Beyond the Xi
MacroH2A CHROMATIN DISTRIBUTION AND POST-TRANSLATIONAL MODIFICATION IN AN AVIAN SYSTEM*

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MacroH2A (mH2A) is a histone variant that is enriched in the inactivated X-chromosomes of mammalian females. To characterize the role of this protein in other nuclear processes we isolated chromatin particles from chicken liver, a vertebrate system that does not undergo X-inactivation. Chromatin digestion and fractionation studies determined that mH2A is evenly distributed at several levels of chromatin structure and stabilizes the nucleosome core particle in solution. However, at the level of the chromatosome, selective salt precipitation showed the existence of a mutually exclusive relationship between mH2A and H1, which may reveal functional redundancy between these proteins. Two-dimensional gel electrophoresis demonstrated the presence of one major population of mH2A containing nucleosomes, which may become ADP-ribosylated. This report provides new clues into how mH2A distribution and a previously unidentified post-translational modification may help regulate the repression of autosomal chromatin.

The incorporation of histone variants into nucleosomes has been causally linked to such events as gene expression, DNA repair, and meiosis (see Ref. 1 for a recent review). For example, H2AX has been labeled the “guardian of the genome” because of its role in the resolution of DNA fragmentation and maintenance of genomic stability (2, 3). Interestingly, the core histone H2A has the largest family of described heteromorphic variants that specialize nucleosomes for defined functions. This family of proteins displays the greatest chemical variability at their C terminus, which has implications for nucleosome stability, the binding of H1, and the formation of higher order chromatin structures (4–6). Accumulating biophysical data suggest H2A variants may indeed exert some function by directly altering the stability and conformation of chromatin complexes (5, 7–10). Within the cell, the direct interplay between histone variants and downstream effector proteins is also proving to be important for nuclear metabolism (11–15).

MacroH2A (mH2A) is a histone H2A variant that was first described 12 years ago (16). Its name was derived from the C-terminal gonhistone region (NHR) that comprises two-thirds of its molecular mass. Recently the crystallographic structure of a protein with homology to the NHR was resolved, which has heightened interest in this histone variant (17). Bioinfomatical analysis of this region suggests that it may function in directly regulating the ADP-ribosylation of histones (17, 18), suggesting for the first time that a histone variant may have inherent enzymatic potential (19).

Because the discovery of mH2A and its early association with X-inactivation most of the literature has explored its potential role in this process and transcriptional repression. Stability assays have shown that mH2A binds with more affinity within chromatin than its H2A counterpart (9, 16). Localization of a NHR-Gal4 fusion protein to a yeast promoter repressed its transcription potential (20). A secondary report by this group determined that mH2A containing nucleosomes are more refractory to SWI/SNF remodeling and the binding of NF-xB near the pseudo-dyad axis (21). In addition, a series of publications have determined that this variant is enriched in the Barr body of mammalian females (22–27), and the XY-body in the testes of adult male mice during spermatogenesis (28–30). Indeed, there is indirect evidence that mH2A may physically associate with Xist (31, 32), a noncoding RNA transcript that helps facilitate sex heterochromatinization by coating the Xi.

Although mH2A contributes to the specialized architecture of the Xi, this protein clearly has implications for chromatin beyond this process. mH2A is present in species that do not undergo X-inactivation (9, 31), highly conserved in vertebrates (9, 31), displays tissue-specific expression at similar levels in both females and males (24, 32), and localizes to other nuclear domains (22, 24, 26, 33, 34). To further characterize the role of mH2A in chromatin folding and stability, we chose to use chicken liver as a model system. In birds, females are heterogametic (ZW) and males are homogametic (ZZ). Although the process remains poorly understood, it is believed that there may be no somatic dosage compensation mechanism in the males of this species (35). Therefore, nucleosome complexes purified from chicken hepatocytes provide an interesting look into the relationship between mH2A and autosomal chromatin.

MATERIALS AND METHODS
Purification of Chicken Hepatocyte Chromatin—Chicken livers were flash frozen in liquid nitrogen after harvesting. Frozen tissue was shattered and then homogenized in 4 volumes 100 mM KCl, 25 mM Tris-HCl (pH 7.5), 1.0 mM MgCl₂ buffer in the presence of 1:100 (v/v) Complete protease inhibitor mixture (Roche). The following purification steps were based upon the protocol described in Ref. 36 with minor changes introduced to optimize the procedure for liver nuclei. Hepatocytes were washed in the buffer described above and then centrifuged at 5,000 × g for 10 min at 4 °C. This process was repeated three times. The final pellet thus obtained was gently suspended to homogeneity with the balance of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: mH2A, MacroH2A; apo-I, apolipoprotein A-I; MNase, micrococcal nuclease; NCP, nucleosome core particle; NHR, nonhistone region; Xi, inactivated X-chromosome; PBS, phosphate-buffered saline.
min. Nuclei were then collected by centrifugation as described above. This step was repeated one time to ensure complete removal of cytoplasmic debris. Purified nuclei were then resuspended in 100 mM KC1, 25 mM Tris-HCl (pH 7.5), 1 mM CaCl2 digestion buffer to a final concentration of 6 mg/ml. The concentration of chromatin was determined from the DNA absorbance at 260 nm in 0.5% SDS as described in Ref. 36 using a Cary-1 UV-Visible Spectrophotometer (Varian Inc., Mississauga, Ontario, Canada). Nuclei were digested with 9 units of MNase (Worthington Biochemical Group, Freehold, NJ) of chromatin for 5 min at 37 °C. Following digestion the sample was centrifuged at 10,000 × g for 4 min at 4 °C. The supernatant (SI) was collected and stored on ice in the presence of Complete protease inhibitor mixture (1:200, v/v) until further use. The pellet was vigorously resuspended in 0.25 mM EDTA (using 0.5 V of that used in the previous digestion) with a sterile glass pipette and then stirred for 1 h at 4 °C to lyse nuclei. The sample was centrifuged as above to generate an EDTA-soluble supernatant and an EDTA-insoluble pellet, which was flash frozen and stored at −80 °C until further use. The EDTA-soluble supernatant chromatin thus obtained was subsequently dialyzed against 25 mM NaCl, 10 mM Tris (pH 7.5), 1.0 mM CaCl2 overnight. The next day, the chromatin was collected and used directly for MNase digestions to generate nucleosome core particles (NCPs) and chromatosomes (see below).

Dissolution and Fractionation of Chromatin—NCPs were generated from digested chromatin stripped of H1 by a secondary digestion with MNase (9 units of MNase/mg of chromatin) (36). These reactions were stopped by adding EDTA to a final concentration of 10 mM. NCPs were purified in a 5–20% sucrose gradient in 25 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer centrifuged at 104,000 × g for 18 h at 4 °C. Following purification, NCPs were dialyzed against 0.3, 0.6, 0.9, 1.2, or 1.5 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer. The samples thus obtained were fractionated in 5–20% sucrose gradients with identical salt and buffer composition. DNA from the gradient fractions was analyzed by 4% native acrylamide gels (37). Aliquots from each fraction were prepared in 0.3% SDS and loaded directly onto the gel.

Chromatosem and oligonucleosome complexes were generated by MNase digestion (30 units of MNase/mg of chromatin) of EDTA-soluble supernatant chromatin (6 mg/ml) at 37 °C for 10 min with gentle shaking. Reactions were stopped by adding EDTA to a final concentration of 10 mM. Digested chromatin was separated by centrifugation in a 5–20% sucrose gradient containing 25 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer at 104,000 × g for 18 h at 4 °C.

Polyacrylamide chromatosem fibers were produced by digesting pre-warmed EDTA-soluble supernatant chromatin (4 mg/ml) with 5 units of MNase/ml of chromatin at 37 °C for 5 min. Following the addition of EDTA to 10 mM to halt digestion, several aliquots were dialyzed overnight against 0, 30, 60, or 80 mM NaCl in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer at 4 °C. Insoluble chromatin was removed by centrifugation at 10,000 × g for 10 min at 4 °C. The chromatin samples thus obtained were dialyzed onto 5–25% sucrose gradients at 4 °C. The gradient buffer composition to that of the dialysis buffers and centrifuged at 83,000 × g for 3 h at 4 °C. The extent of folding of the chromatosem complexes was monitored by analytical ultracentrifuge (see below).

Analytical Ultracentrifuge—To determine the size and extent of folding of chromatin fibers, fractions from sucrose gradients (described above) were selected, dialyzed, and then analyzed by sedimentation velocity. Sedimentation coefficients were determined by the van Holde and Weischat analysis method (57) using XL-I Ultra Scan version 6.0 sedimentation data analysis software (Borries Demeler, Missoula, MT). Sedimentation velocity runs were performed in an An-55 rotor using aluminum-filled epon double sector cells as described in Ref. 38.

Fractionation of Mononucleosome and Chromatosome Complexes—Fractionation of mononucleosome and chromatosem particles was based upon the protocol originally designed by Donald Ollins (90). Briefly, purified nuclei from chicken hepatocytes were prewarmed at 37 °C and digested with 25 units of MNase/mg of chromatin for 12, 24, and 32 min. Each reaction was stopped by adding EDTA to a final concentration of 25 mM EDTA on ice. Digested samples were lysed by overnight dialysis against 0.25 mM EDTA at 4 °C and centrifuged at 8,000 × g for 4 h to yield the supernatant (SI) and a sedimented pellet (P1). SI was subsequently dialyzed against 100 mM KC1, 50 mM Tris (pH 7.5), 1.0 mM EDTA overnight at the same temperature to precipitate the H1-containing nucleosomes. The salt precipitate was collected by centrifugation as above (pellet P2). The supernatant contains the histone H1-depleted nucleosome fraction (SN). P1 and P2 were then resuspended in distilled H2O to an approximate concentration of 2 mg/ml and homogenized in a Dounce by 10 strokes. Samples were aliquoted and flash frozen until further use.

The histone and nonhistone chromosomal components from P1, P2, and SN were analyzed by SDS-PAGE (40) and Western blot. The DNA from each of these samples was phenol-chloroform extracted and analyzed by 4% native-PAGE (37).

To determine the relative distribution of active and inactive genes in SN, P1, and P2, the 24-min digests were probed with the exon 3 of apolipoprotein A-I (apo-AI) and the entire sequence of histone H5, respectively. Apo-AI is a constitutively expressed gene in chicken liver cells (15, 41), and the H5 gene is transcriptionally inactive in differentiated cells (42, 43). The H5 plasmid, PChv2.5B/H, was a generous gift from Dr. James Davie. Apo-AI was generated by PCR amplification of exon 3 using a forward primer: AAGCTTAACCGCTCAGAAC ( HindIII sites underlined) and a reverse primer: AAAGTTGCTCAGGAC ( SacI site underlined). The PCR fragments were cloned into TOPO-pCR 2.1 plasmid (Invitrogen) and sequenced. Verified probes were radiolabeled using [α-32P]deoxyctosine triphosphate random primer DNA labeling according to manufacturer’s instructions (Invitrogen). The concentrations of purified P1, P2, and SN DNA were normalized from the UV absorbance at 260 nm and ethidium bromide staining of agarose gels. Ten micrograms were dot blotted onto a Zeta-Probe GT Genomic blotting membrane (Bio-Rad) by vacuum manifold and UV cross-linked in a UV Stratalinker24 (Stratagene, La Jolla, CA). The probe hybridization was carried out in 0.15 M NaCl, 10% sodium dextran sulfate, 1% SDS with agitation overnight at 65 °C. The membrane was then washed three times for 30 min in 2 × SSC, 0.1% SDS buffer to reduce nonspecific binding. Autoradiographs were performed using Bio-Max X-ray film (Kodak, Rochester, NY).

Western Blot Analysis—Acid-extracted proteins from the different chromatin fractions (9, 44) were analyzed by SDS-PAGE (40), transferred to polyvinylidene difluoride membrane (Bio-Rad), and processed as described in Ref. 9. In the course of this analysis we noticed that acid-extracted histones produced lower mH2A yields and in some cases the disappearance of the modified forms of mH2A in comparison to histones visualized from chromatin that was directly dissolved in SDS sample buffer. The antibody dilutions used in these Western blots were: mH2A1, 1:3700; mH2A2, 1:1000; ubiquitin, 1:3000; poly(ADP-ribos) (Alpha Diagnostics, San Antonio, TX), 1:1500; Xpress, 1:1000 (Invitrogen); Myc, 1:1000 (Invitrogen); and a secondary rabbit horseradish peroxidase conjugate (Abcam, Cambridge, MA), 1:5000.

Two-dimensional Gel Electrophoresis—Sucrose gradient-purified chicken hepatocyte mononucleosomes in 25 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA were concentrated using an YM-10 Centricon (Millipore Corp., Bedford, MA) to a final concentration of ~10 mg/ml. Samples were brought to 10% sucrose with 0.5 volumes of 30% sucrose in the absence of any tracking dyes. In the first dimension, duplicate samples (each: 35–40 μg of nucleosomes) were electrophoresed in 6% polyacrylamide nondenaturing gels to increase the separation of major bands. One lane of each sample was stained with ethidium bromide and photographed, and the other equivalent lane was equilibrated in 2% SDS, 10 mM β-mercaptoethanol, 10 mM Tris (pH 7.5) to dissociate the histones from the nucleosome and to prevent the formation of H3–H3 dimers. The gel strip was next laid horizontally and electrophoresed in a 3% acrylamide stacking, 15% acrylamide separating SDS gel prepared according to Ref. 40.

Transfection and in Situ Expression of mH2A Subtypes—Stably transfected hTERT-RPE1 and HEK-293 clones expressing C-terminal mH2A1.2-Myc, C-terminal mH2A2-Myc, or N-terminal mH2A1.2-Xpress were generated as described previously (24). Cells were grown directly on microscope slides before fixation and extraction for 10 min at room temperature in 1 × PBS buffer containing 4% formaldehyde and 0.1% Triton X-100 (v/v). After washing in 1 × PBS, slides were blocked for 30 min at room temperature in 1 × PBS containing 3% bovine serum albumin and 0.1% Tween 20 (v/v). Cells were stained using a 1:200 dilution of anti-myc (Myc or Invitrogen-Xpress) antibodies in 1 × PBS buffer containing 4% normal goat serum and 0.1% Tween 20 (v/v). Detection was achieved using a 1:200 dilution of goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories) for 60 min at room temperature in 1 × PBS containing 1% bovine serum albumin and 0.1% Tween 20 (v/v). After washing in 1 × PBS, cells were fixed once more as described above. Images were collected using Openlab software (Improvision), with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics) on a Zeiss Axiovert 200 M (Carl Zeiss Microimaging, Inc.).
Previous studies have determined that mH2A binds against 0, 0.6, 0.9, 1.2, and 1.5 M NaCl in 10 mM Tris-HCl (pH 7.5). Chicken liver mononucleosomes were dialyzed and then probed with a mH2A1 antibody. The results indicate that mH2A is evenly distributed in these oligonucleosome particles.

To determine whether mH2A is segregated into more compact higher order structures with different salt-dependent folding properties, a mildly digested sample consisting of a heterogeneous mixture of chromatin particles was subjected to sucrose gradient centrifugation (Fig. 3B). Similar to what was observed for oligonucleosomes, mH2A was also found to be evenly distributed in these larger chromatin complexes. From these results it appears that the presence of mH2A does not significantly contribute to the folding of the chromatin fiber. Furthermore, they suggest that in chicken hepatocyte chromatin, mH2A may be evenly distributed throughout the genome such that all fragments analyzed contain an approximately equivalent mH2A/canonical H2A ratio. Further experiments with homogeneous reconstituted arrays containing exclusively mH2A should be able to provide more sensitive data in regards to the role of this variant in chromatin folding and association.

Stability of Native mH2A Containing Mononucleosomes in Solution—Previous studies have determined that mH2A binds to hydroxyapatite-immobilized chromatin with more affinity than H2A (9, 16). A nagging question that remained following these experiments was whether artificial interactions with the resin contributed to this effect, or if it is a true reflection of an enhanced ionic strength-dependent stability of the nucleosomes containing mH2A. To address this, we analyzed the salt-dependent stability of mH2A containing mononucleosomes in solution. Chicken liver mononucleosomes were dialyzed against 0, 0.6, 0.9, 1.2, and 1.5 M NaCl in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer, and fractionated in sucrose gradients with identical salt concentrations (45) (Fig. 3A). The relative positions of the peaks in the salt-dependent sedimentation profiles (Fig. 3B) are consistent with the expected sedimentation coefficients of native (36, 46) and reconstituted (7, 9, 10) mononucleosomes under the same ionic strength conditions. After normalization of the sucrose gradient fractions for equal H2A content using SDS-PAGE, the distribution of mononucleosome particles containing mH2A was determined by Western blotting (Fig. 3C). At lower salt concentrations, mH2A is uniformly distributed across the entire peak in agreement with previously published data (47). As the ionic strength increases, mH2A remains associated with the mononucleosome under conditions that H2A-H2B dimers start dissociating. Dissociation of mH2A is not observed until 1.5 M NaCl, concomitant with the initial release of (H3-H4)2 tetramers. This result is in agreement with previous results using hydroxyapatite chromatography (9, 16). Therefore, mH2A generates a variant nucleosome structure that displays enhanced ionic strength stability.

MacroH2A and Linker Histones—To gain some insight into the relationship between mH2A and the occurrence of linker histones, we revisited a salt fractionation technique that separates digested chromatin particles into NCPs, chromatosomes, and larger insoluble fragments (39). Dialysis of thoroughly digested chicken liver chromatin against 0.25 mM EDTA produces a soluble fraction and an insoluble fraction (P) that contains a heterogeneous mixture of chromatin particles. When the soluble fraction is dialyzed against 0.1 M KCl, an insoluble fraction containing H1 and H5 (Fraction C) and a soluble linker histone-depleted fraction consisting mainly of NCPs (Fraction N) are obtained (Fig. 4A). Fig. 4B shows the histone composition of these fractions. The presence of some H5 is because of contamination by erythocyte chromatin.

DNA analysis of these samples reveals that the 0.1 M KCl-soluble fraction (N) consists mainly of 146 bp and a smaller fraction ≥168 bp DNA (Fig. 4A). The 0.1 M KCl-insoluble fraction (C) displays a ladder of nucleosome complexes and mononucleosomes consisting mainly of chromatosomal DNA ≥168 bp and fragments ≤131 bp that result from overdigestion (Fig. 4A). This DNA distribution is consistent with previous data (39). The EDTA-insoluble fraction (P) contains a heterogeneous polymonucleosome mixture.

To determine the functional distribution of genes in these populations, DNA from the N, C, and P fractions obtained upon digestion for 24 min were analyzed by Southern dot blotting using apo-I and histone H5 probes (Fig. 4C). Apo-I is constitutively expressed in chicken liver (15, 41) and H5 is silenced in differentiated cells (42, 43). The rationale for this analysis is that the NCP fraction (partially depleted of H1) should be enriched in actively transcribing DNA sequences; whereas, the chromatosome fraction (C) should be enriched in the gene sequences from silenced chromatin domains (H1-containing). The pellet (P) fraction consists of heterochromatin, which is highly resistant to nuclease digestion. Interestingly, it appears that both genes are present in the pellet fraction in approximately equal amounts. However, H5 is preferentially distributed in the linker histone containing (C) fraction; and apo-I is enriched in the linker histone-depleted NCP populations, which is consistent with what was expected.

The distribution of mH2A in fractions N, C, and P was determined by Western blotting (Fig. 4A). Quite unexpectedly, the N and P fractions contain similar levels of mH2A; whereas, in the C fraction mH2A levels are depleted. The opposite is observed in the C fraction, where linker histones are present in approximate stoichiometry (1 per octamer); and mH2A levels are depleted. It appears, however, that the mH2A signal increases with time in the latter fraction, which may correspond...
to either the accumulation of overdigested nucleosomes from the N fraction, or increased accessibility of the enzyme into heterochromatin. Notably, using this fractionation technique the distribution of the two mH2A variants (mH2A1 and mH2A2) appears to exhibit the same distribution patterns.

Very interestingly, an antibody responsive higher molecular weight band for both mH2A1 and mH2A2 is also present in P (see Fig. 4B, arrowheads). The increase in molecular weight resulting in an electrophoretic shift was initially estimated to be 4.5 kDa (results not shown).

Two-dimensional Electrophoretic Analysis of Mononucleosomes—Although NCPs consisting exclusively of 2(mH2A/H2B) dimers and a (H3/H4)2 tetramer have been successfully reconstituted into mononucleosomes (9, 21, 47), whether the histone variant is present in one or two copies in native NCPs remains to be determined. In an attempt to address this question we performed two-dimensional gel electrophoresis on purified native chicken liver mononucleosomes. In the first dimension, mononucleosomes (40–50 μg) were separated from larger nucleosome particles by nondenaturing gel electrophoresis (Fig. 5A). The histone populations of these particles were then resolved by a second SDS-PAGE dimension (Fig. 5B) and probed with mH2A1 antibodies (Fig. 5, C and D). In agreement with previously published data (9) using in vitro reconstituted NCPs consisting of two mH2A, the electrophoretic mobility of native nucleosomes containing mH2A1 is significantly retarded because of the larger mass and conformational asymmetry of the NHR. However, using two-dimensional gel electrophoresis is not possible to distinguish whether native NCP particles consist of one or two copies.

Post-translational Modification of mH2A—Under some experimental conditions, two-dimensional gel electrophoresis revealed the presence of another smaller population of mH2A containing NCPs with higher electrophoretic mobility in the first dimension and containing a mH2A band of lower mobility in the second dimension (Fig. 5D, indicated by black arrows). To determine whether this modified form of mH2A observed in several fractionation experiments was the result of a post-translational event, N-terminal mH2A1.Xpress, and C-terminal mH2A1.2-Myc and mH2A2-Myc constructs were generated and transfected into hTERT-RPE1 and HEK-293 cell lines, respectively. Immunocytolocalization analysis established that these constructs are indeed expressed and form a macrochromatin body (Fig. 6A). Analysis of sucrose gradient-purified mononucleosomes from these cell lines with anti-Xpress and anti-Myc antibodies showed that these cells also produce, in addition to a major mH2A signal, a higher molecular weight band (Fig. 6, B and C). This result confirmed that mH2A is post-translationally modified. Interestingly, during fractionation studies, the modified form of mH2A was observed to be

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**Fig. 2. Analysis of the ionic strength-dependent mH2A distribution in native chromatin fibers.** A, chromatin mildly digested with micrococcal nuclease was fractionated by sucrose gradients at 0, 30, 60, and 80 mM NaCl. The absorbance at 260 nm of the collected fractions is represented as a line graph and cone plot (boxed inset) to highlight the ionic strength-dependent changes in the sedimentation of the samples. As the ionic strength increases, the sedimentation of the main peak increases because of the salt-induced folding of the chromatin fiber fragments. B, SDS-PAGE and Western blot analysis of normalized amounts of histones from the different fractions.
Fig. 3. Ionic strength-dependent stability of native mH2A containing mononucleosomes in solution. Purified mononucleosomes were dialyzed against increasing NaCl concentrations and fractionated by sucrose gradients. A, sedimentation profile of mononucleosomes at 0 M NaCl. B, cone plot representation of the salt-dependent changes in the sedimentation behavior of mononucleosomes. C, SDS-PAGE and Western blot analysis of the fractionated mononucleosomes shown in B. To ensure that the mononucleosome preparation used was free from larger oligonucleosome particles (i.e. dinucleosomes), the DNA component of these complexes was analyzed by native gel electrophoresis (see Fig. 5C, bottom). The amount of histones used for SDS-PAGE were normalized for H2A content and probed with anti-mH2A1. At lower salts (0 and 0.6 M), mH2A exhibits an even distribution across the peak. Under NaCl conditions that disrupt the association of H2A-H2B dimers with the (H3-H4)2 tetramers (0.9 M) (56), mH2A remains stably bound. MacroH2A starts dissociating at 1.5 M NaCl, contaminant with (H3-H4)2 tetramer dissociation from the nucleosomal DNA. Notably, the mH2A1 subtypes, mH2A1.1 and mH2A1.2, display identical salt-dependent stability. N, NCP fraction; C, chromatosome; P, P fraction.
enriched in insoluble chromatin (Fig. 4A). This suggests that the post-translational modification is localized to heterochromatic regions.

In an attempt to characterize the chemical nature of this mH2A modification, Western blots were performed using ubiquitin and ADP-ribose antibodies. Despite our many attempts, we failed to visualize a ubiquitin signal that matched the electrophoretic band corresponding to the modified form of mH2A (Fig. 6D); however, it appears that mH2A may be ADP-ribosylated (Fig. 6D). Moreover, the potential conjugation of ADP-ribose oligomer would introduce two phosphate groups per subunit and may explain the puzzling increase in electrophoretic mobility observed during native-PAGE. In support of these results the electrophoretic shift observed in SDS gels is estimated to be equivalent to 4.5 kDa. This is well below what would be expected for ubiquitin (8.5 kDa) and close to the predicted mass of an ADP-ribose 10-mer. Furthermore, this post-translational modification appears to be acid labile as 0.4 M HCl extraction of histones resulted in the almost complete loss of the low electrophoretic mobility band.

DISCUSSION

Earlier studies suggest that mH2A is present in about 1 of 30 nucleosomes (16), which may be augmented in the Xi of mammalian females. Whether these specialized nucleosomes are clustered in certain regions or randomly distributed has not been determined yet. In this report we have isolated chromatin from chicken, a model system that does not have an identified dosage compensation mechanism for the expression of sex-linked genes (35). This approach is important because the role of mH2A in chromatin function clearly extends beyond the inactivation of mammalian X-chromosomes (9, 22, 24, 26, 27, 31). Indeed, two reports by the same group have suggested that mH2A may act as a transcriptional repressor by lowering the permissibility of DNA to the binding of transcriptional factors and nucleosome remodeling, both of which have functional implications for the general regulation of transcription (20, 21). By characterizing the expression of mH2A subtypes, and their importance for chromatin structure and stability in an avian
compare the behavior of nucleosomes with and without mH2A. Dissociation of the H2A-H2B for the major population of NCPs begins at around 0.9 M NaCl in contrast to what is observed for mH2A (Fig. 3C). Indeed, both mH2A1.1 and mH2A1.2 and presumably their H2B counterparts do not begin to dissociate until 1.5 M NaCl. Under these ionic strength conditions the native (H3H4)2 tetramer has already undergone a significant dissociation from nucleosomal DNA (see Fig. 3C).

At low ionic strengths, the mH2A containing nucleosomes exhibit a sedimentation behavior almost indistinguishable from that of native nucleosomes (Fig. 3C; Ref. 9). However, at 0.9 and 1.2 M the mH2A containing NCP peak sediments faster than that of the major particles. This result clearly shows that mH2A enhances the interaction of the histone octamer with the nucleosomal DNA and probably the interaction between the mH2A-H2B dimers and the (H3H4)2. This enhanced stability within variant NCPs agrees well with the observation that mH2A represses the ability of the nucleosome to be remodeled (21).

The apparent mutually exclusive relationship between H1 and mH2A determined by salt fractionation (Figs. 1 and 4) and generates the differential loading of these two proteins shown in Fig. 4, A and B, represent two distinct but redundant forms of nucleosome stabilization, or 2) does the globular NHR simply occlude the binding site of histone H1? The co-localization of both proteins within the P fraction suggests that these histones may coexist within the nucleosomes of insoluble heterochromatin (Fig. 4A). Whether this observation is representative of individual nucleosomes or whether H1 and mH2A are separately deposited in a mixed population of chromatin fibers within the P fraction remains to be defined.

Alternatively, the globular NHR domain of mH2A (9, 17) may sterically interfere with the binding of H1 to the nucleosome. Indeed, the C-terminal tail of histone H2A variants plays an important role in determining the function of specialized nucleosomes (6). First, these molecules display the greatest primary structure heterogeneity in these domains. Second, the C terminus of H2A projects along the surface and into the gateway of the nucleosome where H1 binds (5, 49). Therefore, the differential structures of H2A C-terminal tails may help regulate linker histone binding. Although it is tempting to conclude that because of its bulky nature the NHR may impede this process, caution must be taken when interpreting these results. Surprisingly, H2A ubiquitination, which is a post-translational modification that conjugates a 76-amino acid protein to the C terminus of H2A, has been recently determined to enhance the interaction of H1 (50). Also, the predicted random coil structure linking the histone and nonhistone region of mH2A may allow for significant flexibility of the globular domain and potential access to the pseudo-dyad axis (9). Indeed, nucleosomes reconstituted with mH2A have an altered DNA structure and display greater sensitivity to DNase I at sites +10 and −6 (super helix location 70 and 64, respectively) (9, 21). However, in support of a steric occlusion model, mH2A is refractory to NF-κB binding to DNA elements in this location (21), suggesting that the NHR remains positioned near the binding site of H1. Reconstitution of mH2A containing chromatosomes or two-dimensional gel analysis of native chromatosomes should help to further clarify this relationship.

Reconstitution of H2A.Z (7, 8), H2AX (51), H2A-Bbd (10, 52), and mH2A (9, 21, 47) variants into nucleosomes have been reported. In each approach, homogeneous nucleosomes were generated using stoichiometric amounts of H2A isoforms, H2B, H3, and H4. These data provide support to the theory that H2A molecules contain a “signature motif” in loop 1 that acts as a
gating mechanism for self-association within nucleosomes (5).

To our knowledge, the successful reconstitution or purification of hybrid nucleosomes containing two different H2As has not yet been reported, although this does not preclude their existence. Two-dimensional gel electrophoresis presented here does not allow us to conclusively address this issue using chicken native mH2A-containing NCPs (Fig. 5C). However, we were able to distinguish between two distinct populations of NCPs. One of these populations, present in smaller amounts, exhibited higher mobility in native PAGE but contained a mH2A form with reduced electrophoretic mobility in SDS (Fig. 5D).

To determine the chemical nature of this mH2A band, tagged forms of mH2A1.2 and mH2A2 were transfected and expressed in cell culture. Both tagged proteins are targeted to macrochromatin bodies (Fig. 6A), co-fractionate with purified mononucleosomes (Fig. 6, B and C), and exist as a canonical major, and a minor band with reduced electrophoretic mobility during SDS-PAGE (Fig. 6, B and C). This result conclusively shows that the low mobility band is not the result of alternative mRNA processing, but rather a post-translational modification. Interestingly, modified forms of both mH2A1 and mH2A2 were observed to be enriched in heterochromatin (Fig. 4B).

To try and determine the identity of this post-translational modification, enriched samples were probed with antibodies against bulky chemical groups known to modify histones (Fig. 6D). Despite repeated attempts, we were unable to positively label an ubiquitinated protein of similar electrophoretic mobility. Also, the estimated molecular mass of the conjugating group is 4.5 kDa, approximately one-half of the mass of ubiquitin. Surprisingly, we did identify a prominent signal with anti-ADP-ribose, suggesting that mH2A may be covalently modified with an ADP-ribose 10-mer (ADPMW451 Da) (Fig. 6D), which may explain the shift in electrophoretic mobility of the nucleosome because of an addition of phosphate groups. In support of this result, this modification appears to be acid-labile (Fig. 6E) (53). This suggests that like H2A, mH2A is conjugated with ADP-ribose moieties through carboxylate ester linkages (54). These results are very exciting in view of the recent suggestion that the NHR of mH2A may play an active role in regulating the ADP-ribose metabolism of histones (17–19). The structural importance of ADP-ribosylation and the differential stoichiometry of mH2A isoforms within the nucleosome present exciting new tracks for further research.
Acknowledgment—We thank Dr. John R. Pehrson for continuous and generous provision of high quality anti-mH2A1 antibody.

REFERENCES

1. Ausiö, J., and Abbott, D. W. (2004) in Chromatin Structure and Dynamics: State of the Art (Zlatanova, J., and Leuba, S. H., eds) pp. 241–290, Elsevier Science, Amsterdam
2. Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002) Curr. Opin. Genet. Dev. 12, 162–169
3. Fernandez-Capetillo, O., Lee, A., Nussenweig, M., and Nussenweig, A. (2004) DNA Repair (Amst.) 3, 859–867
4. Eckbush, T. H., Godfrey, J. E., Elia, M. C., and Moundrianakis, E. N. (1988) J. Biol. Chem. 263, 18972–18978
5. Suto, R. K., Clarkson, M. J., Tremethick, D. J., and Luger, K. (2000) Nat. Struct. Biol. 7, 1121–1124
6. Ausio, J., and Abbott, D. W. (2001) Biochemistry 41, 5945–5949
7. Abbott, D. W., Ivanova, V. S., Wang, X., Bonner, W. M., and Ausio, J. (2001) J. Biol. Chem. 276, 41945–41949
8. Fan, J. Y., Jordan, F., Luger, K., Hansen, J. C., and Tremethick, D. J. (2002) Nat. Struct. Biol. 9, 172–176
9. Abbott, D. W., Laszczak, M., Lewis, J. D., Su, H., Moore, S. C., Hills, M., Dimitrov, S., and Ausio, J. (2004) Biochemistry 43, 1352–1359
10. Gautier, T., Abbott, D. W., Molla, A., Verdel, A., Ausio, J., and Dimitrov, S. (2004) EMBO Exp. 5, 715–720
11. Kauffmann, C. X., Pilch, D., Rogakou, E. P., Suto, R. K., and Bonner, W. M. (2000) Curr. Biol. 10, 886–895
12. Adam, M., Robert, F., Larochelle, M., and Gaudreau, L. (2001) Mol. Cell. Biol. 21, 6270–6279
13. Mahadeviah, S. K., Turner, J. M., Baudat, F., Rogakou, E. P., de Boer, P., Blaize-Rodriguez, J., Jasim, M., Keeney, S., Bonner, W. M., and Burgoyne, P. S. (2001) Nat. Genet. 27, 271–276
14. Ward, I. M., and Chen, J. (2001) J. Biol. Chem. 276, 47759–47762
15. Rajavashisth, T. B., Dawson, P. A., Williams, D. L., Shackelford, J. E., Leber, H., and Lusis, A. J. (1987) J. Biol. Chem. 262, 7058–7065
16. Perche, P. Y., and Fried, V. A. (1992) Science 257, 1398–1400
17. Allen, M. D., Buckle, A. M., Cordell, S. C., Lowe, J., and Bycroft, M. (2003) J. Mol. Biol. 330, 503–511
18. Mestres, M. H., McEwan, T. M., Haggblad, E. M., and Pehrson, J. R. (2000) Development 127, 2283–2289
19. Chadwick, B. P., and Willard, H. F. (2002) J. Cell Biol. 157, 1113–1123
20. Chadwick, B. P., and Willard, H. F. (2003) Hum. Mol. Genet. 12, 2167–2178
21. Chadwick, B. P., and Willard, H. F. (2001) Hum. Mol. Genet. 10, 1101–1113
22. Chadwick, B. P., and Willard, H. F. (2001) J. Cell Biol. 152, 375–384
23. Richier, C., Dhara, S. K., and Wahrman, J. (2000) Cytogenet. Cell Genet. 89, 118–120
24. Turner, J. M., Burgoyne, P. S., and Singh, P. B. (2001) J. Cell Sci. 114, 3367–3375
25. Hoyer-Pender, S., Costanzi, C., and Pehrson, J. R. (2000) Exp. Cell Res. 258, 254–260
26. Pehrson, J. R., and Fuji, R. N. (1998) Nucleic Acids Res. 26, 2837–2842
27. Rasmussen, T. P., Huang, T., Mastrangelo, M. A., Loring, J., Panning, B., and Jaenisch, R. (1999) Nucleic Acids Res. 27, 3685–3689
28. Chadwick, B. P., Valley, C. M., and Willard, H. F. (2001) Nucleic Acids Res. 29, 2699–2705
29. Neelin, J. D., Costanzi, C., Pehrson, J. R., and Brockdorff, N. (1999) J. Cell Biol. 147, 1399–1408
30. Uekiwa, A., Yokomine, T., Sasaki, H., Tsuzuki, M., Tanaka, K., Namikawa, T., and Matsuda, Y. (2002) Cytogenet. Genome Res. 99, 310–314
31. Pehrson, J. R., and Burgoyne, P. S. (1998) J. Mol. Biol. 290, 451–463
32. Yager, T. D., and van Holde, K. E. (1984) J. Biol. Chem. 259, 4212–4222
33. Garcia-Ramirez, M., Dong, F., and Ausio, J. (1992) J. Biol. Chem. 267, 19587–19595
34. Olins, A. L., Carles, B. D., Wright, E. B., and Olins, D. E. (1976) Nucleic Acids Res. 3, 3271–3291
35. Lemaun, U. K. (1970) Nature 227, 680–685
36. Blue, R. M., Ostapchuk, P., Gordon, J. S., and Williams, D. L. (1982) J. Biol. Chem. 257, 11151–11159
37. Jacobson, M. K. (1995) J. Biol. Chem. 270, 11125–11130
38. Siino, J. S., Nazarov, I. B., Zalenskaya, I. A., Yau, P. M., Bradbury, E. M., and Tnomlin, N. V. (2002) FEBS Lett. 527, 105–108
39. Bao, Y., Konesky, K., Park, Y. J., Rosu, S., Dyer, P. N., Rangaswamy, D., Tremethick, D. J., Laybourn, P. J., and Luger, K. (2004) EMBO J. 23, 3314–3324
40. Chadwick, B. P., and Willard, H. F. (2002) J. Mol. Biol. 258, 607–610
41. Chadwick, B. P., Fink, R. M., Lindsey, G., and Ausio, J. (2004) J. Biol. Chem. 279, 179–184
42. Siino, J. S., Nazarov, I. B., Zalenskaya, I. A., Yau, P. M., Bradbury, E. M., and Tomlin, N. V. (2002) FEBS Lett. 527, 105–108
43. Golderer, G., and Grobner, P. (1991) Nucleic Acids Res. 19, 599–601
44. Saitoh, H., Tomiki, J., Cooke, C. A., Rattrie, H., 3rd, Maurer, M., Rothfield, N. F., and Earnshaw, W. C. (1992) Cell 70, 115–125
45. Ausio, J. (2000) Biochim. Biophys. Acta 1475, 1387–1403

Macrotah2a in Avian Chromatin 16445