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An easy assay for histone acetyltransferase activity using a PhosphorImager

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ABSTRACT

A simple radiometric assay for histone acetyltransferase (HAT) activity employing a PhosphorImager is described. In the proposed procedure, following incubation of [1-^{14}C]acetyl coenzyme A (CoA), histones, and HAT enzyme, radiolabeled histones are fixed on GF/F glass microfiber filter while the excess of acetyl CoA is washed out. Afterward, the filter is exposed to a phosphor-screen and the resulting spot signals are quantified with a PhosphorImager. Given the small volumes required, the new assay reduces reagent consumption and contaminated waste. Moreover, the assay can be performed with a large number of samples simultaneously, is applicable on different protein substrates, and is adaptable to the analysis of other protein modifications.

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Histone acetylation, as well as other posttranslational modifications, is involved in gene expression regulation and other essential processes in the life of eukaryotic organisms [1]. Histone acetyltransferases (HATs)[3] catalyze the transfer of acetate from the substrate acetyl coenzyme A (CoA) to histones and possibly other protein substrates. Because of the increasing evidence revealing that HAT enzymes are important players in human pathologies [2], consistent with their essentiality in diverse cellular functions, the determination of the properties and functions of these enzymes continues to be an area of intense investigation. HAT activity can be detected and quantified by different assays [3]. The most sensitive methods employ radioactive, ^{3}H- or ^{14}C-labeled, substrate acetyl CoA. In classical assays, the radiolabeled products are captured by electrostatic binding on phosphocellulose filters [4] or by precipitation on glass microfiber filters [5] and are quantified by liquid scintillation counting. Another radioactive approach makes use of a biotinylated synthetic peptide corresponding to the N terminus of histone H4. The radiolabeled acetylated peptide is captured onto streptavidin-coated beads and quantified [6]. In another adaptation, which is suitable for high-throughput screening, radiolabeled histones are bound to the scintillant surface into the wells of a microplate (FlashPlate) producing a scintillation signal [7]. Nonradioactive assays have also been described. One of them makes use of the reduction of NAD^{+} to NADH, catalyzed by a dehydrogenase-coupled system, to monitor acetylation reaction [8]. Another employs a fluorescent dye to detect the CoA produced in the HAT reaction [9]. Finally, a recent and novel fluorescence strategy detects acetyltransferase activity by using histone H4 N-terminal synthetic peptides bearing an attached fluorophore; changes in the fluorescence signal, caused by the environmental alteration due to the acetylation, report on HAT activity [10]. Although these different HAT assay procedures have their own particular drawbacks, all of them are adequate for the accurate and reliable determination of HAT activity on wide-ranging biological preparations in vitro. However, these methods either are relatively laborious or require specific materials, and so they are not, in general, easily applicable in nonspecialized laboratories. In this article, we describe the use of a PhosphorImager for rapid and easy determination of HAT activity. The new assay, as a radioactivity-based technique, yields high sensitivity while the amount of radioactive compound and other reagents consumed and the residues and materials contaminated are decreased in comparison with more classical radiometric assays. The procedure is versatile enough for a general application in any laboratory with access to a standard PhosphorImager scanner.

Materials and methods

Substrates and recombinant HAT enzymes

Chicken core histones were isolated from nuclei of erythrocytes as described previously [11]. [1-^{14}C]Acetyl CoA (50 mCi/mmol) was obtained from Moravek. Yeast histone acetyltransferase proteins were recombinantly expressed in bacteria. His6-tagged ryGcn5 and ryEsa1 were purified by affinity chromatography on a Ni^{2+} resin [11] and ryHat1 as described previously [12].

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[3] *Abbreviations used:* HAT, histone acetyltransferase; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CPM, counts per minute; PVDF, polyvinylidene fluoride.

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Preparation of yeast enzymatic extracts and anion exchange chromatography

Whole-cell extracts containing acetyltransferase activity from the yeast *Saccharomyces cerevisiae* were prepared by the salt dissociation/ultracentrifugation method described previously [13]. Extracts were dialyzed against buffer B (15 mM Tris–HCl [pH 7.9], 0.25 mM ethylenediaminetetraacetic acid [EDTA], 5 mM 2-mercaptoethanol, 0.05% [v/v] Tween 20, 10% [v/v] glycerol, and 10 mM NaCl) and loaded onto Q-Sepharose FF columns (1.8 × 0.8 cm, bed size, GE Healthcare). After washing, bound proteins were eluted with a linear 80- to 400-mM NaCl gradient in buffer B. Fractions were collected and assayed for protein content (A_{280}) and HAT activity.

HAT assays

The PhosphorImager HAT assay was typically performed as follows. First, 12 μl of enzymatic solutions, such as cell extracts, chromatographic fractions, preparations of recombinant enzymes, or their dilutions, was mixed with 4 μg of chicken erythrocyte core histones and 0.005 μCi [1-^{14}C]acetyl CoA in a final volume of 16 μl. After incubation at 30 °C for 20 min, reactions were terminated by the addition of 4 μl of 5 × sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer (0.312 M Tris–HCl [pH 6.8], 10% [w/v] SDS, 2.5 M 2-mercaptoethanol, 30% [v/v] glycerol, and 0.005% [w/v] bromophenol blue). Portions (typically 5 μl) of each sample were manually spotted across a piece of Whatman GF/F glass microfiber filter paper at 1-cm intervals. After 5 min of air-drying, filter was soaked in fixing solution (46% [v/v] methanol and 8% [v/v] acetic acid). The same fixative with the addition of 0.1% (w/v) Coomassie brilliant blue R250, which is the solution commonly employed for the staining/fixing of polyacrylamide electrophoretic gels, can also be used. The rinsing off of dye from the filters, except on the spotted positions containing proteins, serves as a visual check of appropriate washing during the subsequent steps. Fixative incubation was carried out with shaking at room temperature for 10 min. The filter was washed twice for 10 min each with a solution containing 30% (v/v) methanol plus 5% (v/v) acetic acid, once with 50% (v/v) methanol for 5 min, and finally once with methanol for 2 min. Glass microfiber filters were then dried at 80 °C for 5 min and were exposed to the BAS-SR IP phosphor-storage image plate (FujiFilm) for variable time periods. Phosphor-screens were scanned, at 50 μm resolution, in a FujiFilm FLA-3000 fluorescent imager analyzer. The integrated intensity, representing the sum of the intensities of the pixels within the selected area, for each spot signal was quantified with Imager Gauge software (FujiFilm). The background measurement, taken from an identical area of a nonspotted region on the same filter, was subtracted from the corresponding spot data. The resulting values, in arbitrary units, represent the relative radioactivity incorporated into the histones retained by the glass microfiber filter in each dot. In some experiments, the radioactivity in the spots was determined by means of the electronic autoradiography system, the InstantImager (Packard).

As a filter binding/liquid scintillation counting HAT activity assay, the procedure described by Lopéz-Rodas and coworkers [5] was employed with minor modifications. Briefly, after incubation of the enzymatic fractions with chicken histones (80 μg) and 0.01 μCi of [1-^{14}C]acetyl CoA, in a final volume of 120 μl, reaction solutions were deposited onto GF/F glass microfiber disks. Disks were air-dried for 5 min and then submerged in 25% (w/v) trichloroacetic acid (TCA) for 20 min; washed twice more with 25% (w/v) TCA for 10 min; washed once successively with ethanol, ethanol/ethyl ether (1:1, v/v), and ethyl ether; and finally dried at 80 °C. Radioactivity on the disks was determined by liquid scintillation counting.

Results and discussion

PhosphorImager systems detect and measure radioactivity on two-dimensional probes and can replace time-consuming film autoradiography. In the course of our studies on yeast HAT(s), we frequently use a PhosphorImager detector for the measurement of ^{14}C-labeled acetylated histones resolved on polyacrylamide gels [12,13]. We wanted to try using the PhosphorImager for the quantification of whole HAT activity, present in diverse preparations, after adsorption of the radiolabeled protein products onto a paper support in a manner similar to the immunological dot-blots. We initiated this work using Whatman GF/F glass microfiber filter paper as an adsorbent, which is habitually used in our laboratory as a retention matrix of protein precipitates [14,15]. The radiolabeled acetylated proteins are readily trapped by precipitation on the glass microfiber filter, whereas the excess of free [^{14}C]acetyl CoA is removed by a washing procedure. In the new assay procedure, the [^{14}C]acetate incorporated into the matrix-retained proteins is then quantified with a PhosphorImager.

To establish the reliability of the new HAT assay procedure, our first focus was to determine the capacity of the PhosphorImager detecting ^{14}C-labeled acetylated histones retained on the GF/F glass microfiber paper and to assess whether the signal intensity obtained is linearly dependent on the radioactivity charged on the filter. With these aims, radiolabeled histones, containing [^{14}C]acetate distributed among the different classes of histones, were prepared by incubation of chicken core histones and [1-^{14}C]acetyl CoA in the presence of a mix of the recombinant yeast enzymes Hat1, Esa1, and Gcn5 and were recovered by TCA precipitation [14]. Equal volumes of a series of solutions of radiolabeled histones, with different specific radioactivity, were spotted onto a GF/F glass microfiber filter, which was soaked with fixative, washed, and dried as described above. Filters were exposed to phosphor-screens for various time periods (1–17 h), and the screens were scanned with a PhosphorImager (Fig. 1A). A good signal/background ratio was obtained at all exposure times. A signal was visualized after 10.5 h exposure over dots with only 30 to 60 counts per minute (CPM). For 1 h exposure, a radioactivity between 180 and 300 CPM per spot was necessary to make the label apparent (Fig. 1A). The integrated intensity of the spot signals was determined and plotted against the deposited CPM (Fig. 1B). A clear linear relationship, for all exposure times, was obtained without reaching saturation within the extent of charged radioactivity. Even after only 1 h exposure, it was possible to quantify the spot signals and to observe a linear increasing of the intensity values with the specific radioactivity. These results show the validity of the glass microfiber filter, in combination with the quantification with a PhosphorImager, to determine the relative content of radioactivity of histones.

When various solutions of ^{14}C-labeled acetylated histones at different concentrations (0.01–1.0 mg/ml) were deposited onto the GF/F filter, remarkably a linear response was also obtained (Fig. 1C). Therefore, the efficiency of histone trapping on the glass microfiber paper is apparently not dependent on the protein concentration, at least within the range of concentrations and under the experimental conditions employed. In contrast, the original report describing the phosphocellulose P81 filter for a HAT assay showed a decrease of the retention efficiency of labeled histones on the P81 filters with concentrations lower than 0.5 mg/ml [4]. The ineffective retention could be palliated by adding cold histones, but not bovine serum albumin, as carrier [4].

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