Characterization of Histone H2A and H2B Variants and Their Post-translational Modifications by Mass Spectrometry*

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The nucleosome, the fundamental structural unit of chromatin, contains an octamer of core histones H3, H4, H2A, and H2B. Incorporation of histone variants alters the functional properties of chromatin. To understand the global dynamics of chromatin structure and function, analysis of histone variants incorporated into the nucleosome and their covalent modifications is required. Here we report the first global mass spectrometric analysis of histone H2A and H2B variants derived from Jurkat cells. A combination of mass spectrometric techniques, HPLC separations, and enzymatic digestions using endoprotease Glu-C, endoprotease Arg-C, and trypsin were used to identify histone H2A and H2B subtypes and their modifications. We identified nine histone H2A and 11 histone H2B subtypes, among them proteins that only had been postulated at the gene level. The two main H2A variants, H2AO and H2AC, as well as H2AL were either acetylated at Lys-5 or phosphorylated at Ser-1. For the replacement histone H2AZ, acetylation at Lys-4 and Lys-7 was found. The main histone H2A variant, H2BA, was acetylated at Lys-12, -15, and -20. The analysis of core histone subtypes with their modifications provides a first step toward an understanding of the functional significance of the diversity of histone structures. Molecular & Cellular Proteomics 5:541–552, 2006.

Within the eukaryotic cell nucleus the genetic information is organized in a highly conserved structural polymer, termed chromatin, that supports and controls crucial functions of the genome. The fundamental unit of eukaryotic chromatin, the nucleosome, consists of 146 base pairs of genomic DNA wrapped around an octamer of the core histone proteins H2A, H2B, H3, and H4. The amino-terminal tails of each of the four core histones are subject to several types of covalent modifications, including acetylation, methylation, and phosphorylation. These modifications affect lysines (acetylation, mono-, di-, and trimethylation), serines and threonines (phosphorylation), and arginines (mono- and two types of dimethylation). These particular modifications can alter the global dynamics of chromatin structure and function (1–3). Alternatively incorporation of histone variants into the nucleosome can also introduce variation in chromatin (4).

Each class of histone proteins consists of several subtypes that are encoded by different genes except for the H4 histones where all the different genes encode identical amino acid sequences. These genes are highly similar in sequence, are synthesized primarily during the S phase of the cell cycle, and code for the bulk of the cellular histones. There are also nonallelic variants of the major histones or replacement histones expressed at low but constant levels throughout the cell cycle that have significant differences in primary sequence (5, 6). The histone H3 family can be divided into three subtypes: H3.1, H3.2, and the replacement subtype H3.3 (7, 8). A comparison of the translated amino acid sequences of the known human H2A and H2B variants with their respective molecular masses is shown in Table I (9, 10). Additional potential histone variants retrieved by querying the translated EMBL database are also listed (Table I). There are five replacement H2A histones, including H2AZ, macroH2A, H2A-Bbd, and H2AX, that differ considerably in sequence from the bulk H2A sequences (4, 11, 12). These proteins are present in smaller amounts and have been known as minor variants because of their rarity.

Relative to core histones H3 and H4, considerably less information on variants and post-translational modifications is available for histones H2A and H2B. Recently a mass spectrometric analysis of human core histone identified the variants H2A1 (H2AA, P02261), H2B1 (H2BR, P06899), and H2BF (H2BF, P33778) albeit with no MS/MS analysis confirmation (13). To date, human histone modifications reported for histone H2A include acetylation on Lys-5 and -9 and phosphorylation on Ser-1 (14–16). For histone H2B, multiple acetylation sites were identified previously on Lys-5, -12, -15, and -20 (14–17). Modifications outside the NH2-terminal tails of histone H2A and H2B include ubiquitination in the carboxyl-terminal region on Lys-119 and -120, respectively (14).

In the past, microsequencing was widely used to identify modification sites of histone proteins (17) and to obtain sequence information for the different histone subtypes (7, 18). Immunoblotting with specific antibodies is a convenient technique.
method for detecting known modifications as it requires only small amounts of samples. However, few antibodies against modified H2A and H2B (19, 20) and specific histone subtypes (11, 18) are available. MS provides an ideal tool to overcome these limitations (13, 20–27). Several groups have combined LC-MS analysis and peptide mapping to identify histone proteins and to characterize their post-translational modifications. However, little and incomplete data have been reported for histones H2A and H2B (13, 23, 25). On the other hand, direct structural characterization of intact histone H2B from *Tetrahymena* has recently been achieved by FT ion cyclotron MS using electron capture dissociation. Implemented on a home-built instrument, this technology was used to characterize the two major histone H2B variants differing by only three amino acids and their post-translational modifications (21).

In this study, we combined LC-MS analysis on intact proteins and peptide mapping (MS and MS/MS) to provide a detailed description of histones H2A and H2B subtypes of a human cell line as a basis for the investigation of their potential roles in chromatin structure and function.

**EXPERIMENTAL PROCEDURES**

**Preparation of Nuclei and Extraction of Histones**—Jurkat cells were cultured in RPMI 1640 medium containing 10% FCS, 100 IU of penicillin/streptomycin, and 10 μg/ml gentamycin. Cells were washed twice with PBS, and the cell pellet was suspended in lysis buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaHSO₃, 45 mM sodium butyrate, 10 mM 2-mercaptoethanol, 0.2% Triton X-100) and centrifuged at 800 × g for 15 min to obtain nuclei. For preparing histones (28), nuclei were extracted in 6 volumes 0.2 mM H₂SO₄ (16 h at 4 °C). After centrifugation at 16,100 × g, supernatants were precipitated by trichloroacetic acid (25%, final concentration). The pellet was washed with 50 mM HCl in acetone and then with acetone and subsequently dissolved in β-mercaptoethanol (0.1%) in water.

**HPLC Separation of Histones**—The histone aliquots were acidified with TFA to a final concentration of 0.1% and centrifuged for 2 min at 16,100 × g, and an aliquot of the supernatant corresponding to 11 μg of histones was subjected to micro-LC-ESI-MS analysis. After a desalting step of 3 min with 0.1% TFA in 2% ACN on a Vydac C₁₈ precolumn (1-mm inner diameter × 5 mm, 5-μm particle size), the separation of the histone proteins was achieved by reversed-phase HPLC using a Vydac C₁₈ column (150 mm × 1 mm, 5-μm particle size). Individual histones were eluted from the column by applying a multistep gradient of acetonitrile (0–44% B in 5 min, 44–49% B in 17 min, isocratic gradient 5 min at 49%, 49–62% B in 57 min; solvent A: 0.1% TFA in 2% ACN; solvent B: 0.1% TFA in 80% ACN, 50 μl/min). The flow was split after UV detection (214 nm), 42 μl/min were directed to the automated fraction collector and collected into 96-well plates, and 8 μl/min were directed into the mass spectrometer operated in MS mode.

**Digestion of Histones**—Preparative HPLC fractions containing individual histones according to UV and MS chromatograms were pooled, dried (SpeedVac SC 110, Savant Instruments, Farmingdale, NY), and dissolved in 5–10 μl of 25 mM NH₄HCO₃ or 100 mM Tris-HCl, pH 8. The histones were digested either with endoproteinase Glu-C (sequence grade, Roche Diagnostics) in 25 mM NH₄HCO₃ at an enzyme ratio of 1:20 at 25 °C for 1–2 h, with trypsin (Promega, Madison, WI) at an enzyme ratio of 1:250 at 37 °C for 5 min in 100 mM Tris-HCl, pH 8, or with endoproteinase Arg-C (sequence grade, Roche Diagnostics) at an enzyme ration of 1:250 at 37 °C for 1–2 h in 100 mM Tris-HCl, pH 8. The reaction was stopped by adding formic acid to a final concentration of 0.1%. For desalting, 5–10 μl of peptide mixtures were absorbed on a POROS R3 column, washed with 0.2% formic acid, and desorbed sequentially with 2 μl of 10, 20, 30, and 50% methanol in 0.2% formic acid.

**Mass Spectrometry**—LC-MS of intact histones was conducted using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA) coupled to an LCQ electrospray ion trap mass spectrometer (LCQ, Thermo Finnigan, San Jose, CA). For optimal ESI conditions, spray voltage was set to 4000 V, capillary temperature was 200 °C, capillary voltage was set to 8 V, and the tube lens was set to 16 V. MS spectra were acquired by scanning over m/z range 200–2000 in 350 ms. The mass accuracy of the instrument for intact proteins was estimated to be 100 ppm. The molecular masses of the histones were determined after deconvolution of the multiply charged ion series (BioWorks software, Thermo Finnigan). Interpretation of MS spectra was done manually by comparing measured masses to calculated masses of histone sequences derived from Swiss-Prot (see Table I).

For peptide mass fingerprints, ESI mass spectra were recorded on a QStar Pulsar hybrid quadrupole time-of-flight mass spectrometer equipped with a nanospray ion source (Applied Biosystems, Foster City, CA). The needles (Protana, Odense, Denmark) were loaded with the peptide mixtures and adjusted in front of the orifice, and the spray voltage was set between 900 and 1300 V. The instrument was scanning between 200 and 2000 Da. Parent ions were selected for CID. We achieved a mass accuracy of at least 0.02 Da with external calibration, thus allowing distinction between acetylation and trimethyllysination (Supplemental Table I) as well as between arginine and dimethyllysine (Supplemental Table II). Interpretation of MS and MS/MS spectra was done manually by comparing peptide measured masses to calculated masses derived from histone sequences (see Table I).

**RESULTS**

**General Strategy for Histone Analysis**—We chose a strategy where histones were isolated from nuclei of Jurkat cells by acid extraction and were separated by C₁₈ reversed-phase HPLC (Fig. 1). During separation, 15% of the HPLC effluent was analyzed directly by mass spectrometry with an ion trap LCQ instrument, and the remainder was collected. The fractions were pooled according to the UV and MS chromatograms and digested for detailed analysis by nano-ESI-MS and MS/MS.

The HPLC chromatogram showed nine well resolved peaks that after MS data analysis and deconvolution of the multiply charged ion series were identified as histone proteins H1, H2A, H2B, H3, and H4 (Fig. 1). The assignments were based on the masses predicted from known amino acid sequences and allowing for post-translational modifications. No chromatographic separation was observed for histone H2B isoforms, whereas the H2A variants eluted in two distinct HPLC peaks. Compared with the traditional method using a C₄ column (22–24), reversed-phase HPLC using a C₁₈ column was more efficient in separating the individual histones. The elution pattern was consistent and reproducible with similar results obtained for other human cell lines such as THP1 or HeLa cells (data not shown). To confirm the identity of histone
H2A and H2B variants and to characterize their modifications, the corresponding fractions containing histones were hydrolyzed either with endoproteinase Glu-C, endoproteinase Arg-C, or trypsin. The resulting peptide mixtures were analyzed by nano-ESI-MS, measured peptide masses were compared with calculated masses derived from the histone H2A and H2B variants, and MS/MS analyses were performed. In the following, a detailed account of the peaks containing H2A and H2B histones is presented.

Identification of Histone H2A Variants (First Peak)—The molecular masses of histone H2A in the first peak (see Fig. 1) were determined after deconvolution of the multiply charged ion series (Fig. 2A) and compared with the theoretical masses of the different H2A subtypes described in Table I. Inspection of the mass spectrum of intact H2A histones allowed a first assignment of several potential protein subtypes. The molecular masses at 13,421.8, 13,900.2, 14,006.8, and 14,046.9 Da fitted well with the calculated masses of histone H2AZ (13,421.5 Da), H2AQ (13,899.2 Da), H2AO (14,006.3 Da), and H2AA (14,046.3 Da), respectively (Fig. 2A). The results are compiled in Table II. Final assignments were confirmed by peptide mapping using nano-ESI-MS of digests of pooled histone fractions.

Endoproteinase Glu-C and endoproteinase Arg-C were used as enzymes to achieve 100% sequence coverage of histone H2A. Analysis of Glu-C peptides by MS/MS identified the characteristic peptide 42–56 containing a Met in position 51; this confirmed the presence of histone H2AO and H2AQ in this HPLC fraction (Table III). In contrast, the Glu-C peptide 42–56 and the Arg-C peptide 43–71 containing a Leu in position 51 were never detected in the digests of the second H2A HPLC peak, whereas they were always identified in the digests of the first H2A HPLC peak containing H2AO and H2AQ and H2AZ variants were assigned. The dots represent non-attributed molecular masses. B, nano-ESI-MS spectrum of endoproteinase Glu-C peptide 1–41. The ions at m/z 560.60, 565.85, and 570.59 were identified as [M + H]⁺ of unmodified, acetylated, and phosphorylated peptides 1–41 from histone H2AO variant. Ac, acetylation; P, phosphorylation.
the presence of histone variants H2AO, H2AQ, and H2AZ in
the first HPLC peak.

Characterization of the H2A Histone Post-translational
Modifications (First Peak)—Potential post-translational modifications were searched by screening the masses of proteins or peptides for mass increments of 14, 42, or 80 Da, which are characteristic for methylation, acetylation, or phosphorylation. Post-translational modifications of H2A histones were apparent from the MS spectrum of the full-length proteins. Indeed in the deconvoluted mass spectrum of histone H2A from the first HPLC fraction, the molecular masses at 14,046.9 and 14,086.9 Da matched the acetylated and the phosphorylated forms of H2AO, respectively (Fig. 2A and Table II). No molecular mass for modified histone H2AQ and H2AZ variants were observed in the deconvoluted mass spectrum (Fig. 2A). Because modified H2AQ histone was not observed in the deconvoluted mass spectrum (Fig. 2A), the modifications were most likely predominant on H2AO variant. MS/MS analysis of these modified peptides showed that acetylation occurred at Lys-5 and phosphorylation occurred at Ser-1 (Table III). No molecular mass for modified histone H2AZ variant was observed in the spectrum of the intact protein. Still inspection of the Arg-C digest identified non-modified and acetylated peptides 1–19 from the H2AZ variant (Table III). MS/MS analysis of the modified peptides localized the acetylation on Lys-4 and Lys-7 (Table III).

Identification of Histone H2A Variants (Second Peak)—The mass spectrum of intact H2A histones eluting in the second H2A-containing HPLC peak allowed a first assignment of six histone variants (H2AA, H2AC, H2AE, H2AL, H2AG, and H2AZ) not previously identified in the first peak (Fig. 3A and Table II). No molecular masses for modified histone H2AO and H2AZ variants were observed in the deconvoluted mass spectrum (Fig. 2A). MS analysis of the Glu-C digest identified acetylated (m/z 565.85, [M + 8H]8+) and phosphorylated (m/z 570.59, [M + 8H]8+) peptides 1–41 derived from H2AO and/or H2AQ histones, variants which cannot be distinguished based on this peptide sequence (Fig. 2B). Because modified H2AQ histone was not observed in the deconvoluted mass spectrum (Fig. 2A), the modifications were most likely predominant on H2AO variant. MS/MS analysis of these modified peptides showed that acetylation occurred at Lys-5 and phosphorylation occurred at Ser-1 (Table III). No molecular mass for modified histone H2AZ variant was observed in the spectrum of the intact protein. Still inspection of the Arg-C digest identified non-modified and acetylated peptides 1–19 from the H2AZ variant (Table III). MS/MS analysis of the modified peptides localized the acetylation on Lys-4 and Lys-7 (Table III).
Q96KK5 (13,817.1 Da), H2AE (13,847.1 Da), and H2AC (14,018.3 Da), respectively, and the mass assignments for these variants in the protein mixtures. Inspection of the Arg-C digestion of histone H2A, MS analysis identified the ions at *m/z* 567.61 and 572.29 as acetylated and phosphorylated [M + 8H]^{8+} ions of peptides 1–41 originating from H2AC and/or H2AE histones (Fig. 3B). Because the acetylated form of histone H2AE could not be detected in the deconvoluted mass spectrum, this modification might predominantly be present in histone H2AC. MS/MS analysis of these modified peptides revealed acetylation on Lys-5 and phosphorylation at Ser-1 (Table III). After incomplete Arg-C digestion of histone H2A, MS analysis identified non-modified, acetylated, and phosphorylated peptides 1–17 with Thr in position 16 confirming the results above for H2AC and H2AE but also indicating the potential modification of H2AA/G (Table III). Also peptides 1–17 with Ser in position 16, characteristic of H2AL variant, were identified to be acetylated on Lys-5 or phosphorylated on Ser-1 (Table III). However, modified histone H2AL proteins were not observed in the spectrum of the intact protein, indicating the low abundance of these modifications (Fig. 3).

To summarize, the combination of on-line MS analysis and peptide mapping of the H2A histones identified histones H2AO, H2AQ, and H2AZ exclusively in the first HPLC peak, whereas the other six variants (H2AC, H2AE, H2AL, H2AA, and/or H2AG, Q96KK5) were found in the second HPLC peak. The histones H2AM, Q7L7L0, Q8IUE6, Q96QV6, and Q9BTM1 were not detected. The histone variants H2AO and H2AC appeared to be the most abundant in the H2A protein mixtures.

### Characterization of the H2A Histone Post-translational Modifications (Second Peak)—

In the MS spectrum of intact histone H2A, the molecular masses at 14,044.7 and 14,081.5 Da fitted well with the acetylated and phosphorylated forms of the H2AC variant, respectively (Fig. 3A and Table II). Also histone H2AE was observed in a phosphorylated form at 13,928.0 Da. After Glu-C digestion of histone H2A, MS analysis identified the ions at *m/z* 567.61 and 572.29 as acetylated and phosphorylated [M + 8H]^{8+} ions of peptide 1–41 originating from H2AC and/or H2AE histones (Fig. 3B). Because the acetylated form of histone H2AE could not be detected in the deconvoluted mass spectrum, this modification might predominantly be present in histone H2AC. MS/MS analysis of these modified peptides revealed acetylation on Lys-5 and phosphorylation at Ser-1 (Table III). After incomplete Arg-C digestion of histone H2A, MS analysis identified non-modified, acetylated, and phosphorylated peptides 1–17 with Thr in position 16 confirming the results above for H2AC and H2AE but also indicating the potential modification of H2AA/G (Table III). Also peptides 1–17 with Ser in position 16, characteristic of H2AL variant, were identified to be acetylated on Lys-5 or phosphorylated on Ser-1 (Table III). However, modified histone H2AL proteins were not observed in the spectrum of the intact protein, indicating the low abundance of these modifications (Fig. 3).
mixtures (cf. intensities of peaks in Figs. 2A and 3A) and were identified as acetylated (Lys-5) and phosphorylated (Ser-1). Histone H2AL was also found to be acetylated on Lys-5 or phosphorylated on Ser-1, and histone H2AE was only phosphorylated on Ser-1. There was no evidence for simultaneous phosphorylation and acetylation of these variants. The replacement histone H2AZ variant was identified to be acetylated on Lys-4 and Lys-7. The replacement histone H2AX subtype mentioned in Table I was not observed either as a full-length protein or in peptide mass fingerprints. All MS/MS

| Table III | List of peptides identified by MS/MS for histone H2A contained in the first and second HPLC peaks |
|-----------|-----------------------------------------------------------------------------------------------|
| Proteases | Mass Calculated | Mass Measured | Sequences<sup>a</sup> | Variants<sup>b</sup> |
| First HPLC peak |
| Glu-C | 1–41 | 4476.5 | 4476.77 | SGRGKQGGKARAKAKSRRSSRAQLQFPVGRHRLRLLRKNYAE | H2AO/Q |
| Glu-C | 1–41 | 4518.51 | 4518.73 | SGRGacKQGGKARAKAKSRRSSRAQLQFPVGRHRLRLLRKNYAE | H2AO/Q |
| Glu-C | 1–41 | 4556.46 | 4556.65 | pSGRGKQGGKARAKAKSRRSSRAQLQFPVGRHRLRLLRKNYAE | H2AO/Q |
| Glu-C | 42–56 | 5027.79 | 5027.76 | RVGAAGAPVYMAAVLE | H2AO/Q |
| Glu-C | 60–67 | 936.47 | 936.48 | YLTAEILE | H2AZ |
| Glu-C | 57–64 | 950.49 | 950.5 | YLTAEILE | H2AO/Q |
| Glu-C | 93–121 | 3054.85 | 3054.83 | LNKLQGTVIAGGQVLPNIAVLLPKKT | H2AO/Q |
| Glu-C | 65–92 | 3121.76 | 3121.78 | LGNAARDNKKTRIIPHLQAIRNDEE | H2AO/Q |
| Glu-C | 68–95 | 3127.75 | 3127.71 | LGNASKDLKVRITPRHLQAIRNDEE | H2AZ |
| Glu-C | 96–127 | 3306.94 | 3306.96 | LDLSLITIAAGGQVHPIHKSGLIGKKGQKTVT | H2AO/Q |
| Arg-C | 1–11 | 1142.62 | 1142.64 | SGRGKQGGKAR | H2AO/Q |
| Arg-C | 1–11 | 1184.63 | 1184.64 | SGRGacKQGGKAR | H2AO/Q |
| Arg-C | 1–11 | 1222.58 | 1222.60 | pSGRGKQGGKAR | H2AO/Q |
| Arg-C | 1–19 | 1816.02 | 1816.08 | AGGKAGDSKAGKAK | H2AZ |
| Arg-C | 1–19 | 1858.03 | 1858.08 | AGGacKAGDSKAGKAK | H2AZ |
| Arg-C | 1–19 | 1900.04 | 1900.08 | AGGacKAGDSKAGKAK | H2AZ |
| Arg-C | 82–129 | 5247.01 | 5247.02 | HLQLAIRNDEELNQLKLGTVIAGGQVLPNIAVLLPKKTESHHHKAKGK | H2AO/Q |
| Arg-C | 82–128 | 5139.96 | 5140.01 | HLQLAIRNDEELNQLKLGTVIAGGQVLPNIAVLLPKKTESHHHKAKGK | H2AQ |
| Arg-C | 85–127 | 4568.59 | 4568.49 | HLQLAIRGDEELDSLIKATIAGGQVHPIHKSGLIGKKGQKTVT | H2AZ |
| Second HPLC peak |
| Glu-C | 1–41 | 4476.5 | 4476.89 | SGRGKQGGKARAKAKSRRSSRAQLQFPVGRHRLRLLRKNYAE | H2AL |
| Glu-C | 1–41 | 4490.51 | 4490.89 | SGRGKQGGKARAKAKTSSRAQLQFPVGRHRLRLLRKNYAE | H2AC/E/Q96KK5 |
| Glu-C | 1–41 | 4506.51 | 4506.33 | SGRGacKQGGKARAKAKTSSRAQLQFPVGRHRLRLLRKNYAE | H2AA/G |
| Glu-C | 1–41 | 4570.47 | 4570.25 | pSGRGKQGGKARAKAKTSSRAQLQFPVGRHRLRLLRKNYAE | H2AC/E/Q96KK5 |
| Glu-C | 42–56 | 1484.83 | 1484.83 | RVGAAGAPVYLAAVLE | H2AA/C/G/L/Q96KK5 |
| Glu-C | 57–64 | 950.49 | 950.49 | YLTAEILE | H2AA/C/G/L/Q96KK5 |
| Glu-C | 65–92 | 3211.76 | 3212.08 | LGNAARDNKKTRIIPHLQAIRNDEE | H2AA/C/G/L/Q96KK5 |
| Glu-C | 93–121 | 3054.85 | 3054.85 | LNKLQGTVIAGGQVLPNIAVLLPKKT | H2AC/E/Q96KK5 |
| Glu-C | 93–121 | 3082.85 | 3082.97 | LNKLQGTVIAGGQVLPNIAVLLPKKT | H2AA/L |
| Arg-C | 1–11 | 1142.62 | 1142.64 | SGRGKQGGKAR | All |
| Arg-C | 1–11 | 1184.63 | 1184.64 | SGRGacKQGGKAR | All |
| Arg-C | 1–11 | 1222.58 | 1222.60 | pSGRGKQGGKAR | All |
| Arg-C | 1–17 | 1798.03 | 1798.04 | SGRGKQGGKARAKAK | H2AA/C/G/E/Q96KK5 |
| Arg-C | 1–17 | 1826.03 | 1826.04 | SGRGacKQGGKARAKAK | H2AA/C/G/E/Q96KK5 |
| Arg-C | 1–17 | 1863.98 | 1864.32 | pSGRGKQGGKARAKAK | H2AA/C/G/E/Q96KK5 |
| Arg-C | 82–129 | 5247.01 | 5247.02 | HLQLAIRNDEELNQLKLGTVIAGGQVLPNIAVLLPKKT | H2AC/G |
| Arg-C | 82–129 | 5275.01 | 5274.11 | HLQLAIRNDEELNQLKLGTVIAGGQVLPNIAVLLPKKT | H2AA/L |
| Arg-C | 99–99 | 1299.67 | 1299.69 | NDEELNKLK | H2AA/L |
| Arg-C | 100–129 | 3161.83 | 3161.91 | VTIAGQGQVLPNIAVLLPKKT | H2AA/L |
| Arg-C | 100–129 | 3091.91 | 3091.95 | H2AE |
| Arg-C | 100–127 | 3051.89 | 3051.91 | H2AL |

<sup>a</sup> Bold amino acids represent residues that vary in the sequence, acK indicates a monoacylation on Lys, and pS indicates a phosphorylation on Ser.

<sup>b</sup> List of histone H2A variants identified in the protein mixtures with the same corresponding sequences.
spectra of peptides listed in Table III are shown in the supplemental data.

Identification of Histone H2B Variants—As for histone H2A, on-line MS analysis and peptide mapping were used to identify the histone H2B variants. For the digestion of the H2B proteins, endoproteinase Glu-C and trypsin were used to achieve 100% sequence coverage. The deconvoluted mass spectrum of the histone H2B HPLC peak allowed a first assignment of seven histone variants (H2BA, H2BB, H2BJ, H2BN, H2BQ, H2BX, and Q5QNW6) (Fig. 4A). The results are compiled in Table IV. The calculated molecular masses of H2BB (13,804.9 Da) and H2BJ (13,760.9 Da) variants were matched to molecular masses at 13,805.8 and 13,760.8 Da, respectively; the mass assignments for these histones were unique. However, the calculated masses of H2BA (13,774.9 Da) ...
TABLE IV
Assignments of the variants for histone H2B

| Mass  | Deconvoluted | Calculated | Assignment | Δm  |
|-------|--------------|------------|------------|-----|
| Da    |              |            |            |     |
| 13,760.8 | 13,760.9     | −0.1       | H2BJ       |     |
| 13,770.6 | 13,774.9     | +0.7       | H2BA       |     |
| 13,774.9 | 13,775.9     | −0.3       | H2BN       |     |
| 13,776.6 | 13,776.9     | −1.3       | H2BX       |     |
| 13,788.8 | 13,788.9     | −0.1       | Q5QN9W6    |     |
| 13,805.8 | 13,804.9     | +0.9       | H2BB       |     |

a The molecular masses of the histones were determined after deconvolution of the multiply charged ion series using an automated program.
b The major protein variants were assigned. The other variants may be present at lower levels.

Da) was close to those of H2BN (13,775.9 Da) and H2BX (13776.9 Da), which would fit the molecular masses at 13775.6 Da. Also histone variants H2BQ (13,788.9 Da) and Q5QN9W6 (13,788.9 Da) matched with the molecular masses at 13,788.8 Da.

To identify and confirm the presence of these variants in the histone H2B mixtures, analyses of tryptic and Glu-C digests by MS and MS/MS were performed. As a first evidence, peptides characteristic of variants H2BD, H2BF, H2BS, and Q6NWQ3, variants that were not observed in the deconvoluted mass spectrum, were not detected in the analyses of the corresponding Glu-C and tryptic digests, indicating the absence of these four variants in H2B protein mixtures. Inspection of the predicted amino acid sequences of human H2B described in Table I showed that the majority of the variation on the H2B sequences was located in the first 32 amino acid residues. Therefore, after endoproteinase Glu-C digestion, MS and MS/MS analyses were focused on ions derived from peptide 1–35. MS/MS analysis of the [M + 7H]7+ ions at m/z 546.06, 552.06, and 554.35 allowed identification of peptides 1–35 from histones H2BX, Q5QN9W6, and H2BB, respectively (Fig. 4B and Table V). The unique sequence of these peptides 1–35 confirmed the presence of these three histones in the protein mixtures. On the other hand, [M + 7H]7+ at m/z 552.33 was found by MS/MS as peptide 1–35 derived from either histones H2BC or H2BD. The protein sequences of H2BC and H2BD differ in positions 3, 4, and 75, but for both variants the peptides 1–35 have the same nominal mass. Unfortunately in the MS/MS spectrum of m/z 552.33, no b or y ions were present, which would allow to distinguish between Leu-Ala for H2BC or Pro-Ser for H2BD in positions 3 and 4, respectively (data not shown). To discern between these two variants, incomplete digestion with trypsin was necessary to generate the peptide 1–5. After MS/MS analysis, peptide 1–5 from histone H2BC variant was identified, whereas peptide 1–5 from H2BD variant was not detected (Table V). Thus, histone H2BC variant was clearly identified by peptide mapping; however, its molecular mass was not observed in the intact protein spectrum indicating a low abundance of this variant compared with the other H2B variants. Furthermore MS/MS analysis of m/z 561.92 identified [M + 7H]7+ of peptide 1–35 from histone H2BE variant (Fig. 4B and Table V). Interestingly histone H2BE variant was not observed in the spectrum of intact histone also indicating low abundance of this variant in the H2B variant mixtures. Further inspection of the Glu-C digest identified peptide 1–35 (m/z 548.06 [M + 7H]7+) originating from histones H2BJ and/or H2BN, variants that cannot be distinguished based on this peptide sequence (Fig. 4B). In the intact histone spectrum, the mass assignment for H2BJ at 13,760.8 Da was unique confirming the presence of this variant. Finally MS/MS analysis of the ion at m/z 550.06 ([M + 7H]7+) identified peptides 1–35, which correspond to the most intense signal of the different 1–41 peptide ions. This peptide sequence is shared by histones H2AA, H2BK, H2BQ, and H2AR; however, the latter three variants were absent or of rather low abundance in the deconvoluted mass spectrum compared with the H2BA variant (Fig. 4A). Thus, peptide 1–35 originating from H2BA was probably the main contributor to the peptide ions at m/z 550.06 (Fig. 4B). Identification of the Glu-C peptide 114–125 with Ala in position 124 confirmed the presence of one or both histone H2BR and H2BK variants in the histone H2B mixtures (Table V). Also the tryptic peptide 35–43 with Ile in position 39 was identified by MS/MS confirming the potential presence of histone H2BN, H2BQ, H2BR, and H2BX variants; H2BX was already identified previously. Therefore, neither peptide mapping nor intact histone analysis could exclusively identify variants H2BK, H2BQ, H2BR, and H2BN, indicating the potential presence of one or several of these variants in the H2B histone mixtures.

Characterization of the H2B Histone Post-translational Modifications—In the MS spectrum of the Glu-C digest (Fig. 4B), the ion at m/z 556.06 was identified as [M + 7H]+ of monoacetylated peptide 1–35 probably derived from the most abundant H2BA variant. MS/MS analysis of the monoaecetylated peptide 1–25 (molecular mass, 2631.51 Da) showed the presence of three isomers with acetylation on Lys-12, Lys-15, and Lys-20. All MS/MS spectra compared with the other H2B variants. Furthermore MS/MS analysis of m/z 561.92 identified [M + 7H]7+ of peptide 1–35 from histone H2BE variant (Fig. 4B and Table V). Interestingly histone H2BE variant was not observed in the spectrum of intact histone also indicating low abundance of this variant in the H2B variant mixtures. Further inspection of the Glu-C digest identified peptide 1–35 (m/z 548.06 [M + 7H]7+) originating from histones H2BJ and/or H2BN, variants that cannot be distinguished based on this peptide sequence (Fig. 4B). In the intact histone spectrum, the mass assignment for H2BJ at 13,760.8 Da was unique confirming the presence of this variant. Finally MS/MS analysis of the ion at m/z 550.06 ([M + 7H]7+) identified peptides 1–35, which correspond to the most intense signal of the different 1–41 peptide ions. This peptide sequence is shared by histones H2AA, H2BK, H2BQ, and H2AR; however, the latter three variants were absent or of rather low abundance in the deconvoluted mass spectrum compared with the H2BA variant (Fig. 4A). Thus, peptide 1–35 originating from H2BA was probably the main contributor to the peptide ions at m/z 550.06 (Fig. 4B). Identification of the Glu-C peptide 114–125 with Ala in position 124 confirmed the presence of one or both histone H2BR and H2BK variants in the histone H2B mixtures (Table V). Also the tryptic peptide 35–43 with Ile in position 39 was identified by MS/MS confirming the potential presence of histone H2BN, H2BQ, H2BR, and H2BX variants; H2BX was already identified previously. Therefore, neither peptide mapping nor intact histone analysis could exclusively identify variants H2BK, H2BQ, H2BR, and H2BN, indicating the potential presence of one or several of these variants in the H2B histone mixtures.

Characterization of the H2B Histone Post-translational Modifications—In the MS spectrum of the Glu-C digest (Fig. 4B), the ion at m/z 556.06 was identified as [M + 7H]+ of monoacetylated peptide 1–35 probably derived from the most abundant H2BA variant. MS/MS analysis of the monoaecetylated peptide 1–25 (molecular mass, 2631.51 Da) showed the presence of three isomers with acetylation on Lys-12, Lys-15, and Lys-20. Indeed the observation of two y132+ ions (m/z 715.92 and 694.92; Fig. 5, inset A), two y10+ ions (m/z 1158.66 and 1116.67; Fig. 5, inset C), and two y7+ ions (m/z 860.48 and 818.47; Fig. 5, inset B) with a mass difference corresponding to 42 Da confirmed acetylation on Lys-15, Lys-12, and Lys-20 (Fig. 5). To summarize our findings on H2B histones, on-line MS analysis and peptide mapping identified 11 histone H2B variants (H2BA, H2BB, H2BC, H2BE, H2BJ, H2BK, H2BN, H2BQ, H2BR, H2BX, and Q6NWQ3). The variant H2BA was the most abundant (cf. intensities of peaks and ions in Fig. 4, A and B) in the histone H2B mixtures as compared with the others and was also detected with acetylation on Lys-12, Lys-15, and Lys-20. All MS/MS spectra
Histone variants and their post-translational modifications play an important role in the dynamics of chromatin structure and function. However, no comprehensive analyses of histone H2A and H2B variants have as yet been reported at the protein level. Here a strategy combining mass spectrometric techniques (nano-ESI-MS and MS/MS), HPLC separation (reversed-phase HPLC-MS), and enzymatic digestions (endo-proteinase Glu-C, endoproteinase Arg-C, and trypsin) was used to characterize histone subtypes and the post-translational modifications of human histone H2A and H2B. Our analysis by LC-MS of intact histones combined with peptide mapping (MS and MS/MS) allowed us to show the presence of nine histone H2A variants and 11 histone H2B variants in Jurkat cells. Furthermore, localization of acetylation and phosphorylation sites was characterized on the most abundant H2A and H2B variants. Our results demonstrate that a large number of histone variant genes are expressed at the protein level. We also confirmed the presence of proteins encoded by genes that had as yet not been recognized as bona fide histones.

A key step in the described approach was the separation of individual histone proteins by C18 reversed-phase HPLC prior to on-line MS analysis and peptide mapping to reduce the complexity of the protein mixtures. In previous studies, C4 columns were used for the separation of the core histones, a system in which histone H2A and H2B eluted in the same HPLC peak (22–24). The C18 reversed-phase HPLC column resolved histones H2B and H4 into individual HPLC peaks, and H2A, H1, and H3 each eluted in two or three distinct peaks. Analysis of the two histone H2A peaks showed that

### Table V

| Proteases | Residues | Mass Calculated | Mass Measured | Sequences<sup>a</sup> | Variants<sup>b</sup> |
|-----------|----------|----------------|---------------|-------------------------|---------------------|
| Glu-C | 1–35 | 3815.24 | 3815.36 | PDPKSAPAPKGSKKVAKQKDDKGKKRKRKE | H2BX |
| Glu-C | 1–35 | 3829.25 | 3829.29 | PDPKSAPAPKGSKKVAKQKDDKGKKRKRKE | H2B/J |
| Glu-C | 1–35 | 3843.27 | 3843.29 | PEPKSAPAPKGSKKVAKQKDDKGKKRKRKE | H2BA/Q/K/R |
| Glu-C | 1–35 | 3857.29 | 3857.29 | PEAKSAPAPKGSKKVAKQKDDKGKKRKRKE | H2BC or D |
| Glu-C | 1–35 | 3873.28 | 3873.25 | PEPTKSAPAPKGSKKVAKQKDDKGKKRKRKE | H2BB |
| Glu-C | 1–35 | 3885.28 | 3885.29 | PEAKSAPAPKGSKKVAKQKDDKGKKRKRKE | H2BA/Q/K/R |

<sup>a</sup> Bold amino acids represent residues that vary in the sequence, acK indicates a monoacetylation on Lys.

<sup>b</sup> List of histone H2B variants identified in the protein mixtures with the same corresponding sequence.
each peak contained a mixture of several variants. The variants H2AO, H2AQ, and H2AZ were identified in the first HPLC peak, whereas H2AC, H2AE, H2AL, H2AA, and/or H2AG, Q96KK5 were found in the second HPLC peak. The main difference between these variants appeared in position 51 where a Met residue was observed for H2AO and H2AQ, whereas a Leu residue was found for the other five variants. This single amino acid difference probably dominated the chromatographic behavior of these protein variants. The histone H2AZ differs considerably from the bulk H2A sequences; therefore its elution in the first HPLC peak was not expected. The analysis by nano-ESI-MS/MS of peptides generated by different enzymatic cleavages contributed significantly to the identification of the histone H2A and H2B subtypes. Indeed different proteases were needed to achieve complete sequence coverage of the H2A and H2B histones. Because of

![Fig. 5. MS/MS spectrum of peptide 1–25 from histone H2BA variants acetylated on Lys-15, Lys-12, and Lys-20. The ions at m/z 527.31 corresponding to [M + 5H]5+ of monoacetylated peptide 1–25 (molecular mass, 2631.51 Da) of histone H2BA were subjected to fragmentation, and peptide isomers were observed. The immonium ions at m/z 126.09 indicated the presence of acetyllysine in the peptide (inset A). Acetylation was identified on Lys-15 because the ions y7 at m/z 860.48 (inset C) and y10 at m/z 1158.66 (inset D) showed no mass shifts, whereas y10 at m/z 715.92 (inset B) and the y ions above were shifted by 42 Da, indicating acetylation at Lys-15. In addition, acetylation was assigned on Lys-12 because y13 at m/z 694.93 (inset B) was found with a non-shifted mass, whereas y13 at m/z 779.97 and y ions above were only observed with a mass shift corresponding to an acetylation. Finally acetylation was also localized on Lys-20 because y7 at m/z 818.47 (inset C) and y10 at m/z 1116.67 (inset D) were found with a mass increase of 42 Da, whereas y5 at m/z 589.33 was not observed with a mass shift. Ac, acetylation. ](image)
the scattered distribution of the amino acids that characterize these variants, their identification was only possible by MS analysis of large peptides. For example, the large carboxyl-terminal peptides observed after Arg-C digestion of histone H2A were required to identify or exclude variants with unique amino acid termini. Large fragments generated after Glu-C digestion covering the first 41 amino acids of H2A and the first 35 amino acids of H2B were needed to unambiguously identify variants with unique sequence on these peptides. In addition, modifications (acetylation and phosphorylation) could easily be characterized on these large amino-terminal peptides originating from histone H2A and H2B variants. Acetylation was unambiguously identified in MS/MS spectra by the presence of the characteristic immonium ion at m/z 126, which is produced from peptides containing acetyllysine (22). Furthermore high accuracy mass spectrometry allowed us to distinguish between acetylation and trimethylation (see Supplemental Table I). These modifications were observed at low levels in MS spectra on the most abundant histone H2A and H2B variants (H2AQ, H2AZ, H2AC, H2AL, H2AE, and H2BA). Probably because of their low abundance, no modification was detected on the other variants. Recent studies have shown the existence of H3.3-specific phosphorylation during mitosis that did not occur on the other members of the H3 family, thus indicating a unique function for the modified H3.3 variant (29). In view of such phenomena, the presence of acetylation, methylation, and phosphorylation should also be investigated for the H2A and H2B variants. Several modifications described in the literature for histones H2A and H2B were not identified in this study. The low abundance of ubiquitination, approximately 10% for H2A and 1–2% for H2B, could explain why ubiquitination was not detected (4). Recently ubiquitination on macroH2A1.2 was identified by mass spectrometry but only when upfront enrichment of ubiquitinated protein was performed before analyzing the protein (30). Also acetylation of Lys-9 for histone H2A and Lys-5 for histone H2B was only identified in cells treated with a histone deacetylase inhibitor (which induces histone hyperacetylation) indicating the low abundance of these modification in non-treated cells (16). Acetylation of Lys-4 and Lys-7 was identified here for the first time on the human replacement histone H2AZ. The same modifications were previously reported for H2AZ from *Tetrahymena* (31).

Recently the crystal structure of the nucleosome has been solved (32). The structure provides detailed information about interactions within the histone fold domains as well as between histones and DNA. Multiple electrostatic and hydrophobic interactions and hydrogen bonds are required in the globular domains for nucleosome formation. The amino-terminal tails of the histones do not significantly participate in the nucleosome structure and instead are involved in interactions with other proteins. Acetylation of the histone tails can affect the interactions of transcription factors with chromatin (33). Indeed histone acetylation is one of the better understood histone modifications, and it is now generally accepted that hyperacetylated histones are mostly associated with activated genomic regions, whereas deacetylation mainly results in repression and silencing (34). Could the small difference in the sequences of histone variants, in analogy to the case of the H3.3 variants, affect chromatin function? If so, the individual histone isoforms might differentially mediate biological functions by providing binding sites to selectively interacting partners, such as modifying enzymes or transcription factors and their associated proteins. For the different histone variants described in Table I, the H2B proteins show mainly extensive variation in the first 39 amino acids. The variations at positions 2, 9, 18, and 39 are conservative in nature, whereas the variations at positions 3 and 19 alter the polarity and hydrophobicity of the amino acids. Amino acid variations are also present within the globular domain of histones H2A and H2B, and these may affect nucleosome stability and higher order chromatin structure. In the histone H2A variants listed in Table I, groups of variants can be defined with Leu or Met in position 51, Ser or Thr in position 16, Ala or Ser in position 40, and Lys or Arg in position 99. However, these individual amino acid variations may trigger minor changes in the chromatin structure compared with variations, for example, between replacement H2A histone variants. The replacement histone variants, including H2AZ, macroH2A, H2A-Bbd, and H2AX, are distinguished from the major H2A histones by their carboxyl-terminal tails that diverge in both length and sequence (4). Incorporation of these particular histone subtypes into nucleosomes has significant impacts on transcriptional activation, DNA repair, meiosis, and apoptosis (5, 6, 35). Indeed transcriptionally active and repressed chromatin contains distinct replacement histones, which in turn appear to control transcription. As to the major histone variants investigated here, it should be interesting to study their special distribution along the chromatin, using for example specific antibodies for distinguishing the different histone isoforms, in search for any structural and functional biological roles in the cell.

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