Mitotic-specific methylation of histone H4 Lys 20 follows increased PR-Set7 expression and its localization to mitotic chromosomes

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We describe distinct patterns of histone methylation during human cell cycle progression. Histone H4 methyltransferase activity was found to be cell cycle-regulated, consistent with increased H4 Lys 20 methylation at mitosis. This increase closely followed the cell cycle-regulated expression of the H4 Lys 20 methyltransferase, PR-Set7. Localization of PR-Set7 to mitotic chromosomes and subsequent increase in H4 Lys 20 methylation were inversely correlated to transient H4 Lys 16 acetylation in early S-phase. These data suggest that H4 Lys 20 methylation by PR-Set7 during mitosis acts to antagonize H4 Lys 16 acetylation and to establish a mechanism by which this mark is epigenetically transmitted.

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The nucleosome is the fundamental structural unit of chromatin, which contains 146 bp of DNA wrapped twice around the histone octamer. The highly conserved histone octamer consists of two molecules each of the four core histones: H2A, H2B, H3, and H4. Each histone has a C-terminal histone fold domain that is involved in histone:histone interactions within the nucleosome, whereas the less structured N-terminal tail extends outward from the two superhelical turns of DNA to interact with the nuclear environment (Luger and Richmond 1998). These highly basic histone tails are theorized to be less structured compared with the histone fold regions and are believed to interact with the negatively charged DNA backbone or with other chromatin-associated proteins including neighboring nucleosomes (Hansen et al. 1998; Wolffe and Kurumizaka 1998). In most species, native chromatin is further compacted into higher-order structure and plays a critical role in all processes requiring access of proteins to the DNA (Kornberg and Lorch 1999).

Although the crystal structure of a nucleosome core particle has provided considerable insight into the protein:protein and protein:DNA interactions that govern nucleosome structure (Luger et al. 1997), little is known about how distinct functional domains of chromatin are established and maintained (Wolffe and Guschin 2000). It has become well established that dynamic changes in chromatin structure are directly influenced by the post-translational modifications of the N-terminal tails of the histones (Luger and Richmond 1998; Wolffe and Hayes 1999). Specific amino acids within histone tails are targets for a number of posttranslational modifications including acetylation, phosphorylation, poly(ADP-ribosylation), ubiquitination, and methylation (Zhang and Reinberg 2001). These covalent modifications may likely alter the histone tail interaction with DNA or with chromatin-associated proteins that may be required for different downstream cellular processes (Strahl and Allis 2000; Turner 2000).

A flurry of research on the posttranslational methylation of histone proteins has occurred in the last two years catalyzed by the characterization of the first known histone methyltransferase (HMT; Rea et al. 2000). Since this initial discovery, numerous other HMTs have been identified and found to be involved in various biological processes (Zhang and Reinberg 2001). Each of these HMTs selectively methylates evolutionarily conserved arginine (Arg) or lysine (Lys) residues, mainly in the N-terminal tails of histones H3 and H4 (Rice and Allis 2001). Recently, a new HMT isolated from human cells, named PR-Set7, was found to specifically methylate histone H4 Lys 20 in a nucleosome-dependent context (Nishioka et al. 2002b). This protein has significant homology in species ranging from flies to man; coincident with the conservation of the H4 Lys 20-methyl modification in higher eukaryotes.

In the present study we document histone-specific fluctuations in bulk HMT activity during cell cycle progression in human cells. Under the assay conditions used, relatively high and constant levels of histone H3 HMT activity were detected throughout the cell cycle. In sharp contrast, general H4 HMT activity was only detected during S-phase and G_2/M. Further analysis showed that methylation of H4 Lys 20 increased during S-phase and peaked at mitosis. The increase in H4 Lys 20 methylation closely followed the increased expression of PR-Set7 during cell cycle progression, which peaked at mitosis. PR-Set7 was localized specifically to mitotic chromosomes. Consistent with recent findings, H4 Lys 20 methylation was inversely correlated with H4 Lys 16 acetylation (Nishioka et al. 2002b), suggesting that these two modifications negatively interact during cell cycle progression as predicted by the histone code (Strahl and Allis 2000). The specific association of PR-Set7 with mitotic chromosomes may establish a mechanism by which the H4 Lys 20-methyl mark is epigenetically transmitted.

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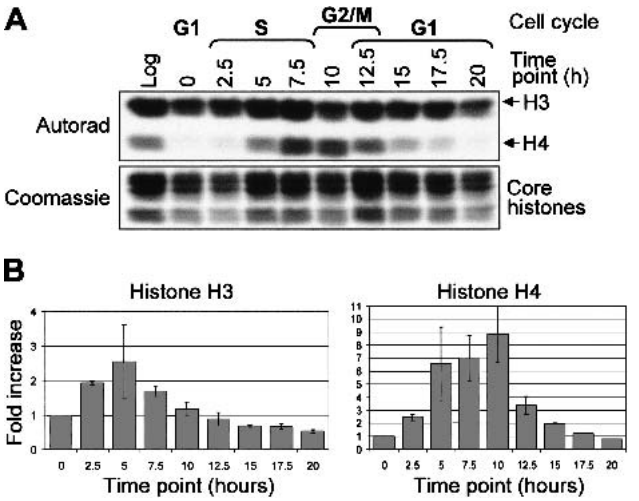

Figure 1. Increased histone H3 and H4 methyltransferase activity during human cell cycle progression. (*A*) In nucleo assay in synchronized HeLa cells. Cells were released from G_1 arrest and analyzed every 2.5 h. The cell cycle phase was confirmed by FACS. Autoradiography indicates the HMT activity specific for histones H3 and H4 at distinct phases in the cell cycle (*top* panel). Coomassie-stained core histones represent the loading control (*bottom* panel). (*B*) Quantitative assessment of HMT activity during the cell cycle. The changes in HMT activity relative to G_1 were determined (see text for details). The *X*-axis is the time in hours after release from G_1 arrest, and the *Y*-axis represents the fold increase in HMT activity relative to G_1 for both histone H3 (*left*) and H4 (*right*). This experiment was performed twice with similar results as shown by the error bars.

Results and Discussion

Increase in histone H3 and H4 methyltransferase activity during distinct phases of the human cell cycle

To determine histone methyltransferase activity during the human cell cycle, HeLa cells were arrested by treatment with thymidine followed by mimosine. Every 2.5 h following release from the G_1 arrest, synchronized cells were isolated for analysis, and the cell cycle phase was determined by fluorescence-activated cell sorting (FACS; data not shown). Nuclei were isolated from the cells at these various time points for the in nucleo HMT assay (Fig. 1) and for Western blot analysis (Fig. 2).

The in nucleo assay was used to provide a preliminary indication of alterations in nuclear methyltransferase activity during specific phases in the cell cycle. The assay was performed by incubating synchronized nuclei in the presence of a radiolabeled methyl donor (^{3}H-*S*-adenosylmethionine), followed by SDS-PAGE and autoradiography (Strahl et al. 1999). As shown in Figure 1A, no apparent changes in the H3 HMT activity occurred during the cell cycle. In contrast, the enzymatic methylation of histone H4 in this assay was greatly increased during mid-S-phase through mitosis and returned to levels observed during G_1. Little, if any, H4 HMT activity was detected outside of this time frame. It is interesting to note that histones H3 and H4 were the only nuclear proteins in this assay that were detectably methylated (data not shown). There are several potential limitations to this assay including occupancy of preexisting methylation sites, complications resulting from neighboring histone modifications, and/or decreased detection of HMT activity caused by as-yet undiscovered histone demethylases. Nonetheless, this assay provided us with a preliminary indication that H4 methylation, as opposed to the H3 methylation, was highly regulated throughout the human cell cycle.

To further characterize the HMT activity from the in nucleo assay, integrated densitometry was used to quantitate the intensity of the radiolabeled histone bands (Fig. 1A, top) and the intensity of the Coomassie-stained histone bands (Fig. 1A, bottom). Once these values were determined, the radiolabeled histone band was divided by the loading control Coomassie-stained histones, yielding an arbitrary number. These values were then standardized by assigning the G_1 value to 1 such that the fold change at each of the time points could be determined (Fig. 1B). Although undetectable by the gels, the analysis indicated a moderate increase in H3 HMT activity that peaked during mid-S-phase (2.5-fold), declined through G_2/M, and reached a baseline at G_1 (Fig. 1B, left). In contrast, the analysis of the histone H4 bands indicated a significant increase in HMT activity during mid-

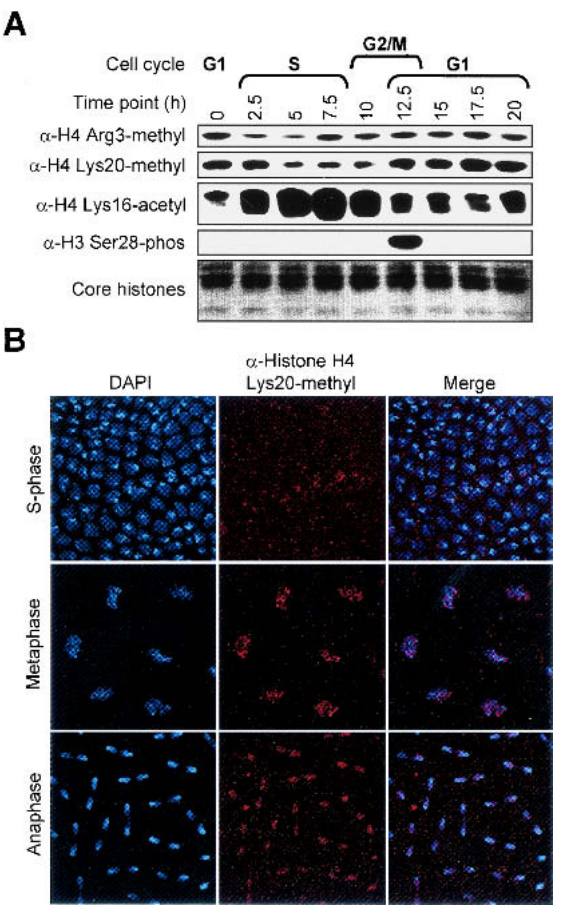

Figure 2. Histone H4 Lys 20 methylation decreases at mid-S-phase and increases during mitosis. (*A*) Analysis of histone H4 modifications in synchronized HeLa cells. Cells were released from G_1 arrest and analyzed every 2.5 h. The cell cycle phase was confirmed by FACS. The relative protein levels of histone H4 arginine 3-methyl, H4 Lys 20-methyl, H4 Lys 16-acetyl, and the H3 serine 28-phos modifications were determined by Western blot. Ponceau-stained core histones represent the loading control (*bottom* panel). This experiment was performed ≥2 times for each antibody. (*B*) Immunofluorescence staining of histone H4 Lys 20 methylation (red) in *Drosophila* embryos. The cell cycle phase was determined by DAPI staining (blue).

S-phase to G_2 (5–10 h) that abruptly declined during mitosis and the transition to G_1 (12.5 h; Fig. 1B, right). The H4 HMT activity then reached a baseline upon return to G_1.

These results show that the enzymatic methylation of histones H3 and H4 occurs during distinct phases in the human cell cycle under these assay conditions. Although there is a modest increase in histone H3 methyltransferase activity during S-phase, it has yet to be determined which Arg or Lys residues are being methylated during this time point. This will be difficult to dissect because many arginines and lysines in histone H3 are known be methylated in vivo (Rice and Allis 2001; Zhang and Reinberg 2001). In contrast, a dramatic increase in histone H4 methyltransferase activity was observed during mid-S-phase and G_2/M in the HeLa cell cycle. Because Arg 3 and Lys 20 are the only histone H4 residues presently known to be methylated in vivo (Rice and Allis 2001; Zhang and Reinberg 2001), we investigated which of these two residues were being methylated during these time points in the human cell cycle.

Histone H4 arginine 3 methylation decreases in early-S-phase and increases during mid-S-phase

Histones from synchronized HeLa cells were fractionated by SDS-PAGE and Western-blotted using antibodies specific for the H4 Arg 3-methyl or Lys 20-methyl modifications. As shown in Figure 2A, methylation of H4 Arg 3 was readily detected in HeLa cells arrested in G_1 (0 h). Upon entry into S-phase (0–2.5 h), there was a decrease in this modification that increased back to the observed G_1 levels during the transition from mid- to late-S-phase (5–7.5 h) and remained constant through mitosis.

One possible explanation for the observed decrease in the H4 Arg 3-methyl modification is the deposition of newly synthesized histone H4, which occurs immediately following DNA replication (Worcel et al. 1978; Smith et al. 1984). Thus, the apparent decrease may be caused by a dilution by the deposition of newly synthesized and unmethylated histone H4 proteins that ultimately become methylated during later points in the cell cycle, most likely mediated by the H4 Arg 3-specific HMT, PRMT1 (Strahl et al. 2001). In addition, the methylation of H4 Arg 3 during mid-S-phase suggests that this modification may be associated with euchromatin, or transcriptionally active regions, which are replicated early in S-phase. This would be consistent with a recent finding that H4 Arg 3 methylation plays a role in transcriptional activation of nuclear hormone receptors (Wang et al. 2001).

Histone H4 Lys 20 methylation decreases at mid-S-phase and increases during mitosis

Although the increase in H4 Arg 3 methylation accounts for the observed increase in H4 HMT activity during S-phase (2.5–7.5 h), it does not explain the increased activity during G_2/M (Fig. 1, 10–12.5 h). Because Lys 20 is the only other histone H4 residue known to be methylated, we hypothesized that its methylation was increased during this phase in the cell cycle. As shown in Figure 2A, Western blot analysis showed that H4 Lys 20 methylation also decreased during S-phase, albeit later in the cell cycle compared with H4 Arg 3 methylation. During the transition from early- to mid-S-phase (2.5–5 h), H4 Lys 20 methylation dropped and remained low through late-S-phase (7.5 h) and G_2 (10 h), which may be caused, again, by dilution of this modification by the deposition of newly synthesized and unmethylated histone H4 proteins. The observed decrease in H4 Lys 20 methylation in mid-S-phase suggests that this modification is associated with heterochromatin, which is known to replicate later in S-phase compared to euchromatin. This theory is supported by a recent report showing that the H4 Lys 20-methyl modification is associated with transcriptionally silent regions of the genome (Nishioka et al. 2002b).

During mitosis (12.5 h), H4 Lys 20 methylation returned to levels similar to those observed in G_1 and stayed constant through the remainder of the cell cycle. Although FACS analysis showed approximately equal numbers of cells in mitosis as G_1 at the 12.5-h time point (data not shown), the single appearance of the mitotic-specific phosphorylation of histone H3 serine 28 (Goto et al. 1999) indicates that H4 Lys 20 methylation occurs during mitosis rather than during the transition to G_1. Furthermore, because histone H3 serine 28 is phosphorylated specifically in the early phases of mitosis and is dephosphorylated abruptly during the metaphase-to-anaphase transition, the results suggest that the methylation of H4 Lys 20 also begins early in mitosis.

To further expand the above observations that H4 Lys 20 methylation decreases in S-phase and peaks at mitosis, immunofluorescence studies were performed in *Drosophila* embryos (Fig. 2B). A recent report shows that the H4 Lys 20-methyl modification is present in *Drosophila* and is essential for *Drosophila* development and viability (Nishioka et al. 2002b). In addition, the embryos provide an excellent model to study H4 Lys 20 methylation during the cell cycle as they rapidly and repeatedly shift from S-phase to mitosis, which can be easily determined by DAPI staining. Consistent with the above findings, H4 Lys 20 methylation was clearly detected on chromosomes during both metaphase and anaphase, whereas staining during S-phase resulted in a faint signal even upon overexposure (Fig. 2B). We hypothesize that the faint signal during S-phase most likely reflects a combination of dilution of the modification by histone deposition (see above) as well as the decrease in chromatin condensation, which could contribute to a dispersion of the signal resulting in a decreased ability to detect the modification. Regardless, these data confirm that H4 Lys 20 methylation is decreased during S-phase and increased specifically during mitosis.

Inverse correlation between histone H4 Lys 20 methylation and H4 Lys 16 acetylation during cell cycle progression

It was recently reported that histone H4 Lys 20 methylation inhibited acetylation of H4 Lys 16, and vice versa (Nishioka et al. 2002b). Based on this, we predicted that these two modifications would occur at distinct points in the cell cycle. Western blot analysis showed that H4 Lys 16 acetylation was low during G_1 (0 h) when H4 Lys 20 methylation was the highest (Fig. 2A). However, H4 Lys 16 acetylation significantly increased and peaked during mid-S-phase, the time when H4 Lys 20 methylation was the lowest. During mitosis (12.5 h), the acetylation of H4 Lys 16 dramatically decreased just as H4 Lys 20 methylation peaked. These observations, taken to-

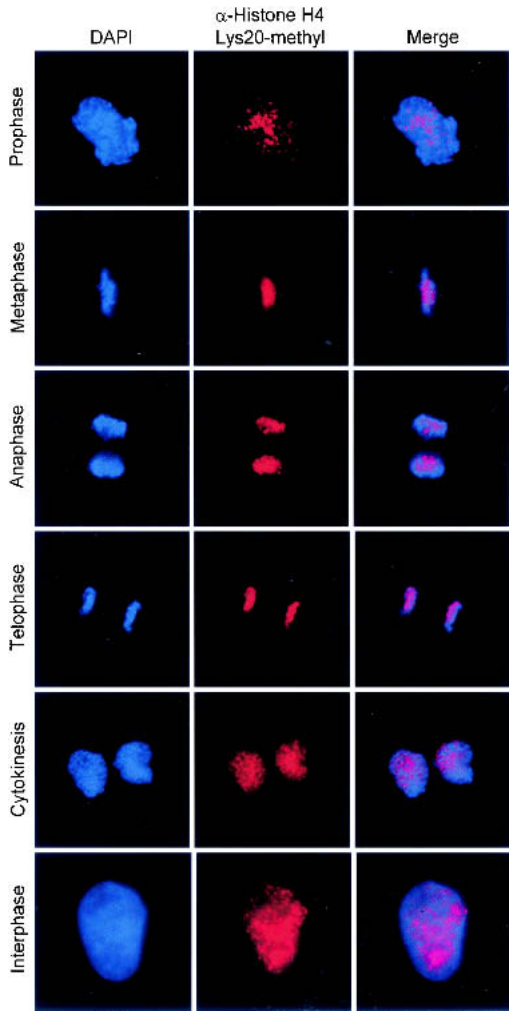

Figure 3. Histone H4 Lys 20 methylation occurs prior to or during metaphase. Immunofluorescence staining of histone H4 Lys 20 methylation (red) in mitotic and interphase HeLa cells. The cell cycle phase was determined by DAPI staining (blue).

gether with the previous findings, show that H4 Lys 20 methylation inhibits H4 Lys 16 acetylation and vice versa.

Histone H4 Lys 20 methylation occurs prior to or during metaphase

Immunofluorescence studies were performed on mitotic HeLa cells to provide a qualitative estimate of the relative phase during mitosis in which H4 Lys 20 methylation increases (Fig. 3). The specific phases of mitosis were determined by staining of DNA with DAPI. At prophase, the H4 Lys 20-methyl modification displayed a more punctate and less intense staining pattern compared with interphase cells that have high levels of H4 Lys 20 methylation. This suggests that the observed decrease in H4 Lys 20 methylation during S-phase and G_2 persists through the early stages of mitosis. It is unlikely that the observed decreased staining at prophase was a consequence of chromatin condensation as H4 Lys 20 methylation is clearly detected at other phases of mitosis when chromatin is even more condensed. In contrast to prophase, there was a visually pronounced increase in H4 Lys 20 methylation at metaphase that coincided directly with alignment of chromosomes on the metaphase plate. This suggests that the increase in H4 Lys 20 methylation occurs prior to or during metaphase. The H4 Lys 20-methyl modification persists through the rest of mitosis. Taken together, these results document the mitotic-specific enzymatic methylation of H4 Lys 20.

PR-Set7 expression is cell cycle-dependent

A novel histone H4 Lys 20-specific methyltransferase, PR-Set7, was recently identified in HeLa cells (Nishioka et al. 2002b). We hypothesized that PR-Set7 expression would increase simultaneously with H4 Lys 20 methylation during cell cycle progression. Northern blot analysis in synchronized HeLa cells indicated that *PR-Set7* mRNA expression was greatly increased during late S-phase and G_2/M and declined during transition to G_1 (Fig. 4A). To determine if PR-Set7 protein expression was also increased during these times, a polyclonal PR-Set7 antibody was developed and confirmed to be specific for PR-Set7 (data not shown). The antibodies detected recombinant as well as endogenous PR-Set7. Consistent with the RNA expression findings, Western blot analysis showed that PR-Set7 protein was not detected during G_1 (Fig. 4A, 0 h). The PR-Set7 protein levels elevated steadily beginning at early S-phase through G_2 (10 h) and peaked during mitosis (12.5 h). The increase during mitosis was confirmed by the appearance of the mitotic-

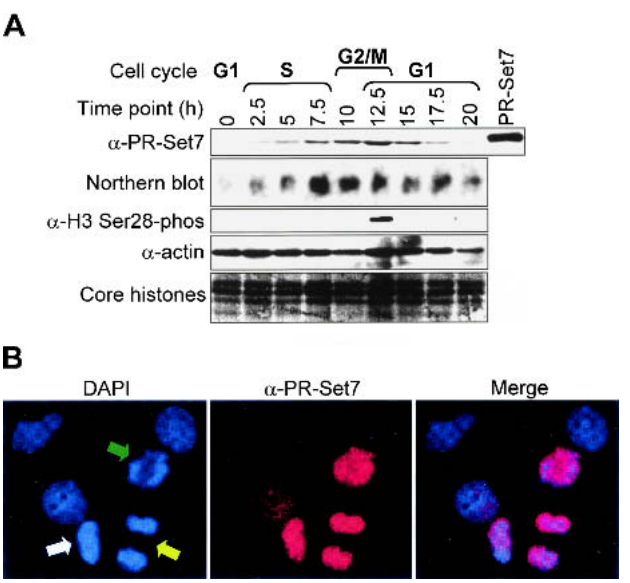

Figure 4. PR-Set7 localizes to mitotic chromosomes and is cell cycle regulated. (*A*) Analysis of PR-Set7 RNA and protein expression in synchronized HeLa cells. Cells were released from G_1 arrest and analyzed every 2.5 h. The cell cycle phase was confirmed by FACS. Northern analysis was performed with a probe generated against the PR-Set7 open reading frame. The relative protein levels of PR-Set7, phosphorylated histone H3 serine 28, and actin (loading control) were determined by Western blot. The shift in molecular weight of the recombinant PR-Set7 protein is owing to its fusion product. This experiment was performed ≥2 times for each antibody. (*B*) Immunofluorescence staining of PR-Set7 (red) in HeLa cells. The cell cycle phase was determined by DAPI staining (blue). The green arrow indicates prometaphase, the white arrow indicates metaphase, and the yellow arrow indicates anaphase.

specific phosphorylation of histone H3 serine 28 (Goto et al. 1999). Moreover, Western blot analysis in G_1-arrested cells confirmed that the PR-Set7 protein was undetectable, whereas in mitotic-arrested cells there were significantly abundant levels of PR-Set7 (data not shown). Subsequent to mitosis, the PR-Set7 protein abruptly decreased and continued to decrease as more cells entered G_1 (15–20 h). These findings indicate that PR-Set7 RNA and protein expression are up-regulated during cell cycle progression, consistent with the observed increase in H4 HMT activity and H4 Lys 20 methylation.

PR-Set7 is specifically localized to mitotic chromosomes

Immunofluorescence studies in HeLa cells were performed to determine the localization of PR-Set7 during different phases in the cell cycle (Fig. 4C). At metaphase (Fig. 4C, white arrow) and anaphase (Fig. 4C, yellow arrow), PR-Set7 is clearly associated with mitotic chromosomes. PR-Set7 was also detected at prometaphase (Fig. 4C, green arrow), although the localization was relatively dispersed compared with metaphase and anaphase. In contrast, two cells in the field failed to stain with the PR-Set7 antibody, suggesting that these cells are in G_1 because PR-Set7 is not detected at this time. One additional cell in the field showed faint staining, which most likely indicates the beginning of mitosis. The localization of PR-Set7 to mitotic chromosomes was specific as the staining was competed by excess PR-Set7 protein, but not by excess Set9 protein (data not shown; Nishioka et al. 2002a).

These data indicate that PR-Set7 expression is cell cycle-regulated and that the PR-Set7 protein is localized to mitotic chromosomes, coincident with the increase in H4 Lys 20 methylation. The steady increase in PR-Set7 expression during cell cycle progression is directly correlated with the observed increase in H4 HMT activity during these same times (Fig. 1). Although these data indicate that there is sufficient enzymatically active PR-Set7 in the nucleus during S-phase and G_2, the methylation of H4 Lys 20 methylation is delayed prior to metaphase (Fig. 3). It is presently unknown what mechanisms account for this delay; however, it is clear that PR-Set7 expression and the PR-Set7 protein must be tightly regulated by, as yet, uncharacterized mechanisms to prevent the premature methylation of H4 Lys 20. Consistent with this theory, studies performed with *Xenopus* egg extracts showed that the *Xenopus* PR-Set7 protein was phosphorylated during mitosis (Georgi et al. 2002). Although phosphorylation of human PR-Set7 is not essential for its HMT activity in vitro, phosphorylation of the enzyme in vivo may serve to regulate its association with mitotic chromosomes.

The cell cycle-regulated methylation of H4 Lys 20 suggests that this modification is localized to specific regions in the genome and inherited in an epigenetic fashion. Using telomere position effect variegation as a model for epigenetic silencing, data in yeast suggest that this repressive chromatin state is disassembled during S-phase and reassembled by G_2/M (Aparicio and Gottschling 1994). This coincides with the decrease in H4 Lys 20 methylation during S-phase and its increase during mitosis. Once established following replication, telomeric silent chromatin is relatively stable, much like histone methylation (Rice and Allis 2001). The similarities between these two suggest that the H4 Lys 20-methyl modification may serve as a stable epigenetic mark that aides in the establishment of discrete chromosomal regions involved in specific chromatin-mediated events. The association of PR-Set7 with mitotic chromosomes may represent a mechanism through which the H4 Lys 20-methyl mark is epigenetically transmitted. It is likely that the localization of PR-Set7 to mitotic chromosomes allows recognition of this mark on the parent chromosomes, which are then duplicated to the daughter chromosomes.

Although methylation does not affect the overall charge of the histone tail, it does increase the hydrophobicity and basicity of the lysine residue, suggesting an increased attraction for the negatively charged DNA. However, a more likely function for this modification is that it serves as a recognition motif for the binding of chromatin-associated proteins that mediate changes in higher-order chromatin structure during mitosis, similar to HP1 binding of methylated H3 Lys 9 to establish heterochromatic regions (Bannister et al. 2001; Lachner et al. 2001). Alternatively, methylation of H4 Lys 20 may serve to prevent the association of factors to the H4 tail, similar to H3 Lys 4 methylation, which prevents the association of the NuRD complex with the H3 tail (Nishioka et al. 2002a; Zegerman et al. 2002). Based on our findings, a likely candidate would be a histone acetyltransferase that modifies H4 Lys 16.

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 G_1, 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. At each time point, ~5×10^5 cells were used for FACS analysis. The in nucleo assay was performed by isolating nuclei from 10^6 cells using nuclear isolation buffer as previously described (Rice and Futscher 2000). Nuclei were pelleted and incubated at 30°C for 1 h in 1× HMT buffer (50 mM Tris-HCl at pH 8.5, 5 mM $MgCl_2$, 4 mM DTT) with 1 µM ^{3}H-*S*-adenosyl methionine (Amersham Pharmacia Biotech), followed by SDS-PAGE and autoradiography, as previously described (Strahl et al. 1999). Histones from the remaining nuclei were acid-extracted as previously described (Strahl et al. 2001).

Western and Northern blot analyses

Western blot analysis was performed as previously described (Strahl et al. 1999). The histone H4 Lys 20-methyl, H4 Arg 3-methyl, H3 Ser 28-phos (Upstate Biotech), and H4 Lys 16-acetyl (Serotec) antibodies were used at a 1:2000 dilution. The PR-Set7 polyclonal antibody was used at a 1:2000 dilution and incubated for 15 min in all cases. Northern blot analysis was performed as previously described (Nishioka et al. 1999). Total RNA was prepared from 10^5 synchronized cells. The hybridization probes were generated using PCR with primers designed from the actin sequence (CLONETECH), or from the full open reading frame of *PR-Set7*. The probes were labeled with [α-^{32}P]dCTP using the Nick Translation System (GIBCO-BRL).

Immunofluorescence

HeLa cells were fixed in 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 15 min. Cells were blocked with 10% normal donkey serum for 30 min before incubation with a 1:80 dilution of the PR-Set7 antibody or a 1:15 dilution of the histone H4 Lys 20-methyl antibody. Cells were then incubated with a rhodamine-conjugated donkey anti-rabbit IgG antibody (Jackson Labs) followed by DAPI (Sigma). *Drosophila* embryo staining was performed as previously described using the histone H4 Lys 20-methyl antibody at a 1:15 dilution (Whalen and Steward 1993). Staining was visualized using a 63× objective

on a Zeiss Axiovert 200M microscope and an AxioCam HRm digital camera. Pictures were analyzed with the AxioVision version 3.0.6 SP3 imaging software.

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Note added in proof

A recent report found that H4 Lys 20 methylation in mouse cells was highest during S phase and lowest during G_2/M (Fang et al. 2002). This discrepancy remains unresolved.

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