Combinatorial Modification of Human Histone H4 Quantitated by Two-dimensional Liquid Chromatography Coupled with Top Down Mass Spectrometry

James J. Pesavento1,2, Courtney R. Bullock5,1, Richard D. LeDuc6, Craig A. Mizzen1, and Neil L. Kelleher1,3

From the 1Center for Biophysics and Computational Biology, 2Department of Cell and Developmental Biology, and 3Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Quantitative proteomics has focused heavily on correlating protein abundances, ratios, and dynamics by developing methods that are protein expression-centric (e.g. isotope coded affinity tag, isobaric tag for relative and absolute quantification, etc.). These methods effectively detect changes in protein abundance but fail to provide a comprehensive perspective of the diversity of proteins such as histones, which are regulated by post-translational modifications. Here, we report the characterization of modified forms of HeLa cell histone H4 with a dynamic range >104 using a strictly Top Down mass spectrometric approach coupled with two dimensions of liquid chromatography. This enhanced dynamic range enabled the precise characterization and quantitation of 42 forms uniquely modified by combinations of methylation and acetylation, including those with trimethylated Lys-20, monomethylated Arg-3, and the novel dimethylated Arg-3 (each <1% of all H4 forms). Quantitative analyses revealed distinct trends in acetylation site occupancy depending on Lys-20 methylation state. Because both modifications are dynamically regulated through the cell cycle, we simultaneously investigated acetylation and methylation kinetics through three cell cycle phases and used these data to statistically assess the robustness of our quantitative analysis. This work represents the most comprehensive analysis of histone H4 forms present in human cells reported to date.

Histones are a class of proteins around which DNA is wrapped and packaged inside a eukaryotic nucleus. Two molecules of each core histone H2A, H2B, H3, and H4 together with ~146 bp of DNA form the fundamental unit of chromatin called the nucleosome. These proteins are heavily modified, with combinations of these enzymatic modifications thought to form a "histone code" orchestrating epigenetic processes such as long-term gene silencing and gene activation (1), higher level chromatin packaging (2), and DNA repair mechanisms (3). All of these activities change with relation to the cell cycle, a sequence of events during which a cell commits to DNA replication (G1), replicates its DNA (S), prepares for mitosis (G2), and undergoes cell division (M) (4). Histone synthesis and deposition are largely coupled to DNA replication during S phase (5). As a cell doubles its nuclear DNA, there is a concomitant doubling of the content of histones and nucleosomes. Even though antibodies have been used to track single modifications, the fates of preexisting histone modifications and the acquisition of new histone modifications during the cell cycle is not well understood because this approach is unable to distinguish previously modified forms from newly modified ones (6–8). However, an epigenetic mechanism presumably exists to faithfully transmit patterns of histone modification and chromatin structure to ensure normal cellular function over successive generations (9).

Dynamic changes in the PTMs4 affecting the N-terminal tails of the core histones, which comprise ~25–30% of their individual masses, are thought to modulate their interactions with both DNA and other proteins (10). Great efforts have been made to catalogue the types and sites of modifications and ascertain their biological contexts. For example, it has been asserted that methylation of lysine 9 in human H3 is a mark for heterochromatin by specifically interacting with heterochromatin-associated protein 1 (11, 12). Nevertheless, the true biological significance of such observations remains to be defined in the absence of quantitative data regarding modification type, co-occurrence with other PTMs on the same histone, and abundance in bulk chromatin. Antibodies have been used to provide qualitative descriptions of the genomic distributions of specific histone PTMs in various organisms (e.g. Refs. 13 and 14). However, a quantitative profile of the intact histone forms that populate the ~32 million nucleosomes packaging the human genome has yet to be reported.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

1 These authors contributed equally to this work.
2 Recipient of a National Institutes of Health Institutional National Research Service Award in Molecular Biophysics ST32 GM 08276. Present address: Dept. of Molecular and Cell Biology; University of California, 16 Barker Hall, Berkeley, CA 94720.
3 To whom correspondence should be addressed: Dept. of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews, 53 Rodger Adams Laboratory, Urbana, IL 61801. Tel.: 217-244-3927; E-mail: kelleher@scs.uiuc.edu.

4 The abbreviations used are: PTM, post-translational modification; TDMS, Top Down mass spectrometry; MS, mass spectroscopy; HILIC, hydrophilic interaction chromatography; RP-HPLC, reverse phase hydrophobic interaction liquid chromatography; FTMS, Fourier transform MS; ECD, electron capture dissociation; PRMT, protein arginine methyltransferase; SWIFT, stored waveform inverse Fourier transform.
42 Forms of Histone H4 by Top Down MS

There are many known sites and types of post-translational modification on human histone H4: N-terminal acetylation (acetylser-1, ser-1 phosphorylation, Arg-3 mono- and dimethylation (mArg-3, 2mArg-3), lysine 5, 8, 12, and 16 acetylation (aLys-5, aLys-8, aLys-12, aLys-16), and lysine 20 mono-, di-, and trimethylation (mLys-20, 2mLys-20, 3mLys-20). Current methods used to characterize and quantify histone H4 PTMs paint only part of the nuclear picture. For example, antibodies, which are specific to one modification, can detect single-site modifications (e.g. mArg-3), the abundance of which in some cell types may be below the detection limit of MS. “Bottom-up MS” allows the detection of combinations of modifications only when they reside on the same peptide (15). The use of peptide mass fingerprinting relies on mass accuracy instead of fragmentation for PTM detection (16). None of these approaches is able to provide a simple measure of percent occupancy of a given PTM or the percentage of its parent histone form in a molecular population. To unambiguously determine the combinations of PTMs on histone proteins, the intact protein must be processed and analyzed such that the identity and regiospecificity of the PTM(s) is restricted to a single known, pre-selected mass. Recent advances in Top Down mass spectrometry (TDMS) and dissociation methods have made this approach possible (17, 18). For example, TDMS has shown that the intact MS profile of reverse-phase purified H2A and H2B are comprised of mainly unmodified variants (19, 20), whereas H3 and H4 are comprised mainly of forms harboring PTMs (21, 22).

Hydrophilic interaction chromatography (HILIC) has been shown to separate allelic variants of histone H1 (23) and modified forms of murine H4 (24, 25). Lindner and co-workers (24, 25) successfully identified the major H4 PTMs present in the most abundant HILIC peaks using bottom-up MS, categorizing and localizing some of the PTMs on H4. More recently, our laboratories have successfully utilized a HILIC/TDMS approach to identify more than 150 different combinations of post-translational modifications on histone H3.2 (26).

Here, we extend previous work by identifying additional H4 forms of human H4 via Top Down MS and validating a quantitative method to assess abundance of human histone H4 from asynchronous and cycling cells. We extend our quantitative approach (27) to combinatorially modified forms of histone H4, detected at <0.02% of the total population. In this way we are able to assess abundance levels of 42 unique combinations of PTMs on H4 in asynchronous HeLa cells. In addition, we report the cell cycle dynamics of seven histone H4 protein forms, identify two novel H4 forms, and uncover correlations between methylation and acetylation in cycling cells.

**EXPERIMENTAL PROCEDURES**

Histone Preparation: Reverse Phase and Hydrophilic Interaction Liquid Chromatography—RP-HPLC-purified H4 was prepared by chromatography of ~100 µg of crude HeLa S3 histone protein (with or without prior oxidation as described previously (28, 29)). Fractions of H4 resolved by HILIC from three individual preparations of asynchronous HeLa cells were analyzed by Fourier transform MS (FTMS). The first experiment was performed without intentional oxidation. For the second experiment, each individual HILIC fraction was intentionally oxidized before MS analysis (29). For the third experiment, crude histone was intentionally oxidized before RP-HPLC (28), and the fully oxidized histone H4 was collected and used for the subsequent HILIC purification. HILIC fractions of H4 were prepared by fractionating ~150 µg of RP-HPLC purified H4 (pooled from several RPLC runs) as described previously (24) on a PolyCAT A column (4.6 mm × 150 mm; 3 µm, 1000 Å, PolyLC, Columbia, MD) with a multistep gradient optimized for the resolution of modified H4 forms from buffer A (70% CH3CN, 20 mm triethylamine (TEA), pH 4.0 with H3PO4) to buffer B (20% CH3CN, 20 mm TEA, 500 mm NaClO4, pH 4.0, with H3PO4). Fractions of interest were partially dried in a SpeedVac. H4 recovered by trichloroacetic acid precipitation, washed extensively with 20% trichloroacetic acid to remove residual salts, washed with acetone/0.1% HCl and then acetone, air-dried, and stored at ~80 °C. For samples that were not oxidized before RP-HPLC, dried samples were resuspended in an electrospray solution (49:49:2, water:methanol:formic acid) supplemented with 3% H3O2 and left at ~80 °C for 2 weeks before FTMS analysis.

Mass Spectral Analysis by Electrospray Ionization-FTMS/MS—All data were acquired on a custom 8.5 Tesla Quadrupole-FTMS hybrid with an electrospray ionization source operated in positive ion mode as described previously (29). Residual chloride or phosphate contaminating some samples was partially removed by applying ~25 V on the accumulation octapoles. Electron capture dissociation (ECD) was performed by applying 5 A through a dispensor cathode filament (Heatwave Technologies, Crescent Valley, BC). During the ECD event, ~10 V were applied on the grid potential whereas ~9 V were sent through the filament for optimal ECD. Typically, 300 cycles of ECD were performed, with individual irradiation times of 5 ms and a 10-ms relaxation time between cycles. All relative molecular weight (Mw) values and fragment masses are reported as neutral, monoisotopic species. ECD MS/MS spectra were internally calibrated using 3–4 identified m/z ions from the unmodified C-terminal region.

Quantitation of Modified Forms of Intact H4—The molecules remain intact so that all possible combinations of modifications are present when analyzed by TDMS. With this in mind, the HILIC chromatogram was integrated, and each fraction was assigned a percent area relative to the entire elution area of H4. This relative area was then multiplied by the percent of each observed mass in the mass spectrum associated with that fraction. If multiple species were present, the MS intensity values were used to calculate the relative ratios for all protein ions observed (so called “PIRR” (27)). Each PIRR was then multiplied by the relative HILIC area percentage. This value, the intact mass percentage, reports solely on the relative percentage of each observed intact mass and does not depend on the absolute abundances in the MS. The intact mass percentage can be further dispersed if isobaric or isomeric components exist (e.g. a mass corresponding to monoacetylated H4 could theoretically be a mixture of acetylation at Lys-5, -8, -12, or -16). Fragmentation of a single intact mass comprised of isobars or isomers will produce fragment ion pairs. As previously demonstrated in our laboratory, the MS/MS intensity values of these fragment ion pairs (so called “FIRRs”) can be used to determine the iso-
meric composition with an error of ≤5% for species generating moderate to high MS signals (27). After isomeric composition is determined, the relative percent of each protein form can be determined for that one fraction. If a form elutes over multiple fractions, multiplying the percentages from MS (PIRRs and FIRRMs) and chromatography gives a value for total percentage for that form. A comprehensive list of all observed histone H4 modifications and their respective abundances from Fig. 1 is shown in Table 1.

For calculating PIRR values for histone H4, the intensity of the four most abundant isotopes (13C5, 13C6, 13C7, and 13C8) were integrated to calculate intact abundance ratios for the 14+ and/or 12+ charge states. When possible, these were combined into a weighted average to report a final PIRR value for the sample, which was then used to calculate the relative amount of the corresponding intact H4 form relative to the sum of the total. ECD generated 1, 2+, 3+, and fragment ions, from which the 13C5, 13C6, and 13C7 (when observed) isotopes were integrated and used to calculate the FIRRMs.

Software—A Web-based software and data base suite, Pro-Sight PTM (21, 30), was used to accelerate the characterization of histone H4 protein forms as previously reported (21). An adapted version of THRASH (31) was used to convert raw data into monoisotopic mass values.

Analysis of H4 from Synchronized Cells—HeLa S3 cells were grown in Jokliks medium supplemented with 10% newborn calf serum and maintained at a density of 2–3 × 105/ml before synchronization using the double thymidine block procedure as described previously (29). The degree of synchrony was assessed by flow cytometry every hour after the second release. The representative G1/S, mid-S, late S/G2, M/G1, and mid-G1 phases were determined to occur 0, 4, 8, 10.5, and 14 h post-release, respectively. To enable a rigorous comparison of H4 from different cell cycle stages, H4 was prepared from cells collected at 0, 8, and 14 h post-release in three independent synchronizations. Approximately 150 μg of RP-HPLC-purified H4 was then further purified for each time point by HILIC as described above. The HILIC chromatogram, MS, and MS/MS data were used to calculate the abundance of unacetylated and monoacetylated H4 as described above. In a few cases, a stored wave-form inverse Fourier transform (SWIFT) isolated window (<1.5 m/z) contained salt-adducted forms of a lower mass species. The amount of contamination was determined by fragment ions larger than c20. The FIRRMs were then corrected by subtracting out this contamination. Of the 93 HILIC fractions analyzed, one showed no measurable protein within the detection limit. The chromatographic area of this peak, representing an estimated ~0.7% of the most abundant peak, was subtracted from the whole and removed from further calculations and analysis.

RESULTS AND DISCUSSION

Hydrophilic-interaction Liquid Chromatography—Using HILIC conditions similar to those described previously (24, 25), we separated multiply modified histone H4 forms from asynchronous HeLa S3 cells. To reduce sample complexity, each 1-min fraction was analyzed individually (shaded bands in Fig. 1A). The MS of each fraction contained on average two distinct mass species typically 14 Da apart (Fig. 1B). A cursory examination of the intact mass in each HILIC fraction revealed an acetylation- and methylation-dependent elution profile. The most highly modified (e.g. the tetraacetylated, dimethylated H4) and, therefore, most hydrophobic form eluted earliest, whereas the least hydrophobic form (e.g. N-terminal acetylation only) eluted last. In general, H4 separated primarily by acetylation state, generating five groups of resolved peaks (Fig. 1A). Each one of these groups was further broken down into a series of peaks which included (in order of elution) tri-, di-, mono-, and unmethylated Lys-20. Mono- and dimethylated Arg-3 (with 2mLys-20) were observed to coelute with Lys-20 trimethylation (see Fig. 1 and Table 1). Intact mass isolation using a SWIFT followed by ECD fragmentation identified the modifications on and relative abundances of 42 H4 forms (Fig. 2, Table 1, and see below).

Unacetylated, Methylated H4—It is well known that all H4 molecules are co-translationally acetylated on the α-NH2 of Ser-1 (56, 57), resulting in a Δm of at least +42 Da for all forms of H4. This +42 Da form will be referred to as the “unacetylated” form of H4 (ααSer-1), whereas the term “monoacetyl-
42 Forms of Histone H4 by Top Down MS

### TABLE 1

**Identification and global quantitation of 42 histone H4 PTMs**

| Quick ID | Modifications | Monoisotopic mass* | Δm | Relative abundance |
|----------|---------------|--------------------|-----|--------------------|
| H4.1     | 2mLys-20      | 11,289.3           | 28  | 0.3                |
| H4.2     | aaSer-1       | 11,303.3           | 42  | 2                  |
| H4.3     | aaSer-1, mLys-20 | 11,317.3       | 56  | 6                  |
| H4.4     | aaSer-1, 2mLys-20 | 11,331.3    | 70  | 65                 |
| H4.5     | aaSer-1, 3mLys-20 | 11,345.3       | 84  | 1                  |
| H4.6     | aaSer-1, mArg-3, 2mLys-20 | 11,345.3  | 84  | 1                  |
| H4.7     | aaSer-1, 2mArg-3, 2mLys-20 | 11,359.3  | 98  | 0.1                |
| H4.8     | aaSer-1, 1lys-16 | 11,345.4      | 84  | 0.6                |
| H4.9     | aaSer-1, 1lys-12 | 11,345.4      | 84  | 0.2                |
| H4.10    | aaSer-1, 1lys-8 | 11,345.4      | 84  | 0.1                |
| H4.11    | aaSer-1, 1lys-5 | 11,345.4      | 84  | 0.4                |
| H4.12    | aaSer-1, 1lys-16, mLys-20 | 11,359.4   | 98  | 1                  |
| H4.13    | aaSer-1, 1lys-12, mLys-20 | 11,359.4   | 98  | 0.1                |
| H4.14    | aaSer-1, 1lys-8, mLys-20 | 11,359.4   | 98  | 0.1                |
| H4.15    | aaSer-1, 1lys-5, mLys-20 | 11,359.4   | 98  | 0.4                |
| H4.16    | aaSer-1, 1lys-16, 2mLys-20 | 11,373.4  | 112 | 13                 |
| H4.17    | aaSer-1, 1lys-12, 2mLys-20 | 11,373.4  | 112 | 1                  |
| H4.18    | aaSer-1, 1lys-8, 2mLys-20 | 11,373.4  | 112 | 0.6                |
| H4.19    | aaSer-1, 1lys-5, 2mLys-20 | 11,373.4  | 112 | 0.9                |
| H4.20    | aaSer-1, mLArg-3, alys-16, 2mLys-20 | 11,387.4 | 84 | 0.003              |
| H4.21    | aaSer-1, mLArg-3, alys-12, 2mLys-20 | 11,401.4 | 84 | 0.001              |
| H4.22    | aaSer-1, alys-16, 3mLys-20 | 11,387.4 | 98 | 0.4                |
| H4.23    | aaSer-1, alys-12, alys-16 | 11,387.4 | 126 | 0.06               |
| H4.24    | aaSer-1, alys-5/aLys-8, alys-16 | 11,387.4 | 126 | 0.06               |
| H4.25    | aaSer-1, alys-5/aLys-8, alys-12 | 11,387.4 | 126 | 0.1                |
| H4.26    | aaSer-1, alys-12, alys-16, mLys-20 | 11,401.4 | 140 | 0.2                |
| H4.27    | aaSer-1, alys-5/aLys-8, alys-16, mLys-20 | 11,401.4 | 140 | 0.08               |
| H4.28    | aaSer-1, alys-5/aLys-8, alys-12, mLys-20 | 11,401.4 | 140 | 0.08               |
| H4.29    | aaSer-1, alys-12, alys-16, 2mLys-20 | 11,415.4 | 154 | 1                  |
| H4.30    | aaSer-1, alys-5/aLys-8, alys-16, 2mLys-20 | 11,415.4 | 154 | 0.6                |
| H4.31    | aaSer-1, alys-5/aLys-8, alys-12, 2mLys-20 | 11,415.4 | 154 | 0.4                |
| H4.32    | aaSer-1, alys-8, alys-12, alys-16 | 11,429.4 | 168 | 0.2                |
| H4.33    | aaSer-1, alys-5, alys-12, alys-16 | 11,429.4 | 168 | 0.2                |
| H4.34    | aaSer-1, mLArg-3, alys-5/8/12, alys-16, 2mLys-20 | 11,429.4 | 168 | 0.06               |
| H4.35    | aaSer-1, alys-8, alys-12, alys-16, mLys-20 | 11,443.4 | 182 | 0.1                |
| H4.36    | aaSer-1, alys-5, alys-12, alys-16, mLys-20 | 11,443.4 | 182 | 0.09               |
| H4.37    | aaSer-1, 2mArg-3, alys-5/8/12, alys-16, 2mLys-20 | 11,443.5 | 182 | 0.03               |
| H4.38    | aaSer-1, alys-8, alys-12, alys-16, 2mLys-20 | 11,457.5 | 196 | 0.4                |
| H4.39    | aaSer-1, alys-5, alys-12, alys-16, 2mLys-20 | 11,457.5 | 196 | 0.4                |
| H4.40    | aaSer-1, alys-5, alys-8, alys-12, alys-16, 3mLys-20 | 11,471.5 | 210 | 0.4                |
| H4.41    | aaSer-1, alys-5, alys-8, alys-12, alys-16, mLys-20 | 11,485.5 | 224 | 0.4                |
| H4.42    | aaSer-1, alys-5, alys-8, alys-12, alys-16, mLys-20 | 11,499.5 | 238 | 0.4                |

* Samples were intentionally oxidized before analysis. Theoretical molecular weights given represent the non-oxidized forms.

Total abundance of all tetraacetylated species was found to be 0.2%.

---

...on the first 20 residues. Therefore, taking all mass shifts into consideration, we can say with confidence that this isomeric species contained a ~1:1 mixture of aaSer-1 + mLArg-3 + 2mLys-20 and aaSer-1 + 3mLys-20. As expected from the area underneath their HILIC peak, the mLArg-3 + 2mLys-20 and the 3mLys-20 were the rarest of the unacylated but methylated H4, each accounting for ~1% of the global abundance of H4 PTMs. Strikingly, no forms with phosphorylated Ser1 were observed. M-phase arrest of HeLa with colchicine does build the occupancy of this PTM up to ~5% (29). Considering this PTM, there are at most ~84 forms of histone H4 present in human cells, with 75 of these detected by the Coon Laboratory from differentiating embryonic stem cells.5

5 J. Coon, personal communication.
+28 Da over the course of elution (Fig. 1B, middle panel), suggesting that the location of the single acetyl group at either Lys-5, -8, -12, or -16 may have an effect on elution of different monoacetylated isomers of H4. Indeed, distinct elution differences between the four monoacetylated sites (with the same number of methylations) were observed (supplemental Fig. 2A). We also found that the abundance of aLys- 5 ≈ aLys-8 < aLys-12 < aLys-16, a trend also noted in other studies of asynchronous and cycling cells (15, 32–34). The “zipper model” states that acetylation occupancies are highest at aLys-16 followed by aLys-12 > aLys-8 > aLys-5. This trend is clearly evident from the data shown in Table 1 and Fig. 5 (15). However, when the acetylation site occupancy is considered relative to the degree of Lys-20 methylation in the same molecule (Fig. 3), it becomes obvious that the widely accepted zipper model of H4 acetylation may not hold the same weight for different Lys-20 methylation states. The most striking example of this is shown by Fig. 3A when comparing the degree of acetylation occupancy of unmethylated and dimethylated Lys-20 molecules. Although the aLys-16 > aLys-12 > aLys-8 > aLys-5 hierarchy exists for both un- and dimethylated Lys-20 molecules, the magnitude of this trend differs dramatically between these two methylated forms. From these data it appears that the frequency of acetylation at sites more distal to Lys-20 (i.e. Lys-5, -8, or -12) is inversely related to the degree of Lys-20 methylation. In contrast, the frequency of acetylation at Lys-16 appears to be directly related to the degree of Lys-20 methylation (Fig. 3). This trend is also observed for monoacetylated forms during G1/S, S/G2, and G1 (discussed below). These findings suggest that site preferences for either acetyltransferases or deacetylases are influenced by the Lys-20 methylation state. 

Methylation at Arg-3 (with dimethylation at Lys-20) and trimethylation at Lys-20 are observed at low levels on unacetylated H4 molecules (Table 1). Methylation of H4 at Arg-3 is associated with transcriptional coactivation by protein arginine methyltransferase 1 (PRMT1) (35, 36), but the nature of this mark has not been unequivocally established. Conventional MS/MS of H4 peptides prepared from asynchronously growing 293T cells detected only monomethyl Arg-3 (35). The extent to which mono- and dimethylation of Arg-3 is attributable to PRMT1 or other PRMTs is unknown. However, because most PRMTs are thought to catalyze dimethylation (37), the monomethyl Arg-3 we detected presumably arises either from a previously unrecognized ability of PRMT1 or other PRMTs to catalyze monomethylation or via partial demethylation of dimethylated Arg-3. Because our analyses revealed that methylation itself was the only alteration made to the structure of arginyl side chains in the aoSer-1 + mArg-3 + 2mLys-20, aoSer-1 + mArg-3 + aLys-16 + 2mLys-20, and aoSer-1 + mArg-3 + aLys-12 + aLys-16 + 2mLys-20 forms, the latter case would have to involve a mechanism other than deimination or demethylimation.
observed in immunocytochemical analyses (46–48). However, hypoacetylated forms of H4 in constitutive heterochromatin correlate with the enrichment of Lys-20 trimethylated and ethylation and hypoacetylation of H4 observed here is consistent with the hypothesis that the effects of Lys-20 trimethylation are distinct from those of mono- and dimethylation. Lindner and co-workers (25) have proposed that Lys-20 trimethylation was readily detected in unacetylated (aaSer-1 + 3mLys-20), monoacetylated (aaSer-1 + Lys-16 + 3mLys-20), and diacetylated (aaSer-1 + Lys-12 + Lys-16 + 3mLys-20) forms of asynchronous HeLa H4, consistent with previous analyses that detected Lys-20 trimethylation on unacetylated and monoacetylated forms of H4 from mouse, rat, and human cells in the absence of histone deacetylation inhibition (24, 25). Together with our evidence that Lys-16 acetylation and Lys-20 mono- and dimethylation are not mutually inhibitory in vivo (Tables 1 and 2), the absence of Lys-20 trimethylation on tri- and tetra-acetylated forms of H4 lends some support for the hypothesis that the effects of Lys-20 trimethylation are distinct from those of mono- and dimethylation. Lindner and co-workers (25) have proposed that Lys-20 trimethylation is coordinated with gene silencing by histone deacetylases to recruit heterochromatin promoting factors involved in maintaining silencing, as suggested previously for trimethylation at Lys-9 and -27 of H3 (43–45). The coincidence of Lys-20 trimethylation and hypoacetylation of H4 observed here is consistent with the enrichment of Lys-20 trimethylated and hypoacetylated forms of H4 in constitutive heterochromatin observed in immunocytochemical analyses (46–48). However, the co-occurrence of Lys-20 trimethylation and Lys-16 and -12 acetylation argues against a heterochromatin-promoting role for the 3mLys-20 mark. Despite these conflicting interpretations, our finding that Lys-20-trimethylated H4 lacks Arg-3 methylation (Tables 1 and 2) further supports the notion that Lys-20-trimethylated H4 is enriched in silent chromatin as Arg-3 methylation is thought to be associated with transcriptional activation (35, 36). The lack of 3mLys-20 and mArg-3 co-occurring on the same H4 molecule suggests that Arg-3 methylation is either precluded from Lys-20-trimethylated forms or is removed upon Lys-20 trimethylation (although it should be noted that we did not identify citrulline or other residues derived from arginine in any of the Lys-20-trimethylated species analyzed).

**Diacetylated, Methylated H4**—The diacetylated forms of H4 eluted between ~36 and 42 min in the Fig. 1 chromatogram. Of the six possible combinations of diacetylated isomers, five distinct combinations were detected: aLys-5 + aLys-12, aLys-8 + aLys-12, aLys-5 + aLys-16, aLys-8 + aLys-16, and aLys-12 + aLys-16. Unfortunately, whenever isomeric forms harboring acetylation at either Lys-12 or -16 are present with an additional acetylation at either Lys-5 or -8, it is impossible to deduce how much acetylation at Lys-5 or -8 occurs in combination with acetylation at Lys-12 or -16 without further analysis such as MS/MS/MS (21). In these cases, we combined the values of aLys-5 + aLys-16 and aLys-8 + aLys-16 into aLys-5/8 + aLys-16, and in a similar fashion we combined aLys-8 + aLys-12 and aLys-5 + aLys-12 into aLys-5/8 + aLys-12. The abundance trends for the diacetylated forms showed that aLys-5/8 + aLys-12 < aLys-5/8 + aLys-16 < aLys-12 + aLys-16. However, as was observed with the monoacetylated molecules, the ratio of acetylation site occupancies changed depending on the methylation state of Lys-20. For example, the aLys-5/8 + aLys-12 form comprised 46% of the unmethylated molecules, but because Lys-20 methylation increased from 0m to 1m and 1m to 2m, this ratio dropped to 21 and 18%, respectively (Fig. 3B). Conversely, aLys-12 + aLys-16 was present at 26% of the unmethylated molecules but then increased to 57 and 51% of the mLys-20 and 2mLys-20 forms, respectively. The relative percentages of aLys-5/8 + aLys-16 did not change significantly in the un-, mono-, and dimethylated Lys-20 species. Nevertheless, we found that Lys-16 was approximately twice as likely to be acetylated in diacetylated forms, which were also mono- or dimethylated at Lys-20 compared with diacetylated forms lacking Lys-20 methylation. These trends were also reproduced in two separate HILIC/FTMS experiments (data not shown). The low abundances of both mArg-3 + aLys-5/8/12 + aLys-16 + 2mLys-20 and 2mArg-3 + aLys-5/8/12 + aLys-16 + 2mLys-20

### TABLE 2

| Modification                       | Monoisotopic mass a | Δm   | % Abd  | 0 h | 8 h | 14 h |
|-----------------------------------|---------------------|------|--------|-----|-----|------|
| 2mLys-20                          | 11,289              | 28   | 0.3 ± 0.1 | ND  | ND  | ND   |
| aaSer-1, mLys-20                  | 11,317              | 56   | 4 ± 0.4 | 14 ± 1.5 | 1 ± 0.3 |
| aaSer-1, 2mLys-20                 | 11,331              | 70   | 65 ± 1.2 | 53 ± 3.6 | 63 ± 9.1 |
| aaSer-1, 3mLys-20                 | 11,345              | 84   | 0.9 ± 0.4 | 0.6 ± 0.3 | 0.5 ± 0.4 |
| aaSer-1, mArg-3, 2mLys-20         | 11,345              | 84   | 0.9 ± 0.4 | 0.3 ± 0.07 | 0.3 ± 0.1 |
| aaSer-1, 2mArg-3, 2mLys-20        | 11,359              | 98   | 0.2 ± 0.1 | 0.05 ± 0.03 | 0.06 ± 0.03 |

### Notes

- a Samples were intentionally oxidized at M84 before analysis (55). Theoretical molecular masses given represent the oxidized forms, +32 Da from unoxidized.
- ND, not detectable. Either the form does not exist or is present at levels below the detection limit of this experiment.
were detected at 0.06 and 0.03%, respectively. The position of acetylation on the diacetylated molecules affected elution order as observed with the monoacetylated molecules (supplemental Fig. 2B). Triacetylated and Tetraacetylated, Methylated H4—The tri- and tetraacetylated forms that eluted between 25 and 35 min were not abundant enough to obtain informative ECD spectra for the HILIC chromatograph shown in Fig. 1. However, after performing another HILIC experiment with ∼2× more protein, we found that these species were abundant enough to be characterized by MS and MS/MS (Table 1 and data not shown). Of the four possible triacetylated isomers, only two were observed: aLys-16 + aLys-12 + aLys-8 and aLys-16 + aLys-12 + aLys-5 (Fig. 3C). The ratio of aLys-16 + aLys-12 + aLys-8 to aLys-16 + aLys-12 + aLys-5 increased as Lys-20 methylation state increased, again showing a correlation between acetylation site occupancy and methylation at Lys-20. Only unmodified Lys-20, mLys-20, and 2mLys-20 masses for the triacetylated forms were observed, with unmodified Lys-20 < mLys-20 < 2mLys-20. No intact masses corresponding to 3mLys-20 or mArg-3 + 2mLys-20 were observed on either triacetylated or tetraacetylated molecules. The tetraacetylated fractions (aSer-1, aLys-16, aLys-12, aLys-8, aLys-5) also exhibited a similar abundance pattern with the unmodified Lys-20 < mLys-20 < 2mLys-20.

Achieving >10⁴ Dynamic Range—One limitation for TDMS detection of H4 forms from reverse-phase purified H4 is its dynamic range of ∼100. This dynamic range translates to a detection limit that precludes complete characterization of forms representing less than ∼3% of the H4 pool (29). By using HILIC to separate rare forms (e.g. fraction 41, Figs. 1 and 2A) from the most highly abundant forms (e.g. fraction 52, Fig. 1), an enrichment of nearly 500-fold was achieved. In fraction 41, a H4 form of mass 11,387 Da was observed with a ∼75 signal-to-noise ratio (S/N, Fig. 2B). After SWIFT isolation and ECD fragmentation, this mass was determined to consist of two isomeric components, resulting in a further increase of the effective dynamic range (Fig. 2C, inset). Using the signal-to-noise ratio of the lowest level forms detected here, the approximate dynamic range of the combined 4-dimensional analysis reported here (RPLC, HILIC, MS, and MS/MS) was determined to be ∼7 × 10⁴. This translates to a projected ability to detect an H4 form present in ∼500 of 32 million total nucleosomes in the human nucleus.
42 Forms of Histone H4 by Top Down MS

A

B

Absolute Abundance of Unacetylated H4 in Bulk Chromatin (% Total)

0 h (G1/S)  8 h (late S/G2)  14 h (G1)

C

Absolute Abundance of Monoacetylated H4 in Bulk Chromatin (% Total)

D

Relative Abundance of Monoacetylated H4 in Bulk Chromatin (% Total)
Two Novel Modifications: 1) 2mLys-20 and 2) aoSer-1 + 2mArg-3 + 2mLys-20—With the increased dynamic range described above, we then interrogated H4 from cells arrested at the G1/S border of the cell cycle (see below), generating the HILIC chromatogram shown in Fig. 4A. Of the many forms described above, two additional forms were observed; one with $\Delta m = 28$ Da and the other with $\Delta m = 98$ Da (Fig. 4). The former comprised dimethylation at Lys-20 with no N-terminal acetylation (Fig. 4, C and E). More experimentation needs to be done to probe possible functions for this unique form, which represented only 0.28% of total histone H4 at the G1/S border (Table 2). A second rare H4 form, $\Delta m = 98$ Da, was determined by ECD to be aoSer-1 + 2mArg-3 + 2mLys-20 and constituted 0.2% of the total H4 population (Figs. 4, B and D, and Table 2). However, although the aoSer-1 + 2mArg-3 + 2mLys-20 species were observed throughout the cell cycle, only at the G1/S time point was this purely unacetylated + 2mLys-20 form observed.

Analysis of Unacetylated, Multiply Methylated H4 during the Cell Cycle—The fraction-by-fraction analysis of each HILIC separation revealed many unique H4 forms, some present at $<1\%$ levels in bulk chromatin. Of these forms, the identification of a high amount of Lys-20 methylation and isomeric acetylation begged the question of how these forms change during the cell cycle, as other groups have reported a correlation between the two events (6, 49). For example, transcription of PR-Set7, the histone methyltransferase responsible for Lys-20 mono- and dimethylation, increases during G2/M phase and declines during the transition to G1 (6). HeLa cells were synchronized by double thymidine treatment and released from G1/S arrest after the passage from the G1/S border to late S/G2. Between late S/G2 and mid-G1, there was a return of aoSer-1 + 2mLys-20 to the level in asynchronous cells. These results indicate an increase in methylation of new, unmethylated H4, creating monomethylated Lys-20 and, subsequently, dimethylated Lys-20 during the cell cycle (29). Both aoSer-1 + 3mLys-20 and aoSer-1 + mArg-3 + 2mLys-20 represented less than 1% of each of the total histone H4 present in the nucleus. These species were present at their highest levels at the G1/S border and subsequently decreased during late S/G2 (Table 2). There was no significant difference in the level of Lys-20 trimethylation or Arg-3 mono- and dimethylation from 8 to 14 h.

Statistical Significance—To test the robustness of HILIC/FTMS as an assay, all cell cycle time points (0, 8, and 14 h) were repeated 3 times. Fractions were collected every minute to allow for the utmost precision in characterization and quantitation of histone H4 forms. After MS and MS/MS analysis, data were generated from each biological replicate for the five major H4 forms listed in Table 2 (species with $\Delta m = 42$ to $\Delta m = 84$ Da). Table 2 contains the mean values of the three replicate experiments. All analysis was conducted in SAS (SAS Institute, Cary, NC). To assess statistical significance, a square root transformation was performed (supplemental Fig. 3B). After this transformation, analysis of variance was conducted to test the differences between the square root of the percent abundance of the observed modification isoforms across the measured time points. The time by protein form interaction effect (i.e. the effect of the cell cycle on the presence of all five forms of H4) was found to be significant ($p < 0.0001$). Therefore, least-squares-adjusted means were calculated for the main effects.

For the particular result of aoSer-1 progressing from 14 to 1% in the 8- to 14-h time points (Table 2), a statistically significant difference ($F = 151.37$ with 1 degree of freedom, Bonferroni corrected $p < 0.0001$) was found. This finding validates the reproducibility of our method and can be understood by the entrance of the aoSer-1 form of histone H4 into the nucleus during S phase (thereby being detected at late S/G2) and then decreasing due to the progressive methylation of Lys-20 as the cell cycles.

Analysis of Monoacetylated, Multiply Methylated H4 during the Cell Cycle—The coupling of HILIC with Top Down mass spectrometry enabled a dynamic range in excess of $>10^4$, which allowed identification and quantitation of rare histone forms. Whereas unacetylated histone H4 represented the most abun-

FIGURE 5. Representative HILIC chromatograms for each of the following cell cycle time points: 0, 8, and 14 h. These time points correspond to the G1/S border, late S/G2, and mid-G1 of the cell cycle, respectively. A, because all histone H4 is co-translationally acetylated at aoSer-1 (54), a form that is acetylated only at the N terminus and lacks any internal acetylations, is referred to as unacetylated (0 Ac). Labeled here are the diacetylated (2 Ac), monoacetylated (1 Ac), and unacetylated (0 Ac) regions. The unacetylated and monoacetylated regions are shaded to delineate fraction collection boundaries. Alternatively, shaded regions reflect sample collection boundaries. Graphical representation of global abundance of unacetylated (B) and monoacetylated (C) of total H4 by methylation state at lysine 20 (0mLys-20, 1mLys-20, and 2mLys-20) is shown. D, the relationship between relative monoacetylation site occupancies of aLys-5 ( ), aLys-8 (A), aLys-12 ( ), and aLys-16 ( ) to the methylation state of Lys-20 during the cell cycle.
dant protein form of nuclear histone H4, monoacetylated histone H4 was far less abundant (Fig. 5A, 1 Ac region and Table 3). This drop in abundance does not correspond to a drop in complexity. Indeed, twice as many monoacetylated isomers were identified as unacetylated isomers. Changing patterns of methylation were also observed on monoacetylated H4, specifically at lysine 20 (0mLys-20, 1mLys-20, 2mLys-20, and 3mLys-20) and arginine 3 (mArg-3). Of the 24 combinations of acetylation-methylation isomers possible, 14 were identified in this study (Table 3). Multiple monoacetylation isomers were detected on molecules with un-, mono-, and dimethylated Lys-20 throughout the cell cycle. The acetylation site occupancies followed a general trend whereby the abundance of aLys-5 ≈ aLys-8 < aLys-12 ≪ aLys-16 as Lys-20 became mono-, di-, and trimethylated (Fig. 5). Indeed, the 3mLys-20 population had Lys-16 as the sole site of acetylation. Furthermore, aLys-16 was the only acetylation site occupied throughout each of the three cell cycle phases. Moreover, this trend remains constant during the cell cycle despite changing abundances, suggesting that H4 acetylation rearrangement occurs rapidly upon chromatin maturation, consistent with other reports (52, 53). However, other factors may contribute to the distribution of acetylation isomers within each Lys-20 modification class, such as the possibility that deacetylation at Lys-12, -8, or -5 is enhanced through increased deacetylase recruitment by increasing degrees of Lys-20 methylation, whereas Lys-16 deacetylation is simultaneously restricted due to steric factors.

Nested within the data of Table 3 are the dynamics of both histone H4 monoacetylation and methylation as the cell cycles. Looking first at acetylation, a number of observations can be made (Fig. 5). From 0 to 8 h, a large increase in monoacetylated, unmethylated molecules (3.3× increase), and monoacetylated, trimethylated molecules (3× increase) was observed. However, from 8 to 14 h, the monoacetylated, unmethylated molecules decreased dramatically (15× decrease), and a small decrease (1.4×) was observed for the monoacetylated, monomethylated molecules. The monoacetylated, dimethylated molecules also decreased by 1.2-fold from 8 to 14 h. These data indicate that methylation at Lys-20 on un- and monoacetylated molecules is progressive as the cell cycles. Interestingly, similar magnitudes of increasing Lys-20 methylation were observed on un- and monoacetylated H4 during the three cell cycle phases studied (compare Fig. 5, B and C, middle graphs). This suggests that acetylation does not have an effect on Lys-20 methylation in vivo, contrary to in vitro data showing mutual antagonism between the two modifications (51). To date there has been no histone methyltransferase identified to be responsible for dimethylating H4 Lys-20. Nevertheless, there is a clear change of Lys-20 during the cell cycle. We were surprised to observe that the frequency of Lys-20 methylation on unacetylated molecules was the same as on monoacetylated molecules, as others have reported that acetylation inhibits Lys-20 methylation (51). To further investigate whether hyperacetylation might antagonize Lys-20 methylation, we released synchronized cells into media containing 10 mM sodium butyrate. The RP-HPLC H4 fraction was then analyzed directly by TDMS (supplemental Fig. 1A).

Newly synthesized H4 was observed as highly acetylated, unmethylated molecules (supplemental Fig. 1A, black isotopes). As the cells cycled, the abundance of the unmethylated mono-, di-, tri-, and tetraacetylated molecules decreased due to increasing Lys-20 dimethylation. Interestingly, at 10.5 h the corresponding intensity ratios of the un-, mono-, and dimethylated forms of highly acetylated molecules were very similar (supplemental Fig. 1). Finally at 15 h virtually all histone H4 was dimethylated. These results suggest that hyperacetylation of H4 has no differential effect on Lys-20 methylation rate in vivo.

This study presents new insights into the abundances of 42 unique H4 forms and their dynamic changes for three cell cycle time points. Future work examining the biological significance of the dynamic methylation at Lys-20 through the cell cycle and specific combinations of acetylation may benefit from the quantitative determinations of H4 PTMs described here. The delineations of the types and abundances of combinatorially modified forms of histone H4 will illuminate future research in chromatin biology.

Acknowledgment—We thank Ryan Fellers for the generation of an in-house data processing program.

REFERENCES

1. Berger, S. L. (2007) Nature 447, 407–412
2. Felsenfeld, G., and Groudine, M. (2003) Nature 421, 448–453
3. Downs, J. A., Nussenzweig, M. C., and Nussenzweig, A. (2007) Nature 447, 951–958
4. Schafer, K. A. (1998) Vet. Pathol. 35, 461–478
5. Kaufman, P. D. (1996) Curr. Opin. Cell Biol. 8, 369–373
6. Rice, J. C., Nishioka, K., Sarma, K., Steward, R., Reinberg, D., and Allis, C. D. (2002) Genes Dev. 16, 2225–2230
7. Turner, B. M. (2002) Cell 111, 285–291
8. Spada, F., Chioda, M., and Thompson, E. M. (2005) J. Cell Biochem. 95, 885–901
9. Lachner, M., and Jenuwein, T. (2002) Curr. Opin. Cell Biol. 14, 286–298
10. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kouzarides, T. (2001) Nature 410, 120–124

TABLE 3
Quantitative analysis of monoacetylated histone H4 forms during the cell cycle (samples from the shaded regions of Fig. 5A)

| Modification | Monoisotopic mass | Δm | % Abd |
|--------------|------------------|----|-------|
| 0 h          | 14 h             | 8 h |
| aLys-5       | 11,345           | 84  | ND    |
| aLys-8       | 11,345           | 84  | 0.5   |
| aLys-12      | 11,345           | 84  | 0.5   |
| aLys-16      | 11,345           | 84  | 0.7   |
| aLys-5, mLys-20 | 11,359 | 98  | ND    |
| aLys-12, mLys-20 | 11,359 | 98  | ND    |
| aLys-16, mLys-20 | 11,359 | 98  | 0.1   |
| aLys-5, 2mLys-20 | 11,373 | 112 | ND    |
| aLys-12, 2mLys-20 | 11,373 | 112 | 0.9   |
| aLys-16, 2mLys-20 | 11,373 | 112 | 0.5   |
| aLys-16, 3mLys-20 | 11,387 | 126 | 0.3   |
| aLys-16, 2mLys-20 | 11,387 | 126 | 0.1   |

a Samples were intentionally oxidized at M84 before analysis. Theoretical molecular masses given represent the oxidized forms, +32 Da from unoxidized.

b ND, not detectable. Either the form does not exist or is present at levels below the detection limit of this experiment.
42 Forms of Histone H4 by Top Down MS

13. Kurdistani, S. K., Tavazoie, S., and Grunstein, M. (2004) Cell 117, 721–733
14. Fischer, J. J., Toedling, J., Krueger, T., Schueler, M., Huber, W., and Sperling, S. (2007) Genomics 49, 91–51
15. Zhang, K., Williams, K. E., Huang, L., Yau, P., Siino, J. S., Bradbury, E. M., Jones, P. R., Minch, M. J., and Burlingame, A. L. (2002) Mol. Cell. Proteomics 1, 500–508
16. Zhang, L., Eugeni, E. E., Parthun, M. R., and Freitas, M. A. (2003) Chromosoma 112, 77–86
17. Zubarev, R. A., Kelleher, N. L., and McLafferty, F. W. (1998) J. Am. Chem. Soc. 120, 3265–3266
18. Kelleher, N. L. (2004) Anal. Chem. 76, 197–203
19. Boyne II, M. T., Pesavento, J. J., Mizzen, C. A., and Kelleher, N. L. (2006) J. Proteome Res. 5, 248–253
20. Siuti, N., Roth, M. J., Mizzen, C. A., Kelleher, N. L., and Pesavento, J. J. (2006) J. Proteome Res. 5, 233–239
21. Pesavento, J. J., Kim, Y. B., Taylor, G. K., and Kelleher, N. L. (2004) J. Am. Chem. Soc. 126, 3386–3387
22. Thomas, C. E., Kelleher, N. L., and Mizzen, C. A. (2006) J. Proteome Res. 5, 240–247
23. Mizzen, C. A., Alpert, A. J., Levesque, L., Kruck, T. P., and McLachlan, D. R. (2000) J. Chromatogr. B Biomed. Sci. Appl. 744, 33–46
24. Sarg, B., Koutzamani, E., Helliger, W., Rundquist, I., and Lindner, H. H. (2002) J. Biol. Chem. 277, 39195–39201
25. Sarg, B., Helliger, W., Talasz, H., Koutzamani, E., and Lindner, H. H. (2004) J. Biol. Chem. 279, 53458–53464
26. Garcia, B. A., Pesavento, J. J., Mizzen, C. A., and Kelleher, N. L. (2007) Nat. Meth. 4, 487–489
27. Pesavento, J. J., Mizzen, C. A., and Kelleher, N. L. (2006) Anal. Chem. 78, 4271–4280
28. Pesavento, J. J., Garcia, B. A., Streek, J. A., Kelleher, N. L., and Mizzen, C. A. (2007) Mol. Cell. Proteomics 6, 1510–1526
29. Pesavento, J. J., Yang, H., Kelleher, N. L., and Mizzen, C. A. (2008) Mol. Cell. Biol. 28, 468–486
30. Taylor, G. K., Kim, Y. B., Forbes, A. J., Meng, F., McCarthy, R., and Kelleher, N. L. (2003) Anal. Chem. 75, 4081–4086
31. Horn, D. M., Zubarev, R. A., and McLafferty, F. W. (2000) J. Am. Soc. Mass Spectrom. 11, 320–332
32. Thorne, A. W., Kniceck, D., Mitchelson, K., Sautiere, P., and Crane-Robinson, C. (1990) Eur. J. Biochem. 193, 701–713
33. Jiang, L., Smith, J. N., Anderson, S. L., Ma, P., Mizzen, C. A., and Kelleher, N. L. (2007) J. Biol. Chem. 282, 27923–27934
34. Turner, B. M., O’Neill, L. P., and Allan, I. M. (1989) FEBS Lett. 253, 141–145
35. Strahl, B. D., Briggs, S. D., Brame, C. J., Caldwell, J. A., Koh, S. S., Ma, H., Cook, R. G., Shabanowitz, J., Hunt, D. F., Stallcup, M. R., and Allis, C. D. (2001) Curr. Biol. 11, 996–1000
36. Wang, H., Huang, Z. Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B. D., Briggs, S. D., Allis, C. D., Wong, J., Tempst, P., and Zhang, Y. (2001) Science 293, 853–857
37. Trievel, R. C. (2004) Crit. Rev. Eukaryotic Gene Expression 14, 147–169
38. Cuthbert, G. L., Daujat, S., Snowden, A. W., Erdjument-Bromage, H., Hagiwara, T., Yamada, M., Schneider, R., Gregory, P. D., Tempst, P., Banister, A. I., and Kouzarides, T. (2004) Cell 118, 545–553
39. Wang, Y., Wysocka, J., Sayegh, J., Lee, Y. H., Perlin, J. R., Leonelli, L., Sonbucner, L. S., McDonald, C. H., Cook, R. G., Dou, Y., Roeder, R. G., Clarke, S., Stallcup, M. R., Allis, C. D., and Coonrod, S. A. (2004) Science 306, 279–283
40. Peters, A. H., Kubicek, S., Mechtler, K., O’Sullivan, R. J., Derijck, A. A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J. H., and Jenuwein, T. (2003) Mol. Cell 12, 1577–1589
41. Rice, J. C., Briggs, S. D., Ueberheide, B., Barber, C. M., Shabanowitz, J., Hunt, D. F., Shinkai, Y., and Allis, C. D. (2003) Mol. Cell 12, 1591–1598
42. Huang, S., Litt, M., and Felsenfeld, G. (2005) Genes Dev. 19, 1885–1893
43. Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002) Cell 111, 185–196
44. Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., and Khorasanizadeh, S. (2003) Genes Dev. 17, 1870–1881
45. Tamaru, H., Zhang, X., McMillen, D., Singh, P. B., Nakayama, J., Grewal, S. I., Allis, C. D., Cheng, X., and Selker, E. U. (2003) Nat. Genet. 34, 75–79
46. Jeppesen, P., and Turner, B. M. (1993) Cell 74, 281–289
47. Kourmouli, N., Jeppesen, P., Mahadevaiah, S., Burgoine, P., Wu, R., Gilbert, D. M., Bongiorni, S., Prantera, G., Fanti, L., Pimpinelli, S., Shi, W., Funede, R., and Singh, P. B. (2004) J. Cell Sci. 117, 2491–2501
48. Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinerb, D., and Jenuwein, T. (2004) Genes Dev. 18, 1251–1262
49. Annunziato, A. T., Eason, M. B., and Perry, C. A. (1995) Biochemistry 34, 2916–2924
50. Knehr, M., Poppe, M., Enulescu, M., Eickelbaum, W., Stoehr, M., Schroeter, D., and Paweletz, N. (1995) Exp. Cell Res. 217, 546–553
51. Nishioka, K., Rice, J. C., Sarma, K., Erdjument-Bromage, H., Werner, I., Wang, Y., Chuiok, S., Valenzuela, P., Tempst, P., Steward, R., Lis, J. T., Allis, C. D., and Reinerb, D. (2002) Mol. Cell 9, 1201–1213
52. Sobel, R. E., Cook, R. G., Perry, C. A., Annunziato, A. T., and Allis, C. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1237–1241
53. Annunziato, A. T., and Seale, R. L. (1983) J. Biol. Chem. 258, 12675–12684
54. Liew, C. C., Haslett, G. W., and Allfrey, V. G. (1970) Nature 226, 414–417
55. Hirs, C. H. W. (1967) Methods Enzymol. 11, 197–199
56. DeLange, R. J., Famborough, D. M., Smith, E. L., and Bonner, J. (1969) J. Biol. Chem. 244, 319–334
57. Song, O. K., Wang, X., Waterborg, J. H., and Sternglanz, R. (2003) J. Biol. Chem. 278, 38109–38112