Kinetics of Re-establishing H3K79 Methylation Marks in Global Human Chromatin*§

Received for publication, May 18, 2010, and in revised form, August 5, 2010 Published, JBC Papers in Press, August 9, 2010, DOI 10.1074/jbc.M110.145094

Steve M. M. Sweet†, Mingxi Li‡, Paul M. Thomas†, Kenneth R. Durbin‡, and Neil L. Kelleher††

From the †Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 and the §Proteomics Center of Excellence, Northwestern University, Evanston, Illinois 60208

We employ a stable isotope strategy wherein both histones and their methylations are labeled in synchronized human cells. This allows us to differentiate between old and new methylations on pre-existing versus newly synthesized histones. The strategy is implemented on K79 methylation in an isoform-specific manner for histones H3.1, H3.2, and H3.3. Although levels of H3.K79 monomethylation are higher than that of H3.2/3.1, the rate of establishing the K79 methylation is the same for all three isoforms. Surprisingly, we find that pre-existing “old” histones continue to be K79-monomethylated and -dimethylated at a rate equal to the newly synthesized histones. These observations imply that some degree of positional “scrambling” of K79 methylation occurs through the cell cycle.

Histones are subject to myriad post-translational modifications. The most prominent among these modifications are acetylation, methylation, phosphorylation, and ubiquitination. Different modifications are preferentially associated with different regions of the genome, such as euchromatin and heterochromatin, or promoter regions and gene bodies (1–3). Some combinations of modifications have been shown to have linked functional outcomes (4), suggesting that the array of modifications associated with a gene is instrumental in the control of transcriptional activity of that gene both then and for progenitor cells (5).

Histone post-translational modifications have been studied for over 30 years (6). The information obtained can broadly be divided into the global and the local; antibodies to a particular modification can provide a global read-out of levels across the genome, and chromatin immunoprecipitation (ChIP) of a particular modification followed by quantitative PCR can give highly local information (7). More recent approaches employ ChIP followed by microarray or deep sequencing, allowing modification mapping across the entire genome with high resolution (5, 8–9). Mass spectrometry (MS) has been applied to the global analysis of histone post-translational modifications and can provide a direct readout of the combinatorial forms present endogenously (10–12).

During S phase, the entire genome is replicated. In order to allow this, existing nucleosomes are removed and then partitioned onto the two nascent DNA strands. New H3/H4 histones are provided by the chaperone CAF-1 or HIRA as dimers (13). Old (H3-H4)2 tetramers are disassembled and reassembled by the same chaperones and therefore are at least transiently split into dimers. However, the original tetramer appears to be reformed upon assembly. This conclusion is drawn from fluorescent labeling and radiolabeling of old histones (14–17). Recently, mass spectrometric analysis of epitope-tagged H3 has confirmed that the majority of (H3-H4)2 tetramers remain intact during replication, although a small proportion of H3.3-containing tetramers are split during replication-dependent nucleosome incorporation (18). It is not known whether old histone tetramers are partitioned evenly or randomly onto the new DNA strands. Random partitioning of clusters of old nucleosomes was observed for SV40 minichromosomes after inhibition of protein synthesis (19). It is also unknown whether old histones retain their position on the DNA strand or whether some mixing of position occurs between neighboring nucleosomes.

After population of both copies of the DNA with nucleosomes, the histones will be undermethylated. In order to regain the initial methylation levels, additional histone modification must occur. If the H3/H4 tetramer remains intact and in the same position on the DNA strand, we would expect this additional modification to occur on new H3/H4 tetramers as the old tetramers would retain their prior modified status. Additional modification could occur on either the new or the old H3, especially if the old nucleosome position is altered upon reinstallation of the old nucleosome.

Recently, two different stable isotope labeling approaches have been applied prior to MS to analyze the rate of formation of H4K20 (20) or H3 methylation during DNA replication and to identify the rates of turnover of different H3 methylation sites in cultured mammalian cells (21, 22). We employ a similar approach to these studies; however, in contrast to this earlier work, we label both histones and methyl groups, allowing us to differentiate between new and old methylation on new and old histones. A combination of protein and methyl labels was previously used to facilitate the identification and quantification of methylated peptides (23). Labeled cells are arrested at the start of S phase, prior to release into unlabeled medium. The combination of two different stable isotope labels measured across five time points and the three H3 variants results in considerable complexity. We therefore focus this initial report on the simple case of H3K79 mono- and dimethyl-
For simplicity, all light labeling is for new material, and all heavy labels arise from parental histone or old methylations using either heavy Arg or heavy methyl isotopic tags. Dot1 is responsible for H3K79 methylation (24, 25), with H2B ubiquitination at K123 required for K79 di- and trimethylation by Dot1 (26–29). H3K79 is unmethylated prior to incorporation into chromatin (30), with no demethylase yet identified for this site.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—For G1/S phase synchronization, a double thymidine block was used. Therefore, HeLa cells were seeded and cultured for 5 days at 37 °C in heavy DMEM supplemented with [13C6]Arg and [13C1,2H3]Met (Cambridge Isotope Laboratories, Inc.). Thymidine (Sigma) was added to a final concentration of 2 mM, and incubation was maintained for 12 h. The block was released by exchanging the thymidine-containing medium with the heavy DMEM medium. The cells were grown for 12 h before adding thymidine again to 2 mM final concentration for a further 16 h to synchronize the HeLa cells at the G1/S border. The arrest was finally released by refeeding the cells with the thymidine-free light DMEM to allow cell cycle progression.

**Fluorescence-activated Cell Sorting Analysis of DNA Content**—Aliquots were taken at the indicated time points throughout the 52-h time course, fixed in 70% ethanol, and incubated with RNase A (1 H9262 g/ml) and 20 H9262 g/ml propidium iodide at 37 °C and then analyzed on a BD LSR flow cytometer. 25,000 cells were counted per time point.

**Histone Preparation and MS**—Histones were recovered from HeLa cells using sulfuric acid extraction, as described previously (31). The histone-enriched samples were fractionated by RPLC using a Jupiter 300-Å C18 4.6-mm column (Phenomenex) at 0.8 ml/min, with MS monitoring (32). A gradient separation was employed: 0–30% B over 30 min; 30–60% B over 100 min.
H3K79 Methylation Kinetics

FIGURE 3. Proportion of new histone H3.1 that is methylated at K79. The averages of [M + 2H]+ and [M + 3H]+ values from two biological and two technical replicates are shown for H3.1 (error bars show S.E.). Signal due to incomplete SILAC labeling of old histone is not included (light histones with old methylation). me1:0, 1 light methyl (new); me2:0, 2 light methyl (new).

Results

After 5–6 divisions in medium with heavy arginine (+6 Da) and heavy methyl groups (+4 Da), cells were arrested at the entrance to S phase and released into medium with normal isotopes, with time points taken at 0, 4, 12, 30, and 52 h (Fig. 1). After 5–6 divisions, a similar divergence from the 50% ideal was observed by Scharf et al. (22), although in their case, the deviation was only 60%. Fluorescence-activated cell sorting (FACS) analysis, in combination with mass spectrometric analysis of the extent of SILAC labeling, indicates that this discrepancy results from 35–40% of the cells that fail to enter S phase after release from the thymidine block (supplemental Fig. 5 and supplemental text). We observe ~93% SILAC label incorporation for both methyl and arginine labels (supplemental Figs. 2–4).

K79 Methylation on Old and New Histone H3—Newly synthesized H3 will be undermethylated at K79. In order for new histones to reach the global monomethylation level (maintained throughout the time course), they need to become ~11% monomethylated. Fig. 3 shows the methylation status of new histones as the experiment progresses; methylation of new histones is at just 5% after 12 h, well below the 11% global monomethylation level. In Fig. 4A, new methylation is shown as a proportion of total monomethyl K79 levels. We see from Fig. 4A that new methylation of old histones contributes significantly to the overall level of monomethylation (15% of the total at 12 h, compared with 11% from methylation of new histones). Fig. 4B shows mass spectra illustrating the significant contribution of new methylation on old histones.

The new methylation on old histones could be due to turnover (demethylation + remethylation) or simply additional, new methylation on the extensive pool of unmodified H3K79. If there is significant methylation turnover, we would expect to see the old histones with old methylation decay faster than the unmodified control peptide. Fig. 5 compares the rates of decay. An H3 peptide that is never modified (YQKSTELLIR) serves as an H3 peptide that is never modified (YQKSTELLIR) serves as a control peptide.
a nice control. The rates of decay of the two species are highly similar (each 1.1%/h). This suggests that levels of K79me1 turnover are not high. The decay of unmodified K79 is slightly more rapid than K79me1 (1.1 versus 1.2% h⁻¹); however, this difference is within the error of measurement.

K79 dimethylation shows similar trends for the formation of new dimethylation on previously unmodified new and old histones (Fig. 6). Interestingly, a significant amount of new methylation occurs on old K79me1, creating mixed heavy/light K79me2.

Rates of Change of Methylation—Rates of change of methylation during the first, synchronized cell cycle were calculated by fitting a straight line to the first three data points (through to 12 h; Table 1). Restricting the analysis to the first cell cycle allows comparison between new and old histones. In the second and subsequent cell cycles, old histones are diluted and degraded, resulting in reduced contribution to the overall pool (Figs. 4 and 6). The rate of increase of unmethylated K79 is greater than that of monomethylated K79, which is greater than that of dimethylated K79. This is consistent with the recent findings of Zee et al. (21). Because the rate of de novo production of all-light dimethylated K79 is lower than that of monomethyl K79, this might suggest that the rate of new dimethylation site creation (i.e. K79me2 turnover) is lower than that of monomethylation. In fact, this is not the case. New K79me2 sites can be formed from existing K79me1 sites on old (heavy) histones (Fig. 6). Any methylation site that incorporates at least one light methyl group is, by definition, “new” (with the caveat that a small proportion of light methyl exists at t = 0 h, due to incomplete, 93–95%, heavy methyl labeling). When this additional source of new methylation is taken into account, the rates for K79me1 and K79me2 production are seen to be similar (Fig. 7).

Histone Variants—From separation of intact histone H3 proteins, we observe the proportions of H3.1, H3.2, and H3.3 to be 50, 42, and 8%. H3.3 was previously reported to show higher levels of monomethylation than H3.1 (30). H3.2 and H3.3 differ only in four positions and partially co-elute in our reverse-phase separation. However, Fig. 8 shows that up to 95% pure H3.3 fractions can be obtained. We find the level of H3.3 monomethylation to be higher than H3.1, which is in turn higher than H3.2 (Fig. 9 and Table 2).

Rates for H3.2 and H3.3 are shown in Tables 3 and 4. All three isoforms show broadly similar rates of new methylation appearance.

DISCUSSION

K79 Methylation Levels throughout the Cell Cycle—Using an antibody raised against an H3 K79me2 peptide, Feng et al. (24) have observed a slight decrease in K79me2 during S phase and
G2 in HeLa cells. By contrast, Schulze et al. (33) found that K79me2 levels in Saccharomyces cerevisiae increased during S phase and peaked at the G2/M phase boundary. We observe approximately constant levels of H3K79 methylation at the 0, 4, 12, and 30 h time points. The fact that no drop in K79 methylation levels is observed at the 4 or 12 h time points suggests that new K79 methylation occurs with S phase or shortly after; K9 methylation is known to be deposited with the insertion of new histones (34).

Re-establishment of K79 Methylation Involves New Methylation of Old Histones—Approximately 35% of the cells do not re-enter the cell cycle. These non-cycling cells do not contribute any significant additional methylation or show additional non-replication dependent H3.3 incorporation (see supplemental text). The non-cycling cells also do not contribute new replication-dependent histones to the mass spectra. Therefore, the intensity of signals from the majority of cells that are cycling will be diluted. The global amount of K79 methylation does not change significantly throughout the 52 h followed in this study. The simplest assumption is that the positioning of the majority of K79 methylation throughout the genome remains approximately constant. Various schemes have been envisaged for the repositioning of old histones and the introduction of new histones after passage of the DNA replication fork. In the simplest model, old (H3-H4)2 tetramers remain intact and are returned to the same loci on the nascent strands, with gaps filled in by new histones (Scheme 1). In this model restoration of K79 methylation status would require only new methylation of new H3. This does not agree with our data. Fig. 3 shows that methylation of new histones is only 5% after 12 h, well below the 11% global monomethylation level. This agrees with the findings of Scharf et al. (22) that K79 monomethylation on new histones after a complete S phase. The labeling strategy of Scharf et al. (22) could not distinguish new from old methylation on old histones, however. We see that a large amount of new methylation occurs on old histones during S/G2 phase: 15% of total monomethyl at 12 h, compared with 11% of the total on new histones (Fig. 4). This would fit

**TABLE 1**

| Species                  | Gradient | Days to half-maximum (S.E.) |
|--------------------------|----------|-----------------------------|
| New unmodified K79       | 1.7      | 1.3 (0.2)                   |
| New K4YQKSTELLIR5a       | 1.6      | 1.4 (0.1)                   |
| New and old K79me1:0     | 1.5      | 1.4 (0.1)                   |
| New and old K79me2:1, me2:0 | 1.2   | 1.8 (0.2)                   |
| New K79me1:0             | 0.7      | 2.9 (0.2)                   |
| Old K79me1:0             | 0.8      | 2.7 (0.3)                   |
| New K79me2:0             | 0.3      | 8.1 (1.3)                   |
| Old K79me2:1             | 1.0      | 2.2 (0.1)                   |
| Old K79me2:0             | 0.1      | 14.1 (0.3)                  |
with a simple model where new chromatin is methylated at K79 evenly on new and old histones, with the additional methylation of old histones being due to turnover of existing K79 methylation and/or underlabeled old histones with old methyl.

Because there is 10-fold more unmodified K79 than K79me1, additional methylation of a small fraction of unmodified K79 can account for a relatively large increase in K79me1. We observe a 0.1% h$^{-1}$ faster rate of decay for the unmodified K79 peptide (Fig. 5). If the 0.1% h$^{-1}$ faster rate of decay of unmodified old K79 compared with the control heavy peptide is due to new methylation, this would contribute 12% of the total monomethyl pool by 12 h. This very small increased rate of decay is sufficient to account for the additional methylation of old histones shown in Fig. 4.

Equal methylation on new and old histones implies “scrambling” of methylation position; if new and old tetramers remain distinct and remain in the same positions, then the exact position of K79 monomethylation changes on the new chromatin, and if the nucleosomes do not remain in exactly the same positions after DNA replication, this also implies that the position of methylation (old methylation, in this case) is altered. Analysis of K79 dimethylation also provides evidence for position scrambling and robust methylation of old histone H3 at K79. A large proportion of the total dimethyl K79 pool at 12 h (24%) is due to new additional methylation of old K79me1 H3 (Fig. 6). This implies that either the nucleosome localization is altered after replication, or the dimethyl/monomethyl location is altered. Scheme 2 shows alter-
scrambling explanation requires portions old and new histones. This "scrambling" explanation requires new methylation of old and new histones. This "scrambling" explanation requires portions old and new histones. This "scrambling" explanation requires portions of histones.

The rate of increase of new unmodified K79 and the control peptide YQKSTELLIR are combined for each methylation state and normalized according to the total signal at each time point. The averages of $\Delta M^2H\]2$ and $\Delta M^3H\]3$ values from two biological replicates are shown for H3.2 and H3.3. Average of $\Delta M\]2H\]2$ and $\Delta M\]3H\]3$ values from two biological and two technical replicates are shown for H3.1 (error bars show ± S.E.).

### TABLE 2
Overall methylation levels of H3.1, H3.2, and H3.3

All contributions from different stable isotope-labeled forms of the $^{73}$EIAQDFKTLR$^{35}$ peptide are combined for each methylation state and normalized according to the total signal at each time point. The averages of $[M + 2H]^++$ and $[M + 3H]^++$ values at all time points from two biological replicates are shown for H3.2 and H3.3. The averages of $[M + 2H]^++$ and $[M + 3H]^++$ values at all time points from two biological and two technical replicates are shown for H3.3.

| Variant     | 0 methyl | 1 methyl | 2 methyl |
|-------------|----------|----------|----------|
| H3.1        | 87.8     | 10.7     | 1.5      |
| H3.2        | 91.7     | 7.3      | 1.0      |
| H3.3        | 76.5     | 21.0     | 2.5      |

### TABLE 3
Rate of increase of new methylation on either old or new histone H3.2

The rate of increase of new unmodified K79 and the control peptide YQKSTELLIR are shown at the top for comparison. Rates are calculated from 0–12 h data from H3.2 (the numbers after the colons indicate the number of heavy, "old" methyl groups).

| Species                   | Gradient %/h | Days to half-maximum (S.E.) |
|---------------------------|--------------|-----------------------------|
| New unmodified K79        | 1.6          | 1.3 (0.1)                   |
| New $^{35}$YQKSTELLIR$^{35}$ | 1.7          | 1.3 (0.1)                   |
| New and old K79me1:0      | 1.7          | 1.2 (0.1)                   |
| New and old K79me2:1. me2.0 | 2.0          | 1.2 (0.4)                   |
| New K79me1:0              | 0.7          | 3.0 (0.3)                   |
| Old K79me1:0              | 1.0          | 2.1 (0.4)                   |
| New K79me2:0              | 0.1          | 17.9 (4.7)                  |
| Old K79me2:1              | 1.5          | 1.6 (0.5)                   |
| Old K79me2:2              | 0.4          | 6.6 (2.3)                   |

The rate of increase of new unmodified K79 and the control peptide YQKSTELLIR are shown at the top for comparison. Rates are calculated from 0–12 h data from H3.2 (the numbers after the colons indicate the number of heavy, "old" methyl groups).

The rate of increase of new unmodified K79 and the control peptide YQKSTELLIR are shown at the top for comparison. Rates are calculated from 0–12 h data from H3.2 (the numbers after the colons indicate the number of heavy, "old" methyl groups).
with earlier reports and with K79 methylation being associated with gene expression (9, 30, 38). The global level of K79 methylation is considerably higher in S. cerevisiae; in this case, only one H3 is present (H3.3-like), and a high proportion of the genome is actively transcribed (25). One could use yeast H3 to verify the initial finding here that robust methylation occurs on old histones.

In general, the rates of production for the various K79 methylated species are similar across the three H3 isoforms. This is consistent with the same methyltransferase, Dot1, acting on all three isoforms.

Overall Perspective—The use of heavy and light labels for methylation studies using stable isotopes produced 12 different MS signals for just K79 unmodified, K79me1, and K79me2 in this study. For other sites, hundreds of data channels are formed for the Lys and Arg methylation sites on histones H3 and H4, with the general data work-up and interpretation presented here now representing a clarified route to measure the turnover rates of all major lysine and arginine methylations on histones. It will be interesting to see how much heterogeneity exists in turnover rates among the 12 major sites of methylation in human cells. Such data will have a major impact on plans for epigenetic treatment in terms of target selection, dosages, and off-target effects (39).

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