Analytical Biochemistry 457 (2014) 51–58

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Nucleosome acetylation sequencing to study the establishment of chromatin acetylation

Chitvan Mittal, Melissa J. Blacketer, Michael A. Shogren-Knaak *

*Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA*

ARTICLE INFO

*Article history:*
Received 26 February 2014
Received in revised form 15 April 2014
Accepted 18 April 2014
Available online 24 April 2014

*Keywords:*
Chromatin
Acetylation
Enzymology

ABSTRACT

The establishment of posttranslational chromatin modifications is a major mechanism for regulating how genomic DNA is utilized. However, current *in vitro* chromatin assays do not monitor histone modifications at individual nucleosomes. Here we describe a strategy, nucleosome acetylation sequencing, that allows us to read the amount of modification at each nucleosome. In this approach, a bead-bound trinucleosome substrate is enzymatically acetylated with radiolabeled acetyl CoA by the SAGA complex from *Saccharomyces cerevisae*. The product is digested by restriction enzymes that cut at unique sites between the nucleosomes and then counted to quantify the extent of acetylation at each nucleosomal site. We find that we can sensitively, specifically, and reproducibly follow enzyme-mediated nucleosome acetylation. Applying this strategy, when acetylation proceeds extensively, its distribution across nucleosomes is relatively uniform. However, when substrates are used that contain nucleosomes mutated at the major sites of SAGA-mediated acetylation, or that are studied under initial rate conditions, changes in the acetylation distribution can be observed. Nucleosome acetylation sequencing should be applicable to analyzing a wide range of modifications. Additionally, because our trinucleosomes synthesis strategy is highly modular and efficient, it can be used to generate nucleosomal systems in which nucleosome composition differs across the array.



In eukaryotes, the utilization of genomic DNA for processes such as transcription, replication, and repair is usually preceded by posttranslational modification of its chromatin [1]. Typically, these modifications occur over multiple nucleosomes and include different types of modifications on distinct histone residues. For example, during the transcription of inducible genes, nucleosomes of promoter regions can exhibit histone H3K4 methylation, H3 and H4 histone tail acetylation at multiple lysines, and histone H3S10 phosphorylation [2–8]. These modifications can be highly dynamic and can affect the establishment of additional modifications. For example, H2BK123 ubiquitination precedes and is required for H3K4 methylation [9,10].

*In vitro* assays offer a potentially powerful tool for elucidating how chromatin modifications are established by controlling the composition of the reaction components and by providing quantitative information on reaction rates and distributions. However, current *in vitro* assays do not provide information on how histone modifications are established across a span of nucleosomes. On one hand, assays have been used to carefully define the sites and kinetics of enzymes toward histone tail peptides [11–14], but provide a relatively limited approximation of the physiological substrates, chromatin. One the other hand, biochemical methods can generate nucleosomal arrays, but discerning the extent of modification across different nucleosomes is difficult.

To remedy these problems, we have established a method, nucleosome array acetylation sequencing, which allows us to read the extent of nucleosome modification across a well-defined oligonucleosome substrate. The enzyme we have chosen to focus on for our initial studies is the Spt-Ada-Gcn5-acetyltransferase (SAGA)[1] complex from *Saccharomyces cerevisae*. This multisubunit complex, first isolated in budding yeast and subsequently found to be present in a wide range of eukaryotes [15], facilitates the transcriptional initiation of inducible genes, such as developmental genes in humans [16–18]. One way it accomplishes this is by acetylating histone lysines in chromatin, where four lysine residues in histone H3 are the major sites of acetylation [19,20]. Further, SAGA contains domains that recognize histone acetylation, which potentially helps mediate the spread of SAGA-mediated nucleosome acetylation

* Corresponding author. Fax: +1 5152940453.
*E-mail address:* knaak@iastate.edu (M.A. Shogren-Knaak).

[1] *Abbreviations used:* SAGA, Spt-Ada-Gcn5-acetyltransferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

http://dx.doi.org/10.1016/j.ab.2014.04.024
0003-2697/

across multiple nucleosomes in inducible gene promoters [21]. Studying how SAGA acetylates a multiple nucleosome system offers the potential to generate new insights into the mechanism by which histone modifications are established across a chromatin region.

## Materials and methods

### Chemicals

Buffer components were purchased from either Fisher Scientific or Sigma Aldrich. [H3]-Acetyl CoA with a specific activity of 16.9 Ci/mmol was purchased from Moravek. Restriction endonucleases, MNase, T4 DNA ligase, and streptavidin-coated magnetic beads were purchased from NEB.

### Histone octamer preparation and mononucleosome assembly

Recombinant wild-type and tetra-alanine (K8A, K14A, K18A, K23A) mutant H3 *Xenopus laevis* histones were expressed in *Escherichia coli* and purified as described previously [22]. The purity of the histones was assessed by SDS–PAGE and quantitated using UV spectroscopy. Histone octamer was prepared using the four canonical histones H2A, H2B, H3, and H4, as described previously [23]. The 601-177-1 strong positioning DNA sequence was prepared, as described previously [24,25]. Mononucleosomes were assembled by rapid dilution as described previously [26,27] and analyzed by 4% native PAGE to assess the degree of homogeneity and saturation. We observed that the innermost nucleosome, nucleosome 1, could adopt two different positions, with respect to the DNA.

### SAGA expression and purification

Endogenous *S. cerevisiae* SAGA complex containing Spt7-TAP was affinity-purified as described previously [28]. The purified enzyme was quantitated by Western blotting, using recombinant Gcn5 as a standard. The activity of the purified complex was tested on H3 peptide using a filter binding assay [29].

### Biotinylated adapter

Oligonucleotides 5′-/5PCBio/ACGACGGCCAGTGAACCACGATT-3′ and 5′-/5Phos/GTGGTTCACTGGCCGTCGT-3′ were purchased from IDT. To form a double-stranded adapter, the oligos were mixed, each at a final concentration of 30 μM, in 1X NEB T4 DNA Ligation buffer (50 mM Tris–HCl at pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT), in a final volume of 50 μl. The mixture was heat denatured at 96–98 °C in a heating block for 10 min. Subsequently, the heating block was turned off and the temperature was allowed to cool to 33 °C. The oligos were then placed at RT for 5 min before transfer to ice and subsequent storage at −20 °C. The annealed product, a 19-bp double-stranded adapter with a 4 nucleotide overhang on the nonbiotinylated end, was analyzed by 20% SDS–PAGE to assess the purity.

### Synthesis of the trinucleosome on beads

For a typical synthesis, 4.66 pmol of biotinylated adapter was attached to 50 μl of streptavidin-coated Dynabeads slurry (4 mg/ml suspension) by incubation in 1X binding buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl) at room temperature for 10 min with occasional agitation. The beads were washed 3 times with 50 μl 1X binding buffer prior to adding the adapter. The adapter-bound beads were pulled to the side of the tube using a magnet and the supernatant was removed. The amount of 6.99 pmol of 601-177-1 mononucleosomes was added one at a time in a set of sequential ligations in the following order: mononucleosome 1, mononucleosome 2, mononucleosome 3. Each ligation was done in a final volume of 40 μl in 1X ligation buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT; pH 7.5) and 800 U of T4 DNA ligase. The ligation reactions were allowed to proceed at room temperature for 12–16 h, after which excess unbound nucleosome was washed off.

This first nucleosome, referred to as nucleosome 1, has 80 bp of additional sequence upstream in the linker DNA as compared to nucleosomes 2 and 3, and the upstream end forms a BstXI restriction site upon ligating with the adapter. The linker DNA between ligated nucleosomes 1 and 2 contains a BglI restriction site, whereas the linker DNA between ligated nucleosomes 2 and 3 contains a PflMI site.

The resultant trinucleosome was digested with BstXI, BglI, or PflMI at 37 °C for 5 h and analyzed by 4% native PAGE to assess the quality of the ligation product. The trinucleosome was stored, on beads, at 4 °C at a final concentration of 150 nM.

### Acetylation sequencing of trinucleosome

WT or mutant trinucleosome, at a final concentration of 75 nM of trinucleosome, was acetylated under saturating or subsaturating conditions by the SAGA complex under standard HAT assay conditions [30]. For both saturation and subsaturation conditions, the substrate was acetylated at 30 °C in 1X HAT buffer (25 mM Tris–Cl, pH 7.5, 50 mM KCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 2.66 μM unlabeled, 5.33 μM radiolabeled acetyl CoA with a specific activity of 8.45 Ci/mmol) at a final volume of 50 μl. For saturation conditions, acetylation was allowed to occur for 4 h by 60 nM final SAGA, whereas the reaction took place for 10 min for subsaturation conditions by 10 nM final SAGA. The beads were washed 5 times at RT with 50 μl 1X washing buffer (100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100). They were then washed an additional 11 times, with a 7-min incubation at 37 °C between washes, for saturation conditions and an additional 7 times, with 37 °C incubation as above, for subsaturating conditions. After washing, the beads were resuspended in 50 μl 1X digestion buffer (100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100). The 10-μl aliquots were transferred to 4 fresh tubes. In order to minimize handling and reduce the total time for the assay, we chose to count nucleosomes generated from a single round of digestion. The samples were either digested with PflMI (4 U), BglI (4 U), BstXI (4 U), or mock digested at 37 °C for 5 h in a final volume of 20 μl. Because 10–20% of nucleosomes were not liberated by BstXI digestion alone, these samples were further digested with MNase (1 U) under conditions similar to the other digestions, for 10 min. The supernatants were added directly to vials containing 9 ml of Scintiverse BD LSC cocktail (Fisher Scientific). All samples were counted using a Packard tri-carb 1600 TR liquid scintillation analyzer. Results were analyzed from 4 independent trials of each experiment.

### Testing of nucleosome stability under assay conditions

Wild-type trinucleosome was subjected to the assay conditions described above with one exception: only cold acetyl CoA was used. The trinucleosomes were then digested with PflMI or BglI. Following removal of the supernatant, additional digestions were performed on the remaining bead-bound product. The beads were resuspended in 1X digestion buffer and digested with BstXI or mock digested in a final volume of 20 μl, at 37 °C for 5 h. The supernatants were then analyzed by 4% native PAGE.

*Comparison of nucleosome acetylation on bead and in solution*

DNA for mononucleosome 1 and the biotinylated adapter were ligated and purified. Mononucleosome was then prepared with this DNA and wild-type octamer. For acetylation assays, 7.5 nM SAGA was used with 55 nM mononucleosome under the reaction conditions described above. For the solution assay, all components were mixed and the reaction was allowed to proceed for 8 min before streptavidin beads were added. After 2 min, the beads were washed extensively and then counted as above. For the bead assay, mononucleosomes were first incubated with the streptavidin beads for 2 min. The remaining reaction components were then added and the reaction was allowed to proceed for 10 min. Beads were then washed and counted. It was found that 2 min of incubation was sufficient for complete nucleosome binding. To determine background signal, mock reactions were performed without SAGA.

## Results

To better understand how chromatin-modifying enzymes establish histone acetylation across a chromatin substrate, we developed an on-bead acetylation sequencing assay (Fig. 1). In this strategy, a standard histone acetyltransferase assay is performed on a trinucleosome substrate that is attached to streptavidin-coated magnetic beads, where the SAGA complex is the enzyme and radiolabeled acetyl CoA is the acetyl donor. After removing unincorporated acetyl CoA, the acetylated trinucleosome is digested with different restriction endonucleases to liberate specific nucleosomes. The amount of radioactivity is quantified by liquid scintillation counting, allowing us to ultimately measure the extent of acetylation for each nucleosome.

*Synthesis of cleavable trinucleosome substrate*

As a first step, we needed to generate a bead-bound trinucleosome substrate with unique restriction sites between each nucleosome (Fig. 1). In previous work [26], we had generated short nucleosomal arrays by ligating mononucleosomes with different nonpalindromic overhangs together in solution. While this strategy was effective, it was difficult to separate ligated products that were incomplete from the desired array. As an alternative, we investigated a solid-phase nucleosomal array synthesis approach (Fig. 2A). The first step in this strategy was to immobilize a biotinylated, double-stranded DNA adapter to streptavidin-coated magnetic beads. The binding between the biotin and the streptavidin occurred very rapidly and completely (data not shown). Following

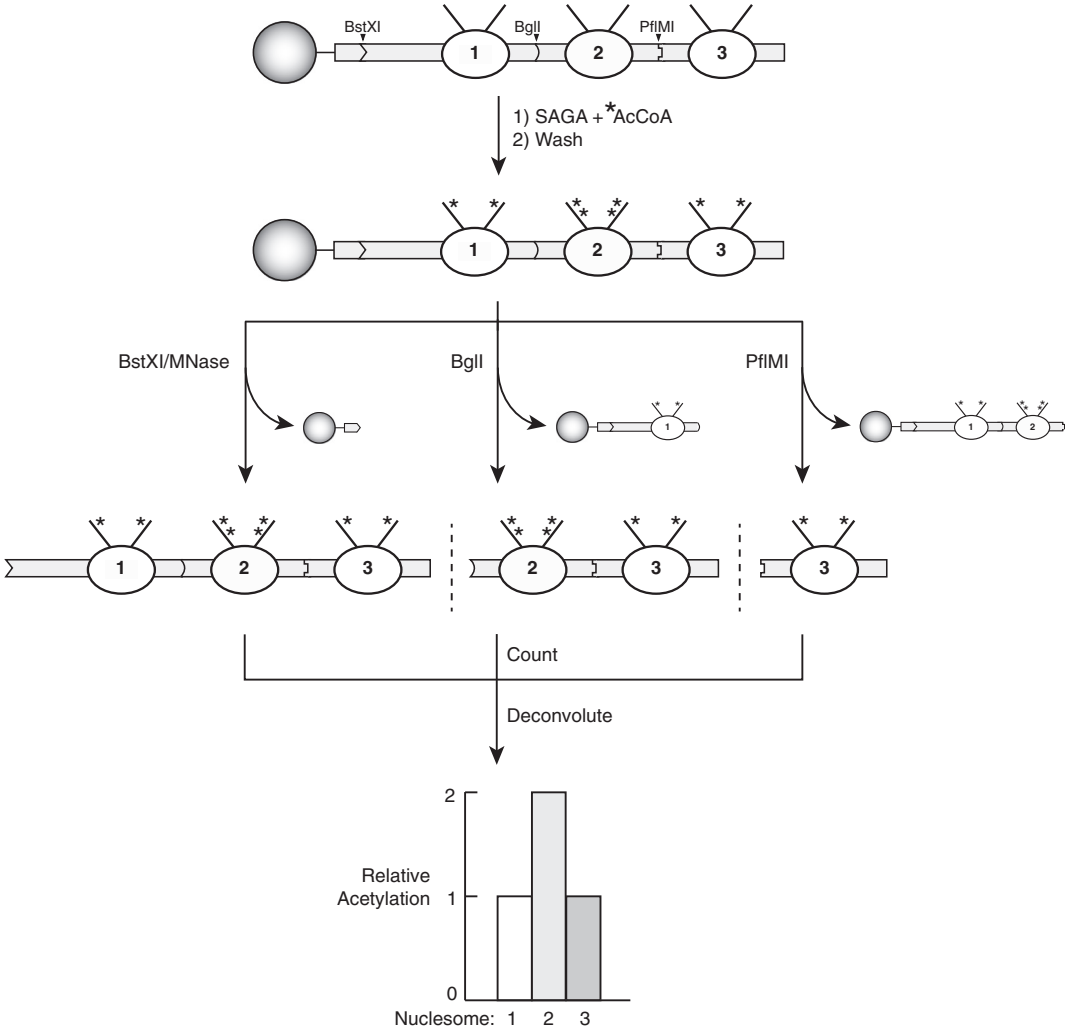


**Fig.1.** Nucleosome acetylation sequencing strategy. The immobilized oligonucleosomal substrate is enzymatically acetylated with radiolabeled acetyl CoA and subsequently washed to remove unincorporated acetyl CoA. Desired nucleosomes are then cleaved off of the bead using specific restriction endonucleases to yield mono-, di-, and trinucleosomes. Released fragments are counted by scintillation to quantify the extent of nucleosome acetylation. The acetylation pattern shown is meant as a possible example of differential nucleosome acetylation.

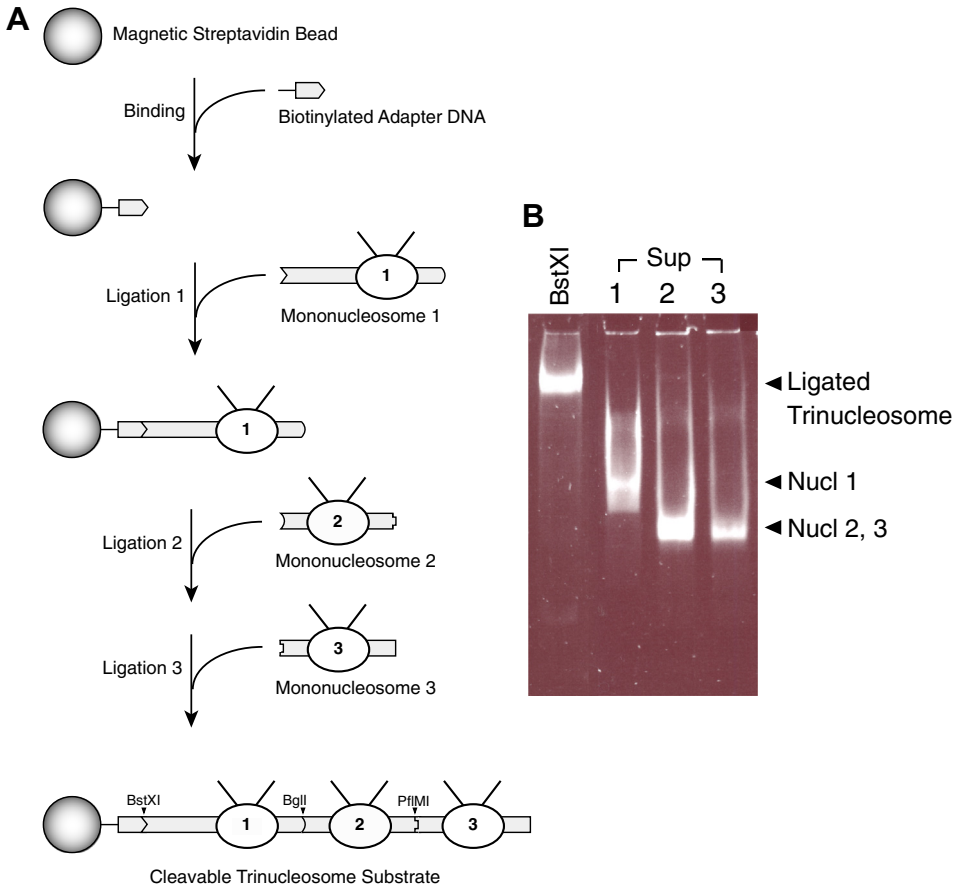


**Fig.2.** Solid-phase synthesis of bead-bound trinucleosome substrates. (A) Schematic representation of substrate synthesis. A double-stranded 19-bp biotinylated adapter with a 4 nt single-stranded overhang opposite the biotinylated end is first bound to streptavidin-coated magnetic beads. Mononucleosomes are then sequentially ligated, allowing different kinds of nucleosomes to be linked together. Unique nonpalindromic restriction endonuclease sites are present in the linker DNA between each nucleosome. (B) Characterization of reactants and products in the solid-phase synthesis of bead-bound trinucleosome substrate. A 4% native PAGE was used to resolve mononucleosome in the supernatant from the ligation steps (Lanes 2–4) and to characterize the fully ligated product liberated from the bead by digestion with BstXI (first lane). DNA-containing components were visualized by SYBR Gold staining. The mononucleosome attached to the bead first contains 80 bp more DNA than the other two mononucleosomes.

binding of the adapter, mononucleosomes were then ligated sequentially, first to the adapter and then to each other, with extensive washing between ligation steps. An excess of mononucleosomes was used in each ligation step to drive ligations to completion and could be observed in the ligation supernatant (Fig. 2B, lanes "Sup 1–3").

To characterize the fully ligated product, the trinucleosome was liberated from the bead by BstXI digestion. Native gel analysis of this product (Fig. 2B) suggests that it is a single product with no incomplete ligation intermediates present. Further, it appears that using mononucleosome with different nonpalindromic overhangs prevents incorporation of more than one nucleosome per round of ligation.

*Validation of acetylation sequencing assay*

For nucleosome acetylation sequencing to be useful, a number of criteria must be fulfilled. One requirement is that acetylation signal should be due to enzyme-mediated acetylation of the nucleosomes, not because of nonspecific acetylation or acetyl CoA retention. Additionally, this acetylation should be detectable with high sensitivity and reproducibility. To this end, we looked at acetylation levels of bead-based substrates with different components of the acetylation reaction present. In the absence of acetyl CoA, SAGA, or nucleosome, we saw that the level of acetylation was relatively low on the bead prior to digestion (Fig. 3A, left panel) and in the supernatant after digestion (Fig. 3A, right panel). This indicates that there was not significant signal from something besides the acetyl group, nonenzymatic substrate acetylation, bead acetylation, or nonspecific acetyl CoA retention. On the other hand, when all components were present, a robust signal was seen on the bead prior to digestion (Fig. 3A, left panel) and in solution after digestion (Fig. 3A, right panel). This signal was significantly greater than those observed in our control reactions, indicating that enzyme-mediated nucleosome acetylation could be detected with good specificity and sensitivity. The amounts of signal on the bead prior to digestion and in solution after digestion were relatively similar, suggesting that countings on bead and in solution are comparable. However, because initial results with the liberated nucleosomes were more reproducible, as judged from the relatively small size of the error bars relative to the total signal (Fig. 3A), all subsequent experiments were done in solution.

Another requirement of the acetylation sequencing assay is that each nucleosome be removable from the bead quantitatively, specifically, and without disturbing the integrity of the nucleosomes. After wild-type trinucleosomes were acetylated by SAGA, they were subjected to restriction enzyme digestion and analyzed by native gel electrophoresis (Fig. 3B). Digestion of the acetylated trinucleosome by PflMI liberated nucleosome 3 specifically, as no other digestion products were observed. The liberated mononucleosome was also stable to the assay conditions, as no free DNA was seen. Moreover, the digestion was complete. Following PflMI digestion and removal of the liberated product, further digestion of the

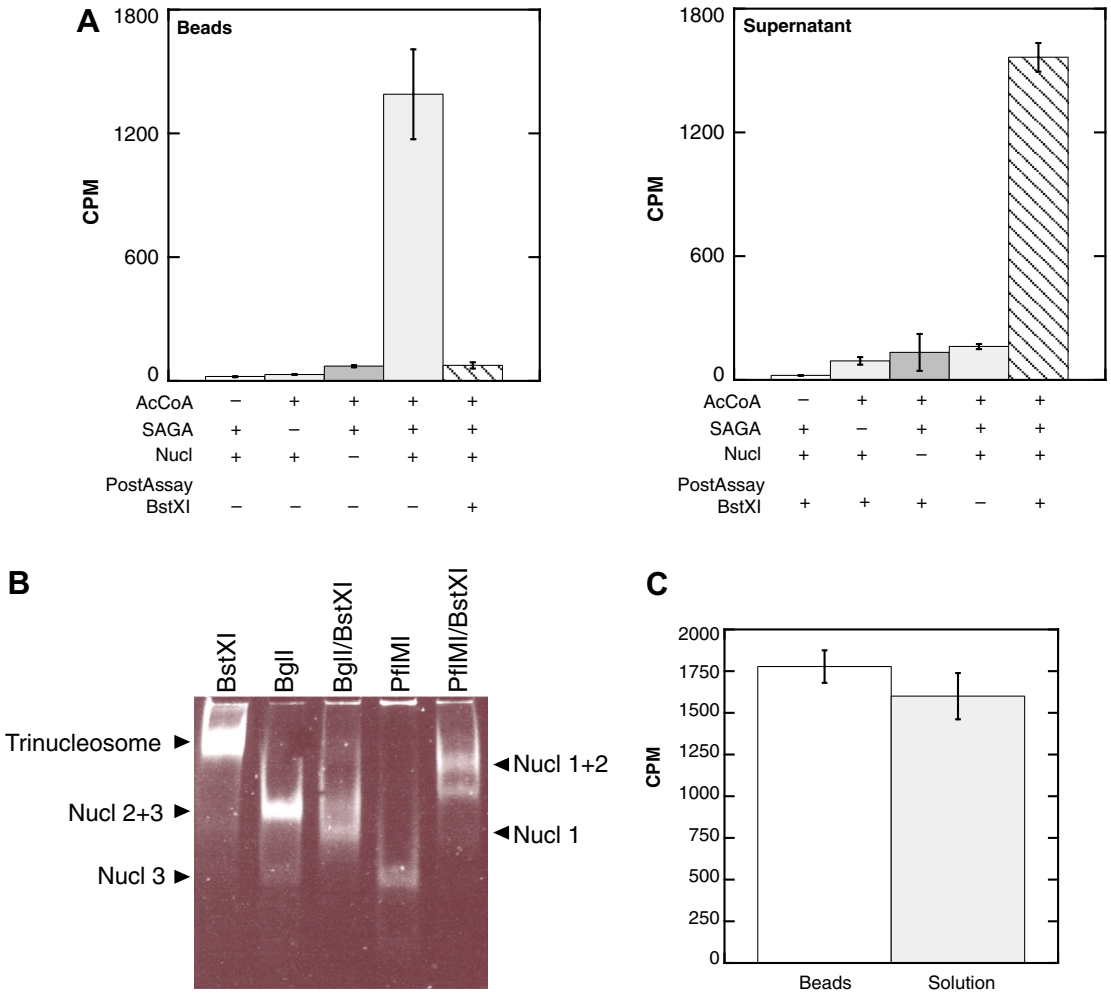


**Fig.3.** Demonstration of the specificity and robustness of the acetylation sequencing assay. (A) Investigation of the specificity of radiolabeled acetyl CoA incorporation. Shown are the amounts of radioactive signal after acetylation assays run with different components. The left panel shows signal on the beads, while the right panel shows the signal in the supernatant fractions. Error bars represent standard deviations from three independent trials. (B) Characterization of the extent of nucleosome liberation, as well as nucleosome stability. Following restriction enzyme digestion, products were analyzed by 4% native PAGE. Products of a single digestion are shown in lanes labeled with a single restriction enzyme. Lanes labeled with two restriction enzymes indicate that after the beads were treated with the first enzyme and washed, the beads were then digested with the second enzyme, and this liberated product was analyzed. The two bands in lane PflMI/BstXI are consistent with variable nucleosome positions on the same dimer DNA. (C) Comparison of nucleosome acetylation reaction efficiency on beads versus in solution. Shown is the total amount of nucleosome acetylation on the bead, where nucleosomes were either first acetylated and then bound to beads, or first bound to beads and then acetylated. Acetylation experiments were performed in triplicate. Background reactions (no SAGA) were performed once and subtracted from the acetylation bead counts.

bead by BstXI generated dinucleosome product. If the PflMI digestion had been incomplete, some trinucleosome product would have been expected in the subsequent BstXI digestion. Similarly, digestion of the acetylated trinucleosome product between the first and the second nucleosomes with BglI resulted exclusively in dinucleosome product (nucleosomes 2 + 3) with only mononucleosome (nucleosome 1) left on the bead. BstXI digestion of the acetylated trinucleosome product generated only trinucleosome product. Thus, our restriction digestion analysis indicates that digestion at all three restriction sites is highly efficient and nondisruptive. Additionally, they further support the integrity of the synthesized trinucleosome substrate. While these results suggest that performing multiple digestions is possible, subsequent experiments were performed with single digestion for consistency and to minimize the handling of the substrates.

To determine if acetylation is affected by attachment of the trinucleosome to the bead, assays were performed on mononucleosomes in solution and immobilized on a bead (Fig. 3C). Comparison of the total acetylation between the two substrates shows that they are identical, suggesting that attachment of the nucleosomes to a bead does not significantly change how SAGA acetylates its substrate.

### *Characterization of model trinucleosome acetylation*

Because our initial experiments demonstrated the feasibility of our acetylation sequencing strategy, we applied this strategy to studying SAGA-mediated nucleosome acetylation with different nucleosomal substrates. Our initial studies were performed under saturating acetylation conditions, where acetylation reactions were run with concentrations of SAGA near those of the nucleosome substrate (60 and 75 nM, respectively), and over long periods of time (4 h). Under these conditions, we expected that nucleosome acetylation should be largely complete, and thus not differ significantly between the three nucleosomes. Indeed, all three nucleosomes were labeled to nearly the same extent under these conditions (Fig. 4A). However, acetylation sequencing could also report on differences in the pattern of nucleosome acetylation. When assays were performed under saturating acetylation conditions on a substrate in which the central nucleosome lacked the

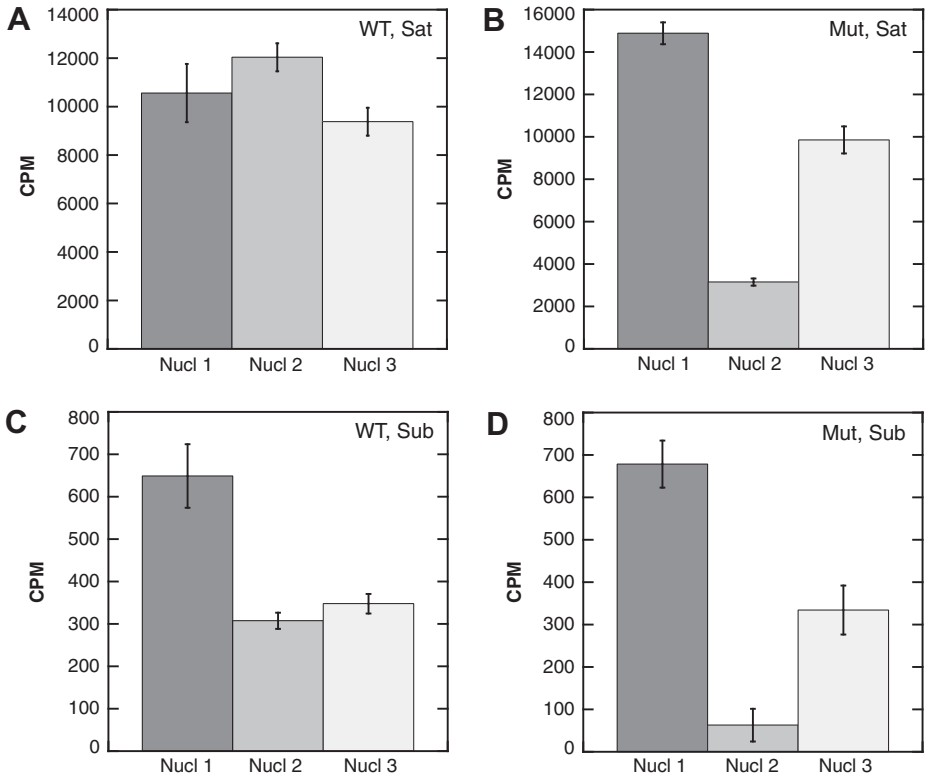


**Fig.4.** Acetylation sequencing of wild-type and mutant trinucleosomes under saturating and subsaturating acetylation conditions. Trinucleosomes were acetylated by the yeast SAGA complex and then subjected to acetylation sequencing. The trinucleosome substrate contained either three wild-type nucleosomes (A and C) or two wild-type nucleosomes flanking a central nucleosome in which the four major sites of SAGA acetylation (K9, K14, K18, K23) were mutated to alanine in the histone H3 tail. Assays were performed under saturating acetylation conditions (A and B) or subsaturating acetylation conditions (C and D). Error bars represent standard deviations from four independent trials.

major sites of SAGA acetylation (Fig. 4B), nucleosome 2 was acetylated significantly less, while the other two nucleosomes were acetylated to comparable levels.

These experiments were also performed under subsaturating conditions. For the wild-type trinucleosome (Fig. 4C), the total amount of acetylation is significantly diminished, as is expected because the acetylation assay was performed with significantly less enzyme (6-fold decrease) and time (24-fold decrease) than for the saturating conditions. The pattern of nucleosome acetylation is also altered. There is a preference for nucleosome 1, the innermost nucleosome (closest to the bead) with the other two nucleosomes, nucleosomes 2 and 3, showing roughly half as much acetylation. For experiments under similar conditions with the trinucleosome with the mutated central nucleosome, the central nucleosome (nucleosome 2) is poorly acetylated, while nucleosome 3 is again acetylated to a level of about half that of nucleosome 1.

## Discussion

### *Interpretation of acetylation sequencing patterns*

While insightful from a qualitative perspective, the acetylation distribution can also provide quantitative kinetic information. During the course of an acetylation reaction, each nucleosome within an array competes as a substrate, where the extent of acetylation of a particular nucleosome depends on the strength with which it binds to the enzyme, its acetylation turnover, and the concentration of the unacetylated nucleosome [31]:

$$\frac{v_{\mathrm{nuc}_x}}{v_{\mathrm{nuc}_y}} = \frac{\left(\frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}}\right)\mathrm{nuc}_x[\mathrm{nuc}_x]}{\left(\frac{k_{\mathrm{cat}}}{K_{\mathrm{M}}}\right)\mathrm{nuc}_y[\mathrm{nuc}_y]}.$$

Here $v_{\mathrm{nuc}}$ is the instantaneous rate of nucleosome acetylation under steady-state conditions ($d[\mathrm{acet}]_{\mathrm{nuc}}/dt$), $k_{\mathrm{cat}}/K_{\mathrm{M}}$ is the specificity constant, and [nuc] is the equilibrium concentration of free nucleosome.

Under certain circumstances, it is possible to directly compare the relative amounts of nucleosome acetylation at two nucleosomes to determine their relative ratio of specificity constants, thereby quantifying relative substrate preference. However, under saturating acetylation conditions (Fig. 4A and B), this is not possible. In these experiments, large amounts of enzyme are used (1:1.25 enzyme to each nucleosome) and reactions are allowed to proceed for long times (4 h total). Under these conditions, the total amount of radioactive signal corresponds to nearly 8 acetylations/nucleosome, suggesting complete acetylation of the major nucleosomal sites (four sites in each of the two H3 tails). Thus, the total amount of acetylation at each nucleosome does not reflect its acetylate rate ($v_{\mathrm{nuc}}$), and it would be expected that little difference in acetylation distribution between nucleosomes would be observed even with differences in specificity constants. Interestingly, under exhaustive acetylation conditions, some acetylation is observed for the nucleosome in which the major sites of H3 acetylation have been mutated to alanine (Fig. 4B). Others have observed that additional lysines on H2B can be acetylated by SAGA, although the extent of this acetylation tends to be relatively modest [19]. However, under exhaustive acetylation conditions the modification of these sites could account for acetylation signal above background.

In contrast to the assays performed under exhaustive acetylation conditions, it should be possible to determine approximate specificity constant information for the assays performed under subsaturating acetylation conditions (Fig. 4C and D). In these experiments significantly less enzyme is used (1:7.5 enzyme to

each nucleosome) and the reaction is run for a much shorter time (10 min). Under these conditions, the amount of acetylation is roughly 5% of that observed under the saturating conditions. Thus, we can approximate the equilibrium concentration of unbound, unacetylated nucleosome as the initial nucleosome concentration (i.e., $[nuc_x] = [nuc_x]_o$). Further, because the initial concentrations of each unacetylated nucleosome in the trinucleosomal array are the same ($[nuc_x]_o = [nuc_y]_o$), the ratio of their concentrations is unity (i.e., $([nuc_x]_o/[nuc_y]_o = 1$). Since only 5% of the major acetylation sites appear to be acetylated, the ratio of the instantaneous rates for each nucleosome can also be approximated as the ratio of total acetylation per nucleosome ($v_{nucx}/v_{nucy} = (d[acet]/dt)_{nucx}/(d[acet]/dt)_{nucy} = (\Delta[acet]/\Delta t)_{nucx}/(\Delta[acet]/\Delta t)_{nucy} = \Delta[acet]_{nucx}/\Delta[acet]_{nucy}$). Using this information, our data suggest that nucleosomes 2 and 3 have identical specificity constants, while the specificity constant for nucleosome 1 is roughly 2-fold greater than the other nucleosomes. It should be also noted that, because of the approximations made and because not all acetylation sites within a nucleosome have identical acetylation kinetics, the specificity constants should more appropriately be referred to as apparent specificity constants.

Because the specificity constant is composed of both $k_{cat}$ and $K_M$, we cannot tell which kinetic parameter contributes to the apparent difference in specificity constant between innermost nucleosome 1 and the others. It is expected that the chemical step in acetylation does not differ between nucleosomes; making it likely that $k_{cat}$ is unaltered. This would suggest that the difference is due to altered $K_M$s between nucleosomes and is consistent with an increased binding affinity for nucleosome 1. Unpublished data from our lab suggest that SAGA has a strong affinity for free DNA, and nucleosome 1 does contain a longer stretch of DNA relative to the other nucleosomes. Additionally, a number of chromatin-interacting factors are known to show preferential binding of nucleosomes with large regions of free DNA [32,33]. Future studies will be necessary to conclusively establish the basis for this difference in specificity.

### *Potential extensions of acetylation sequencing*

Our acetylation sequencing assay can be extended a number of different ways. By using a more extensive range of time points, it should be possible to view the progression of nucleosome acetylation from initial establishment to completions to better understand how intermediate nucleosome acetylation states affect the course of the acetylation reaction. With more initial time points and variation in substrate concentrations, it may also be possible to perform more traditional initial rate steady-state kinetics to obtain separate $k_{cat}$ and $K_M$ values for each nucleosome. To do so requires rapid quenching of acetylation, which should be possible because magnetic beads can be removed from solution relatively quickly (less than 10 s). Currently, our assay does not report on the distribution of acetylation within a given nucleosome, but our ability to isolate individual nucleosomes should make this feasible. Specifically, resolving the histones electrophoretically and detecting which bands are radiolabeled can determine which histones are acetylated [30]. Microsequencing of the isolated histones can determine which amino acids are acetylated and their extent [24]. Finally, we note that acetylation sequencing reactions do not require that either substrate or enzyme concentration be below the $K_M$ of the reactions. This is because substrate nucleosomes are in direct competition with one another, and acetylation activity will simply partition between substrates according to the specificity factor equation described above.

The nucleosome acetylation sequencing strategy should also be applicable to other substrates and enzymes. Because the on-bead solid-phase ligation strategy is highly modular and efficient, it should be suitable for a wide range of oligonucleosome products and lengths, including those incorporating mononucleosomes with different histone variants, posttranslational modifications, DNA sequences, and DNA lengths. Studies with different histone-modifying enzymes should be feasible, assuming that the high degree of specificity and sensitivity exhibited by SAGA-mediated acetylation is demonstrated. Finally, it should be possible to study how other chromatin-associated factors, such as transcriptional activators and ATP-dependent chromatin remodeling complexes, affect the establishment of chromatin modifications.

## Acknowledgment

This work was supported by a grant from the American Cancer Society – United States (RSG-12-065-01-DMC).

## References

[1] A.J. Bannister, T. Kouzarides, Regulation of chromatin by histone modifications, Cell Res. 21 (2011) 381–395.
[2] B.D. Strahl, R. Ohba, R.G. Cook, C.D. Allis, Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in *Tetrahymena*, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 14967–14972.
[3] T.R. Hebbes, A.W. Thorne, C. Crane-Robinson, A direct link between core histone acetylation and transcriptionally active chromatin, EMBO J. 7 (1988) 1395–1402.
[4] D.Y. Lee, J.J. Hayes, D. Pruss, A.P. Wolffe, A positive role for histone acetylation in transcription factor access to nucleosomal DNA, Cell 72 (1993) 73–84.
[5] P. Cheung, K.G. Tanner, W.L. Cheung, P. Sassone-Corsi, J.M. Denu, C.D. Allis, Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation, Mol. Cell 5 (2000) 905–915.
[6] W.S. Lo, R.C. Trievel, J.R. Rojas, L. Duggan, J.Y. Hsu, C.D. Allis, R. Marmorstein, S.L. Berger, Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14, Mol. Cell 5 (2000) 917–926.
[7] S.J. Nowak, V.G. Corces, Phosphorylation of histone H3 correlates with transcriptionally active loci, Genes Dev. 14 (2000) 3003–3013.
[8] S. Thomson, A.L. Clayton, L.C. Mahadevan, Independent dynamic regulation of histone phosphorylation and acetylation during immediate-early gene induction, Mol. Cell 8 (2001) 1231–1241.
[9] A. Wood, J. Schneider, J. Dover, M. Johnston, A. Shilatifard, The Paf1 complex is essential for histone monoubiquitination by the Rad6–Bre1 complex, which signals for histone methylation by COMPASS and Dot1p, J. Biol. Chem. 278 (2003) 34739–34742.
[10] B. Zhu, Y. Zheng, A.D. Pham, S.S. Mandal, H. Erdjument-Bromage, P. Tempst, D. Reinberg, Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation, Mol. Cell 20 (2005) 601–611.
[11] K.G. Tanner, M.R. Langer, Y. Kim, J.M. Denu, Kinetic mechanism of the histone acetyltransferase GCN5 from yeast, J. Biol. Chem. 275 (2000) 22048–22055.
[12] K.G. Tanner, R.C. Trievel, M.H. Kuo, R.M. Howard, S.L. Berger, C.D. Allis, R. Marmorstein, J.M. Denu, Catalytic mechanism and function of invariant glutamic acid 173 from the histone acetyltransferase GCN5 transcriptional coactivator, J. Biol. Chem. 274 (1999) 18157–18160.
[13] Y. Kim, K.G. Tanner, J.M. Denu, A continuous, nonradioactive assay for histone acetyltransferases, Anal. Biochem. 280 (2000) 308–314.
[14] R.C. Trievel, F.Y. Li, R. Marmorstein, Application of a fluorescent histone acetyltransferase assay to probe the substrate specificity of the human p300/CBP-associated factor, Anal. Biochem. 287 (2000) 319–328.
[15] E. Koutelou, C.L. Hirsch, S.Y. Dent, Multiple faces of the SAGA complex, Curr. Opin. Cell Biol. 22 (2010) 374–382.
[16] A. Johnsson, Y. Xue-Franzen, M. Lundin, A.P. Wright, Stress-specific role of fission yeast Gcn5 histone acetyltransferase in programming a subset of stress response genes, Eukaryot. Cell 5 (2006) 1337–1346.
[17] Z. Nagy, A. Riss, C. Romier, X. le Guezennec, A.R. Dongre, M. Orpinell, J. Han, H. Stunnenberg, L. Tora, The human SPT20-containing SAGA complex plays a direct role in the regulation of endoplasmic reticulum stress-induced genes, Mol. Cell. Biol. 29 (2009) 1649–1660.
[18] S. Rodriguez-Navarro, Insights into SAGA function during gene expression, EMBO Rep. 10 (2009) 843–850.
[19] P.A. Grant, L. Duggan, J. Cote, S.M. Roberts, J.E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C.D. Allis, F. Winston, S.L. Berger, J.L. Workman, Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex, Genes Dev. 11 (1997) 1640–1650.
[20] P.A. Grant, A. Eberharter, S. John, R.G. Cook, B.M. Turner, J.L. Workman, Expanded lysine acetylation specificity of Gcn5 in native complexes, J. Biol. Chem. 274 (1999) 5895–5900.
[21] L. Zeng, M.M. Zhou, Bromodomain: an acetyl-lysine binding domain, FEBS Lett. 513 (2002) 124–128.

[22] K. Luger, T.J. Rechsteiner, T.J. Richmond, Expression and purification of recombinant histones and nucleosome reconstitution, Methods Mol. Biol. 119 (1999) 1–16.
[23] K. Luger, T.J. Rechsteiner, T.J. Richmond, Preparation of nucleosome core particle from recombinant histones, Methods Enzymol. 304 (1999) 3–19.
[24] B. Dorigo, T. Schalch, K. Bystricky, T.J. Richmond, Chromatin fiber folding: requirement for the histone H4 N-terminal tail, J. Mol. Biol. 327 (2003) 85–96.
[25] P.T. Lowary, J. Widom, New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning, J. Mol. Biol. 276 (1998) 19–42.
[26] M.J. Blacketer, S.J. Feely, M.A. Shogren-Knaak, Nucleosome interactions and stability in an ordered nucleosome array model system, J. Biol. Chem. 285 (2010) 34597–34607.
[27] H.R. Widlund, H. Cao, S. Simonsson, E. Magnusson, T. Simonsson, P.E. Nielsen, J.D. Kahn, D.M. Crothers, M. Kubista, Identification and characterization of genomic nucleosome-positioning sequences, J. Mol. Biol. 267 (1997) 807–817.
[28] P.Y. Wu, F. Winston, Analysis of Spt7 function in the *Saccharomyces cerevisiae* SAGA coactivator complex, Mol. Cell. Biol. 22 (2002) 5367–5379.
[29] S. Li, M.A. Shogren-Knaak, Cross-talk between histone H3 tails produces cooperative nucleosome acetylation, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 18243–18248.
[30] A. Eberharter, S. John, P.A. Grant, R.T. Utley, J.L. Workman, Identification and analysis of yeast nucleosomal histone acetyltransferase complexes, Methods 15 (1998) 315–321.
[31] A. Ferscht, Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding, W. H. Freeman and Company, New York, 1998. pp. 116–117.
[32] A. Ranjan, G. Mizuguchi, P.C. FitzGerald, D. Wei, F. Wang, Y. Huang, E. Luk, C.L. Woodcock, C. Wu, Nucleosome-free region dominates histone acetylation in targeting SWR1 to promoters for H2A.Z replacement, Cell 154 (2013) 1232–1245.
[33] J.G. Yang, T.S. Madrid, E. Sevastopoulos, G.J. Narlikar, The chromatin-remodeling enzyme ACF is an ATP-dependent DNA length sensor that regulates nucleosome spacing, Nat. Struct. Mol. Biol. 13 (2006) 1078–1083.