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Quantitative profiling of histone post-translational modifications by stable isotope labeling

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Abstract

Methods for accurately quantitating changes in histone post-translational modifications are necessary for developing an understanding of how their dynamic nature influences nuclear events involving access to genomic DNA. This article describes methods for the use of *in vivo* stable isotope label incorporation for quantitating the levels of modification at specific residues in histone proteins. These methods are applicable to a wide variety of model systems and examples of their use in both mammalian cells and *Saccharomyces cerevisiae* are presented.

Keywords

Histone; Histone modifications; Chromatin; Mass spectrometry; Stable isotope labeling

1. Introduction

Post-translational modifications on histones play an intimate role in cellular processes requiring access to the genome. These modifications include small molecule modifications such as acetylation, methylation and phosphorylation as well as larger polymeric modifications such as poly ADP-ribosylation, ubiquitylation and sumoylation. In order for these modifications to actively participate in the regulation of events in the cell it is essential that they not be static. The dynamic nature of histone modifications results from the presence of specific enzyme complexes that are responsible for their addition or removal. The ability to accurately monitor how the levels of specific modifications change in response to different conditions, such as cell cycle changes, disease states or treatment with therapeutic agents, is essential for fully understanding the mechanisms by which histone modifications act. This article will describe the use of stable isotope labeling and mass spectrometry as tools for monitoring changes in levels of histone modifications *in vivo*.

A number of techniques are available for assessing changes in the levels of histone modifications. The most widely used of these techniques involves the probing of Western blots with antibodies that specifically recognize the modification of interest. A significant advantage of this method is that it can be highly sensitive. In addition, this method does not require specialized equipment and can be routinely performed in most laboratories. However, there

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are also a number of significant drawbacks. First, the critical determinant for this type of analysis is the specificity of the antibody used. While there are a large number of commercially available antibodies that were raised against peptides containing specific sites of modification, in many instances the precise specificity of these antibodies is not known. Of particular concern is the influence that modifications to residues near the site of interest can have on the specificity of the antibodies [1,2]. Hence, changes in signal on a Western blot may not be entirely due to changes in the abundance of a single modification. In addition, the use of Western blots to comprehensively assess the modification state of the core histones would require a large amount of sample, further development of antibodies and be quite labor intensive.

Direct protein sequencing has also been used to quantify levels of modification at specific residues. In this technique, the histone protein is subjected to NH₂-terminal sequencing via Edman degradation. At cycles corresponding to sites of modification, the cleaved amino acid can be analyzed for the presence of modifications and the ratio of the modified and unmodified forms determined. This method has been particularly useful when coupled with radioactive pulse-labeling to assess the modification status of newly synthesized histones [3-5]. The use of direct protein sequencing to quantitate levels of modification has been limited due to several factors. These include a requirement for relatively large amounts of sample, the presence of blocked NH₂-termini on many histone species and the difficulty in analyzing internal modification sites.

More recently, mass spectrometry has emerged as an important new tool for the quantitation of histone post-translational modifications [6,7]. The mass spectroscopic analysis of intact histone proteins that have been separated by reverse phase HPLC (LC/MS) allows for determination of the distribution of differentially modified isoforms within a sample. This approach is rapid and requires relatively small amounts of sample. However, its use for quantification of histone modifications is limited by the fact that histones containing differing levels of modifications are chemically distinct and thus may have differing ionization properties. Therefore, quantitation using LC/MS is restricted to measuring the abundance of each modified isoform relative to the total of all the isoforms present in that sample. None the less, this type of information has proven useful in identifying global patterns of histone modifications that are altered in association with a number of malignancies [8]. In addition, LC/MS analysis has shown that specific alterations in the distribution of histone isoforms occur when patients are treated with histone deacetylase inhibitors in phase I clinical trials [9].

A second limitation to the LC/MS analysis of intact histones is that modifications cannot be localized to specific residues. This can be overcome by proteolytic digestion of the histones. Precise mass analysis of the resulting peptides can localize post-translational modifications to a specific stretch of amino acids. Tandem mass spectrometry (MS/MS) can then be used to verify the sequence of the peptide and definitively pinpoint the site of modification. This is a very powerful method for the identification of histone post-translational modifications but there are critical elements that need to be controlled before it can be used to reliably quantitate levels of modification. Namely, the variable presence of modifications can influence proteolytic digestion. For example, the most commonly used protease, trypsin, cleaves proteins COOH-terminal to lysine or arginine residues unless the lysine is acetylated or di- or tri-methylated. In addition, variations in the level of modification can result in significant differences in ionization in the mass spectrometer, particularly when the charge state is affected by the modification. Both of these difficulties can be lessened by derivatization of histone lysine residues with organic anhydrides such as acetic anhydride or propionic anhydride *in vitro* [2, 10-16]. Trypsin cleavage is therefore prevented at all lysine residues regardless of their *in vivo* modification status. In addition, *in vitro* acetylation or propionylation of histone lysine residues decreases the charge of the lysine residue-containing peptides and increases chemical uniformity between peptides derived from samples whose original levels of modification

differed. Thus, there is less mass discrimination due to difference in ionization efficiency resulting in more reliable quantitation of peptide abundance.

The ability to quantitate histone modifications can be significantly enhanced by the incorporation of stable isotopes during derivatization. For example, the use of deuterated acetic anhydride induces a shift of 3 mass units in peptides for each unmodified (or mono-methylated) lysine residue present. This mass difference then provides a tag for distinguishing peptides during subsequent mass analysis. Two strategies have been used to exploit *in vitro* stable isotope labeling of histones to quantitate levels of post-translational modifications. Smith and colleagues quantitated the *in vivo* level of acetylation on lysine residues in the histone H4 NH₂-terminal tail domain by modifying the protein *in vitro* with deuterated acetic anhydride. Following this treatment, all lysine residues acetylated *in vivo* had a protiated acetyl group (42 Da) and those modified *in vitro* (unacetylated *in vivo*) had a deuterated acetyl group (45 Da). *In vitro* acetylation allowed histone H4 digested with trypsin to liberate a peptide encompassing amino acids 4–17, containing all four NH₂-terminal sites of lysine acetylation. Analysis of this peptide by MS/MS allowed determination of the ratio of protiated to deuterated acetyl groups at each lysine. This ratio then provided a quantitative measure of the level of acetylation that occurred in the cell at each of these lysine residues. This method proved crucial in quantitating the differential impact of histone H4 point mutations on specific sites of acetylation [11,12].

A second strategy involving the *in vitro* incorporation of stable isotopes was used to quantitate the enrichment of specific post-translational modifications among mammalian histone H3 variants [2]. Separate pools of histones H3.1, H3.2 and H3.3 were isolated chromatographically. All histones were first propionylated (to normalize cleavage and ionization). Following proteolysis, carboxylic acids were then converted to methyl esters with one variant being protiated and a second variant deuterated. This stable isotope labeling allows for the identification of the source of a peptide during subsequent mass analysis. Equal quantities of the variants were then mixed and analyzed by mass spectrometry. Equivalent peptides from the two variants, which contain identical modifications, appear as doublets separated by 3 Da for each ester. By comparing the abundance of each component of the doublet, the relative enrichment of specific modifications in the variants can be deduced. For example, if the doublet peaks are of equal abundance, the modifications found on that peptide are present at equal levels in both variants. Using this technique, Hake and colleagues demonstrated that histone H3.3 tends to be enriched for modifications associated with transcriptionally active chromatin, H3.2 is enriched for modifications linked to silent chromatin structure and H3.1 is enriched for both types of modifications [2].

While the incorporation of stable isotopes provides an important means for identifying and quantitating peptides containing post-translational modifications, the accuracy of these methods critically depends on the reproducibility of the *in vitro* derivatization. This issue can be circumvented through metabolic incorporation of stable isotopes. In addition, the use of stable isotopes incorporated through specific amino acids provides a mass tag that can be used to unambiguously identify peptides solely by mass without the need for MS/MS analysis [17-23]. The metabolic labeling of proteins with amino acids containing stable isotopes for quantitative proteomics has been termed either SILAC (Stable Isotope Labeling with Amino acids in Cell culture) or AACM (Amino Acid Coded Mass tagging). For clarity, we will refer to this technique as SILAC.

SILAC is likely to emerge as the method of choice for quantitating changes in histone modification levels between two conditions. Examples of the types of conditions that can be analyzed are normal vs. disease states, wild type vs. mutant backgrounds, and control vs. drug treatment. The key consideration for the use of SILAC is that this technique requires that the histones be isolated from cells that are growing and that will take up exogenously added amino

acids. The specific amino acid that is labeled can be varied depending on the specific site or modification to be examined. For the analysis of histones, deuterated lysine is a convenient choice given its abundance in histones and its role as a site of acetylation and methylation.

The basic procedure for characterizing histone post-translational modification levels using SILAC is outlined in Fig. 1. Duplicate cultures of cells are grown in either normal media or media that has been supplemented with a stable isotope labeled amino acid (heavy media). Each culture represents one of the conditions to be compared. For example, if the goal of the experiment is to quantitate the changes in histone modification that occur in a cell line when it is treated with an HDAC inhibitor, the normal culture would be left untreated while the heavy media culture would receive drug treatment. Following the appropriate treatment time, equal numbers of cells from each culture are mixed and histones are isolated from the cells. By combining cells prior to histone isolation, any error inherent in the extraction, purification and analysis of the histones is eliminated. Purified histones are proteolyzed and peptide mass mapping is performed. Again, by having histones from the experimental conditions mixed, equal levels of digestion and ionization between the samples are assured. Changes in histone modification levels induced by the drug treatment can be determined by calculating the ratio of the labeled and unlabeled component of the doublets that arise from the stable isotope incorporation. This ratio can be normalized to correct for experimental variation by using a duplicate set of cultures grown in normal and heavy media in which neither culture is drug treated.

This article will provide detailed methods for the use of SILAC to quantitatively profile changes in histone post-translational modifications. As an indication of the versatility of this procedure, we will present examples of its use to study histone modification levels in both mammalian cells and in the yeast *Saccharomyces cerevisiae*.

2. Methods

2.1. Growth and labeling of mammalian tissue culture cells

When D₄ lysine is to be used to incorporate the stable isotope label, cells are grown for at least four doubling times in lysine- and glutamine-free RPMI 1640 media (custom made by JRH Biosciences, Lenexa KS) supplemented either with standard L-lysine (Sigma, St. Louis MO) or deuterium-labeled lysine (L-lysine-4,4,5,5-D₄ HC1, Isotech Inc. Miamisburg, OH). Media is also supplemented with L-glutamine (final concentration 1 mM) and with fetal bovine serum dialyzed to remove inherent lysine (final concentration 10%) (both from Gibco, Grand Island, NY). Four doubling is sufficient to achieve full incorporation of the heavy isotope into the histone proteins. Fig. 2A shows LC/MS analysis (described below) of histone H4 isolated from cells grown in normal media or media containing D₄-lysine for 4–7 doubling periods. Each of the peaks represents a distinct modified form of H4. Human histone H4 contains 11 lysine residues and it is clear that each of the H4 peaks has a mass shift of 44 Da corresponding to full incorporation of D₄-lysine after 4 doublings and that further growth in D₄-lysine does not increase the level of incorporation.

Following labeling, cells are carefully counted, brought to equal concentrations, and then exposed to a specific experimental treatment (such as addition of a drug). Following the experimental treatment, equal numbers of control and experimental cells are mixed. Histones can then be isolated immediately or the cells can be flash frozen and stored at –80 °C. As an example of how this approach can be applied, we have used SILAC to assess the effect of the histone deacetylase inhibitor depsipeptide (DDP) on histone modifications in a lymphoblastoid cell line. For this experimental design we used four separate incubations for each cell line: **A**: Media with normal lysine; **B**: Deuterium-labeled lysine media plus DDP; **C**: Deuterium-labeled lysine media; **D**: Media with normal lysine plus DDP. At least 2×10^8 cells for each

timepoint and treatment were used. At 0, 2, 4, 8, 12 and 24 h following addition of drug, equal volumes/cell numbers of **A + B** (mix 1) and of **C + D** (mix 2) were combined and immediately centrifuged. Media was aspirated off and the cell pellets were frozen in liquid nitrogen for later histone extraction. At the zero hour timepoint only, a third mixture **A + C** was prepared. Also at 4 and 24 h cells were evaluated by annexin-FITC/propidium iodide flow cytometry to ensure that there was not significant cell death that would alter the ratios present [24]. In these experiments, there were no differences in cell growth or apoptosis between the media containing standard or deuterium-labeled lysine.

2.2. Isolation of histones from mammalian cells

Following the mixing of equal numbers of cells from the normal and isotope labeled cultures, histones are isolated. Combining the cells prior to histone isolation eliminates any error during variations during sample preparation. Fresh or frozen cell pellets are resuspended in Nuclei Isolation Buffer (NIB: 10 mM Tris-HCl pH 7.5/10 mM Na-Bu-tyrate/1.5mM MgCl₂/1.0mM CaCl₂/0.25 M Sucrose/0.5% Triton X-100/0.2mM PMSF/3 µg/mL Aprotinin/1.0 mM Benzamidine). Cell membranes are primed for lysis via a 10 min incubation on ice. Cells are then lysed using 20 gentle strokes of a tight glass/glass Dounce Homogenizer. Intact nuclei are collected by centrifugation (2000g, 5', 4 °C) and washed twice with NIB without Triton. Acid soluble proteins are extracted from the nuclei via resuspension in 0.4 N H₂SO₄ and incubation on ice for 30 min. The insoluble fraction is pelleted (16,000g, 15–, 4 °C) and the histone-rich supernatant transferred to a fresh tube. The histones are then acid-precipitated at a final concentration of 20% TCA allowing the samples to incubate, with occasional inversion, for at least 1 h (on ice or at 4 °C). The precipitated protein is collected by centrifugation as before and pellets washed twice, without resuspension, with ice-cold acetone in order to remove residual acid. The histones are then stored as pellets at –20 °C.

2.3. Growth and labeling of yeast cells

We use cells that are derived from strain BY4705 which is a commonly used laboratory strain bearing deletions for several auxotrophic marker genes [25]. One of these markers is *LYS2*, which encodes an α -aminoacidopate reductase essential for lysine biosynthesis. The lack of an endogenous *LYS2* gene ensures that the cells must take up lysine from the media in order to survive. Media (YPD/SC) and growth conditions used are typical and have been described previously with the exception that 50 ng/mL of Ampicillin is added freshly to all cultures to deter bacterial contamination [26]. A 5–10 mL seed culture is used to inoculate 100 mL cultures of synthetic complete media containing either normal L-lysine or an equal amount of D₄-lysine (0.12mg/ml). When inoculating the larger cultures, the appropriate volume of seed culture is spun down and the cells resuspended in the new media. Alternatively, if seed cultures are quite dense direct additions on the order of 1:1000 are not problematic. Cultures are grown overnight at 30 °C to an OD₆₀₀ of 0.6–1.0. It is important to note that no observable growth defects occur in the presence of D₄-lysine, as reflected by cell size, appearance and growth rate. Equal numbers of unlabeled and labeled cells, calculated from density measurements, are then combined and prepared for immediate histone extraction. We have also prepared histones from unlabeled and labeled cells individually to confirm that overnight growth is sufficient for complete incorporation of the heavy isotope label. For example, Fig. 2B shows the deconvoluted ESI mass spectra for histone H2B (HTB1) from labeled and unlabeled cells. Yeast histone H2B contains 20 lysine residues and complete incorporation of D₄-lysine would shift the mass of the protein by 80 Da. As seen in Fig. 2B the H2B peaks, each representing a differentially modified isoform, shift 79 Da after overnight growth in D₄-lysine containing media. Importantly, it is clear that incorporation of D₄-lysine does not alter the variety or relative abundance of the H2B isoforms.

2.4. Isolation of histones from yeast cells

Yeast, having a thick cell wall, must be treated differently than mammalian cells in order to isolate their nuclei. Yeast cultures are spun down (~4700g, 5', 4 °C), washed once with water to remove residual media and then resuspended in 50 mM Tris-HCl pH 7.5 containing 30 mM DTT (made fresh). The cells are then incubated for 15 min at 30 °C allowing DTT reduction of the cell wall. Cells are collected via centrifugation (2000g, 5', RT) and washed once with pre-warmed (30 °C) Spheroplasting Buffer (1.2 M Sorbitol, 20 mM HEPES pH 7.4). Cells are again collected by centrifugation, weighed and resuspended in 30 °C Spheroplasting Buffer containing 2 mg of Zymolyase 100T (US Biologicals) per gram of cells. Cells are then incubated at 30 °C with gentle agitation. During this time the progress of spheroplasting is periodically checked. Spheroplasting is assessed by diluting an aliquot of cells 1:20 into either water or spheroplasting buffer. Cell integrity is then monitored by examination under a microscope where spheroplasted cells will lyse after being diluted into water but not Spheroplasting Buffer. Spheroplasting is stopped when roughly 90% of the cells have lysed. This generally takes 30–40 min. Following spheroplasting, twice the volume of ice-cold Buffer B (1.2 M Sorbitol, 20 mM PIPES pH 6.8, 1.0 mM MgCl₂, 1 mM PMSF, 5 µL/mL Protease Inhibitor Cocktail, PIC (Sigma)) is added to the suspension and spheroplasts harvested at 2500g (5', 4 °C). Cells are lysed by resuspension in ice-cold yNIB (0.25 M Sucrose/60 mM KCl/14mM NaCl/5 mM MgCl₂/1 mM CaCl₂/15mM MES, pH 6.6/0.8% Triton X-100/1 mM PMSF/5 µL/mL PIC). Following a 20 min incubation on ice, the lysate is clarified via centrifugation as before. Isolated nuclei are then washed twice more in this fashion. While the isolation of histones from nuclei by acid extraction is rather straight forward for mammalian cells, in yeast, many proteins share a similar acid-solubility which necessitates additional washing steps to minimize the level of contaminating species. Isolated yeast nuclei are therefore incubated on ice for 15 min, three successive times in Wash Buffer A (10 mM Tris-HCl, pH 8/30 mM Na-Butyrate/ 0.5% NP-40/1.0 mM PMSF/5 µL/mL PIC/75 mM NaCl), followed by two, 10 min washes on ice with Wash Buffer B (10 mM Tris-HCl, pH 8/30 mM Na-Butyrate/0.5% NP-40/1.0 mM PMSF/5 µL/mL PIC/400 mM NaCl). Histones are then acid extracted exactly as described above for mammalian cells.

2.5. LC/MS analysis of histones

Once isolated, procedures for the analysis of histones are essentially identical regardless of their origin. The degree of stable isotope incorporation can be readily monitored by the LC/MS analysis of the intact histones proteins. Acid extracted histone mixtures are separated on a Discovery Bio wide pore C18 column (1.0 mm I.D. × 150 mm, 5 µm, 300 Å, Supelco, Bellefonte, PA, USA) by reversed phase HPLC (Waters 2690, Waters, Milford, MA). The mobile phase A is 0.1% TFA in water and B is 0.1% TFA in ace-tonitrile at a flow rate of 50 µL/min. All mobile phase reagents are used unfiltered to avoid contamination by polymeric membranes. The gradient is increased linearly from 30% B to 45% B over 2 min and then increased to 60% B over 20 min. The mobile phase is held at 60% B for 4 min and then increased to 95% B over 2 min. After washing at 95% B for 2 min, the gradient is reset to 30% B and the column is re-equilibrated for 29 min prior to injection of the next sample.

Fractions obtained from the chromatography are mass analyzed with a Micromass LCT (Micromass, Wythenshawe, UK) mass spectrometer with an orthogonal electro-spray source (Z-spray) coupled to the outlet of the HPLC. Electrospray ionization (ESI) is performed at capillary voltage of 3 kV, source temperature of 100 °C and a cone voltage of 50 V. Data are acquired in continuum mode at the rate of 1 spectrum s⁻¹. All spectra are obtained in the positive ion mode. NaI was used for external mass calibration over the *m/z* range 500–2500 Da.

2.6. Proteolytic digestion and peptide analysis

Isolated histone mixtures are resolved by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) prior to protease digestion and mass analysis of peptides. While SDS-PAGE separates the individual histone species, if necessary, additional resolution of modified isoforms can be obtained through the use of Triton-Acid-Urea (TAU) or Acid-Urea gel electrophoresis [27]. For SDS-PAGE, we use standard Tris-glycine buffers with resolving gel acrylamide concentrations of 18% (30:0.8 acrylamide:bis) and stacking gel acrylamide concentrations of 10%. The use of an 18% resolving gel can lead to difficulties with protease digestion. Reducing the acrylamide concentration to 16.5% can overcome these problems.

Following Coomassie staining of the gels, bands containing each of the core histones are excised separately into small pieces and placed into a well of a 96-well Zip-Plate (Millipore, Bedford, MA, USA). They are individually digested with trypsin by use of the Montage In-Gel Digestion Kit (Millipore, Bedford, MA). While trypsin is the protease that we use most frequently, other proteases may be more appropriate for specific applications. The gel pieces are washed twice (1 h each) in freshly made wash solution (50% methanol/5% acetic acid) followed by dehydration with acetonitrile and rehydration with 100 mM ammonium bicarbonate twice (5 min each). They are then dried in a speedvac for 2–3 min. The dried samples are rehydrated with 30 μ L of a freshly prepared solution of trypsin (20 ng/ μ L in 25 mM NH_4HCO_3) on ice for 10 min. An additional 20 μ L of 25 mM ammonium bicarbonate is added into the mixture and the samples are incubated at 37 °C for 1 h. The tryptic peptides are extracted with 50% ACN/5% formic acid three times, dried with a speedvac and dissolved in 8 μ L of HPLC grade water.

2.7. Mass analysis of peptides

The peptide solution obtained from in-gel digestion is mixed in the ratio 1:5 with a saturated solution of the matrix, α -cyano-4-hydroxy cinnamic acid (HCCA) (Bruker; Billerica, MA) in 50% ACN/0.1% TFA/50% H_2O . An aliquot of 1.0 μ L of the mixture is then spotted on the target plate and air dried prior to MS analysis. The MALDI-TOF MS instrument (Bruker Reflex III, Bruker Daltonics Inc; Bremen, Germany) is operated in linear, positive ion mode with the accelerating voltage set to 28 kV. The N_2 laser is set at the lowest threshold level necessary to generate signal. The instrument is calibrated by use of a mixture of bradykinin fragment 1–5, angiotensin I, fibrinopeptide B and ACTH fragment 18–39 (Sigma Chemical Co., St. Louis, MO, USA). The data are analyzed by use of the FlexAnalysis. Peak areas are determined using the first three isotopic peaks. To obtain good statistics from MALDI-TOF MS, each of the tryptic digests is spotted three times on the MALDI target and analyzed twice resulting in a total of 6 spectra per sample. The Dixon's Q-test is performed to remove outliers. The ratios for labeled to unlabeled peptides are then calculated. All the peptide sequences are further confirmed by nano-LC-MS/MS experiments.

2.8. Data analysis

Stable isotope labeling with D_4 -lysine facilitates peptide assignment with peptide mass fingerprinting (PMF) because the isotope distribution allows for the determination of the number of lysine residues in the peptide. Even at relatively low mass accuracy, sequence assignments can be made with higher confidence than without labeling. For example, digestion of yeast histone H4 generates a peptide with monoisotopic mass at 1178.72⁺ Th that has 4 possible assignments based on the *in silico* digest (allowing for acetylation, mono-, di- and trimethylation and phosphorylation) in protein prospector: (1) $^{68}\text{DSVTYTEHAK}_{\text{Me}2}^{77}$; (2) $^{46}\text{ISGLIYEEVR}^{55}$; (3) $^9\text{GLGK}_{\text{Ac}}\text{GGAKRHR}^{19}$; (4) $^9\text{GLGK}_{\text{Me}3}\text{GGAKRHR}^{19}$. However, this peptide had no labeled partner indicating that no lysine residues were present (data not

shown). Therefore, we can assign this peptide as $^{46}\text{ISGLIYEEVR}^{55}$ without the use of high mass accuracy instrument or tandem mass spectrometry.

For peptides that do show a labeled partner, the mass difference gives the number of lysines present in the peptide and the area of the respective peaks gives an accurate measure of the abundance of that particular species for each experimental condition. For example, a wild-type yeast strain was grown in normal synthetic media and a strain deleted for the histone acetyltransferase *Hat1p* (Δhat1) was grown in D_4 -lysine media. The *Hat1p* enzyme is highly specific for the acetylation of histone H4 lysine residues 5 and 12 and is thought to be involved in the acetylation of newly synthesized histones [28,29]. After mixing equal quantities of cells, analysis of peptides generated by the trypsin digestion of histone H4 showed a pair of peptides with masses of 530.3 and 534.4 (Fig. 3). The 4 Da mass difference indicates the presence of a single lysine and identifies this peptide as $^{13}\text{GGAK}_{\text{Ac}}\text{R}^{17}$ representing the acetylation of H4 lysine 16. Consistent with the specificity of *Hat1p*, the abundance of this peptide is not altered by deletion of the *HAT1* gene (Fig. 3). The absolute quantitation of the change in abundance of this peptide that results from the loss of *Hat1p* can be obtained by normalizing the peak areas to the control where the wild type strain is grown in both normal and D_4 -lysine media (data not shown). By analyzing all of the peptides isolated following proteolysis, a comprehensive picture of the changes occurring in histone post-translational modifications can be rapidly developed.

Using this strategy, changes in the abundance of specific histone modifications can be quantitated in a variety of experimental conditions. Using a model system like yeast readily allows for the analysis of mutants to determine the impact on histone modifications of factors that act on chromatin structure. In addition, alterations in histone modification associated with changes in cell growth or cell cycle are readily addressed. Similar questions can be approached using this strategy in mammalian cells. Importantly, the impact of potentially therapeutic agents, such as HDAC inhibitors, on histone modifications in tumor cell lines can be quantitatively and comprehensively determined through the combination of stable isotope labeling and mass spectrometry (Su, X., et al., submitted for publication).

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Fig. 1. Quantification of histone modifications by SILAC. A schematic diagram outlining the use of stable isotope labeling to quantitate changes in histone post-translational modification.

Fig. 2.

Full incorporation of stable isotopes into histone proteins in yeast and mammalian cells. (A) Deconvoluted mass spectra of human histone H4 following reverse phase HPLC separation. Histones were isolated from 697 cells grown in normal media (black) or in media containing D₄-lysine (red) for the indicated number of doubling periods. (B) Deconvoluted mass spectra of histone H2B following reverse phase HPLC separation. Histones were isolated from yeast cells grown in normal synthetic media (bottom) or after overnight growth (~8 doublings) in synthetic media containing D₄-lysine.

Fig. 3. The use of stable isotope labeling to assess levels of histone modification. MALDI-TOF MS spectra of peptides isolated following trypsin digestion of histone H4. Histone H4 was isolated from a mixture of equal quantities of wild type cells grown in normal synthetic media and Δ hat1 cells grown in D₄-lysine. Consistent with the known specificity of the Hatlp, loss of this enzyme does not effect acetylation of histone H4 lysine 16.