Establishment of Histone Modifications after Chromatin Assembly

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ABSTRACT

Every cell has to duplicate its entire genome during S-phase of the cell cycle. After replication, the newly synthesized DNA is rapidly assembled into chromatin. The newly assembled chromatin ‘matures’ and adopts a variety of different conformations. This differential packaging of DNA plays an important role for the maintenance of gene expression patterns and has to be reliably copied in each cell division. Posttranslational histone modifications are prime candidates for the regulation of the chromatin structure. In order to understand the replication of histone modification patterns, we have pulse-labeled synchronized cells with an isotopically labeled arginine (15N4) that is 4 Da heavier than the naturally occurring 14N4 isoform. As most of the histone synthesis is coupled with replication, the cells were arrested at the G1/S boundary, released into S-phase and simultaneously incubated in the medium containing heavy arginine, thus labeling all newly synthesized proteins. This method allows a comparison of modification patterns on parental versus newly deposited histones. Experiments using various pulse/chase times show that particular modifications have considerably different kinetics until they have acquired a modification pattern indistinguishable from the parental histones.

INTRODUCTION

The packaging of DNA into chromatin plays a crucial role in regulating its accessibility for RNA polymerases and transcription factors. The level of chromatin condensation is dependent on the differentiation state of a cell, ranging from a hyperdynamic, highly accessible structure in embryonic stem cells (1,2) to a less accessible more condensed form in senescent cells (3). The posttranslational modification of histones can alter the properties of chromatin fibers and is therefore a prime candidate to mediate the stable inheritance of chromatin structures. The histone code hypothesis (4,5) is further supported by genome-wide mapping studies of histone modifications (6,7), which show a high degree of correlation between particular histone modifications and RNA–Polymerase II occupancy. Based on these data, a complex set of modifications has been postulated to stably mark specific regions of the genome with regard to their further activity. Experiments in yeast (8), however, suggest that a putative histone code may reflect a relatively simple binary signal (9). An alternative model to explain the generation of complex histone modification patterns has been put forward by Schreiber and Bernstein who suggested that histone modifications function similar to modification networks involving receptor tyrosine kinases (10).

In order to distinguish between an inheritable histone code and the generation of modification patterns in response to external signals, it is crucial to investigate the histone modifications that occur during and immediately after histone deposition. During S-phase, newly synthesized and parental histones are distributed randomly on the two daughter strands (11,12) resulting in a dispersive replication of histone modification patterns (13). Newly synthesized histones have a distinct modification pattern (14,15) that matures after assembly into a pattern similar to the one observed on parental histones (16–18). As the histone modifications are considered to be causal for the maintenance of particular chromatin structures (5), mechanisms have to exist that allow a faithful copying of modification patterns from ‘old’ paternal histones to new ones. It is striking that many enzymes that catalyze a posttranslational modification of histones also carry domains known to bind to modified histone molecules or interact with factors containing such a domain (19–24). These domains could target histone-modifying or demodifying enzymes to particular regions within the
genome by recognizing a modification pattern on the 'old' histones and copying it to the new ones.

The kinetics with which the modifications are copied from old histones to the new ones may define the time frame during which a given cell is susceptible for incoming signals and therefore its epigenetic plasticity. We investigated the kinetics of how fast modification patterns on new histones resemble the one on old ones by pulsed stable isotope labeling with amino acids in cell culture (pSILAC) and mass spectrometric analysis of histones. In accordance with previously published work, we observed a rapid adjustment of lysine acetylation patterns within the first 2 h after deposition (14,25,26). In contrast to this rapid acetylation and deacetylation, the methylation of lysine residues requires much more time to become indistinguishable from the old histones. In fact, some of the modification patterns on newly incorporated histones remain different through most of the next cell cycle after deposition indicating that the histone code needs one full cell cycle in order to become fully re-established. Our findings have a major implication in our view for how histone modification marks may mediate epigenetic inheritance.

EXPERIMENTAL PROCEDURES

Synchronization of HeLa cells

For G1/S-phase synchronization, a double thymidine block was used. Therefore, HeLa cells were seeded and cultured for 24 h at 37°C in the R⁰ SILAC medium (Invitrogen). Thymidine (Sigma) was added to a final concentration of 2 mM and incubation was maintained for 16 h. The block was released by exchanging the thymidine-containing medium with the R⁰ culture medium. The cells were grown for 9 h before adding thymidine again to 2 mM final concentration for further 16 h to synchronize the HeLa cells at the G1/S border. The arrest was finally released by refedding the cells with the thymidine-free R² SILAC medium (Invitrogen) to allow cell cycle progression. Whenever indicated, 10 mM NaBu was added directly into the medium and the cells were washed twice with PBS before transferring them into the medium without NaBu. The cells were maintained in a 37°C incubator with a humidified atmosphere of 5% CO₂.

SILAC labeling

We used three different SILAC DMEM media: R⁰ SILAC (L-arginine), R⁴ (L-¹²C₆ ¹⁵N₄-arginine) and R¹⁰ (L-¹³C₆ ¹⁵N₄-arginine). HeLa cells were synchronized at the G1/S border in the R⁰ SILAC medium and released into the cell cycle in the R² SILAC medium in order to label all newly synthesized histones. For all pulse-chase experiments, we fed the synchronized cells with the R⁴ SILAC medium for 6 h and chased with the R¹⁰ SILAC medium. All materials for SILAC labeling were purchased from Invitrogen and prepared according to the manufacturer’s instructions.

Flow cytometric analysis of the cell cycle

For fluorescence activated cell sorting (FACS) analysis, the cells (1 × 10⁶) were harvested, washed twice in PBS followed by fixation in 70% ethanol at −20°C for a minimum of 1 h. Fixed cells were washed in PBS and incubated with 100 µg/ml of RNase A in PBS for 30 min at 37°C. Afterward, propidium iodide (Sigma) to a final concentration of 50 µg/ml was added and the cells were incubated at 37°C for 30 min. The samples were stored at 4°C in the dark until analysis on BD Biosciences FACSCanto. A minimum of 10 000 cells were counted, and the raw data were analyzed and histograms plotted using the FlowJo software.

RT–PCR

RNA was extracted from snap-frozen HeLa cell pellets using the RNeasy kit (Qiagen) according to the manufacturer’s manual and dissolved in RNase-free water. Total RNA concentration was quantified using a spectrophotometer (Peqlab, Nanodrop). Reverse transcription was primed with 250 µg of random primers (Promega) with 1 µg of total RNA per sample at 70°C for 5 min. The samples were then incubated with 20 U MuLV in 20 µl of buffer containing 1000 µM dNTP and manufacturer’s RT buffer for 10 min at 25°C, then heated up to 37°C for 1 h, 70°C for 10 min, chilled on ice and frozen at −20°C. PCR reaction was conducted with the following primers: H3.2 (5'-GTACCAAGTGCCGAG, 5'-GATGTCCTTGCGCCATTG) and 18S (5'-TGGTTTTCTCGGAACG, 5'-CATTCTTATGCTCGGACTACG).

Histone extraction

The cell pellets were redissolved in 0.4 N HCl in a total volume of 0.5 ml per 1 × 10⁶ cells and centrifuged for a minimum of 1 h at 4°C. After centrifugation at 13,000 rpm for 30 min, the supernatant was dialyzed at 4°C against 100 mM ice-cold acetic acid for three times for 1 h using 6–8000 MWCO. The sample was concentrated using a speed vac and redissolved in SDS-loading buffer and applied to a SDS–PAGE gel for further analysis.

MALDI-TOF analysis

Histones were separated by 18% SDS–PAGE. G-250 Coomassie blue stained bands were excised and destained with 50 mM ammonium bicarbonate in 50% ACN (Sigma) for 1 h at 4°C. After centrifugation at 13,000 rpm for 30 min, the supernatant was dialyzed at 4°C against 100 mM ice-cold acetic acid for three times for 1 h using 6–8000 MWCO. The sample was concentrated using a speed vac and redissolved in SDS-loading buffer and applied to a MALDI–TOF–MS spectrometer (APM).
Biosystems) and analyzed. Peptide mass fingerprint covered the mass range from 500–2000 amu.

**Quantification of MALDI signals**

To determine all modifications occurring on histones, the Manueltio software was used. Spectra were processed and analyzed with the Data Explorer software (Applied Biosystems). For quantification, the integrated area of the peaks was used with a signal-to-noise ratio of 2. The sum of the area from all peaks derived from a single peptide was defined as 100%. Charts were drawn in Excel.

**RESULTS**

Pulse labeling can be used to mark newly synthesized histones

Most of the new histones that are incorporated during replication are synthesized during S-phase [(27) and Figure 1B and C]. To study the dynamics of histone modifications on the newly synthesized histones in comparison to the parental histones, we designed a method to selectively label new histones by isotopic labeling (Figure 1A).

To do this, we arrested HeLa cells at the G1/S boundary using a double thymidine block (28) and verified the cell cycle arrest using fluorescence-activated cytometry (Figure 1B). After removal of the block, the cells were placed into the labeling medium containing isotopically labeled arginine (15N4). To control the correct replication are synthesized during S-phase [(27) and Figure 1B and C]. To study the dynamics of histone modifications on the newly synthesized histones in comparison (Figure 1B), (ii) not all histones are incorporated into chromatin or that (iii) residual pools of light arginine are in vivo.

Histone acetylation

One of the best-known modifications on histones is the acetylation of lysines, which is considered to lead to a more open and hence to a more active chromatin structure (30). Histones are acetylated at specific chromatin structure (30). Histones are acetylated at specific sites before deposition (14,15), which get deacetylated after histone assembly (16,18,25). We wanted to determine, whether our analysis would allow us to dissect the process of acetylation and deacetylation coupled with histone deposition in vivo.
Therefore, we compared the levels of lysine acetylation on newly synthesized histones with that on old histones. When histones were analyzed 6 h after the release into S-phase, we observed a small but reproducibly increased proportion of the diacetylated isoform in the new histones (Figure 2C). As free H4 is exclusively diacetylated before deposition (14,15,25), this increased level of diacetylated H4 reflects the higher percentage of pre-deposition forms with the pool of the diacetylated isoform in the new histones (Figure 2C).
of new histones. In order to get an estimate of how quickly the pre-deposition markers disappear, we used the broad-range deacetylase inhibitor sodium butyrate (NaBu) (Figure 2A). When treating the cells with NaBu throughout the labeling period, we observed an increase in acetylation on the old as well as on the new histones, suggesting that histone acetyltransferases as well as histone deacetylases do not distinguish between old and new histones. However, whereas NaBu treatment leads to similar amounts of all acetylated isoforms among old histones [mono-, di- and triacetylated; tetraacetylated H4 could not be analyzed by MALDI-TOF due to an overlap of the heavy peptide H4ac4 (4–17) with an unmodified H4 peptide], in the case of the new histones, H4ac2 is the most abundant isoform (4–17) with an unmodified H4 peptide], in the case of the new histones. In order to get an estimate of how quickly the pre-deposition modifications are removed within <2 h after assembly (Figure 2E). The higher percentage of diacetylated H4 on the new histones compared to the old ones is very likely due to the ongoing assembly at 4–6 h, after the beginning of S-phase. If, after treatment, the histones were incubated for another 2 h in the absence of NaBu, the acetylation patterns become indistinguishable between the old and new histones (Figure 2F). In summary, we conclude from the analysis of acetylation kinetics of new and old histones that the acetylation is rapidly adjusted to equalize the modification patterns between old and new histones. Due to the high turnover of acetyl groups (32), the pattern of acetylation does not seem to be well suited to confer stable epigenetic memory. Moreover, as acetylation events occur independent from each other, we have no evidence for a regulated establishment of a specific modification pattern that is based on distinct pre-existing modifications. This is in agreement with what has been previously described in yeast (33) and human cells (8).

**Histone methylation**

In contrast to acetylation, the methylation of lysine residues on histone tails has been suggested to constitute a major factor in the establishment and inheritance of stable chromatin states (4,5,34). We therefore wondered whether the methylation of histones might show a different behavior than acetylation. We focused our studies mainly on the most prominent methylations on H3 and H4 (K4, 9, 27, 36 and 79 on H3 and K20 on H4). In contrast to the highly dynamic lysine acetylation, lysines on the histone tails of newly synthesized histones are methylated with a much slower kinetic. When we compared the methylation levels of H3 and H4 after 6 h of pSILAC labeling, we observed a striking difference in modification patterns between old and new histones (Figure 3). As the cells progress through the cell cycle, methylation patterns on new histones gradually adopt those on the old histones. This is most obvious for the methylation of lysine 20 within histone H4 (Figure 3A). On newly synthesized H4 molecules, K20 is usually not modified (15,16) and becomes monomethylated after incorporation into chromatin (16,26). This is in sharp contrast to the methylation pattern on the pre-existing H4 molecules that are mainly dimethylated at K20 (Figure 3A).

We observed a similar difference for the methylation on the H3 tail. Like in the H4 molecule, the replication-dependent H3 variants are not methylated to a measurable extent before incorporation (15,16). We concentrated our analysis on K9, K27, K36 and K79, as these have been suggested to play important roles during the inheritance of epigenetic states (5,34). In contrast to yeast cells, most of the H3 molecules remain unmodified at K79 in human cells (35,36). This makes it difficult to quantitatively compare the methylation levels of K79 on old and new histones. However, we observed a small but discernible difference between old and new histones with regard to K79 methylation. Interestingly, we saw more dimethylation of K79 on the new histones at 6 h after synthesis, whereas the old histones contain a higher percentage of monomethylated K79 (Figure 3B). The other lysines that carry methyl groups within H3 are K4, K9, K27 and K36. We limited our analysis on K9, K27 and K36, as the peptide containing K4 (3–8) gave only a very weak signal and appeared to be mostly unmodified in new as well as in old histones. Like H4K20me, the differences in methylation levels between old and new histones 6 h after the release into S-phase were clearly visible (Figure 3C and D). In the case of H3K9, the degree of mono- or dimethylation was considerably lower in the new histones when compared to the old ones and a large percentage of the new H3 molecules were still unmodified at that time point (Figure 3D). As we used MALDI-TOF for quantification, we could not distinguish between trimethylation and acetylation of the peptide, but based on earlier experiments (15) we suggest that most of the peptides contributing to this mass carry an acetyl group at K14. The methylation profiles of the peptides carrying K27 and K36 that were derived from the old and new histones are shown in Figure 3D and also show a marked difference between the two histone types. In contrast to the methylation at K9, we observed a higher percentage of monomethylation of this peptide, which was predominant due to monomethylation at K27 (data not shown). Interestingly, within the first 6 h after release, this monomethylation does not seem to get further methylated to the trimethyl state on the new histones, and we do not observe a strong trimethylated peptide, which is the predominant one in the pre-existing histones (Figure 3D). In light of recent reports that show a localization of the main H3K27-specific histone methyltransferase EZH2 to sites of replication (37), the rapid monomethylation of H3K27 is very likely a product of the enzyme bound to replication foci.
Pulse chase labeling to follow histone modifications over one cell cycle

Our findings that the methylation patterns of lysine residues does not adopt the identical modification patterns immediately after deposition prompted us to ask how long it would take until the two modification patterns become indistinguishable. To do this, we pulse labeled the newly synthesized histones for 6 h using heavy arginine (R4) and subsequently chased the culture using super-heavy arginine (R10). This procedure is crucial for the analysis as it prevents the interference of histones incorporated at late S-phase, outside S-phase or during the next S-phase. As for R4 labeling, we measured incorporation by quantifying the peptides H3 64–69 and H4 68–78 (Figure 4 shows only the quantification of the H3 peptide; a representative spectrum is shown in Figure 4C). As the HeLa cells partly lost their synchrony during the second cell cycle, the incorporation of R10 into histones is not as clearly restricted to a defined time window but increases over the whole analysis time (Figure 4B). Therefore, we focused our analysis on the comparison between the old pre-existing histones and the ones incorporated during the first 6 h of S-phase. The samples were taken every 4 h after the end of the labeling period until the end of the following S-phase as judged by FACS analysis (~30 h after

Figure 3. Differences in methylation patterns of ‘old’ and ‘new’ histones 6 h after release in G1/S-phase. (A) Methylation patterns of H4 peptide 20–23 of ‘old’ and ‘new’ histones analyzed by MALDI-TOF. 1ac, monoaetylation; me1, monomethylation; me2, dimethylation; me3, trimethylation. (B) H3 73–83 (C) H3 27–40 (D) H3 9–17. Error bars indicate the SEM of three independent biological replicates. The *P-values are calculated using Student’s unpaired t-test. The significance of the differences in methylation levels were assessed by unpaired Student’s *t*-tests and indicated on the top of the bars：“**P < 0.01: a highly significant difference; “*P < 0.05: a significant difference; and no bracket means no significant difference.

Figure 4. Double labeling using different SILAC media allows to follow histones for a period longer than one cell cycle. (A) Experimental scheme. After synchronization, HeLa cells are released into S-phase under the R4-medium conditions. After 6 h, the cells were transferred into a medium containing R10 and then harvested at indicated time points. time (h); time after release. (B) Incorporation efficiency of double-labeled histones for R4 and R10 showing H3 peptide aa 64–69. Error bars indicate the SEM of three independent biological replicates. (C) MALDI-TOF spectrum of H3 aa 64–69 from HeLa cells that were R4 labeled for 6 h and afterward R10 labeled for additional 6 h.
release from the G1/S block). During this time, we only see minor changes in the global methylation patterns of the old histones arguing for a relatively stable marking system (Figure 5A, left panels). The methylation patterns of the newly synthesized histones on the contrary change slowly during the course of one cell cycle (Figure 5A, right panels). Similar to what had been reported before (26), H4K20 gets transiently monomethylated after deposition and subsequently dimethylated. At the beginning of the next S-phase, the H4K20 methylation levels of the new histones are very similar to the old ones (Figure 5B).

**Figure 5.** Establishment of posttranslational modification patterns of histones differ in their kinetics. Pulse-chase experiments as described in Figure 4A. Underneath the charts, a cell cycle scheme is depicted, time (h); time after release. Three independent biological experiments are shown and analyzed by MALDI-TOF. Error bars indicate standard deviation of a mean. Grey square indicates 20 h after release (A) top left: H4 peptide aa 20–23 is shown of the ‘old’ histones. Top right: H4 peptide aa 20–23 is shown of the ‘new’ histones. Bottom left: R0 (‘old’)-labeled histones showing H3 peptide 27–40. Bottom right: R4 (‘new’)-labeled histones showing H3 peptide 27–40. (B) Methylation patterns after 20-h release of H4 peptide 20–23 (left) and H3 peptide 27–40 (right) of ‘old’ and ‘new’ histones analyzed by MALDI-TOF. 1ac, monoacetylation; me1, monomethylation; me2, dimethylation; me3, trimethylation. Error bars indicate the SEM from three independent biological replicates.
DISCUSSION

Histone modifications are considered to constitute a second (epi)genetic code that operates to establish distinct chromatin structures and maintain them throughout several cell divisions. In order for this to happen, the histone modifications on the pre-existing old histones have to be copied on the newly deposited histones, which have a fundamentally different modification pattern when placed onto DNA (15,18). It has been shown that histone modifications indeed change during the cell cycle (38). However, no distinction has been made with regard to modifications present on old and new histones except for H4 (26). In order to investigate the mechanisms and the kinetics of modification pattern inheritance, we used a pulsed SILAC labeling technique that enabled us to selectively label the newly synthesized histones and subsequently compare modifications on old and new histones. In agreement with previous results (32), we found that the acetylation of histones is highly dynamic and regulated by a tight equilibrium of acetyltransferases and deacetylases. The situation is different in the case of the observed methylations. For all lysines investigated (except H3K79), we observed a transient peak in the monomethylated isoform, suggesting that the mono-methylation is put onto the histones either at or immediately after histone deposition. Several histone methyltransferases have been shown to interact with the replication machinery (37,39,40) where they probably catalyze the monomethylation of histones (41,42). This early burst of monomethylation is followed by a relatively slow progression to higher levels of methylation. This is in accordance with earlier observations, which suggest that monomethylation occurs first and may indeed be a prerequisite for further methylations (16,43). This is especially interesting in the case of H3K27, where the methylase EZH2 is responsible for all degrees of methylation. The stepwise methylation suggests a highly regulated progression from lower to higher methylated forms. Such a regulation of the generation of higher methylation states has been reported for several HMT complexes, where histone-binding subunits are required to achieve these states (44,45). The trimethylated forms are much better binding partners for the structural proteins HP1 and Polycomb (46-48) that are thought to condense chromatin by preventing nucleosome remodelers from acting on them (49). It is therefore tempting to speculate that the slow trimethylation on newly deposited histones prevents a premature chromatin condensation and allows chromatin to adopt a structure that is more susceptible for external signals. This window of opportunity could allow cells to stably shift their gene expression profile when they are exposed to changing external signals, such as stem cells leaving their specific niche. On the other hand, the methylation state could also provide a means for the cell to detect the cellular age, as trimethylated isoforms will increase when senescent cells do not undergo continuous cell division (3,50). Especially, as no demethylase has been characterized so far that is able to remove methyl groups from H4, H4K20 may have an exquisite function in measuring cellular age (26).

We and others (26) have analyzed the modification kinetics in immortalized human cells (HeLa) of a tumorigenic origin. Many tumor cells have a markedly different histone modification pattern when compared to normal cells (51). This has to be taken into account when interpreting our data. It will be interesting to see whether primary cells have a similar kinetic or whether the fast replication of tumor cells may in fact prevent the cells from fully replicating the epigenome, which could in fact explain the epigenetic instability that is frequently observed in tumors.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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