Free and chromatin-associated mono-, di-, and trimethylation of histone H4-lysine 20 during development and cell cycle progression

Dmitry Karachentsev, Marina Druzhinina, Ruth Steward *

Waksman Institute, Department of Molecular Biology and Biochemistry, Cancer Institute of New Jersey, Rutgers University, 190 Frelinghuysen Road, Piscataway, NJ 08854, USA

Received for publication 22 June 2006; revised 23 November 2006; accepted 8 December 2006
Available online 13 December 2006

Abstract

Methylation of specific amino acids in histone tails is responsible for packaging DNA into condensed, repressed chromatin, and into open chromatin that is accessible to the transcription machinery. Monomethylation and trimethylation of histone H4-lysine 20 (H4-K20) control the formation of repressed chromatin. Using antibodies that specifically recognize the three methyl marks of histone H4-K20, we characterized their regulation during the cell cycle and throughout development. We find free mono- and trimethylated histone H4-K20 in unfertilized Drosophila eggs and in S2 tissue culture cells. Soluble mono- di-, and trimethylated H4-K20 are also found in HeLa cells. These soluble modified histones may represent a pool of free histones that can rapidly be incorporated into chromatin. The three methyl marks are each regulated differentially during development and their detection on western blots does not overlap with their detection on chromosomes. Monomethylated H4-K20 is detected on condensed chromosomes throughout development, while di- and trimethylated H4-K20 are detected on metaphase chromosomes at specific stages. Our results suggest that the detection of methylated H4-K20 on chromosomes may reveal chromatin packaging rather than the distribution of the methyl marks.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Histone methylation; Chromatin; Free histones; Cell cycle and mitosis; Drosophila

Introduction

Histones have long been recognized as forming the core of nucleosomes, the basic unit of chromatin. It is now evident that the modification of histone tails has a major effect on higher order chromatin structure, controlling the activation or silencing of particular genes by rendering chromatin accessible or inaccessible to DNA binding proteins like the transcriptional machinery (Reinberg et al., 2004). One of these modifications, histone methylation, is regulated by histone methyl transferases (HMTs) that can modify either arginine or lysine residues. Under the control of specific HMTs, lysine residues can be mono-, di-, or trimethylated (Peterson and Laniel, 2004).

Histone H4-K20 monomethylation is controlled by the PR-Set7 (or Set8) HMT (Nishioka et al., 2002; Fang et al., 2002; Couture et al., 2005; Xiao et al., 2005), and the Suv4-20 HMT controls the trimethylation of the same lysine (Schotta et al., 2004). Both enzymes are conserved from flies to humans, but in S. pombe mono-, di-, and trimethylation of H4-K20 are controlled by one enzyme, the Set9 HMT. In HeLa cells, the expression of PR-Set7 was found to be cell cycle regulated with highest levels at G2/M phase and with lower levels in G1 and S phases. Methylation of H4-K20 was also found to peak during mitosis (Rice et al., 2002).

By antibody staining, methylation of H4-K20 in HeLa cells was observed specifically on mitotic chromosomes (Rice et al., 2002). On salivary gland chromosomes, all three methyl marks overlap, staining heterochromatin and euchromatin, except for trimethylated H4-K20, that is enriched in the pericentric heterochromatin. On the euchromatic chromosome arms, the three methyl marks are found on condensed regions, a distribution distinct from the staining pattern of H3-K4, and a mark of transcriptionally active genes. Co-staining of the three methyl marks and transcriptionally active RNA polymerase also showed no overlap, indicating that the marks are associated with
silent chromatin (Nishioka et al., 2002; Rice et al., 2002; Karachentsev et al., 2005).

Consistent with these staining results above, mutants in PR-Set7 and Suv4-20 were found to suppress position effect variegation, indicating that both genes function in silencing gene expression (Schotta et al., 2004, Karachentsev et al., 2005). Monomethylation is also essential for the normal progression through the cell cycle. Imaginal discs from mutants lacking PR-Set7 have only ~25% as many cells as wild-type discs and the cells are larger (Karachentsev et al., 2005).

The monomethyl mark is stable over several cell generations, because in homozygous PR-Set7 animals the enzyme cannot be detected on western blots from the 1st instar larval stage onward. On the other hand, monomethylated H40-K20 can be detected on salivary glands of early third instar larvae and disappears only in late third instar (Karachentsev et al., 2005).

Histone methylation was originally thought to be a stable modification controlling gene expression, with the marks set at specific stages of development, and inherited from mother to daughter cells. But now lysine, demethylases have been identified, suggesting that the histone methylation marks are reversible and that the marks may have a more transient function (Shi et al., 2004; Trojer and Reinberg, 2006).

We chose a developmental approach to determining the regulation and distribution of the three methylated states of the same lysine of histone H4. We investigated if the modifications are stable during development and the cell cycle, if their distribution on chromosomes is linked, and if different cell types show distinct patterns of distribution.

Materials and methods

Cell culture and synchronization

HeLa cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen). To arrest cells in G1, cells were treated with 2 mM thymidine (Sigma) for 16 h, released into fresh media for 8 h, and blocked again by addition of 0.4 mM mimosine overnight (Sigma). Cells were released into fresh media and time points were taken every 2.5 h (Rice et al., 2002). At each time point, 10^5 cells were used for western blot analysis. Drosophila S2 cells (ATCC) were grown in SFM (Gibco) supplemented with 10% fetal bovine serum.

Cell fractionation

Drosophila S2 cells were pelleted and resuspended in small volume of cold 4 °C, 10 mM Tris–HCl pH 8, 5 mM 2-mercaptoethanol, and protease inhibitors cocktail. Cells were mechanically lysed with few strokes in Dounce homogenizer. Nuclei were collected by centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant containing soluble histones was dialyzed against 10 mM Tris–HCl pH 8. Samples were mixed with SDS loading buffer and separated on 14% PAGE.

Results

Methylation of histone H4-K20 occurs cell cycle-specifically; in HeLa cells, PR-Set7 levels and methylated H4-K20 change during the cell cycle and the detection of methylated histone H4-K20 on chromosomes is cell cycle-specific (Rice et al., 2002). These observations raise two basic questions: are all three methyl-forms of H4-K20 coordinately regulated during the cell cycle and during development, and what happens to the methyl marks in interphase chromatin?

The three methyl marks of histone H4-K20 are developmentally regulated

To determine possible diverse functions of the three methyl marks of H4-K20, we investigated their regulation during development. On salivary gland chromosomes, the distribution of each, the mono-, di-, and trimethylated forms of histone H4-
K20, are almost identical (Karachentsev et al., 2005). But we find that the appearance and levels of the methylated histones are developmentally controlled and are variable.

On developmental western blots (Fig. 1), both monomethylated and trimethylated histone H4-K20 are present in adults, in ovaries, early embryos, and throughout larval and pupal stages. The dimethylated form of histone H4-K20 is not detected in ovaries or in early embryos, and first becomes apparent only between 4 and 8 h of embryogenesis, then remains present throughout development.

We also performed immunofluorescence studies to determine when the three methyl marks were demonstrable on chromosomes. While the staining patterns of mono- and trimethylation were apparent at different stages of development (see below), dimethylation was detected clearly only at the extended germ-band stage about 7 h into development, when mono- and trimethylation are also seen (Figs. 2A–C). Di- and trimethylated H4-K20 are generally detected on few nuclei, specifically in metaphase, and are not detected on interphase nuclei (Figs. 2A–C, insets). Monomethylated H4-K20 is detected on a larger number of chromosomes and, as in ovaries and younger embryos, is present on condensed chromosomes. As expected, the dimethylation staining was not seen during oogenesis or in early embryos, and neither could we detect it on larval tissues, is present on condensed chromosomes. As expected, the dimethylation staining was not seen during oogenesis or in early embryos, and neither could we detect it on larval tissues, indicating that the highest levels of dimethylated H4-K20 are observed in mid-gastrula stage embryos. Lower levels present at other stages of development may have been missed because of the quality of the anti-dimethylated H4-K20 antibody.

**H4-K20 methylation during the cell cycle**

Because of the cell cycle-specific detection of the H4-K20 methyl marks on HeLa cell chromosomes (Rice et al., 2002), we decided to determine the distribution of the mono-, di-, and trimethyl H4-K20 marks throughout the cell cycle by western blot analysis. HeLa cells were arrested in G1 by thymidine followed by mimosine treatment. After release of the cells into fresh medium, aliquots were collected every 2.5 h (Rice et al., 2002). Total cell extracts were fractionated on SDS/PAGE and the western blot was probed with different antibodies.

**Fig. 2. Distribution of the H4-K20 methyl marks during embryogenesis.** (A–C) Gastrulation stage embryos stained with anti-monomethylated histone H4-K20 (A), anti-dimethylated histone H4-K20 (B), anti-trimethylated histone H4-K20 (C). The inset in each panel shows a higher magnification, note that the majority of nuclei in all animals do not stain. (D–F) Cleavage and blastoderm stage embryos stained with anti-monomethylated histone H4-K20. Embryo at first mitosis (D), cleavage stage embryo (E), cleavage stage embryo in mitosis (F), syncytial stage blastoderm stage embryo (G). Anti-methylated H4-K20 antibodies are shown in red and DNA dye, Hoechst, in blue.
Fig. 4. Distribution of the H4-K20 methyl marks during oogenesis. (A) Germarium stained with anti-monomethylated H4-K20. (B) Germarium stained with anti-trimethylated H4-K20. (C) Stage 5–6 egg chamber stained with anti-monomethylated H4-K20. (D) Stage 14 oocyte at metaphase of meiosis 1 stained with anti-monomethylated H4-K20. Anti-methylated H4-K20 is shown in red, DNA dye in blue, and anti-α-tubulin in green.

Fig. 5. The monomethyl mark is present throughout the cell cycle in early embryos, while the trimethyl mark is not detected. Cleavage stage embryos stained with anti-monomethylated and anti-trimethylated H4-K20 antibodies (red) and anti-H3P antibodies (green).
antibodies (Fig. 3). The cell cycle-specific detection of H3-Ser10 phosphorylation shows that the cells were synchronized successfully.

As previously observed, the levels of PR-Set7 are lowest in G1/S and culminate at G2/M (Rice et al., 2002). Mono- and trimethylation of histone H4-K20 levels appear relatively stable during the cell cycle, while dimethylation of H4-K20 follows a cyclical pattern with maximum levels in S phase.

The monomethyl and trimethyl marks are both cell cycle-specifically detected on chromosomes

Our western blots show that monomethylated histone H4-K20 is detected throughout development and throughout the cell cycle. Nevertheless, it is only detectable by antibody staining on chromosomes at specific stages. Fig. 4A shows a germarium containing the early stages of oogenesis when groups of cells (cystocytes) undergo synchronous mitoses (for a review of oogenesis see Spradling, 1993). Monomethylation is seen in clusters of cystocytes. Screening of more than 100 germaria shows that detection of the monomethyl mark is not developmentally specific but cell cycle-specific. Staining of monomethylated H4-K20 is observed in ~30% of the germaria and sometimes in more than one cyst.

Trimethylated histone H4-K20 (Fig. 4B) is observed only in early germaria. This staining is always associated with metaphase chromosomes and is only observed in one cyst in ~5% of ovarioles. It overlaps with the detection of phosphorylated histone H3-S10P (H3P) by a monoclonal antibody, specific for metaphase chromosomes (data not shown). This is strong indication that in oogenesis the trimethyl mark is specific for metaphase chromosomes.

Monomethylation of histone H4-K20 is observed throughout oogenesis in dividing cells, but it is not detected on pachytene chromosomes of stage 14 oocyte (Figs. 4C, D). Immediately upon fertilization, the monomethyl mark is clearly visible (Fig. 2D). The cleavage stage cell cycle only consists of S and M phases and lasts 8 to 9 min (Foe et al., 1993). During these early stages of embryonic development, the monomethylated H4-K20 is detected continuously on all chromosomes (Figs. 2E, F and 5), unlike H3P staining that is detected specifically on metaphase chromosomes (Fig. 5). At the blastoderm stage, when the cell cycle incorporates G phases and lasts 2 to 3 times longer, anti-monomethylated H4-K20 staining is again associated specifically with condensed chromosomes, and interphase nuclei show no or only very low levels of staining. This cell cycle specificity of the monomethyl mark is also observed in pole cells (Fig. 2G). Some pole cells stain strongly and others not at all, presumably because their divisions are asynchronous.

Trimethylated H4-K20 is undetectable at any stage of early development until mid-gastrulation. Blastoderm stage metaphase chromosomes positive for H3P staining do not stain with anti-trimethylated H4-K20 (Fig. 5). This result is surprising since western blots of chromatin isolated from 0–3 h embryos show that trimethylated H4-K20 is clearly associated with chromatin in early, 0–3 h embryos (Fig. 1S).

Free methylated histone H4-K20

Histone methyltransferases are thought to function when bound to DNA and to methylate histones when they become incorporated onto the nucleosomes (Zhang and Reinberg, 2001). But on our developmental western blots, we detected mono- and trimethylated histones H4-K20 present in unfertilized eggs, while no dimethylated H4-K20 was apparent (Fig. 1). At this stage, the oocyte is in pachytene of the first meiotic division. Each oocyte contains only 4 copies of each chromosome and the chromosomes are condensed. Thus, the bulk of the mono- and trimethylated forms of histone H4-K20 we observe on the western blots are not associated with DNA.

We wondered if methylated, soluble histones are present in mammalian and fly tissue culture cells. S2 Droso phila and HeLa cells were fractionated into nucleosomes and free histones using a 0.4 M salt buffer, followed by centrifugation (see Material and methods; Schwartz and Ahmad, 2005). The pellet containing the chromatin fraction and the supernatant containing the free histones were separated by SDS/PAGE (Fig. 6). On western blots most of the methylated H4-K20 is found in the chromatin fraction, as expected. But in both the S2 and HeLa extracts, substantial amounts of monomethylated H4-K20 are present in the free fraction. Traces of trimethylated H4-K20 are also detected in the soluble histone fractions from S2 cells, while low levels of di- and trimethylated H4-K20 are observed in HeLa cells. As a control, we found that, as expected, H4-K8 acetylation is only observed on chromatin but not in the soluble fraction (Sobel et al., 1995).

Discussion

Stability of the three H4-K20 methyl marks

The histone code theory posits that epigenetic marks are stable, inherited from one cell generation to the next, and that they affect gene expression. Bulk chromatin is assembled during S phase and it is assumed that most HMTs function at that time to modify lysines of histones on specific nucleosomes (Jenuwein and Allis, 2001).

In agreement with the histone code hypothesis, we found previously that the monomethyl mark of histone H4-K20 is
stable over several days. Animals lacking the PR-Set7 HMT die at the larval-to-pupal transition, and monomethylated histone H4-K20 is still present in third instar larvae until about 1 day before the animals die (Karachentsev et al., 2005). We now find that the mono- and trimethylation marks of histone H4-K20 are present throughout development and are also relatively stable throughout the cell cycle. It is unlikely that demethylases exist to remove these methyl marks before they are reset in each new cell generation, but low levels of exchange of the methylated histones may occur.

The dimethyl mark behaves differently. It is not present during oogenesis and only appears in the gastrula stage ~8 h into development. In HeLa cells, the levels of dimethylated histone H4-K20 peak during S phase and are reduced during mitosis, suggesting that a demethylase regulating the levels of the H4-K20 dimethyl mark could exist.

Pre-deposition mono- and trimethylated H4-K20

It has been thought that histones are methylated when they are assembled into nucleosomes. Our finding of free mono- and trimethylated histone H4-K20 in the ooplasm indicates that the Drosophila mono-HMT Pr-Set7 and tri-HMT Suv4-20 can modify free histones. This modification probably occurs when the histones are first synthesized and deposited in the oocyte. The Drosophila egg contains high levels of maternally deposited histones incorporated into chromatin during the rapid cell cycles in the early embryo. It is therefore not surprising that some of these pre-deposition histones are modified (Walker and Bownes, 1998).

Even though the methyl marks are relatively stable, we unexpectedly find consistent levels of free, pre-deposition monomethylated histones in S2 cells. Similarly, in HeLa cells free mono- and trimethylated H4-K20 are readily detected, and lower levels of free dimethylated histones are apparent. The presence of pre-deposition histones in Drosophila and mammalian tissue culture cells suggests that the modification of free histones by HMTs is occurring in most if not all tissues, and that it is conserved from flies to vertebrates. Soluble, methylated histones in S2 and HeLa cells are likely to represent a pool of free histones that can rapidly be incorporated into chromatin.

Pre-deposition mono- and trimethylated H4-K20

Sims et al. (2006) found that the three H4-K20 methyl marks show distinct distributions in HeLa cell nuclei. It is possible that the pools of pre-deposition modified mono-, di-, and trimethylated histones are sequestered to specific areas of the nucleus.

Stability of the three H4-K20 methyl marks and their cell-cycle-specific detection on chromosomes

On western blots of HeLa cells, all three methyl marks are present throughout the cell cycle. The preponderance of the three modified histones H4-K20 is associated with chromatin in HeLa and S2 cells. It is therefore likely that the methylated histones are associated with chromatin throughout the cell cycle.

Monomethylated histone H4-K20 and trimethylated H4-K20 can each be detected by antibody staining at specific and overlapping stages of the cell cycle. The monomethyl mark is present from late G2 throughout mitosis and represents an excellent marker for condensed chromosomes during mitosis at all stages of development. Di- and trimethylated histones H4-K20 are visible on metaphase chromosomes, but only at specific stages of development. Further, on western blots of early embryos, trimethylated H4-K20 is found in the chromatin fraction, but the trimethyl mark is not detected on chromosomes by antibody staining.

Together, these western and antibody staining results suggest that when the methyl marks are not detected they are too diluted, or are obscured by additional and reversible modifications of histone H4, or that they are buried by chromatin packaging. It is possible that the distribution of the H4-K20 methyl marks is general and that antibody staining reflects higher order chromatin packaging rather than the distinct localization of each modification.

In early embryos, antibody staining reveals the monomethyl mark throughout the shortened cell cycle, and the trimethyl mark is not detected. This result is consistent with the idea that higher order packaging is different in the nuclei of early embryos than in nuclei during oogenesis or in gastrulating embryos. Similarly, the banding pattern of the three forms of methylated H4-K20 detected on salivary gland chromosomes may reflect the packaging of chromatin rather than the distribution of the marks. Just as specific histone lysine methyl marks show different stabilities (Trojer and Reinberg, 2006), some methyl marks may be associated with specific DNA sequences, while others may be more uniformly distributed.

That the mono- and trimethyl marks are detected on chromosomes at different stages of the cell cycle and development implies that they are not interspersed but are associated with separate domains of chromatin accessible to antibodies at specific stages.

Acknowledgments

We thank Danny Reinberg and Kavitha Sarma for anti-PR-Set7 antibodies, and Michael Hampsey and Girish Deshpande for critical reading of the manuscript. We also thank Le Nguyen for technical help and fly food. This work was supported by a grant from the NIH and by the Horace W. Goldsmith Foundation.

Appendix A. Supplementary data


References


