleosome-containing fractions were separated by gel electrophoresis on an 18% SDS PAGE gel before transfer to polyvinyldifluoride membrane as previously described (Harlow and Lane, 1989). The blot was probed with a 1:5,000 dilution of anti–myc mAb in 5% nonfat milk, PBS, 0.1% Tween-20, followed by incubation with a horseradish peroxidase–conjugated goat anti–mouse secondary antibody. Detection was performed using the ECL chemiluminescence substrate (Amersham Pharmacia Biotech).

Results

Isolation of a Novel Histone H2A Variant

Using the nucleotide sequence of members of the human histone H2A family, we searched the public databases and identified a group of overlapping ESTs with distant homology to H2A that matched a predicted gene present in three intronless copies in Xq28 (Naylor et al., 1995). Two representative clones were obtained for the group and completely sequenced (AF254576). An open reading frame was identified from nucleotides 9–356, with the ATG at nucleotide 9 having a good match to the translation initiation start site consensus for vertebrates (CCCAGCAUGC versus GCCA/GCCAUGG) (Kozak, 1991). To demonstrate expression of the gene, the coding sequence was used to probe a Northern blot, which identified a signal of ~900 bp in testis (Fig. 1 a). The presence of the mRNA in a variety of cell lines and tissues was demonstrated by reverse transcription PCR (Fig. 1 b). Unlike conventional histone H2A genes (Dominski and Marzluff, 1999), the cDNA sequence contained a polyA tail and has a consensus polyadenylation sequence of AATAAA. In addition, no vertebrate Histone Downstream Element

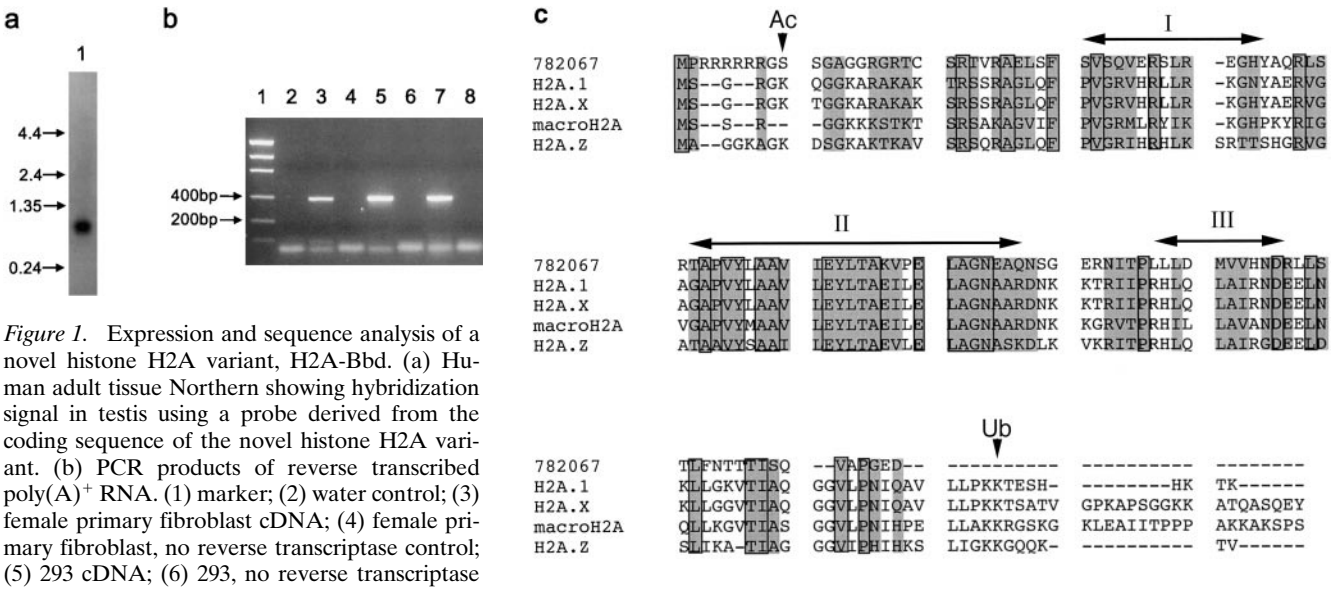

Figure 1. Expression and sequence analysis of a novel histone H2A variant, H2A-Bbd. (a) Human adult tissue Northern showing hybridization signal in testis using a probe derived from the coding sequence of the novel histone H2A variant. (b) PCR products of reverse transcribed poly(A)$^+$ RNA. (1) marker; (2) water control; (3) female primary fibroblast cDNA; (4) female primary fibroblast, no reverse transcriptase control; (5) 293 cDNA; (6) 293, no reverse transcriptase control; (7) female lymphoblast cDNA; (8) female lymphoblast, no reverse transcriptase control. (c) Sequence of the novel histone H2A variant (782067), the H2A region of macroH2A (AAC39908), and three members of the human H2A family: H2A.1, a replication-linked H2A (CAB06031), the histone variant H2A.X (P16104), and the histone variant H2A.Z (P17317). The location of residues modified by acetylation (Ac) and ubiquitination (Ub) are indicated. The three α-helices (I, II, and III) of the histone fold domain are indicated. Alignments were made using GeneWorks® release 2.2.1 (IntelliGenetics).

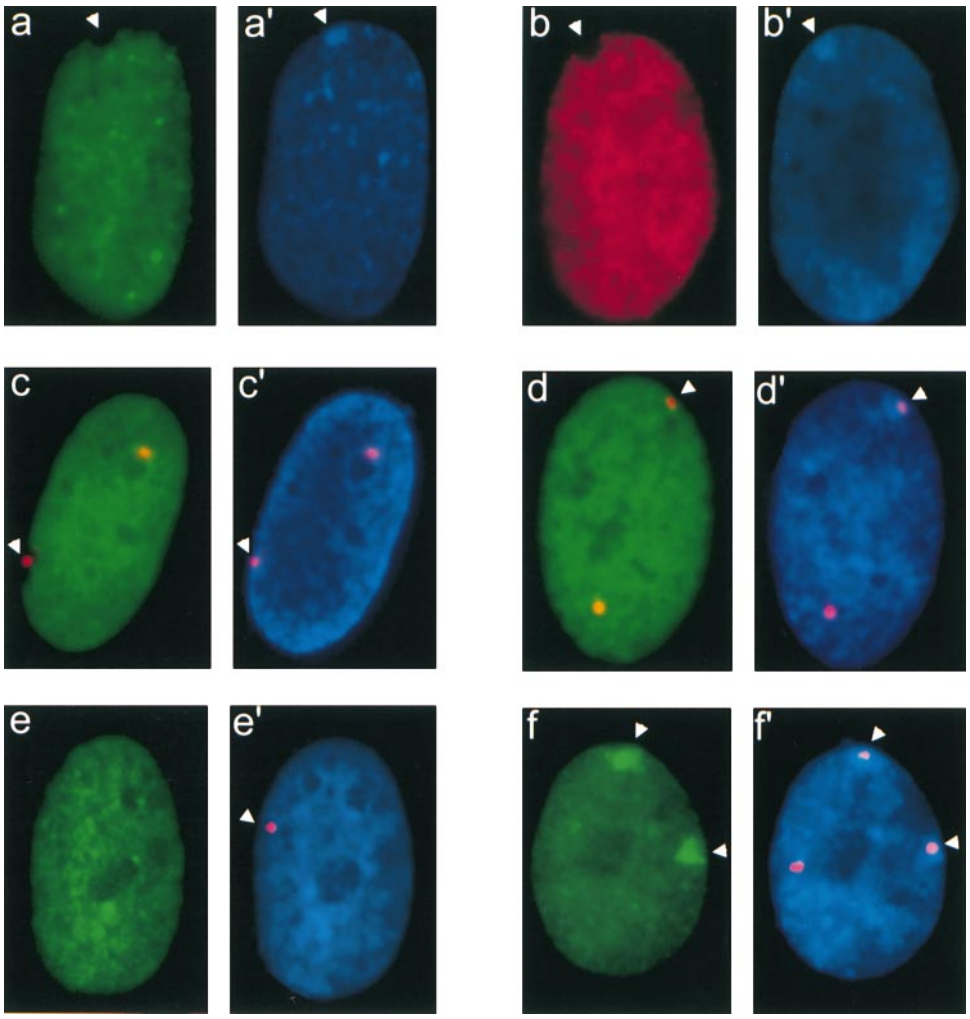

Figure 2. Nuclear distribution of H2A-Bbd at interphase in primary female fibroblast cells showing the zone of exclusion around the Barr body and X chromosome, and the distribution of macroH2A in 46,XY and 47,XXX primary fibroblast cell nuclei. (a) Transfected female cell showing the nuclear distribution of a COOH-terminal GFP-tagged H2A-Bbd by indirect immunofluorescence (green). The region of exclusion is indicated with the white arrow. (a′) DAPI staining of the same nucleus reveals the condensed Xi in the form of the Barr body at the periphery of the nucleus (white arrow). (b) Indirect immunofluorescence of a transfected female cell showing the nuclear distribution of a COOH-terminal myc-tagged H2A-Bbd (red, TR), stained with a anti–myc mAb followed by goat anti–mouse IgG conjugated with Texas red. The exclusion is indicated with a white arrow. (b′) The DAPI image of the same nucleus indicates the position of the Barr body. (c) Female cell transfected with myc-tagged H2A-Bbd showing the nuclear distribution of H2A-Bbd by indirect immunofluorescence (green, FITC) merged with the FISH signals for a human X alpha satellite probe (orange, rhodamine). One of the two X centromere signals is located within a deficient region and is indicated with a white arrow. (c′) The DAPI image of the same nucleus indicates the location of the X alpha satellite FISH probe that is contained within the H2A-Bbd exclusion zone. (d) Transfected female cell showing the even nuclear distribution of a COOH-terminal myc-epitope–tagged H2B construct by indirect immunofluorescence (green, FITC) superimposed with two X alpha satellite FISH signals (orange, rhodamine). Neither X chromosome is located within a region deficient in H2B staining. (d′) DAPI stain of the same nucleus is shown. (e) Transfected interphase male cell showing the nuclear distribution of a COOH-terminal myc-epitope–tagged macroH2A construct by indirect immunofluorescence (green, FITC). (e′) The location of the single X chromosome is shown merged with the DAPI stain of the same nucleus (orange, rhodamine). No MCBs characteristic of an Xi can be seen in the nucleus. (f) Indirect immunofluorescence of a female interphase triple X cell transfected with macroH2A showing the nuclear location of two MCBs (green, FITC) indicated by white arrows. (f′) The DAPI stain of the same nucleus merged with the X alpha satellite FISH (orange, rhodamine) shows two X signals to be coincident with the MCB indicative of Xi's. The third X chromosome is not associated with an MCB.

consensus sequence of AAAGAG is obvious in the 3′ untranslated region (Williams and Marzluff, 1995), nor is there a match to the consensus stem–loop sequence, suggesting that polyadenylation is the only form of 3′-end processing for this transcript. The presence of the polyA tail was confirmed by 3′RACE using a primer specific to the polyA start site in the ESTs in the database.

The cDNA is predicted to encode a 115 amino acid protein, with a molecular weight of 12.7 kD and a pI of 10.8. The predicted protein is 48% identical to histone H2A. Fig. 1 c shows an alignment of the predicted protein with human histone H2A (Albig et al., 1999), two replication-independent H2A variants, H2A.X (Mannironi et al., 1989) and H2A.Z (Hatch and Bonner, 1988), and the histone region of human macroH2A (Pehrson and Fried, 1992). The novel histone is considerably shorter than other H2A family proteins, but still contains a highly basic NH_2- and a COOH-terminal tail that is unique to H2A histones. Histone H2A is subject to several forms of posttranslational modification, including acetylation, phosphorylation, and ubiquitination (Luger and Richmond, 1998; Wolffe and Hayes, 1999). None of the residues that are the targets of these modifications is conserved in the novel H2A variant, suggesting that this protein is not regulated in the same fashion as H2A; modification of alternative residues in the protein cannot be ruled out. The region of the protein that shows the highest level of sequence identity lies within the α helices of the histone fold (Argents and Moudrianakis, 1993; Luger et al., 1997). Residues within this domain are important for protein–protein and

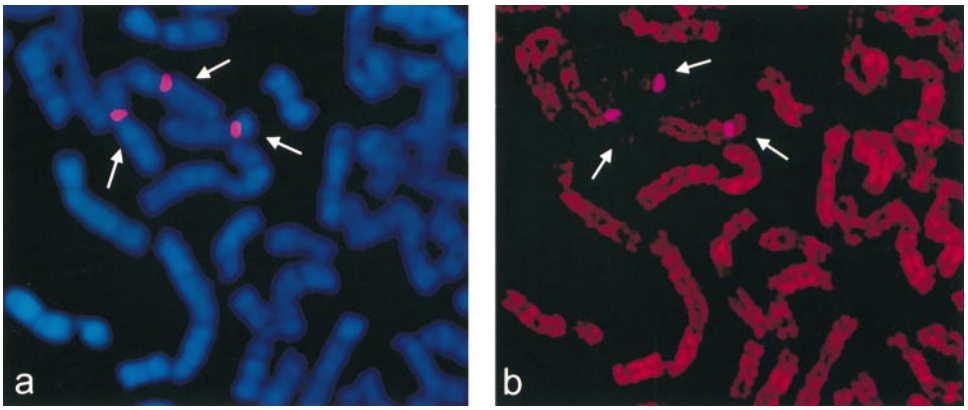

Figure 3. Chromosomal localization of H2A-Bbd on metaphase chromosomes from the female embryonic kidney carcinoma cell line 293. (a) Partial metaphase spread of a H2A-Bbd–transfected 293 cell showing the DAPI staining of the chromosomes (blue) merged with X alpha satellite cyanine 5.18 (Cy5) FISH signals (pink). Three X chromosomes are indicated with white arrows. (b) Indirect immunofluorescence of H2A-Bbd distribution on the same partial metaphase spread. Two of the X chromosomes do not stain with H2A-Bbd, while one (the cytologically marked Xa) shows a similar pattern to the surrounding autosomes (red, TR). The X alpha satellite Cy5 signal is shown (pink) and the positions of the X chromosomes are indicated with white arrows.

protein–DNA interactions within the nucleosome (Ichimura et al., 1982; Argents and Moudrianakis, 1995; Luger and Richmond, 1998), suggesting that the protein may be incorporated into a proportion of nucleosomes, replacing one or more histone H2A molecules.

The Novel Histone Variant Localizes to the Nucleus and Is Largely Excluded from the Barr Body in Interphase Nuclei

To investigate the subcellular localization of the protein, a GFP fusion to the COOH terminus of the histone variant was constructed and transfected into primary human fibroblasts. In 100% of transfected 46,XY cells, a uniformly distributed GFP signal was observed in the nucleus. However, in female cells, counterstaining of the nucleus with DAPI revealed a clear excluded region in the distribution of the protein at the periphery of the nucleus, in a region corresponding to the Barr body (Fig. 2 a). To address the possibility that the protein was being excluded from the Barr body because of the large GFP fusion, we performed similar experiments using a myc-tagged version of the protein. The myc-tagged protein also localized to the nucleus and again, an exclusion zone was observed where the Barr body was found (Fig. 2 b). We have named the protein H2A-Bbd, for Histone H2A variant, Barr-body deficient, to reflect this pattern.

To confirm that the exclusion zone observed in the nucleus coincided with an X chromosome, an alpha satellite probe specific for the X was used to FISH the transfected cells. A clear signal was obtained for both X chromosomes, with one of the two signals coinciding with the Barr body and with the excluded region in the H2A-Bbd distribution (Fig. 2 c).

As a further control to ensure that H2A-Bbd was not being excluded from the Barr body because of the epitope tag, we generated a myc-tagged histone H2B construct. When transfected into normal female cells, the resulting nuclear staining pattern was largely homogeneous (Fig. 2 d). Notably, there was no obvious H2B exclusion zone consistently associated with an X chromosome.

To further address association of H2A-Bbd exclusion zones with the Xi, we determined the number of H2A-Bbd exclusion zones in normal male, normal female, and 47,XXX female cells, containing 0, 1, or 2 Xi chromosomes, respectively. A strong correlation was seen between the number of H2A-Bbd exclusion zones and the number of Xi's in each cell line. As a control to establish that transiently expressed H2A variants could associate with the Xi in these cell lines, we used an epitope-tagged macroH2A1.2 construct to unambiguously identify the Xi in normal female and 47,XXX cells. As illustrated in Fig. 2, e and f, male cells never showed a macroH2A MCB associated with the X (n = 50 cells), whereas 47,XXX cells showed two distinct MCB's in the majority (42/50) of cells scored.

Inactive X Chromosomes Are Deficient in H2A-Bbd Staining in Metaphase

As shown above, H2A-Bbd clearly localizes to the nucleus of interphase cells and is largely excluded from the Xi chromosome in female cells. To investigate whether H2A-Bbd remains associated with metaphase chromosomes during mitosis, we examined the female embryonic kidney carcinoma cell line 293, which contains a single Xa (marked cytogenetically by a deletion of the short arm) and a variable number of Xi's (one to four copies in different cells). The 293 cells were transfected with myc-tagged H2A-Bbd and metaphase chromosomes were prepared. Immunolocalization followed by FISH to identify the X chromosomes revealed a distinct H2A-Bbd staining pattern. There was no obvious enrichment for any chromosomal regions; however, there was an almost complete absence of signal on the Xi (Fig. 3). Over 90% of metaphase

Table I. Metaphase Chromosome Analysis of Male and Female Cells Transfected with H2A-Bbd or H2B Construct, and Scored for the Presence or Absence of Signal Associated with an X Chromosome

		X chromosome staining			
Cells	Construct	All Xs positive		≥1 X deficient	
			%		%
293	H2A-Bbd	13	9	126	91
HT1080	H2A-Bbd	266	97	8	3
293	H2B	116	100	0	0

Numbers were obtained from cytospun metaphase chromosomes labeled by indirect immunofluorescence using an anti–myc mAb followed by a FITC-conjugated goat anti–mouse IgG. X chromosome was identified using an X alpha satellite probe directly labeled with rhodamine.

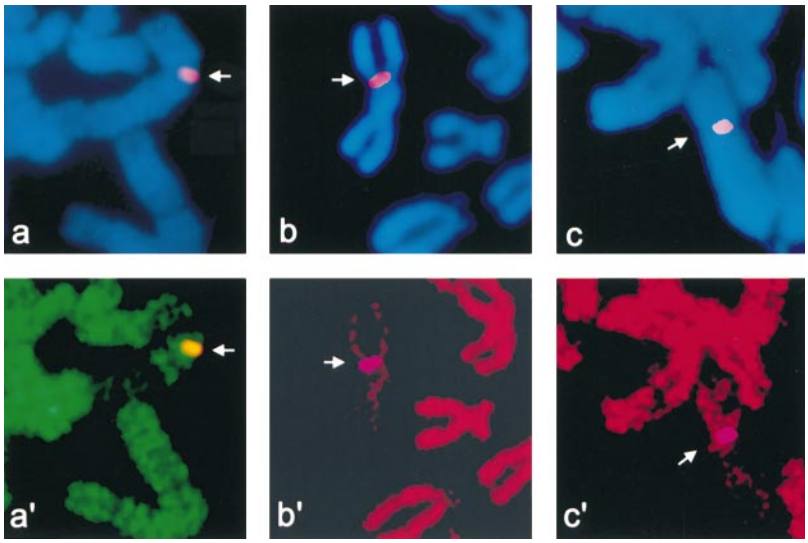

Figure 4. Indirect immunofluorescence of the H2A-Bbd banding pattern on the inactive X chromosome. Partial metaphase spreads of H2A-Bbd–transfected 293 cell showing the DAPI staining of the chromosomes (blue) merged with X alpha satellite rhodamine signal (orange) (a and a′) or Cy5 signal (pink) (b, b′, c, and c′). In all panels, the X chromosome is indicated with a white arrow. Indirect immunofluorescence of the H2A-Bbd distribution along the metaphase chromosomes was detected with FITC (green, a′) or rhodamine (red, b′ and c′).

spreads showed the same pattern; staining of the Xa was indistinguishable from autosomes, while all additional Xs stained poorly (Table I). A control experiment using a myc-tagged H2B control construct showed all X chromosomes and autosomes having an identical homogeneous staining (data not shown). As an additional control, metaphase spreads were made from the male tetraploid tumour line HT1080 transfected with H2A-Bbd. In the vast majority of spreads, the X chromosomes showed homogeneous H2A-Bbd staining (Table I).

In some metaphase spreads from female cells, close examination of the weakly staining X chromosome by H2A-Bbd revealed a slight banding pattern on the Xi (Fig. 4). The staining was most prominent on several regions of the short arm of the chromosome and in the pericentromeric region, with occasional staining on the proximal long arm.

The Distribution of H2A-Bbd Protein Completely Overlaps with Regions of H4-Acetylation in Interphase and Metaphase

Histone H4 is posttranslationally modified by acetylation at four lysine residues in the NH_2-terminal tail of the protein (Luger and Richmond, 1998; Wolffe and Hayes, 1999; Spencer and Davie, 1999). Euchromatic regions of the genome are enriched for acetylated H4 isoforms (O'Neill and Turner, 1995), while heterochromatic domains (including the Xi) lack acetylation (Jeppesen and Turner, 1993; O'Neill and Turner, 1995; Boggs et al., 1996; Gilbert and Sharp, 1999). Using antisera to H4Ac12, we compared the pattern of H4-acetylation to the distribution of H2A-Bbd. Both H2A-Bbd and acetylated H4 localize throughout the nucleus, with the exception of the exclusion zone corresponding to the Xi and the Barr body (Fig. 5). Overlaying the two distributions reveals almost identical patterns, indicating that H2A-Bbd is tightly associated with acetylated euchromatic regions of the genome.

Metaphase spreads were prepared from H2A-Bbd–transfected 293 cells and coimmunostained for regions of H4 acetylation. The Xi has previously been shown to be hypoacetylated at H4 at metaphase (Jeppesen and Turner, 1993), indicating acetylation status as a reliable marker for the Xi. While one X chromosome (the cytologically identifiable Xa) showed staining equivalent to the autosomes, additional X chromosomes in each spread lacked any H4Ac12 signal, identifying them as Xi's. As evaluated in >120 metaphase spreads, the same X chromosomes were also deficient for H2A-Bbd staining (Fig. 6). In no case was H2A-Bbd found to stain the underacetylated Xi chromosome. An overlay of both H2A-Bbd and H4Ac12 shows an identical pattern of distribution at metaphase (Fig. 6 d).

H2A-Bbd Associates with Core Histones and Nucleosomes

To address the question of association of H2A-Bbd with core histones in the nucleosome, the myc epitope-tagged version of the protein was transfected into 293 cells, and a stable cell line established. Chromatin was isolated from the cells, and histones were extracted under acidic conditions

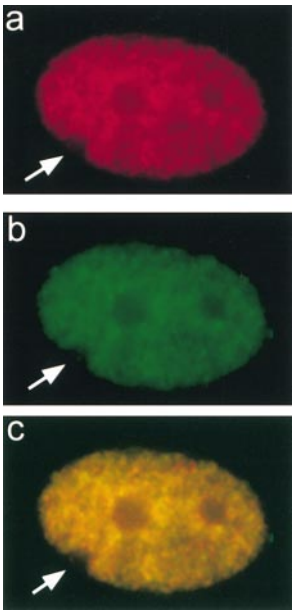

Figure 5. Female primary fibroblast interphase cell showing the nuclear distribution of H2A-Bbd and histone H4 acetylation. (a) Female cell transfected with myc-tagged H2A-Bbd showing the nuclear distribution by indirect immunofluorescence (red, TR). The white arrow indicates the location of the Xi-associated exclusion. (b) Indirect immunofluorescence showing the distribution of acetylation of histone H4 at lysine-12 (green, FITC). A distinct region lacking acetylation is indicated with an arrow. (c) Merge of the H2A-Bbd and H4Ac12 staining patterns. The nucleus has an orange appearance due to complete overlap of the two distributions. The exclusion zone indicated with the white arrow is clearly underacetylated and deficient for H2A-Bbd.

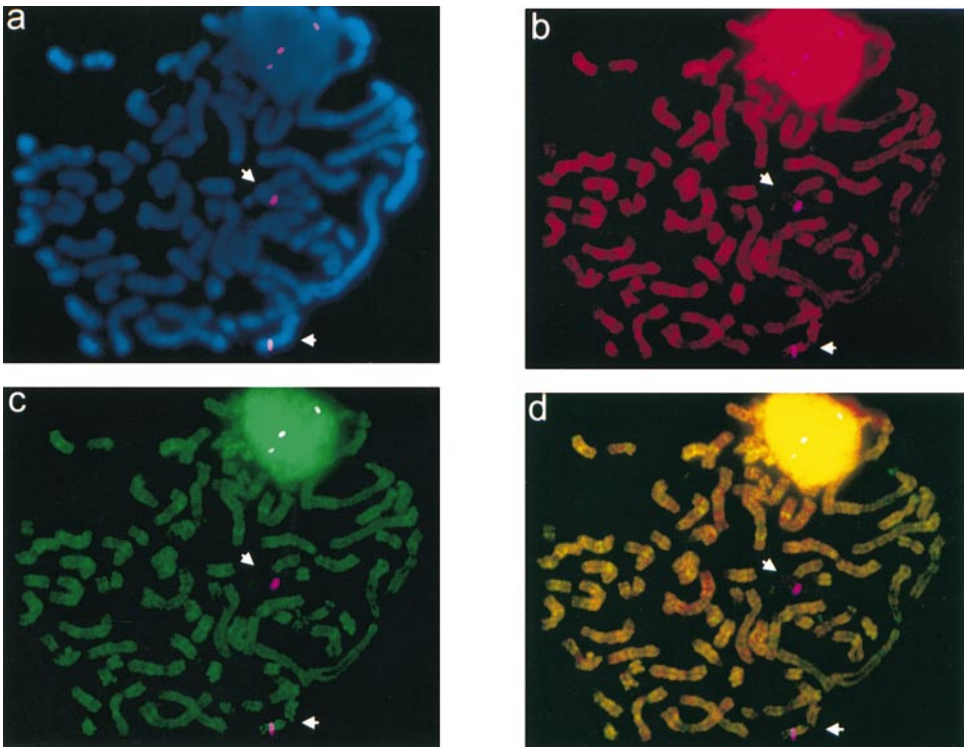

Figure 6. Female metaphase chromosomes from a H2A-Bbd–transfected 293 cell, counterstained for histone H4 acetylation at lysine-12. (a) DAPI stain of metaphase chromosomes merged with the FISH signal of an X alpha satellite probe (pink, Cy5). The positions of the two X chromosomes is indicated with white arrows. (b) Indirect immunofluorescence of the H2A-Bbd distribution along the metaphase chromosomes (red, rhodamine). The location of the two X chromosomes is shown with the white arrow, and the centromere is marked by the FISH signal from X alpha satellite probe (pink, Cy5). One of the two X chromosomes is deficient for H2A-Bbd when compared with the other X chromosome and the surrounding autosomes. (c) Indirect immunofluorescence showing the distribution of H4Ac12 on metaphase chromosomes (green, FITC). The location of the X chromosomes is indicated with the white arrows and the centromere is marked by the FISH signal from X alpha satellite probe (pink, Cy5). One of the two X chromosomes lacks H4Ac12 signal, indicative of the Xi. This is the same X shown in b to be deficient for H2A-Bbd. (d) Merge of the staining patterns of H2A-Bbd and H4Ac12. The orange color indicates a direct overlap of the H2A-Bbd and H4Ac12 signals.

and separated from the remaining chromatin by centrifugation. By Western analysis, H2A-Bbd was found to cofractionate with histones, indicating that H2A-Bbd behaves like core histones (Fig. 7, a and b). The same result was obtained using an anti–myc mAb (data not shown). The chromatin fractions were tested for the presence of a number of nonhistone chromatin proteins in the same way, and none was found to cofractionate with the histones (data not shown).

To further investigate the relationship of H2A-Bbd with histones in the nucleosome, we looked for the cosedimentation of H2A-Bbd with nucleosomes through a sucrose gradient. The presence of H2A-Bbd was detected in all nucleosome fractions tested (Fig. 7, c and d), indicating that H2A-Bbd is likely to be nucleosomal and associates with core histones in the nucleosome.

Discussion

The identification of a novel histone variant, macroH2A (Pehrson and Fried, 1992), which is enriched on the inactive X chromosome (Costanzi and Pehrson, 1998), suggested that, in addition to posttranslational modification of conventional histone tails in the nucleosome, histone variants may be an additional means of altering chromatin structure. Although macroH2A is for the most part found associated with the Xi chromosome, staining has also been observed throughout the nucleus, suggesting that macroH2A may also be incorporated into some nucleosomes on the autosomes. We set out to identify a counterpart to macroH2A that might be found in a mutually exclusive distribution throughout the nucleus.

H2A-Bbd Is a Novel Histone Variant that Is Physically Associated with Core Histones

The composition and organization of the nucleosome is well known (Ramakrishnan, 1997). The nucleosome core consists of a heterotetramer of two H3 and two H4 histone molecules flanked on either side by an H2A-H2B dimer. All core histones are extremely basic proteins due to their high proportion of arginine and lysine residues. A common feature of histones is the histone fold and histone tails (Argents and Moudrianakis, 1993, 1995). The histone fold is a α-helical structure that mediates protein–protein interactions within the nucleosome as well as interactions with the DNA. The NH_2-terminal tail regions of histones are the sites of several forms of posttranslational modification, including phosphorylation, ADP-ribosylation, methylation, and acetylation (Spencer and Davie, 1999; Wolffe and Hayes, 1999). In addition to an NH_2-terminal tail, histone H2A is unique in that it also has a COOH-terminal tail. The exact role of the COOH-terminal tail is unknown, although it can be posttranslationally modified by ubiquitination (Kleinschmidt and Martison, 1981).

Nearly two thirds of the histone variant macroH2A is made up of an extended COOH-terminal tail. Little is known about the function of the tail, but a leucine zipper motif in this region may be involved in protein–protein interactions (Pehrson and Fried, 1992). In addition to macroH2A, two other human H2A variants exist, H2A.X and H2A.Z (Hatch and Bonner, 1988; Mannironi et al., 1989). H2A.X and H2A.Z exhibit a high level of amino acid identity to the major isoforms, with most sequence variation

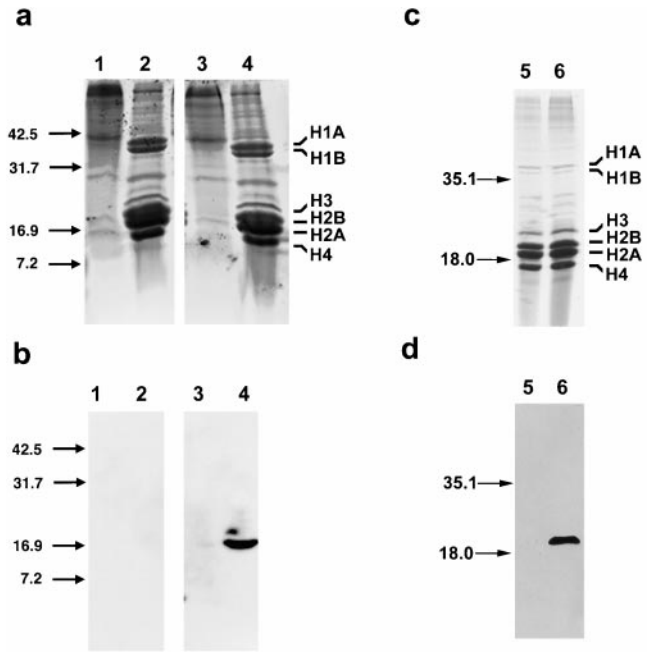

Figure 7. Association of H2A-Bbd with acid-extracted histone proteins in chromatin fractions and copurification with nucleosomes by sucrose gradient ultracentrifugation. (a) Coomassie stain of an 18% polyacrylamide gel of chromatin fractions. (1) Proteins from the chromatin pellet fraction from 293 cells. (2) Proteins extracted from the 293 chromatin pellet under acidic conditions. (3) Proteins from the chromatin pellet fraction from a stable H2A-Bbd–transfected 293 cell line. (4) Proteins extracted from the stable H2A-Bbd–transfected 293 chromatin pellet under acidic conditions. Sizes are given in kilodaltons. The location of histone H1 and the core histones (H2A, H2B, H3, and H4) are indicated. (b) Immunoblot analysis of chromatin fractions from 293 and a stable H2A-Bbd–transfected 293 cell line. A clear 17-kD signal can be seen for the epitope-tagged H2A-Bbd in the acid-extracted fraction of the H2A-Bbd–stable transfected 293 cell line only (4). (c) Coomassie stain of an 18% polyacrylamide gel of nucleosome containing sucrose gradient fractions. (5) Nucleosomes from a nontransfected 293 cell line. (6) Nucleosomes from a stable H2A-Bbd–transfected 293 cell line. (d) Immunoblot analysis of chromatin fractions from 293 and a stable H2A-Bbd–transfected 293 cell line. A clear 17-kD signal can be seen for the epitope tagged H2A-Bbd in the nucleosomal fraction of the H2A-Bbd stable transfected 293 cell line only (6).

occurring outside of the histone fold region. Unlike expression of the major H2A isoforms, expression of H2A.X and H2A.Z is not coupled to replication (Hatch and Bonner, 1990; Bonner et al., 1993). Their transcripts are polyadenylated (Hatch and Bonner, 1988; Mannironi et al., 1989), and their genes are located outside of the major histone gene cluster at chromosome 6p21.3-22 (Albig et al., 1999).

Using the sequence of H2A genes, we have identified a novel histone variant that we have termed H2A-Bbd on the basis of its near exclusion from the Barr body and the Xi. This novel histone variant is expressed in a wide variety of tissues and is also polyadenylated. H2A-Bbd is more distantly related to histone H2A (48% amino acid sequence identity) than either H2A.X (95% identity), H2A.Z (75% identity), or the histone region of macroH2A (68% identity). An alignment of H2A-Bbd with H2A, H2A.X, and macroH2A (Fig. 1 c) reveals that the highest region of amino acid conservation (58%) is within the histone fold that may be necessary for correct positioning of H2A-Bbd within the nucleosome and for interactions with the DNA helix. Unlike all histone H2A family members, H2A-Bbd does not have an extensive COOH-terminal tail, and sites for acetylation and ubiquitination of H2A are not conserved, suggesting that H2A-Bbd is not regulated in the same manner as other H2A molecules. The amino terminus of H2A-Bbd, like that of H2A proteins, is extremely basic, but is unique in lacking lysine residues, a common site of modification.

We established a stable epitope-tagged H2A-Bbd cell line and showed by chromatin fractionation that H2A-Bbd cofractionates with histones and copurifies with core histones in the nucleosome by sedimentation (Fig. 7). This, in combination with its structural similarity to histone H2A molecules, suggests that H2A-Bbd is likely to be an integral component of the nucleosome core. MacroH2A was originally isolated directly from mononucleosomes (Pehrson and Fried, 1992) and was estimated to be present in 1 of every 30 nucleosomes. With macroH2A enriched on the Xi (Costanzi and Pehrson, 1998), the level of this protein within nucleosomes contained on the Xi is likely to be far higher than this estimate, and considerably lower on the autosomes. The frequency of H2A-Bbd representation in nucleosomes is yet to be determined.

The Inactive X Chromosome Is Deficient for H2A-Bbd Staining at Interphase and Metaphase

Subcellular localization of H2A-Bbd revealed an exclusively nuclear location for the protein, with a uniform distribution throughout the nucleus in male cells. In female nuclei, a similar pattern was observed with the exception of a clear region of exclusion that coincides with the Barr body (Fig. 2). Whenever a localized deficiency of H2A-Bbd was observed in female interphase nuclei, one X chromosome was contained within the exclusion zone. In keeping with the Lyon hypothesis (Lyon, 1961), we observed a direct correlation between the number of exclusion zones and one less than the total number of X chromosomes in 46,XY, 46,XX and 47,XXX cell lines, supporting the lack of association of H2A-Bbd with the Xi (Fig. 2).

Deficiency of H2A-Bbd on the Xi is most striking at metaphase (Fig. 3). Close examination of the Xi revealed occasional localized staining as a banding pattern for H2A-Bbd (Fig. 4), particularly on the short arm. A similar observation was made with the Xi when antisera raised to various acetylated forms of histone H4 were used on female metaphase chromosomes (Jeppesen and Turner, 1993). It is possible that the banding patterns observed reflect H2A-Bbd (and acetylated H4) associated with regions on the Xi that escape X inactivation (Carrel et al., 1999). The possibility that H2A-Bbd is associated with regions of the Xi that escape X inactivation may provide a useful handle to identify transcriptionally active genes on the Xi by chromatin immunoprecipitation (Braunstein et al., 1993; Alberts et al., 1998). On close examination of H2A-Bbd chromosome staining shown in Fig. 6 b, it is apparent that there is a slight deficiency for the protein at the pericentromeric heterochromatin of chromosome 1, and also possibly chromosomes 9 and 16. This may indicate that H2A-Bbd is deficient in heterochromatic regions in

general. The possible absence of H2A-Bbd from centromeres is not a consistently notable feature of its distribution, however.

H2A-Bbd Colocalizes with Regions of H4-Acetylation at Interphase and Metaphase

Previous studies using antisera to acetylated forms of histone H4 revealed that the Xi is markedly underacetylated, thus providing a good cytological marker for the Xi (Jeppesen and Turner, 1993; Boggs et al., 1996). Coimmunolocalization experiments using antisera to acetylated lysine-12 of histone H4 and to the epitope-tagged H2A-Bbd show an indistinguishable pattern of distribution at interphase and metaphase (Figs. 5 and 6). This strongly supports the finding that H2A-Bbd is deficient on the Xi. It will be interesting to determine the relationship of core histone acetylation and the presence of H2A-Bbd within the same mononucleosome and to evaluate whether levels of histone acetylation differ between H2A-Bbd–incorporated and –deficient nucleosomes. Acetylation of the histone tails of H3 and H4 is thought to destabilize the higher-order structure of chromatin, allowing protein access to the DNA for processes such as transcription or replication (Spencer and Davie, 1999; Wolffe and Hayes, 1999). The presence of the histone variant H2A-Bbd within the nucleosome core may alter the conformation of the nucleosome, decreasing or increasing the accessibility of protein complexes involved in silencing or activation. The presentation of an alternative NH_2-terminal tail from H2A-Bbd may also provide a docking site for protein complexes that distinguish H2A-Bbd–containing chromatin domains.

Histone Variants and X Inactivation

The process of X inactivation can be subdivided into a number of major stages, including initiation, promulgation, and maintenance (Heard et al., 1997; Willard, 2000). Despite the association of macroH2A with the inactive X chromosome, the protein is detected on the Xi only after the choice of which X chromosome to inactivate has occurred (Pehrson et al., 1997; Mermoud et al., 1999; Rasmussen et al., 1999, 2000). Therefore, it is unlikely to be involved in the early processes of initiation of X inactivation. MacroH2A is also not required exclusively for the maintenance of X inactivation (Csankovszki et al., 1999). Thus, the exact role the protein plays in X inactivation is unknown.

Onset of X inactivation requires an increase in the steady state levels of Xist from the chromosome that will be inactivated (Panning et al., 1997; Sheardown et al., 1997). Subsequently, the chromosome acquires a number of features that appear to be involved in "locking in" that inactive state (Lock et al., 1987; Singer-Sam et al., 1990; Grant et al., 1992; Kay et al., 1993, 1994; Keohane et al., 1996; Mermoud et al., 1999). The maintenance of the inactive state likely requires a highly redundant system including methylation, acetylation, and Xist RNA localization and probably involves a combination of proteins. After inactivation, removal of a single component doesn't result in complete reactivation of the chromosome (Mohandas et al., 1981; Driscoll and Migeon, 1990; Singer-Sam et al., 1992; Brown and Willard, 1994; Gartler and Goldman, 1994; Rack et al., 1994; Csankovszki et al., 1999). Is exclusion of H2A-Bbd yet another mechanism to remodel chromatin and lock in a transcriptionally repressed state? Determining the timing of expression and the spatial distribution of H2A-Bbd in relation to these other events during the early stages of X inactivation will help to elucidate any role this protein may play in the process.

We anticipate that many proteins will be involved in the heterochromatic condensation of the Xi and that several more will be identified that show either an enriched or deficient association with the Barr body and the X chromosome. It is likely that many of the same factors and modifications that associate and condense regionalized heterochromatin on the autosomes will be involved in the same process on the inactive X chromosome. By virtue of its sheer size, the heterochromatin present in the Xi provides an attractive target to monitor proteins involved in remodeling and maintenance of chromatin states.

We are grateful to Drs. Kevin Sullivan, Teru Kanda, and Geoffrey Wahl for providing pEGFP-H2B, and to Drs. Todd Gray, Rob Nicholls, and Stuart Schwartz for providing various cell lines. We are indebted to Anne Higgins and Karen Gustashaw for technical advice and to Drs. Laura Carrel and Peter Harte for critical examination of the manuscript.

This work was supported by research grant GM 45441 to H.F. Willard from the National Institutes of Health and an award from the Board of Regents of the State of Ohio.

Submitted: 24 October 2000
Revised: 7 December 2000
Accepted: 14 December 2000

References

Alberts, A.S., O. Geneste, and R. Treisman. 1998. Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation. *Cell.* 92:475–487.

Albig, W., R. Trappe, E. Kardalinou, S. Eick, and D. Doenecke. 1999. The human H2A and H2B histone gene complement. *Biol. Chem*. 380:7–18.

Argents, G., and E.N. Moudrianakis. 1993. Topography of the histone octamer surface: repeating structural motifs utilized in the docking of nucleosomal DNA. *Proc. Natl. Acad. Sci. USA*. 90:10489–10493.

Argents, G., and N.N. Moudrianakis. 1995. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. *Proc. Natl. Acad. Sci. USA*. 92:11170–11174.

Barr, M.L., and E.G. Bertram. 1949. A morphological distinction between neurones of the male and female, and the behaviour of the nucleolar satellite during accelerated nucleoprotein synthesis. *Nature.* 163:676–677.

Boggs, B.A., B. Connors, R.E. Sobel, A.C. Chinault, and C.D. Allis. 1996. Reduced levels of histone H3 acetylation on the inactive X chromosome in human females. *Chromosoma.* 105:303–309.

Bonner, W.M., C. Mannironi, A. Orr, D.R. Pilch, and C.L. Hatch. 1993. Histone H2A.X gene transcription is regulated differently than transcription of other replication-linked histone genes. *Mol. Cell. Biol.* 13:984–992.

Braunstein, M., A.B. Rose, S.G. Holmes, C.D. Allis, and J.R. Broach. 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev*. 7:592–604.

Brockdorff, N., A. Ashworth, G.F. Kay, V.M. McCabe, D.P. Norris, P.J. Cooper, S. Swift, and S. Rastan. 1992. The product of the mouse Xist gene is a 15kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell.* 71:515–526.

Brown, C.J., A. Ballabio, J.L. Rupert, R.G. Lafreniere, M. Grompe, R. Tonlorenzi, and H.F. Willard. 1991. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature.* 349:38–44.

Brown, C.J., L. Carrel, and H.F. Willard. 1997. Expression of genes from the human active and inactive X chromosomes. *Am. J. Hum. Genet.* 60:1333–1343.

Brown, C.J., B.D. Hendrich, J.L. Rupert, R.G. Lafreniere, Y. Xing, J. Lawrence, and H.F. Willard. 1992. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell.* 71:527–542.

Brown, C.J., and H.F. Willard. 1994. The human X-inactivation centre is not required for maintenance of X-chromosome inactivation. *Nature.* 368:154–156.

Carrel, L., A.A. Cottle, K.C. Goglin, and H.F. Willard. 1999. A first-generation X-inactivation profile of the human X chromosome. *Proc. Natl. Acad. Sci. USA*. 96:14440–14444.

Costanzi, C., and J.R. Pehrson. 1998. Histone macroH2A1 is concentrated in

the inactive X chromosome of female mammals. *Nature.* 393:599–601.
Csankovszki, G., B. Panning, B. Bates, J.R. Pehrson, and R. Jaenisch. 1999. Conditional deletion of Xist disrupts histone macroH2A localization but not maintenance of X inactivation. *Nat. Genet.* 22:323–324.
Disteche, C. 1995. Escape from X inactivation in human and mouse. *Trends Genet.* 11:17–22.
Dominski, Z., and W.F. Marzluff. 1999. Formation of the 3′ end of histone mRNA. *Gene.* 239:1–14.
Driscoll, D., and B. Migeon. 1990. Sex differences in methylation of single-copy genes in human meiotic germ cells: implications for X chromosome inactivation, parental imprinting, and origin of CpG mutations. *Somat. Cell Mol. Genet.* 16:267–282.
Gartler, S.M., and M.A. Goldman. 1994. Reactivation of inactive X-linked genes. *Dev. Genet.* 15:504–514.
Gilbert, C.W., S. Muldal, L.G. Lajthal, and J. Rowley. 1962. Time-sequence of human chromosome duplication. *Nature.* 195:869–873.
Gilbert, S.L., and P.A. Sharp. 1999. Promoter-specific hypoacetylation of X-inactivated genes. *Proc. Natl. Acad. Sci. USA*. 96:13825–13830.
Gilbert, S.L., J.R. Pehrson, and P.A. Sharp. 2000. XIST RNA associates with specific regions of the inactive X chromatin. *J. Biol. Chem*. 275:36491–36494.
Grant, M., M. Zuccotti, and M. Monk. 1992. Methylation of CpG sites of two X-linked genes coincides with X-inactivation in the female mouse embryo but not in the germ line. *Nat. Genet*. 2:161–166.
Harlow, E., and D. Lane. 1989. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 471–510.
Hatch, C.L., and W.M. Bonner. 1988. Sequence of cDNAs for mammalian H2A.Z, an evolutionarily diverged but highly conserved basal histone H2A isoprotein species. *Nucleic Acids Res*. 16:1113–1124.
Hatch, C.L., and W.M. Bonner. 1990. The human histone H2A.Z gene. Sequence and regulation. *J. Biol. Chem*. 265:15211–15218.
Heard, E., P. Clerc, and P. Avner. 1997. X-Chromosome inactivation in mammals. *Annu. Rev. Genet.* 31:571–610.
Holt, C., H.J. Brandt, G.G. Greyling, J.D. Lindsey, J.A. Retief, S. Rodrigues, S. Scwager, and B.T. Sewell. 1989. Isolation and characterisation of histones. *Methods Enzymol*. 170:431–523.
Ichimura, S., K. Mita, and M. Zama. 1982. Essential role of arginine residues in the folding of deoxyribonucleic acid into nucleosome cores. *Biochemistry.* 21:5329–5334.
Jeppesen, P., and B.M. Turner. 1993. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell.* 74:281–289.
Kanda, T., K.F. Sullivan, and G.M. Wahl. 1998. Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol*. 8:377–385.
Kay, G.F., S.C. Barton, M.A. Surani, and S. Rastan. 1994. Imprinting and X chromosome counting mechanisms determine *Xist* expression in early mouse development. *Cell.* 77:639–650.
Kay, G.F., G.D. Penny, D. Patel, A. Ashworth, N. Brockdorff, and S. Rastan. 1993. Expression of *Xist* during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell.* 72:171–182.
Keohane, A.M., L.P. O'Neill, N.D. Belyaev, J.S. Lavender, and B.M. Turner. 1996. X-Inactivation and histone H4 acetylation in embryonic stem cells. *Dev. Biol*. 180:618–630.
Kleinschmidt, A.M., and H.G. Martison. 1981. Structure of nucleosome core particles containing uH2A. *Nucleic Acids Res.* 9:2423–2431.
Kozak, M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115:887–903.
Lahn, B.T., and D.C. Page. 1997. Functional coherence of the human Y chromosome. *Science.* 278:675–680.
Lennox, R.W., and L.H. Cohen. 1989. Analysis of histone subtypes and their modified forms by polyacrylamide gel electrophoresis. *Methods Enzymol*. 170:532–549.
Lock, L.F., N. Takagi, and G.R. Martin. 1987. Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. *Cell.* 48:39–46.
Luger, K., A.W. Mader, R.K. Richmond, D.F. Sargent, and T.J. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature.* 389:251–260.
Luger, K., and T.J. Richmond. 1998. The histone tails of the nucleosome. *Curr. Opin. Genet. Dev*. 8:140–146.
Lyon, M.F. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature.* 190:372–373.
Lyon, M.F. 1962. Sex chromatin and gene action in the mammalian X-chromosome. *Am. J. Hum. Genet.* 14:135–145.
Mannironi, C., W.M. Bonner, and C.L. Hatch. 1989. H2A.X. a histone isoprotein with a conserved C-terminal sequence, is encoded by a novel mRNA with both DNA replication type and polyA 3′ processing signals. *Nucleic Acids Res*. 17:9113–9126.
Mermoud, J.E., C. Costanzi, J.R. Pehrson, and N. Brockdorff. 1999. Histone MacroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. *J. Cell Biol*. 147:1399–1408.
Mohandas, T., R.S. Sparkes, and L.J. Shapiro. 1981. Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science.* 211:393–396.
Morishma, A., M.M. Grumbach, and J.H. Taylor. 1962. Asynchronous duplication of human chromosomes and the origin of sex chromatin. *Proc. Natl. Acad. Sci. USA*. 48:756–763.
Naylor, J.A., D. Buck, P. Green, H. Williamson, D. Bentley, and F. Giannelli. 1995. Investigation of the factor VIII intron 22 repeated region (int22h) and the associated inversion junctions. *Hum. Mol. Genet*. 4:1217–1224.
O'Neill, L.P., and B.M. Turner. 1995. Histone H4 acetylation distinguishes coding regions of the human genome from heterochromatin in a differentiation-dependent but transcription-independent manner. *EMBO (Eur. Mol. Biol. Organ.) J*. 14:3946–3957.
Panning, B., J. Dausman, and R. Jaenisch. 1997. X chromosome inactivation is mediated by Xist RNA stabilization. *Cell.* 90:907–916.
Pehrson, J.R., C. Costanzi, and C. Dharia. 1997. Developmental and tissue expression patterns of histone macroH2A1 subtypes. *J. Cell Biochem*. 65:107–113.
Pehrson, J.R., and V.A. Fried. 1992. MacroH2A, a core histone containing a large nonhistone region. *Science.* 257:1398–1400.
Pfeifer, G., R. Tanguay, S. Steigerwald, and A. Riggs. 1990. In vivo footprint and methylation analysis by PCR-aided genomic sequencing: comparison of active and inactive X chromosomal DNA at the CpG island and promoter of human PGK-1. *Genes Dev*. 4:1277–1287.
Rack, K.A., J. Chelly, R.J. Gibbons, S. Rider, D. Benjamin, R.G. Lafreniere, D. Oscier, R.W. Hendriks, I.W. Craig, H.F. Willard, et al. 1994. Absence of the XIST gene from late-replicating isodicentric X chromosomes in leukaemia. *Hum. Mol. Genet.* 3:1053–1059.
Ramakrishnan, V. 1997. Histone structure and the organization of the nucleosome. *Annu. Rev. Biophys. Biomol. Struct*. 26:83–112.
Rasmussen, T.P., T. Huang, M.A. Mastrangelo, J. Loring, B. Panning, and R. Jaenisch. 1999. Messenger RNAs encoding mouse histone macroH2A1 isoforms are expressed at similar levels in male and female cells and result from alternative splicing. *Nucleic Acids Res*. 27:3685–3689.
Rasmussen, T.P., M.A. Mastrangelo, A. Eden, J.R. Pehrson, and R. Jaenisch. 2000. Dynamic relocalization of histone macroH2A1 from centrosomes to inactive X chromosomes during inactivation. *J. Cell Biol*. 150:1189–1198.
Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1.21–1.82 and 6.22–6.34.
Sheardown, S.A., S.M. Duthie, C.M. Johnston, A.E.T. Newall, E.J. Formstone, R.M. Arkell, T.B. Nesterova, G.-C. Alghisi, S. Rastan, and N. Brockdorff. 1997. Stabilization of Xist RNA mediates initiation of X chromosome inactivation. *Cell.* 91:99–107.
Singer-Sam, J., L. Goldstein, A. Dai, S.M. Gartler, and A.D. Riggs. 1992. A potentially critical Hpa II site of the X chromosome-linked PGK1 gene is unmethylated prior to the onset of meiosis of human oogenic cells. *Proc. Natl. Acad. Sci. USA*. 89:1413–1417.
Singer-Sam, J., M. Grant, J.M. LeBon, K. Okuyama, V. Chapman, M. Monk, and A.D. Riggs. 1990. Use of a *Hpa*II-polymerase chain reaction assay to study DNA methylation in the *Pgk-1* CpG island of mouse embryos at the time of X-chromosome inactivation. *Mol. Cell. Biol.* 10:4987–4989.
Spencer, V.A., and J.R. Davie. 1999. Role of covalent modifications of histones in regulating gene expression. *Gene.* 240:1–12.
Sullivan, B.A., and P.E. Warburton. 1999. Studying the progression of vertebrate chromosomes through mitosis by immunofluorescence and FISH. *In* Chromosome Structural Analysis. W.A. Bickmore, editor. Oxford University Press, Oxford, UK. 81–101.
Waye, J.S., and H.F. Willard. 1985. Chromosome-specific alpha satellite DNA: nucleotide sequence analysis of the 2.0 kilobasepair repeat from the human X chromosome. *Nucleic Acids Res*. 13:2731–2743.
Willard, H.F. 2000. Sex chromosomes and X chromosome inactivation. *In* The Molecular and Metabolic Bases of Inherited Disease. 8th ed. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill Publishing Co., New York. In press.
Williams, A.S., and W.F. Marzluff. 1995. The sequence of the stem and flanking sequences at the 3′ end of histone mRNA are critical determinants for the binding of the stem-loop binding protein. *Nucleic Acids Res*. 23:654–662.
Wolffe, A.P., and J.J. Hayes. 1999. Chromatin disruption and modification. *Nucleic Acids Res*. 27:711–720.