Protein Expression and Purification 118 (2016) 92–97

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression and purification of histone H3 proteins containing multiple sites of lysine acetylation using nonsense suppression

Isaac A. Young, Chitvan Mittal, Michael A. Shogren-Knaak*

Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, 1210 Molecular Biology Building, Ames, IA, 50011, USA

ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form
30 September 2015
Accepted 13 October 2015
Available online 19 October 2015

Keywords:
Histone
Acetylation
Chromatin
Nonsense suppression

ABSTRACT

Lysine acetylation is a common post-translational modification, which is especially prevalent in histone proteins in chromatin. A number of strategies exist for generating histone proteins containing lysine acetylation, but an especially attractive approach is to genetically encode acetyl-lysine residues using nonsense suppression. This strategy has been successfully applied to single sites of histone acetylation. However, because histone acetylation can often occur at multiple sites simultaneously, we were interested in determining whether this approach could be extended. Here we show that we can express histone H3 proteins that incorporate up to four sites of lysine acetylation on the histone tail. Because the amount of expressed multi-acetylated histone is reduced relative to the wild type, a purification strategy involving affinity purification and ion exchange chromatography was optimized. This expression and purification strategy ultimately generates H3 histone uniformly acetylated at the desired position at levels and purity sufficient to assemble histone octamers. Histone octamers containing four sites of lysine acetylation were assembled into mononucleosomes and enzymatic assays confirmed that this acetylation largely blocks further acetylation by the yeast SAGA acetyltransferase complex.

1. Introduction

Lysine acetylation is a pervasive post-translational protein modification, where approximately 3600 sites of lysine acetylation in over 1700 different proteins have been identified through proteomic studies of human cells [1]. This modification helps to regulate proteins with a wide range of functions, including those involved in metabolism [2], cell structure [3], and cell signaling [4].

Histone proteins that comprise chromatin constitute an especially large class of acetylation targets. Histone proteins H2A, H2B, H3 and H4, are highly basic proteins that contain a high density of lysine residues. Two copies of each of these proteins can form a histone octamer, around which DNA can wrap to form a nucleosome [5], the fundamental structural unit of chromatin. In nucleosomes, the bulk of the histone sequence is contained within the wraps of the DNA in the globular histone domain. However, less structured regions of each histone extend past the DNA to form histone "tails." At least thirty different sites of lysine acetylation have been identified in histones, with multiple sites identified in each [6]. Modifications occur both in the globular and tail regions of the histone, although a significantly greater number of sites are present in the histone tails.

One strategy, to better understand how histone acetylation affects chromatin structure and function, is to reconstitute chromatin *in vitro* using acetylated histones. In this strategy, the main challenge is to obtain histones that contain lysine acetylation at the desired sites. Direct purification of uniformly acetylated histones is generally impractical, because the heterogeneity of acetylation sites coupled with their similar physical properties makes isolation difficult. Enzymatic acetylation of histones has had some success. However, ensuring that only desired lysines are acetylated, and are acetylated fully, is often not easy [7]. As an alternative, we have previously adapted the strategy of native chemical ligation to incorporate a range of histone modifications in the H3 and H4 tails [8,9]. In this strategy, a histone tail peptide containing desired modifications is generated by standard solid-phase peptide synthesis strategies, and then ligated to a recombinantly expressed histone fragment corresponding to the remaining sequence. This strategy yields uniformly modified, full-length histone in reasonable yields. However, because peptide synthesis is relatively costly, and the steps involved are laborious, we were interested in

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

http://dx.doi.org/10.1016/j.pep.2015.10.006
1046-5928/

exploring alternative strategies for incorporating different patterns of lysine acetylation into the histone tails.

A promising strategy that has recently been applied to generating acetylated histone is nonsense suppression expression. In nonsense suppression expression [10], the idea is to genetically encode for a non-standard amino acid that becomes incorporated during translation. In its most common form, the non-standard amino acid is introduced into a specific site in a protein by first mutating its sequence to an amber stop codon. By including a modified tRNA that contains an amber anticodon and a site for attaching a desired nonstandard amino acid, as well as a tRNA synthetase that can charge the tRNA with the desired amino acid, the nonstandard amino acid becomes incorporated into the protein during translation. Chin and coworkers showed that a modified pyrrolysyl-tRNA synthetase derived from *Methanosarcina barkeri* can efficiently charge a tRNA with acetyl-lysine, and that this residue can be incorporated into histone H3 with good yields [11]. With this strategy acetyl-lysine was incorporated at multiple sites within the H3 histone. However, in all cases, each histone contained only a single acetyl-lysine.

In vivo, acetylation of multiple sites within the same histone is common [12], where the pattern of this acetylation can change the binding of chromatin-associated proteins or directly change chromatin structure and stability. For examples, dual bromodomains found in a number of nuclear proteins recognize and bind to specific pairs of lysine acetylation, thereby targeting these proteins to specific chromatin regions [13]. Similarly, because lysine acetylation reduces the total change on histone tails, multiple acetylations can work synergistically to reduce the folding of chromatin into 30 nm fibers and reduce its stability [14,15]. Because of our interest in how different combinations of lysine acetylation can modulate the structure and function of chromatin, we were interested in exploring to what extent this strategy could be extended to incorporating multiple sites of acetylation within a single H3 histone.

2. Materials and methods

2.1. Plasmid production

Xenopus laevis H3 histone in a pET3c expression plasmid [16] was mutated to incorporate 1–4 amber codons by sequential application of Quikchange mutagenesis (Strategene). The mutated H3 histone ORFs were then cloned into a pCDF-PylT-H3K14amb plasmid to replace the single amber codon containing H3 histone [11].

2.2. Expression of acetylated histone H3

Expression methods were adapted from those previously published [11]. BL21 DE3 cells were co-transformed with the pAcKRS-3 plasmid containing the tRNA synthetase and the pCDF-PylT plasmid containing both the H3 histone with the desired number of amber codons and the amber suppressor tRNA. Transformed cells were grown overnight at 37 °C in 50 mL standard Luria Broth media under selection of 50 μg/mL kanamycin and 50 μg/mL spectinomycin. 250 mL of prewarmed non-standard 2xYT broth (2xYT-KS: 0.5% w/v sodium chloride, 1% w/v yeast extract, 2% w/v tryptone), containing 50 μg/mL kanamycin and 50 μg/mL spectinomycin, was inoculated with overnight culture to a final OD_{600} of 0.08 at 37 °C. The dilute 2xYT-KS culture was grown to 0.7 OD_{600}. N-ε-Acetyl-L-lysine (≥98% pure, Novabiochem) was then added to a final concentration of 10, 20, 40, or 80 mM. Nicotinamide (NAM) was added to a final concentration of 20 mM. After 30 min, protein expression was induced with the addition of 0.5 mM IPTG. After 4 h, 250 mL cultures of cells were pelleted and resuspended in 30-ml of Wash Buffer (WB: pH 7.5, 50 mM Tris–HCl, 100 mM NaCl, 1 mM Na-EDTA, 1 mM benzamidine, 1 mM DTT, 20 mM NAM) then frozen at −80 °C overnight.

2.3. Purification of acetylated histone H3

Inclusion bodies from 250 mL of culture were isolated as previously described [17]. The insoluble final pellet containing histone was macerated with 0.25 mL DMSO and then thoroughly resuspended in 15 mL unfolding buffer (UB: 6 M guanidine hydrochloride, 20 mM Tris hydrochloride, 5 mM β mercaptoethanol, pH 8.0) and stirred at room temp for 1 h. The mixture was clarified via centrifugation (12,000 RCF, 10′). The supernatant was added to 2.5 mL of pre-equilibrated and drained Ni^{2+}-NTA Bead Resin (Qiagen) in a 30-ml disposable column and stirred at room temperature for 1 h. The column was drained and the resin washed two times with 30-ml Guanidine Wash Buffer (GWB; 6 M guanidine hydrochloride, 100 mM monosodium phosphate, 5 mM β-mercaptoethanol, pH 6.3). The resin was then washed with 100 mL 1X TEV Cleavage buffer (50 mM Tris hydrochloride, 50 mM imidazole, 0.5 mM EDTA, 5 mM β mercaptoethanol, pH 8.0). The resin was resuspended in 23 mL 1X TEV cleavage buffer and 10,000 units of TURBOTEV protease (Nacalai USA) were added. Digestion was carried out with nutation for 16 h at room temperature. The column was then drained and resuspended in 15 mL UB and stirred at RT for 1 h. UB eluent containing histones was dialyzed into SAU buffer (7 M urea, 20 mM sodium acetate, 1 mM DTT, 1 mM sodium EDTA, pH 5.2). The histone sample was then purified via cation exchange on a salt gradient from 0 mM to 600 mM NaCl over 225 mL using a HiTrap SP HP 5 mL column (GE Life Sciences). Histones eluted in a highly pure state and were concentrated and desalted using Sep-Pak C-8 reverse phase purification columns (Waters) before lyophylization. Purified acetylated histone H3 was checked via MALDI-TOF analysis to verify the correct number of acetylations were present (John Leszyk at University of Massachusetts Medical School). Histone was quantified using densitometry against standard curves of H3-tetra-alanine containing histone.

2.4. Assembly of mononucleosomes

Wild type histones and the tetra-alanine H3 histone were expressed, purified, and quantified according to literature protocols [17]. 177 bp DNA templates containing the 601 strong positioning sequence [18] and BglI non-palindromic sticky ends were prepared as previously described [19]. Octamers were assembled from the appropriate recombinant histones and purified via size exclusion chromatography as published previously [17]. Mononucleosomes were reconstituted from the DNA template and histone octamer via rapid dilution methods [20], and then were characterized via 4% native PAGE Gel with staining for the DNA. The assembled nucleosomes were dialyzed into native buffer (2.5 mM NaCl, 10 mM Tris, 0.25 mM EDTA, pH 7.4) and concentrated 7–10 fold by volume using 30 kDa MWCO concentrators (EMD Milipore). Mononucleosomes were immobilized onto beads largely as previously described [19]. Briefly, 4.66 pmol of the biotinylated adapter were bound to 200 μg of paramagnetic streptavidin beads (NEB) at RT for 20 min in 1X binding buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl) to get a final reaction volume of 100 μl. The supernatant was removed and beads were washed three times with 50 μl of 1X ligation buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT; pH 7.5). 6.99 pmol of WT, TA or TAc nucleosomes were added to the beads in 1X ligation buffer, in a final reaction volume of 100 μl. 800 U of T4 DNA ligase (NEB) were used to ligate the nucleosomes to the adapter. The reactions were carried out at RT for 5–6 h. Excess unbound nucleosomes were then washed off. The beads were resuspended in 31 μl of 1X ligation buffer to get a final

nucleosome concentration of 150 nM. The integrity of the assembled substrates was analyzed by digesting the beads with PstI at 37 °C, for 5 h. The liberated nucleosomes were analyzed on 4% native PAGE.

2.5. Histone acetylation assay

To compare the extent of acetylation of WT, TA and TAc nucleosomes, substrates were subjected to standard acetylation assays by ySAGA (Spt-Ada-Gcn5-acetyltransferase complex from *Saccharomyces cerevisiae*), under initial rate, sub-saturating nucleosome concentrations, as previously described [19]. Briefly, 1.5 nM ySAGA was used to acetylate 10 nM of each kind of nucleosome in a 50 μl reaction volume of 1X HAT buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 4.0 μM acetyl CoA with a specific activity of 5.78 Ci/mmol (Moravek Biochemicals)). The acetylation reaction was carried out at 30 °C for 10 min. The beads were washed 4 times at RT with 50 μl 1X wash buffer 1 (WB1-100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% triton X-100) and for additional 5 times at 37 °C with 15 μl 1X WB2 (50 mM NH_2OH, pH 7.5, 0.1% triton X-100), with 25 min incubation between each wash. The beads were then resuspended in 15 μl of 1X WB1 and added directly to 6 mL of scintillation cocktail. The samples were counted in Tri carb 4910 TR Liquid Scintillation Analyzer (Perkin Elmer). To account for non-H3 nucleosomal acetylation, the counts obtained from the WT and TAc nucleosomes were subtracted from those obtained from TA nucleosomes. The resultant extent of acetylation obtained from TAc substrate was compared to that of WT, which was normalized to 100%.

3. Results

We have been interested in how histone H3 tail acetylation is established by the SAGA family of transcriptional co-activitors [9,19,21], and how this acetylation affects chromatin structure and function, such as subsequent histone acetylation [21]. The SAGA complex from budding yeast has been shown to target four major residues on the H3 tail, with lysine 14 being the most prominent acetylation site, followed by lysine 9, lysine 18, and then lysine 23 (Fig. 1) [22]. Nonsense suppression has been successfully employed to generate H3 histones containing lysine 14 acetylation [11], and we sought to determine if such a strategy could be applied to generating histones containing as many as all four primary acetylations. Histone proteins can be recombinantly expressed in *Escherichia coli* [16], and H3 K14 acetylated histone was previously generated from an overexpression plasmid containing an amber codon at amino acid residue 14 (Fig. 1) [11]. Building off this sequence, we introduced additional putative acetyl-lysine residues by using site directed mutagenesis to convert lysine codons to amber stop codons. Plasmids were constructed to contain up to four amber codons at the major sites of SAGA acetylation, where histones with intermediate levels of acetylation were made to match the known preference of lysine acetylation – i.e. H3 histone containing one acetylation contained H3 K14 acetylation; K14 and K9 acetylation for two acetylations; and K14, K9, and K18 acetylation for three acetylation sites (Fig. 1).

To test the efficiency of acetyl-lysine incorporation, we first over-expressed singly acetylated H3 K14 acetylated histone according to literature protocols [11]. In this protocol, H3 protein expression is induced by IPTG addition in *E. coli* BL21 DE3 cells. However, for suppression of the amber stop codons, the cells also must contain a plasmid that constitutively expresses a tRNA containing an amber anticodon and a tRNA synthetase that can charge the tRNA with acetyl-lysine. Acetyl-lysine is added to the media, as well as NAD, which inhibits acetyl-lysine deacetylation. As expected, we observed visible amounts of H3 histone in whole cell extracts (data not shown). In the inclusion bodies, where overexpressed histones localize, the full-length histone was especially prevalent (Fig. 2A). However, when the same conditions were used for overexpression of the tetra-acetylated H3 histone, no H3 histone was observed in the inclusion body (Fig. 2A). Because full length expression of the tetra-acetylated histone requires efficient suppression of all four non-sense codon, and suppression involves a number of components, lack of expression could be due to a number of factors. During optimization, we found that increasing the concentration of acetyl-lysine in the cell media dramatically increased the amount of full-length histone expressed (Fig. 2B). Further, expression of histones with fewer than four acetyl-lysines was also feasible, with greater expression occurring when fewer amber codons are suppressed (Fig. 2C).

With improved tetra-acetylated H3 histone expression, we set out to purify the histone. Standard purification protocols for histones overexpressed in *E. coli* involve isolating inclusion bodies and then further purifying denatured histone by size exclusion and cation exchange chromatography. With these steps, we were able to increase the purity of the H3 histone, but not enough to obtain pure protein (data not shown). In the literature protocol for the singly

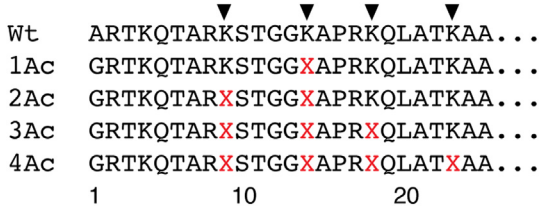

Fig. 1. Sequences of acetylated histone H3 proteins. Top row shows the sequence of the N-terminal tail of histone H3. Triangles point to the major sites of SAGA-mediated lysine acetylation. Subsequent rows show the acetylated histones generated, where 'X' denotes a site of amber codon replacement in the histone expression vector and acetyl-lysine residues in the expressed protein. The C-terminal portion of the histone sequence is not shown. The N-terminal portion of the histone reflects the sequence present after TEV cleavage of the 6-His affinity tag: HHHHHHSQDPENLYFQG, with TEV cleavage between the last two residues, leaving the first H3 residue glycine.

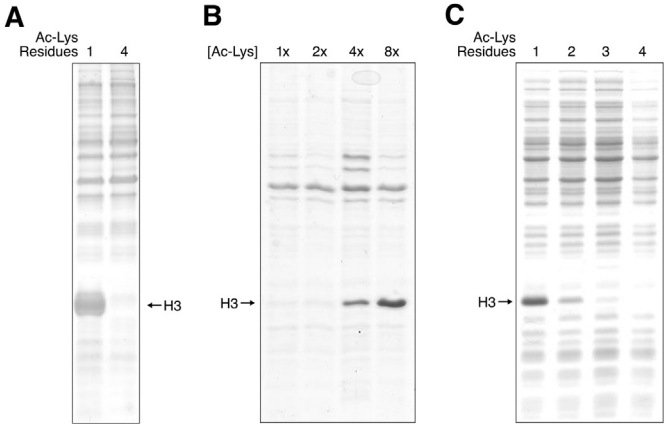

Fig. 2. Expression of acetylated histone H3 proteins. Proteins were resolved on an 18% SDS-PAGE gel and stained with Coomassie Blue. A) Comparison of amounts of full-length mono- and tetra-acetylated histone protein in inclusion bodies under published nonsense suppression conditions. B) Comparison of amounts of full-length tetra-acetylated histone protein in inclusion bodies with increasing concentrations of acetyl-lysine in the media. 1X represents previously utilized acetyl-lysine concentrations of 10 mM. C) Comparison of expression of full-length histone containing one to four acetyl-lysines in whole cell extracts using 40 mM acetyl-lysine concentrations.

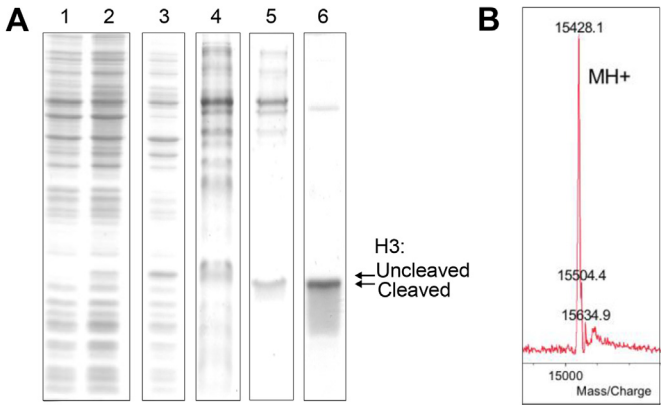

Fig. 3. A) Analysis of tetra-acetylated H3 histone purity at various steps of the modified purification protocol. Proteins were resolved on an 18% SDS-PAGE gel and stained with Coomassie Blue. Pre- and post-induction of whole cell extracts are shown in lanes 1 and 2, respectively. Lane 3 shows protein composition following inclusion body purification. Lanes 4 and 5 show proteins bound to nickel-NTA-bead and then released by TEV protease, respectively. Lane 6 shows the protein composition following cation exchange chromatography. B) MALDI-TOF mass spectrometry analysis of purified tetra-acetylated H3 protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acetylated histone [11], a TEV-cleavable, six-His affinity tag was fused to the N-terminus to facilitate purification, and a similar tag was investigated for the multiply acetylated histones. For the tagged, tetra-acetylated histone, induction and inclusion body purification proceeded to enrich the amount of full-length H3 histone (Fig. 3A, lanes 1–3). Histone could then be unfolded and captured on nickel-NTA beads under denaturing conditions to further enrich purity (Fig. 3A, lane 4). After solvent exchange into a non-denaturing buffer compatible with TEV protease, H3 histone was liberated from the nickel beads (Fig. 3A, lane 5). We found that to get complete cleavage required optimization of the both the binding and cleavage steps. For histone binding, we utilized just enough nickel-NTA beads to get complete histone binding, because additional beads decreased cleavage efficiency. Because commercially available TEV protease is also His-tagged, we believed that the nickel-NTA beads could be sequestering away TEV protease. Consistent with this idea, we found that addition of up to 50 mM imidazole improved the cleavage efficiency of the immobilized histone (higher concentrations of imidazole resulted in elution of uncleaved histone). The amount of TEV protease was also optimized to minimize the amount of protease required to give complete cleavage of the tetra-acetylated histone. Despite full cleavage, the histone could only be eluted from the beads under denaturing conditions, where a significant amount of contaminating proteins coelute. A cation exchange chromatography step under denaturing conditions resulted in histone protein sufficiently pure for subsequent applications. (Fig. 3A, lane 6). Because we were working with relatively small amounts of protein, we found that reversed phase C8 Sep-Pak purification was an efficient way to concentrate and desalt the purified tetra-acetylated histone after cation exchange chromatography.

While the purification step generated full-length H3 histone, additional characterization was necessary to confirm the acetylation state of the histone. Full-length H3 protein lacking tetra-acetylation could result from amber suppression by non-acetyl-lysine codons, or by enzymatic deacetylation of the acetyl-lysine residues. To rule out these possibilities, the mass of the purified histone was determined by MALDI mass spectrometry (Fig. 3B), and this analysis confirmed that the isolated protein was tetra-acetylated (15,425 Da expected, 15,428 Da observed, 42 Da per acetyl group). The ultimate yield of the tetra-acetylated H3 histone was 0.06 mg/g of cells. While this is significantly less than the 0.82–2.45 mg/g of cells that is typically obtainable for unacetylated H3, it is not significantly worse than the 0.17 mg/g of cells that was obtained from original single-site nonsense suppression studies [11]. A similar purification protocol was applied to each of the other acetylated histones, and yielded 2.45 mg/g of cells, 0.65 mg/g of cells, and 0.24 mg/g of cells for H3 histones containing one, two and three acetylated lysines, respectively.

The purity and amount of tetra-acetylated H3 was sufficient to incorporate into a histone octamer and then a mononucleosome. To generate histone octamer, the tetra-acetylated H3 was combined with recombinant H2A, H2B, and H4 histones under denaturing conditions and dialyzed into a high salt (2 M NaCl) solution [16]. Histone octamer was resolved from incomplete assembly products by size exclusion chromatography, and eluted identically to wild-type octamers. Denaturing protein gel electrophoresis of the wild type, tetra-acetylated H3, and tetra-alanine H3 octamers look similar (Fig. 4A), with some changes in electrophoretic mobility of acetylated and alanine-containing H3 histone relative to the wild-type H3. Equal amounts of octamers were then incorporated into mononucleosomes by deposition onto a 177 base pair 601 double stranded DNA template by rapid dilution [20], and all three mononucleosomes appear largely similar in their extent of assembly and their electrophoretic mobility (Fig. 4B). Each mononucleosome was immobilized onto a paramagnetic bead, and then used as a substrate for a SAGA-mediated acetyltransferase assay (Fig. 4C). As expected, the nucleosome containing acetyl-lysine at the major SAGA acetylation sites showed a significant reduction of new acetylation relative to a nucleosome that was not pre-acetylated. The reduction in the amount of acetylation was similar to that observed for a nucleosome in which the four major H3 tail acetylation sites were mutated to alanine, indicating that the tetra-acetylated histone prevents SAGA-mediated histone acetylation in

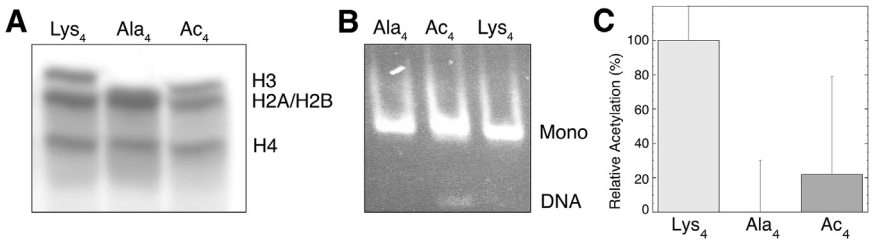

Fig. 4. Utilization of tetra-acetylated histone H3 protein. A) Denaturing protein gel analysis of histone octamers containing unacetylated, tetra-alanine, or tetra-acetylated H3 histones. Histones were resolved on an 18% SDS PAGE gel with Coomassie Blue staining. B) Native gel analysis of mononucleosomes containing tetra-alanine, tetra-acetylated, or unacetylated H3 histone. Species were resolved on a 4% native PAGE gel with ethidium bromide staining. C) Comparative extent of SAGA-mediated mononucleosome acetylation relative to mononucleosomes in which the major H3 tail acetylation sites were mutated to alanine. To determine the extent of acetylation, the amount of radioactive acetyl incorporation was subtracted from that of the tetra-alanine mononucleosome and then normalized to the wild-type mononucleosome. Data represents four independent trials.

a manner similar to loss of the lysine residue.

4. Discussion

We have shown that nonsense suppression can be used to incorporate multiple acetyl-lysine residues into the tail of H3 histone protein. One major hurdle we encountered was expressing full-length protein. This difficulty presumably arises because full-length expression requires efficient nonsense suppression at every amber codon. Consistent with this idea, we observed that the amount of full-length protein decreases with increasing numbers of amber mutations to suppress (Fig. 2C). Thus, conditions to optimize nonsense suppression were necessary. We found that increasing the amount of acetyl-lysine present in the media increases full-length protein expression (Fig. 2B), suggesting that a key difficulty in nonsense suppression in this system is charging the nonsense suppression tRNA with acetyl-lysine. It is likely that acetyl-lysine is limiting in the cell and by increasing its concentration in the media, intracellular concentrations increase to drive the action of the synthetase. For our purposes, a four-fold increase in acetyl-lysine concentrations was sufficient for generating amounts of tetra-acetylated histone we needed. However, for the increasing acetyl-lysine concentrations used (Fig. 2B), we did not observe saturation of histone expression, suggesting that even greater levels of expression should be possible. In this case, the only trade-off may ultimately be the cost of acetyl-lysine. Further, because nonsense suppression is a complicated process, other steps in the reaction might also benefit from optimization. This could include improving the expression levels and properties of the tRNA and synthetase, as well as optimizing the length and timing of histone induction relative to acetyl-lysine addition.

The other major hurdle encountered was in the purification of the multiply acetylated histones. Inclusion body preparations, nickel-NTA beads binding, and cation exchange chromatography were highly efficient, while proteolytic cleavage of the histone from the nickel-NTA required significant optimization in the bead, imidazole, and TEV protease amounts. Even under these optimized conditions, a significant amount of TEV protease was required. The inefficiency of TEV cleavage could be due in part to sequestration of the protease to the nickel-NTA beads, and alternative forms of TEV-protease that do not contain a His-tag might improve cleavage efficiency. Another potential contributor could be the inability of H3 histone to form a well-behaved globular structure under native conditions. In such a case, it is likely that affinity captured histones form aggregates on the surface of the nickel-NTA beads when solvent is exchanged for proteolysis, making the cleavage site less accessible. A cleavage strategy that can be performed under denaturing conditions would likely avoid this issue.

In our studies, we focus on incorporating multiple acetyl-lysine residues into the H3 histone at known sites of SAGA-mediated acetylation. However, we feel our strategies should be applicable to other sites within the H3 histone, to other acetylation sites in other histones, and even to acetylation of other proteins. Within the H3 histone, Chin and coworkers have previously shown that many different sites within the H3 histone can be individually acetylated, suggesting that, in general, nonsense suppression is not highly sequence dependent in the H3 histone. This idea is further supported by the fact that we observe a similar degree in decrease in histone expression with every amber codon added. Thus, we expect that other combination of multiple lysine acetylations in H3 histone should be possible. With respect to other histones, multiple acetylations may also be possible. For example, while the H4 histone had proven recalcitrant to single site acetyl-lysine nonsense suppression, recent studies have shown that with codon optimization, H4 K16 acetylated histones can be generated at level comparable to those found for single sites acetylation H3 histones in the original Chin study. Our improvements in H3 expression and purification may be directly applicable to H4 histones, as well as others. Finally, for non-histone proteins, especially those that can be purified more effectively than histone, increasing acetyl-lysine concentrations might prove sufficient to allow for improved incorporation of single or multiple acetyl-lysine residues.

5. Conclusion

We have shown that we can extend a nonsense suppression strategy for incorporating acetyl-lysine into H3 histones to generate histones containing up to four acetyl-lysine residues. Key to achieving this is improved histone expression via an increase in the amount of acetyl-lysine added to the cell media, and a combined affinity capture and ion exchange chromatography purification strategy. The tetra-acetylated H3 histone is generated in sufficient yield and purity to be incorporated into histone octamers and nucleosomes, and we expect that the insight gained from our study could aid in utilizing nonsense suppression to incorporate acetyl-lysine residues into other histones and non-histone proteins.

Acknowledgments

This work was supported by American Cancer Society Research Scholar Grant 1206501 to M. A. S.-K.

References

[1] C. Choudhary, C. Kumar, F. Gnad, M.L. Nielsen, M. Rehman, T.C. Walther, J.V. Olsen, M. Mann, Lysine acetylation targets protein complexes and co-regulates major cellular functions, Science 325 (2009) 834–840.
[2] Y. Xiong, K.L. Guan, Mechanistic insights into the regulation of metabolic enzymes by acetylation, J. Cell Biol. 198 (2012) 155–164.
[3] D. Perdiz, R. Mackeh, C. Pous, A. Baillet, The ins and outs of tubulin acetylation: more than just a post-translational modification? Cell. Signal. 23 (2011) 763–771.
[4] K.A. Mowen, M. David, Unconventional post-translational modifications in immunological signaling, Nat. Immunol. 15 (2014) 512–520.
[5] K. Luger, A.W. Mader, R.K. Richmond, D.F. Sargent, T.J. Richmond, Crystal structure of the nucleosome core particle at 2.8 A resolution, Nature 389 (1997) 251–260.
[6] L. Zhang, E.E. Eugeni, M.R. Parthun, M.A. Freitas, Identification of novel histone post-translational modifications by peptide mass fingerprinting, Chromosoma 112 (2003) 77–86.
[7] P.J. Robinson, W. An, A. Routh, F. Martino, L. Chapman, R.G. Roeder, D. Rhodes, 30 nm chromatin fibre decompaction requires both H4-K16 acetylation and linker histone eviction, J. Mol. Biol. 381 (2008) 816–825.
[8] M.A. Shogren-Knaak, H. Ishii, J. Sun, M.J. Pazin, J.R. Davie, C.L. Peterson, Histone H4-K16 acetylation controls chromatin structure and protein interactions, Science 311 (2006) 844–847.
[9] 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.
[10] T.S. Young, P.G. Schultz, Beyond the canonical 20 amino acids: expanding the genetic lexicon, J. Biol. Chem. 285 (2010) 11039–11044.
[11] H. Neumann, S.M. Hancock, R. Buning, A. Routh, L. Chapman, J. Somers, T. Owen-Hughes, J. van Noort, D. Rhodes, J.W. Chin, A method for genetically installing site-specific acetylation in recombinant histones defines the effects of H3 K56 acetylation, Mol. Cell 36 (2009) 153–163.
[12] N.L. Young, P.A. DiMaggio, M.D. Plazas-Mayorca, R.C. Baliban, C.A. Floudas, B.A. Garcia, High throughput characterization of combinatorial histone codes, Mol. Cell Proteomics MCP 8 (2009) 2266–2284.
[13] A.G. Ladurner, C. Inouye, R. Jain, R. Tjian, Bromodomains mediate an acetyl-histone encoded antisilencing function at heterochromatin boundaries, Mol. Cell 11 (2003) 365–376.
[14] C. Tse, T. Sera, A.P. Wolffe, J.C. Hansen, Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III, Mol. Cell Biol. 18 (1998) 4629–4638.
[15] A. Gansen, K. Toth, N. Schwarz, J. Langowski, Structural variability of nucleosomes detected by single-pair Forster resonance energy transfer: histone acetylation, sequence variation, and salt effects, J. Phys. Chem. B 113 (2009) 2604–2613.
[16] K. Luger, T.J. Rechsteiner, T.J. Richmond, Expression and purification of recombinant histones and nucleosome reconstitution, Methods Mol. Biol. 119 (1999) 1–16.

[17] K. Luger, T.J. Rechsteiner, T.J. Richmond, Preparation of nucleosome core particle from recombinant histones, Methods Enzymol. 304 (1999) 3–19.
[18] 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.
[19] C. Mittal, M.J. Blacketer, M.A. Shogren-Knaak, Nucleosome acetylation sequencing to study the establishment of chromatin acetylation, Anal. Biochem. 457 (2014) 51–58.
[20] 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.
[21] S. Li, M.A. Shogren-Knaak, The Gcn5 bromodomain of the SAGA complex facilitates cooperative and cross-tail acetylation of nucleosomes, J. Biol. Chem. (2009) 9411–9417.
[22] 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.