Comprehensive analysis of dynamics of histone H4 acetylation in mitotic barley cells

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Nucleosomal histones are covalently modified at specific amino acid residues. In the case of histone H4, four lysines (K5, K8, K12, and K16) are acetylated. In the current studies, we examined the dynamics of histone H4 acetylation at K8 and K12 in mitotic barley cells using a three-dimensional immunofluorescent method. Based on the results and previous studies on the dynamics of K5 and K16 acetylation, we provide a comprehensive view of the dynamics of H4 acetylation. Interphase nuclei exhibit strong acetylation in the centromeric region at K5, K8 and K12. In the case of K12, strong acetylation at nucleolar organizing regions was observed from prophase to anaphase. The dynamics of K12 were closely related to those of K5. On the other hand, K8 exhibited a pattern of almost uniform acetylation from prophase to telophase and strong acetylation in distal regions of chromosomes at both metaphase and anaphase, which is very similar to the dynamics of K16 acetylation. Thus, it appears that there is pair-wise acetylation of K12 and K5 in the nucleolar organizing regions and of K8 and K16 in the gene-rich regions. Together, these results suggest that pair-wise dynamics of H4 acetylation regulate chromosomal structure and function during the cell cycle.

Key words: barley, histone H4 acetylation, mitotic chromosome, nucleolar organizing region (NOR)

INTRODUCTION

Higher-order chromatin structure consists of nucleosomes, which are fundamental units made up of DNA and a histone octamer (two heterodimers of H2A/H2B and a tetramer of H3/H4). Each histone has a protruding N-terminal tail that is subject to various post-translational modifications, including acetylation, phosphorylation, and methylation, at specific amino acid residues. Histone acetylation is a well-documented modification that occurs at N-terminal lysine residues of core histones and plays an important role in various cellular functions (Spencer and Davie, 1999, Strahl and Allis, 2000). Histone acetylation participates in transcription activation and is regulated by histone acetyltransferases (HATs), which are subunits of transcription factors (Marmorstein and Roth, 2001). The reverse reaction, histone deacetylation, leads to transcription silencing and is mediated by histone deacetylases (HDACs), which are found in transcription repressors (Marks et al., 2004). Specific acetylation occurs on the newly synthesized histones in the cytoplasm (Sobel et al., 1995) and is required for DNA double-strand break repair (Bird et al., 2002). Replication-related histone acetylation is also reported in flow-sorted nuclei from field bean, barley, and Arabidopsis (Jasencakova et al., 2000, 2001, 2003).

Distinct combinations of covalent modifications of histone tails are thought to work as epigenetic code, referred to as a “histone code”. These combinations of modifications regulate the interactions of histones with DNA strands, chromatin-associated proteins, or protein complexes, which, in turn, control chromatin function (Strahl and Allis, 2000, Jenuwein and Allis, 2001). For example, there is evidence that methylation of histone H3 works in concert with other modifications to mediate transcription silencing (Jenuwein and Allis, 2001).

Chromosome structure is known to change dynamically during the cell cycle. Because nucleosomes are the fundamental units of a chromosome, the modifications of core histones could affect the higher-order chromosome structure. Indeed, during mitosis of barley, chromosomal mor-
physiology and H4 acetylation at both K5 and K16 change dynamically in a stage-dependent manner at certain chromosomal regions (Wako et al., 2002). Moreover, detailed analysis has revealed that combinational hyperacetylation occurs at the centromeres in a barley nucleus during interphase (Wako et al., 2003). Histone H4 has two other acetylatable lysine residues at K8 and K12 in the N-terminal region. Thus, in the current studies, we examined the changes in acetylation at K8 and K12. We discuss the results with respect to our previous findings on K5 and K16, and we present a comprehensive model of the dynamics of H4 acetylation and their putative function in chromosomal morphology.

MATERIALS AND METHODS

Plant materials and sample preparation. Interphase nuclei and chromosome samples were obtained from barley, *Hordeum vulgare* L. cv. Minorimugi (2n = 14). Seeds were germinated in the dark at 25°C. The root tips were fixed with 4% formaldehyde, macerated with an enzymatic cocktail (Fukui, 1996) and then spread on glass slides by the tapping method (Wako et al., 1998). The slides were stored at –80°C until use.

Immunostaining and fluorescence in situ hybridization (FISH). Indirect immunofluorescence for the detection of individual acetylated histone H4 residues was performed as described previously (Wako et al., 1998). One of two antibodies, anti-acetylated histone H4 at K8 and K12, was used as the primary antibody (Upstate Biotechnology, USA). These antibodies showed the same pattern in interphase nuclei (data not shown) as our previous data (Wako et al., 2003) with another antibodies (Turner et al., 1989, White et al., 1999). This was followed by detection with an FITC-conjugated second antibody (SIGMA). After immunostaining and microscopic observation, some slides were subjected to FISH to determine the acetylated region in interphase nuclei. This technique was performed as previously described (Wako et al., 2003). The slide was incubated with the digoxigenin-labeled centromeric probe, CEREBA (Courtesy of Drs. I. Schubert and G. Presting, Institut für Pflanzengenetik und Kulturpflanzenforschung; Presting et al., 1998), or the digoxigenin-labeled telomere probes, (TTTAGGG), (Richards and Ausubel, 1998), washing and fluorescence detection with Texas-Red procedures were described as previously (Ohmido and Fukui, 1997, Ohmido et al., 2001).

Image Analysis. Three-dimensional fluorescent signals from histone acetylation, centromeric and telomere repetitive sequences were captured and deconvolved using DeltaVision deconvolution microscope system (Applied Precision Inc., USA). Regions specific acetylation levels were quantitatively measured based on standardized intensity of fluorescence from more than five representative nuclei at each stage. Briefly, following deconvolution, sets of optical sections (0.2 μm-thick; approximately 50 sectioned images per nucleus) from each nucleus were analyzed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) with a macro-program (Wako et al., 2002) from an extended subset of CHIAS III (http://www2.kobe-u.ac.jp/~ohmido/cl/chiasIII/; Kato and Fukui, 1999).

RESULTS

Acetylated histone H4 in interphase nuclei. Regions of interphase nuclei containing acetylation of K8 and K12 were identified by a combination of immunostaining and FISH experiments (Fig. 1). Blue, green, and red colors indicate DAPI-stained nuclei, histone H4 acetylated at K8 or K12, and fluorescent signals from the centromere or telomere repeats, respectively. Interphase nuclei were divided into strongly (green arrowheads) and less acetylated regions. K8 was strongly acetylated at the centromere cluster and the surrounding area (Fig. 1A),
and the region of K8 acetylation did not overlap with the telomeric hemisphere containing the telomere sequences (Fig. 1B). These findings indicate that K8 was strongly acetylated in the centromeric region. Strong acetylation of K12 was also detected in the centromeric region (Fig. 1C), and the telomeric hemisphere was less acetylated (Fig. 1D). These results show that K8 and K12 have identical patterns of acetylation in interphase nuclei. This is the same pattern as that previously reported for K5 (Wako et al., 2002), suggesting that the centromeric region in interphase nuclei is highly acetylated at K5, K8 and K12.

**Dynamics of acetylated histone H4 through mitosis.** We examined the dynamics of histone H4 acetylation at K8 and K12 during the cell cycle. The patterns of K8 and K12 acetylation differed at all stages except at interphase when both K8 and K12 were acetylated at the centromeric region. Immunostaining showed dot-like signals of acetylated histone H4 in the centromeric region of interphase nuclei both K8 and K12 (arrowheads, Figs. 2A and G). When the samples were prepared without fixation, the dot-like pattern could be further resolved into individual hyperacetylated regions (Wako et al., 2003). The K8 acetylation in the centromeric region of interphase nuclei (Fig. 2A) disappeared before prophase (Fig. 2B) and was absent at prometaphase (Fig. 2C). Thereafter, strong acetylation occurred in distal regions of chromosomes at metaphase and anaphase (arrows); however, the other chromosomal regions were almost uniformly less acetylated (Figs. 2D and 2E). Moreover, strong acetylation, even at the distal regions, was not observed in approximately half numbers of the chromosome spreads analyzed (data not shown). Finally, strong acetylation at the distal region disappeared at telophase (Fig. 2F).

In the case of K12, there was acetylation in the centromeric region at interphase (Fig. 2G). Thereafter, the

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Fig. 2. Dynamics of histone acetylation at K8 and K12 in barley mitotic cells. The strongly acetylated regions at the K8 (A–F) and K12 (G–L) were visualized by indirect immunofluorescence staining using deconvolution microscopy. Figures show projection images constructed based on each set of the optical sections. The upper panel shows the DAPI-stained DNA images, and the lower panel shows the immunostained signals (green). (A) Histone H4 acetylated at K8 is unevenly distributed in the nucleus at interphase. (B–C) Almost uniform acetylation is observed in prophase and prometaphase. (D) The distal region is acetylated at metaphase. (E) Anaphase chromosomes are also acetylated at distal region. (F) Deacetylation is occurs at the distal region in telophase and results in uniform acetylation. (G) In the case of acetylation at K12, interphase nuclei are unevenly acetylated. (H) Deacetylation at the centromeric region and acetylation at NORs occurs at prophase. (I–K) This status is maintained in prometaphase, metaphase and anaphase. Inner panel of (J) is a optical section and indicates strong acetylated signals are located on a secondary constriction. (L) The centromeric regions are again strongly acetylated at telophase, but the acetylation in the NORs disappears. Arrowheads in A, G and L indicate centromeric region, and arrows in D and E indicate distal region. Inner panels of D, E and J indicate single optical sections. Bar indicates 5 µm.
centromeric region was deacetylated, and the nucleolar organizing regions (NORs) were strongly acetylated at prophase. Barley possesses two pairs of NORs, which explain the presence of the four distinct spots in Fig. 2H. The strong acetylation of NORs was maintained from prometaphase to anaphase (Figs. 2I-K). After this, the NORs were deacetylated and the centromeric region was reacetylated at telophase (arrowheads, Fig. 2L). These results clearly show that, with the exception of interphase, the dynamics of K8 and K12 acetylation are distinct.

Quantitative image analysis of histone acetylation. Stage- and region-specific acetylation and deacetylation were observed for both K8 and K12. The acetylation levels were analyzed quantitatively by image analysis. Strongly and less acetylated regions were separated by setting the appropriate threshold in the fluorescent images. Quantitative analysis to ascertain the degree of acetylation revealed specific changes of acetylation in each chromosomal region.

Fig. 3A shows the dynamics of K8 acetylation during the cell cycle. Strongly acetylated regions could be identified in interphase, metaphase, and anaphase chromosomes, and their acetylation levels were quantitatively measured. The filled bars indicate the basal level of acetylation during the cell cycle. The basal level was approximated using the level of weakly acetylated chromosomal regions, which did not change significantly during cell cycle. The other bars indicate the difference of

![Quantitative analysis of histone acetylation](image-url)
Histone acetylation in mitotic cells of barley

Acetylation level between the basal level and certain chromosomal region such as centromeric region. The meshed bars indicate the level of acetylation in the centromeric region, and reveals that the maximum was found at interphase. Acetylation level at centromeric regions was the same as the basal level from prophase to telophase. Prophase and prometaphase chromosomes did not show any measurable difference in acetylation between the centromeric regions, indicating that the acetylation of the centromeric region in prophase and prometaphase was reduced to the basal level. At metaphase, distal regions showed the maximum level of acetylation (striped bars), and the level was maintained even until the end of anaphase. Thereafter, chromosomes in telophase were almost uniformly acetylated.

Fig. 3B shows the region-specific dynamics of chromosome acetylation at K12. The acetylation levels of the centromeric region and NORs are shown with the meshed bars and gray bars, respectively. The acetylation level in the centromeric region of interphase nuclei was slightly higher than the basal level. The centromeric region was then deacetylated to the basal level at prophase, and NORs began to be acetylated at a higher level. The level of acetylation in NORs suddenly increased at prophase and reached a maximum at metaphase. Thereafter, NORs began to be rapidly deacetylated to the basal level at telophase. Rapid acetylation and deacetylation at the NOR were also shown for K5 (Wako et al., 2002). The centromeric region was acetylated again at telophase to a level similar to that at interphase, indicating that reacetylation was completed at this stage. In the case of K5, quantitative analysis revealed that acetylation at the centromeric region increased gradually towards interphase. In the case of K5, quantitative analysis revealed that acetylation at the centromeric region increased gradually towards interphase (Wako et al., 2002). Finally, the basal level did not change through mitosis.

**Combinational dynamics of histone H4 acetylation.** Dynamic changes in acetylation are the most clearly observed in three chromosomal regions: the NOR, the distal, and the centromeric regions. The NOR is strongly acetylated at K5 and K12 from prometaphase to anaphase and is not acetylated during telophase or interphase. A slight difference is observed at prophase, wherein K12 is acetylated earlier than K5. In barley, replication-related acetylation at K5 and K12 of histone H4 is also reported during the mitotic S phase stages (Jasencakova et al., 2001). On the other hand, the distal regions show high acetylation only at K8 and K16. Their acetylation dynamics, however, are distinct. From interphase to prometaphase, only K16 is acetylated; towards metaphase, the level of acetylation decreases gradually; finally, the level of acetylation begins to increase again at anaphase. Based on these results, it appears that the acetylation of K8 might compensate for the decrease in K16 acetylation at these stages. Thereafter, K8 and K12 are deacetylated at prophase, and K5 is deacetylated at prometaphase. Reacetylation at K5 and K12 is detected at telophase prior to the acetylation of K8. This indicates that, in the centromeric regions, the dynamics of K5 and K12 acetylation are similar to those of K8.

These data demonstrate lysine residue-specific and chromosomal region-specific acetylation patterns as well as chromosomal region-specific combinations of lysine residues. The acetylation at interstitial regions always remains at a basal level. The NOR exhibits dynamic pair-wise changes in acetylation of K5 and K12, and the distal regions also show a pair-wise change of K8 and K16. In addition, a combination of three lysine residues, K5, K8 and K12, seems to be involved in the acetylation of the centromeric region.

**DISCUSSION**

Chromosomes change their structure dynamically during mitosis by condensation and decondensation. In addition, during mitosis, histones in the nucleosomes are subjected to various modifications at their N-terminal tails. To determine the correlation between the structural changes of chromosomes and histone modification, we previously performed a comprehensive analysis of the dynamics of histone H4 acetylation during the cell cycle in barley (Wako et al., 1998, 2002, 2003). Two patterns were identified as a result of this analysis. First, during mitosis, the K5/K12 pair is highly acetylated in the NORs; and, second, the K5/K16 pair is highly acetylated in the distal regions. In addition, three lysine residues of K5, K8 and K12 are acetylated in centromeric regions.

Pair-wise dynamics of histone H4 acetylation at K5 and K12 have been found in the NORs. These two residues are highly acetylated from prophase to anaphase and including metaphase. Then they are simultaneously deacetylated during telophase and interphase. Even when microscopic observation indicates that the whole chromosomes are fully condensed, the NORs containing the 28S-5.8S-18S rRNA gene repeats escape from full condensation. These structural characteristics agree with the high level acetylation at K5 and K12 in the NORs. Acetylated chromatin fraction is less compacted than the non-acetylated counterparts (Wang et al., 2001). Thus, as found in other chromosomal regions, strong acetylation of histone H4 at K5 and K12 in the NORs likely prevents tight chromosomal condensation during telophase. Consistent with this, transcription of rRNAs has been reported even at telophase (Roussel et al., 1996). In fact, strong acetylation in the NORs at metaphase has been reported in marsupials (Wakefield et al., 1997, Keohane et al., 1998) and *Vicia faba* (Houben et al., 1996, Belyaev et al., 1997, Jasencakova et al., 2000). In barley metaphase chromosomes, the NORs are strongly acetylated on histone H4 at K5 and K12 (Jasencakova et al., 2001).
Like NORs, nucleoli have been reported to be acetylated (Jasencakova et al., 2000, 2001). However, we were unable to detect the strong acetylation of nucleoli in barley. This suggests that the nucleoli were weakly or rarely acetylated during interphase.

The primary amino acid sequences of histone H4 in the vicinity of K5 and K12 are very similar (Kimura and Horikoshi, 1998), suggesting that they are modified by the same enzyme. Indeed, both residues are acetylated by the cytoplasmic histone acetyltransferase, yHAT1, and its homologues in human and maize (Parthun et al., 1996, Verreaut et al., 1996, Kölle et al., 1998). Furthermore, Rpd3 histone deacetylase removes acetyl groups from both K5 and K12 (Rundlett et al., 1998, Kölle et al., 1999). These results provide a molecular basis not only for the synergistic changes in acetylation but also for the pair-wise acetylation of K5 and K12 in various organisms.

A second pair of acetylations occurs on histone H4 at K8 and K16. These acetylations are observed in the distal regions, which have a high rate of recombination and are rich in genes (Fukui and Kakeda, 1990, Künzel et al., 2000). However, K8 and K16 have relatively distinct dynamics. Strong acetylation of K8 was observed at metaphase and anaphase in this study, whereas acetylation of K16 decreased slightly during these stages (Wako et al., 2002). The different dynamics of K8 and K16 in barley suggest that acetylation of K8 compensates for the decrease in K16 acetylation, thereby maintaining the similar level of acetylation in the distal region. Acetylation of K8 and K16 has been reported during transcription (Strahl and Allis, 2000), suggesting that they have similar functions. Therefore, similar to K16, which we discussed in our previous report (Wako et al., 2002), acetylation at K8 may help prevent over-condensation of gene-rich regions.

In addition to these pair-wise acetylations, we found a third combination of acetylations in the centromeric region at K5, K8, and K12. Unlike Jasencakova et al. (2001), we did not separate the interphase nuclei into its sub-stages, but our current and previous findings (Wako et al., 2002, 2003) suggest that these three lysines are continuously acetylated during interphase. Centromeres in barley possess a different structure than the other chromosomal regions (Iwano et al., 1997), and, in fission yeast (Bownen et al., 2003) and rice (Nagaki et al., 2004), they contain some transcribed sequences. However, all barley chromosomes have centromeric heterochromatin (Fukui and Kakeda, 1990). Therefore, the combination of hyperacetylation in the centromeric region at K5, K8 and K12, mainly during interphase, could help protect centromeres from the over-condensation of heterochromatin. Consistent with this possibility, abnormal acetylation at the centromere during mitosis results in missegregation of chromosomes in yeast and mammals (Ekwall et al., 1997, Taddei et al., 2001). Furthermore, deacetylation of K5, K8, and K12 at the centromeric region is observed at prophase or prometaphase and is thought to be required for the progression of normal mitosis by promoting condensation of the centromeric region.

It has been proposed that combinations of single modifications, modifications on the same histone molecule, or both act as an epigenetic code. This is referred to as the “histone code” and is involved in specific functions (Strahl and Allis, 2000, Jenuwein and Allis, 2001). There are well-known histone codes for transcriptional silencing and activation and for condensation and decondensation of chromatin. In the case of transcriptional silencing, tri-methylation of K9 and/or K27 on histone H3 is essential (Lachner et al., 2003), and phosphorylation of S10 on this histone is associated with mitotic condensation (Houben et al., 1999). Our findings clearly demonstrate that the acetylation of H4 exhibits residue-, stage-, and chromosomal region-specific dynamics during the cell cycle. We also suggest that the acetylation occurs at distinct combinations of lysine residues at specific chromosomal regions, including K5/K12 and K8/K16. Thus, K18 and K16 may function as a histone code for transcriptional activity in NORs and gene-rich regions, respectively. Moreover, the pair-wise acetylation occurred in less or uncondensed region of mitotic chromosomes, whereas less acetylated chromosomal regions were fully condensed. This suggests that histone acetylation correlates with mitotic chromosome condensation, and that the pair-wise acetylation may also act as histone code for preventing the over-condensation of chromosomes. Similarly, the additional combination of acetylation observed at the centromeric region likely helps prevent chromosomal over-condensation, thus maintaining transcriptional activity even in the centromeres. In fact, fission yeast has tRNA genes in its centromeres (Bownen et al., 2003), and in rice, the centromere of chromosome 8 contains active genes (Nagaki et al., 2004). Therefore, our results suggest that the combinations of histone H4 acetylation that occur in specific chromosomal regions in barley serve as novel histone codes for regulating chromosome structure and related functions during the cell cycle.

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REFERENCES


Ohmido, N. and Fukui, K. (1997) Visualisation of close disposition between a rice A genome-specific DNA sequence (TssA) and the telomere sequence. Plant Mol. Biol. 35, 963–968.