**INTRODUCTION**

In eukaryotic cells, the DNA is packaged into chromatin. The basic module of chromatin, the nucleosome, consists of 146 base pairs of DNA wrapped around a histone octamer (Luger et al., 1997). The octamer is composed of two each of the core histones H2A, H2B, H3 and H4. Each of the four core histones has a central “histone fold” domain that consists of three alpha helixes connected by two loops (Arents & Moudrianakis, 1995; Luger et al., 1997). An array of nucleosomes generally contains the additional histone, H1, which is also known as a linker histone. The amino acid sequence of canonical core histones is highly conserved in eukaryotes.

The N-terminal tails of the core histones protrude from the nucleosomal surface and are subject to multiple covalent modifications including methylation, phosphorylation and acetylation. These modifications have the potential to regulate the chromatin architecture and can thereby affect all aspects of DNA processing. According to the so-called “histone code” hypothesis, these modifications could be read by proteins that bind to specific modifications and can then regulate downstream events (Turner, 1993; Strahl & Allis, 2000).

With the exception of H4, all canonical core histone proteins are known to have variant counterparts, which often differ in surprisingly few amino acids (Pusarla & Bhargava, 2005). The incorporation of histone variants into nucleosomes as a mode of marking chromatin regions has been proven to have a high impact on gene regulation, DNA repair and meiotic events. These histone variants have been implicated in the epigenetic inheritance mechanisms of chromatin markings (Ahmad & Henikoff, 2002a; Henikoff et al., 2004) and shown to play significant roles in gene expression, antisilencing, heterochromatinization and the formation of specialized regions of chromatin (Ausio & Abbott, 2002; Smith, 2002; Kamakaka & Biggins, 2005; Sarma & Reinberg, 2005). H2A has the largest macro-heterogeneous family of variants, and all of them were found to have a crucial role in gene expression and nuclear dynamics (Ausio & Abbott, 2002). Homologues of H2AX are found across all phyla, including fungi, animals, plants and the most primitive eukaryotes such as Giardia (Malik & Henikoff, 2003). H2AX is randomly incorporated into nucleosomes and represents 10-15% of total cellular H2A. Phosphorylation of H2AX provides an epigenetic mark for broken DNA to recruit repair machineries. The carboxy terminus of H2AX differs from that of bulk H2A by being longer and having a four-amino-acid sequence element SQEL at the extreme C-terminal end of the protein. H2AZ, which is sometimes referred to as H2A.Z/F, is the most conserved variant during eukaryotic evolution. H2AZ has different functions in various organisms that include maintenance of pericentric and telomeric heterochromatin, transcriptional activation and viability. H3.3, one of the histone H3 variants, is almost identical to H3 and differs at only four positions: one in the N-terminal tail (Ala31) and three in the histone...
fold domain (Ser87, Val89 and Met90) (Ahmad & Henikoff, 2002b). Compared to H3, H3.3 shows several-fold enrichments of modifications found on active genes that serve as a significant mark for active chromatin (Bulger et al., 2003; McKittrick et al., 2004).

B. mori is known to have holocentric chromosomes (Murakami & Imai, 1974), and the roles of histone variants and their modification are of great interest. In B. mori, however, molecular cloning and characterization have been performed only for the histone H2A variant H2AZ, which analyzed the expression patterns of BmH2AZ (Furukawa et al., 2007). To date, it has not even been reported whether histone modifications are present in the silkworm. Here we have cloned and characterized the other canonical core histones and some variants including H2A, H2B, H2AX, H2AZ, H3.2, H3.3, and H4 to shed light on the regulation mechanisms of the chromatin structure in B. mori.

MATERIALS AND METHODS

Cloning of histones
We designed the primer pair for each histone based on the nucleotide sequences registered in the B. mori sequence database (silkworm EST database constructed by the Bombyx Genome Database Working Group, Toru Shimada, Kazuei Mita and co-workers) and performed PCR amplification using cDNA derived from the total RNA (isolated from the testes of silkworm strain r06 stocks at our university) as a template (See Table 1 for the primer sequence). The amplified histones cDNA were digested with XhoI (or SalI) and subcloned into the NcoI/blunt-XhoI site of pENTR™11 (Invitrogen), and their nucleotide sequence were determined via dye-terminator cycle sequencing using a DNA sequencer (ABI PRISM).

RT-PCR analysis
Total RNA was isolated from the silkworm tissues and blood cells by Isogen (Nippon Gene) according to the manufacturer’s protocol. The total RNAs were reverse-transcribed by SuperScript II™ (Invitrogen) in the presence of oligo (dT) primers. The resulting cDNAs were used as templates for RT-PCR by the same primers used for the cloning of histones cDNAs, except for H2A. The amplified products were separated by electrophoresis through a 1% agarose gel in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) and stained with ethidium bromide.

Cell culture and transfection
B. mori BmN4 cells provided from Dr. Aoki of Kyushu University were maintained in IPL-41 medium (Gibco) with 10% fetal bovine serum at 27°C. These cells were seeded in 24-well plates at a density of 1 × 10^5 cells/well.

Table 1. The sequence of primers for cloning and RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>H2A</td>
<td>5’-TCCGGTCGCGGAAAAGGCGGAAAG-3’ 5’-CCCTCGAGTTAACTTCTCTCCTCCTTC-3’</td>
</tr>
<tr>
<td>H2B</td>
<td>5’-CCGCCCTGCTAAGCAAGTGGAAGGCCGCC-3’ 5’-AAGGGCCTTTTTCGACGTAATCGCAACCC-3’</td>
</tr>
<tr>
<td>H3.2</td>
<td>5’-GGCCGCGGCAAAAGGAGGCACCAGGCC-3’ 5’-GTCATGCAGGCCTCTCTCCTTCTCTTCT-3’</td>
</tr>
<tr>
<td>H3.3</td>
<td>5’-TCCCTCGAGGTCGATGAACACCTCTTTATAACGCCC-3’ 5’-CCCCTCGAGTCGATGAAACTTCTTTACCGCC-3’</td>
</tr>
<tr>
<td>H2AX</td>
<td>5’-AATCGTGGAGGCATTGTAAGACTGAG-3’ 5’-CCCTCGAGTTAGTAGTCTTGTGATGATGAG-3’</td>
</tr>
<tr>
<td>H2AZ</td>
<td>5’-GGCCGCGGCTAAGCAAGTGGAAGGCCGCC-3’ 5’-CCCTCGAGTTAACTTCTCTCCTCCTTC-3’</td>
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One day after seeding, the cells were overlaid with a lipid-DNA complex (200 μl/well) for transient transfection of the vectors. Shortly before transfection, the lipid-DNA complex was prepared by mixing 200 ng of plasmid expression vector with 5 μl of PDD111 solution (Maeda et al., 2006) in a final volume of 30 μl, followed by incubation on the ice for 30 min and supplementation with 170 μl of SF-900 II SFM (Gibco). The cells were incubated for 6 h and maintained for another 36 h in the medium replaced with 1 ml of IPL-41.

Live cell imaging
We previously constructed a vector that produces GFP-fused proteins using Gateway technology (Mitsunobu et al., 2006). The GFP-fused histones were expressed in BmN4 cells using the above vector. Five days after transfection, the transfected cells were transferred to a glass-bottom dish the day before imaging. One hour before imaging, the medium was changed from regular IPL-41 medium to IPL-41 diluted Hoechst 22243. Images were acquired using a BIOZERO microscope (KEYENCE).

Extraction of Histones
Cultured cells were washed twice with PBS, and the cell pellet was suspended in an extraction buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 0.2% NP-40) supplemented with protease inhibitors, phosphatase inhibitors (Complete and PhosSTOP, respectively, Roche Diagnostics Ltd.) and 10 mM sodium butyrate. The suspension was centrifuged at 6,500 g for 5 min to obtain the nuclei as a pellet. The pellet (the nuclei) were dissolved in extraction buffer
Fig. 1. Comparison of H2A and variants across species. (A) Phylogenetic tree constructed from H2A family amino acid sequences using the Tree view X program. All sequence data were retrieved from the NCBI/GenBank database. The scale bar represents the branch length in terms of amino acid substitution per site. The database accession numbers are as follows: H2A homologues, *B. mori* (BAH22590.1), *D. melanogaster* (P84051), *H. sapiens* (Q6Fl13), *M. musculus* (Q6GSS7), *S. cerevisiae* (P04911), *S. pombe* (P04909), *C. elegans* (P09588); H2AX homologues, *B. mori* (BAH22618.1), *D. melanogaster* (P08985), *H. sapiens* (P16104), *M. musculus* (P27661); H2AZ homologues, *B. mori* (Q1HPV7), *H. sapiens* (P0C0S5), *M. musculus* (P0C0S6), *S. cerevisiae* (Q12692), *S. pombe* (P48003), *C. elegans* (Q27511). Alignment of the amino acid sequences of H2AX (B) and H2AZ (C) of *B. mori* with the homologues of *H. sapiens*, *S. cerevisiae* and *D. melanogaster*. The bold letters in the H2AX and H2AZ sequences indicate the SQ motif and Gly106 and His112/His114, respectively. The asterisks represent positions that have a fully conserved amino acid residue. Single dots and double dots represent weak similarity and strong similarity among the four organisms, respectively.
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Fig. 2. Expression of histones in diverse tissues from normal larvae (r06) on day 3 of the fifth instar. RT-PCR was performed using the same primers used for cloning except for H2A, and the RT-PCR of H2A was performed using primers for RT-PCR listed in Table 2. TE, testis; OV, ovary; SG, silk gland; MG, midgut; MT, malpighian tubule; FB, fat body; BC♂, blood cell male; BC♀, blood cell female. TCTP, which stands for translationally controlled tumor protein and is expressed ubiquitously in diverse tissues (Lee et al., 2004), was used as an internal control.

RP-HPLC Separation of Histones

Separation of the histone proteins was achieved by reversed-phase HPLC using a C8 column (Aquapore RP-300, 220 mm × 4.6 mm i.d., 7-mm particle size, Perkin Elmer) as described by Shechter et al. (2007). For the RP-HPLC separation, the histone extract was dissolved in the histone extraction buffer, which contained 2.5 M NaCl, because the dialyzed histone extract is prone to aggregation. Individual core histones were eluted from the column by application of a multistep gradient of acetonitrile (0-35% B in 10 min, isocratic gradient 10 min at 35%, 35-65% B in 75 min, 65-100% B in 20 min; solvent A: 0.1% trifluoroacetic acid (TFA) in 5% acetonitrile (ACN); solvent B: 0.1% TFA in 90% ACN, 0.8 ml/min).

Antibodies and Western blotting

Antibodies against mono-, di-, tri-methylated and acetylated H3K9 were kindly provided by Dr. Hiroshi Kimura (Osaka University). Proteins were resolved by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Blots were probed with the primary antibodies described above and incubated with rabbit anti-mouse-coupled alkaline phosphatase as a secondary antibody and visualized with CDP-Star chemiluminescent substrate (Tropix).

RESULTS AND DISCUSSION

Cloning of canonical core histones and their variants

We have cloned the cDNA encoding silkworm homologues of the canonical core histone (H2A, H2B, H3 and H4) and their variants (H2AX, H2AZ and H3.3). The H2A cDNA contains a complete ORF of 372 bp encoding 124 amino acid residues with a predicted molecular weight of 13.4 kDa. The complete ORF, amino acid residues and the predicted MW of the other homologues are as follows: H2B (372 bp, 124 a.a., MW 13.8 kDa), H3 (H3.2; 408 bp, 136 a.a., MW 15.4 kDa), H4 (309 bp, 103 a.a., MW 11.4 kDa), H2AX (408 bp, 136 a.a., MW 14.7 kDa), H2AZ (387 bp, 129 a.a., MW 13.4 kDa), H3.3 (408 bp, 136 a.a., MW 15.3 kDa). The amino acid sequences of the canonical histones from the silkworm are nearly identical to those of their counterparts in other eukaryotes. H2A and H2B have a homology of more than 92% and 84% even when compared to Homo sapiens. The homologies of H3 and H4 are even higher, with more than 99% identity to their mammalian counterparts. A phylogenetic tree was made using the amino acid sequence data of H2A family proteins registered in a database (Fig. 1A). B. mori H2AX had a highly conserved motif, termed the SQ motif, in the C-terminal (Fig. 1B). In B. mori, H2AZ also has the important residues Gly106 (relevant to the docking with (H3-H4)2) and His112/His114 (relevant to the binding of metal ions and the stabilizing it) (Raisner & Madhani, 2006) (Fig. 1C). The amino acid sequence of H3.3 was almost identical to that of H3 (H3.2) except for only four positions (Ala to Ser 31, Ser to Ala 87, Val to Ile 89, Met to Gly 90) (data not shown).

Table 2. The sequence of primers for RT-PCR to H2A

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>H2A</td>
<td>5’- GTGAAGGGCAAGGTCAAGTCCGTCG-3’</td>
</tr>
<tr>
<td></td>
<td>5’- GCTTTTCTTCTCGGTGTTGGGAGTAG-3’</td>
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Histone gene expression in diverse tissues

The expression levels of each canonical core histone in a variety of tissues were determined by RT-PCR using cDNA derived from the total RNA of larval tissues including the testes, ovaries, silk glands, midguts, fat bodies, malpighian tubes and blood cells. Histones except for H2AX showed ubiquitous expression in every tissue tested. The expression level of every histone mRNA in the fat bodies was lower than in the other tissues. The expression pattern of H2AZ was inconsistent with the previous report by Furukawa et al. (2007), in which the silkworm H2AZ gene was expressed mainly in the fat body and hemocytes of the 5th instar larvae.

Surprisingly, in the silkworm H2AX was expressed only in the genital organs, whereas in the mouse it is expressed in the other tissues including the thymus, spleen, uterus and intestine (Nagata et al., 1991). As H2AX is known to serve as a sensor protein for DNA double strand breaks, the restricted expression might suggest specific roles for BmH2AX in the maintenance of chromosomes during meiosis.

**Fig. 3.** Cell-cycle distribution of histone proteins. BmN4 cells were transiently transfected with EGFP-H2A (A), and the green signal represents EGFP-histones (a, b, c, d), whereas the blue signal represents the Hoechst-stained nuclei (e, f, g, h). Overlays of both pictures are presented in (i, j, k, l) and, moreover, overlays merged with bright-field images are shown in (m, n, o, p). Additionally, EGFP-H2A, EGFP-H2B (B), -H3.2 (C), -H4 (D), -H2AX (E), -H2AZ (F) or -H3.3 (G) were overexpressed in BmN4. Each stage of the cell cycle indicates the interphase (a, e, i, m), prophase (b, f, j, n), metaphase (c, g, k, o) and late anaphase (d, h, l, p).
Fig. 4. Characterization of silkworm histones. (A) Histones extracted from cultured cells (1, BmN4; 2, Bm5; 3, S2; 4, HeLa) were resolved on a 15% SDS-PAGE gel and Coomassie stained. S2 and HeLa histones were used as a mammalian control. The letter M represents a marker that has bands of 100, 67, 55, 37 and 14 kDa. (B) Chromatogram and Coomassie-stained gel from reversed-phase HPLC (RP-HPLC) separation of extracted histones. Extracted histones from BmN4 cells were separated on a C8 reversed-phase column in a ddH2O/acetonitrile gradient. The chromatogram shows the retention of proteins (solid line) on the column over the course of the acetonitrile gradient program shown in Table 3 (dashed line). Peak fractions were run on an SDS-PAGE gel and Coomassie stained. The Coomassie-stained gels correspond to the eluted fraction range as depicted in the chromatogram. The locations of the various histone proteins as identified by mass spectroscopy are noted under the gel. (C) Modification of the histone H3 at lysine 9 in the silkworm. The modifications were detected by Western blot analysis with the antibodies against the modifications indicated.
Localization of Histones during the cell cycle

To investigate the subcellular localization of histones, GFP-fused histones were expressed in cultured BmN4 cells. As expected, all histones were present in the nucleus throughout the cell cycle. In interphase, they basically colocalized with the diffused DNA in the nucleoplasm, and some of them assembled at regions of extensive staining by Hoechst 22243, i.e., the heterochromatin domain (Fig. 3A-G). During prometaphase, histones were highly concentrated along with the chromosomes, which were arrayed at the equatorial plane of the cell at metaphase. In anaphase, the histones diffused into the nucleoplasm as they did in the interphase. The accumulation of H2AX and H3.3 differs from that of each canonical histone with respect to the GFP fluorescence intensity, particularly in H2AX, a H2AX and H3.3 required a three- and two-fold increase in the camera sensitivity, respectively, to obtain the similar signal intensity.

H2AX and H3.3 are known to have specific functions of chromatin remodeling for DNA repair (Rogakou et al., 1998; Paull et al., 2000) and transcription regulation (Ahmad & Henikoff, 2002c; Chow et al., 2005). Thus, their expression or incorporation into chromatin was regulated due to their specific functions. In contrast to H3.3 and H2AX, the histone variant H2AZ exhibited localization and signal intensity that were similar to those of the canonical core histones. In other organisms, H2AZ is known to function in the regulation of heterochromatin formation (Fan et al., 2004), marking both active and inactive genes in euchromatin (Raisner et al., 2005). Therefore H2AZ is constitutively expressed due to its diverse roles in chromatin remodeling.

Purification and Characterization of Silkworm Core Histones

To confirm the above results and characterize the molecular profile of B. mori histones, canonical core histones were isolated from the cultured BmN4 silkworm cells and were resolved by SDS-PAGE. The mobilities of H3 and H4 from BmN4 cells were similar to those from other species. The mobilities of H2A and H2B from BmN4 cells were almost identical to those from the Drosophila melanogaster cell line (S2), but they were different from those from a human cell line (HeLa) (Fig. 4A). This result coincided with the degree of amino acid sequence homology among the canonical histones described above. Thus, the homologies of H3 and H4 (≥ 99% identity to mammalian H3 or H4) are higher than those of H2A and H2B among eukaryotes (98% and 99% similarity with D. melanogaster and 92% and 84% similarity with Homo sapiens, respectively). Moreover, this propensity was observed even when the sequences of core histones described here were compared to their counterparts from rice or yeast; H2A, H2B, H3 and H4 share more than 83%, 80%, 95% and 96% amino acid sequence similarity, respectively, among the organisms described above. A tetramer that contained two H3 and two H4 molecules would play important roles in chromatin regulation mainly via diverse post-translational modifications (PTMs). Therefore, H3 and H4 are highly conserved across organisms in contrast to other core histones such as H2A and H2B.

The separation of each histone protein by RP-HPLC and the characterization of the peak fractions on a 15% SDS-PAGE gel revealed the abundance of individual histones in silkworm cells. The silkworm histone H2B was found in the earliest fraction in RP-HPLC as well as in humans. Similarly to human H2A, silkworm histone H2A variants were also eluted in two distinct HPLC peaks (Fig. 4B) (Bonenfant et al., 2006). Mass spectrometry (MS) data analysis revealed that the first peak includes H2AZ and the second peak contains canonical histone H2A (Izumi M et al., manuscript in preparation). In contrast to the pattern in humans, the relative amount of H2AZ in the silkworm is lower than that of canonical H2A.

The peaks of the H3 variants appeared after the other core histones were eluted. The MS analysis of these fractions showed that that of the 70 min fraction that contained only H3.2, the 71-73 min fraction included both H3.2 and H3.3 and the 79-82 min fraction contained only H3.3 (Fig. 4B). This elution pattern of H3 variants is consistent with that of human H3 variants except for H3.1; no fraction containing H3.1 was obtained (Hake et al., 2005).

### Table 3. The buffer conditions for RP-HPLC separation of histones

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent B (%)</th>
<th>Step</th>
</tr>
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<tbody>
<tr>
<td>0 – 5</td>
<td>0</td>
<td>Injection</td>
</tr>
<tr>
<td>5 – 15</td>
<td>0 (to 35)</td>
<td>Ramp-up</td>
</tr>
<tr>
<td>15 – 25</td>
<td>35</td>
<td>Non-histones and H1s</td>
</tr>
<tr>
<td>25 – 100</td>
<td>35 (to 65)</td>
<td>Core histone separation</td>
</tr>
<tr>
<td>100 – 120</td>
<td>65 (to 100)</td>
<td>Typically no proteins (wash)</td>
</tr>
<tr>
<td>120 – 130</td>
<td>100</td>
<td>Wash</td>
</tr>
<tr>
<td>130 – 135</td>
<td>100 (to 0)</td>
<td>Wash</td>
</tr>
<tr>
<td>135 – 145</td>
<td>0</td>
<td>Wash</td>
</tr>
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</table>
Modifications of Histone H3 in the silkworm

The silkworm is known to have holocentric chromosomes. To determine whether the modifications of histone H3 occur as well as those of monocentric H3, we performed a western blotting analysis using specific antibodies against mono-, di- and tri-methylated as well as acetylated H3K9. Mono-, di- and tri-methylation as well as acetylation of H3K9 occurred in the silkworm (Fig. 4C). H3 bears more amino acid residues that are modified to regulate the function of chromatin. Various chromatin remodeling factors recognize modification sites including H3K9 and initiate events that ultimately lead to downstream gene activation or silencing. In the silkworm, homologues of such remodeling factors are found, suggesting that the modifications of histones might be conserved, at least in part, in the silkworm, although the structure of the chromosomes is markedly different between the silkworm and other organisms that bear monocentric chromosomes.

ACKNOWLEDGEMENTS

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REFERENCES