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Introducing multiple sites of acetylation to histone H3 via nonsense suppression

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by

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TABLE OF CONTENTS

ABSTRACT

A common post-translational modification (PTM) of proteins is lysine acetylation. This is an especially ubiquitous PTM in the histones of chromatin, and is important for helping to regulate both structural and mechanistic aspects of chromatin. The fundamental unit of chromatin is called the nucleosome and is made up of DNA that wraps around a histone protein octamer. Protruding from the nucleosome are 10 unstructured “tails” which protrude into the aqueous environment. A number of strategies exist for generating acetylated nucleosomes for the in-vitro study of chromatin including: Purification from eukaryotic organisms, chemical acetylation, amino acid analog incorporation, enzyme mediated acetylation, native chemical ligation of peptides, and enzyme mediated ligation of peptdes. 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, it is worth while to determine whether this approach could be extended.

The results in this thesis show that recombinantly expressed histone H3 proteins that incorporate up to four sites of lysine acetylation on the histone tail can be produced in good yields. 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.

In the future, multiply acetylated histones may be applied in many ways including two that are of primary interest. Firstly, they can be used to mechanistically probe the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex in yeast which is known to play an active part in the activation of transcription in silent inducible genes. Secondly, they may be used to investigate acetylation's role in destabilizing nucleosomes.

CHAPTER 1

INTRODUCTION TO CHROMATIN ACETYLATION

Chromatin: Eukaryotic Cell DNA Organization and Regulation

How genetic information in living things is organized and regulated is one of the central questions of biology, and has been a long standing question in science. It was initially found that in eukaryotes, the genetic instructions of life, that material which is passed from one generation to the next to allow the survival of a species, was held within “chromatin” inside the nucleus based on research of many scientists including Walter Flemming in the 1880’s. Chromatin got its name based on the fact that DNA binds well to many dyes; the root word chrom is Greek for color. The proteins in chromatin were named histones and the protease resistant fraction (later called DNA) was called nuclein. Work from the early 1880’s up until the late 1960’s showed that DNA, as a continuous linear molecule for each chromosome, was the main information carrier in chromatin. Studies in the last two decades have revealed that chromatin plays a central regulatory role in how the long linear DNA molecules are utilized. This has resulted in an explosion of interest in chromatin structure and function (Olins and Olins, 2003).

At its most basic structural unit, the nucleosome (which was discovered by Kornberg and others in 1975) is a protein core made of 2 copies each of histones H3, H4, H2A, and H2B, around which 147 base pairs of DNA are wrapped (Figure 1). The first crystal structure of the nucleosome was published in 1984 by Tim Richmond based on his work in the Klug lab. That work showed a crystal structure with a 7 angstrom resolution that indicated an octameric core of histones with DNA wrapped around roughly 2 times in a left handed helix (Richmond et al., 1984). Since then, the Richmond lab has continued to refine the structure of the mononucleosome. Utilizing alpha-satellite palindromic DNA sequences from the Bunick lab, they have obtained high resolution crystal structure at 1.9 Å (Davey et al., 2002; Luger et al., 1997; Vence, 2015).

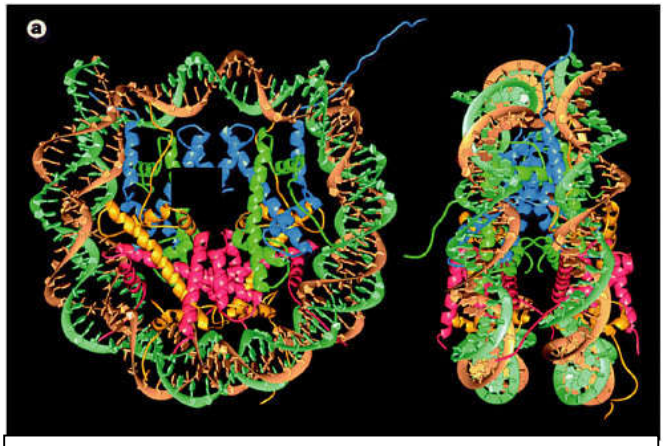

Figure 1 Cartoon image of first high resolution crystal structure (Luger et al., 1997) of the nucleosome in two views. Left: the DNA wrapping around can be seen with Histone h3 in blue, Histone H4 in green, Histone H2B in yellow, and Histone H2A in red. Right: the DNA wrapping around makes the nucleosome's general shape that of a disk and the DNA makes two wraps around the Histone core.

One function of chromatin is to organize DNA in the nucleus. The sheer length of DNA found to be inside each cell of a eukaryotic organism is staggering. Every human cell contains about 2 meters of nucleic acid. So a primary function of chromatin is to compact DNA. Individual nucleosomes in chromatin are linked like beads on a string as shown by EM images (Woodcock et al., 1976). The nucleosomes are not uniformly spaced throughout the genome and are seen in a wide variety of different levels of compaction. The defined levels of chromatin

compaction are that of Euchromatin (open, loose and more accessible to proteins) and Heterochromatin (closed, tight and less to proteins). The chromosome is a macrostructure of chromatin and is composed of both euchromatin and heterochromatin.

A second important function of chromatin is that it regulates how the underlying DNA is used, and nature has evolved strategy to both contend with and exploit the regulatory opportunities chromatin provides. DNA replication, repair, and transcription do not happen in eukaryotic cells in the absence of chromatin. For the sake of simplicity, fields of DNA research must initially be confined to systems which exclude chromatin. The presence of histones which bind very tightly to DNA cannot be ignored, so it becomes necessary to investigate how chromatin functions to modulate and change the initial mechanisms discovered in all fields of DNA research.

H3 Histone Acetylation: a Mechanism of Chromatin Regulation

One mechanisms for regulating chromatin is through post-translational modification (PTM) of histone proteins. Histone modifications occur at many different sites within histone, especially on the histone tails, and occur with high frequency throughout the genome. Histone acetylation was one of the first discovered histone PTMS (Allfrey et al., 1964). It is well documented that acetylated domains of chromatin are more commonly associated with highly transcribed areas found in the loosely packed euchromatin (Choudhary et al., 2009; Tse et al., 1998). The acetylation of chromatin occurs through catalysis by many lysine acetyl-transferase enzyme complexes. Acetyl-CoA and lysine are the substrates which form products sCoA and Acetyl-lysine. One found in yeast is of particular interest in the Shogren-Knaal lab: The Spt-Ada-

Gcn5-acetyltransferase complex (SAGA). This complex is found to be involved with activation of transcription in genes which are inducible. It predominantly targets four loci on the histone H3 n-terminal tail: Lysine residues 9, 14, 18, and 23 (Grant et al., 1999) (Figure 2). Histones targeted by saga are not uniformly acetylated at all 4 sites. There is, in fact, a preferential order of acetylation. Lysine 14 is most heavily acetylated, followed by 9, then 18, and least preferred is residue 23. Every combination of acetylation possible may have specific unique effects in-vivo and it is of interest in our lab to investigate biophysical differences generated by these various combinations.

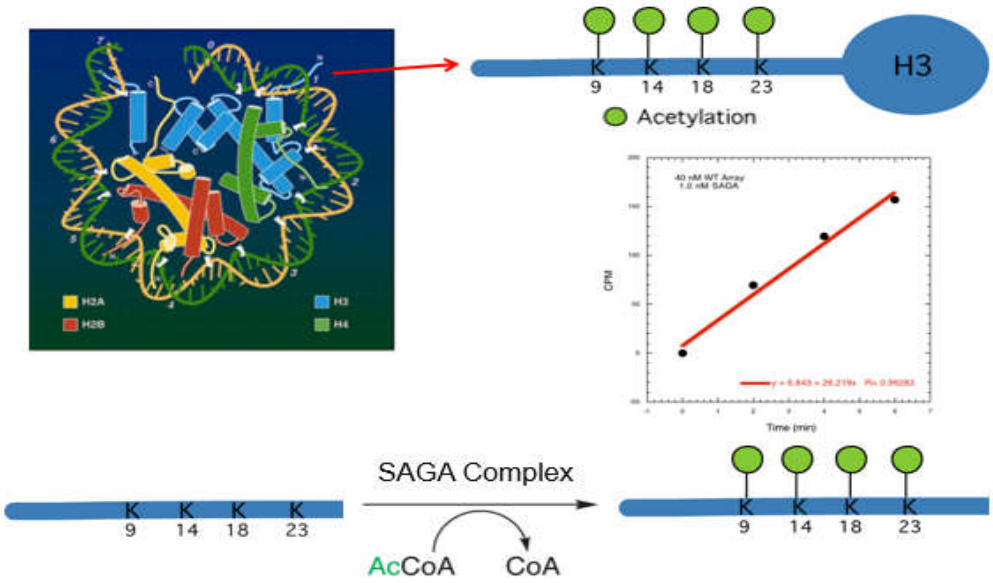

Figure 2 Major targets of acetylation by the SAGA complex. The N-terminal tail of Histone H3 protrudes from the core near the dna entry/exit point and is acetylated primarily at sites 9, 14, 18 and 23. Lower: Yeast SAGA complex is routinely assayed for activity using the N-terminal tail peptide and radioactive acetyl-CoA. Inset graph: Kinetics of SAGA under saturating levels of substrate. Activity is measured by scintillation counting.

Acetylation of lysine in a histone is a common post-translational modification (PTM) and causes two chemical changes to the histone: One, the histone loses a positive charge under all physiological pH levels. And two, the histone has a different size and shape. The change in ionic

charge of the histone has obvious implications when the highly negatively charged polyphosphate backbone of DNA is considered; the histones bind strongly to DNA thanks in part to the attraction between positively charged histones and negative charges of DNA. Indeed, it has been shown that acetylated nucleosomes are less stable than unacetylated nucleosomes (Gansen et al., 2009; Tóth et al., 2006; Tse et al., 1998). The change in size and shape of the histone also allows the acetylated lysine to serve as a binding site for chromatin modifying enzymes, transcription factors, as well as proteins involved in DNA replication (Wang et al., 2012), and DNA repair (Das et al., 2009). In this way, the acetylation mark may recruit DNA machinery to a specific genetic locus in the organism. Specifically, any protein with one or more bromodomains may be recruited to acetylated nucleosomes in-vivo.

A major method by which the function of acetylated histones can been determined is through in-vitro biochemical and biophysical studies with systems containing acetylated lysine. Early studies focused on using acetylated peptides of the H3 tail. While such peptides can be quite useful, in many cases it has been revealed that chromatin-associated proteins interact with domains outside of isolated histone tails. For example, in the Shogren-Knaak lab, acetylated histone h3 tails in nucleosomal arrays (a common in-vitro chromatin model system) have been studied to ascertain information about SAGA preference for acetylating one tail in a nucleosome when the other tail is already acetylated (Li and Shogren-Knaak, 2009).

In-vitro Techniques to Obtain Histones with Acetylation

Because of the utility of acetylated histones for understanding their function, a number of strategies have been explored, each with their strengths and limitations.

Purification from natural and unnatural sources

Histones from various eukaryotic tissues are full of unique post-translational patterns that include recognizable epigenetic acetylation patterns. It is in large part thanks to post-translational chromatin patterns that the cells of multicellular organisms are able to differentiate. It can be useful to study reconstituted nucleosomes using histone octamers purified from specific tissues. Novel properties might be elucidated by nucleosomes isolated from different tissue types. Immortal cell lines such as helacells and cancer cell lines will also have histones that may be purified and used for study (Tóth et al., 2006).

The primary disadvantage of this technique is that the histone samples will always be highly heterogeneous. The histone code implies the idea that the differential expression is highly location specific within chromatin and technologies for studying a single length of chromatin from a single cell give limited information.

Chemical acetylation of histones from recombinant sources

E-coli was developed as a tool to express proteins from a plethora of different organisms. Expressing in e-coli has the advantage of generating proteins that are largely PTM free. This makes the resulting proteins an excellent substrate for subsequent experiments to be performed that study any enzyme that catalyzes the generation of PTMs on proteins. In order to study Histones that have only acetylation present, histones must first be produced in e-coli. Subsequently, it has been shown that mixing the histones with acetyl phosphate at 50 deg C will produce histones with lots of acetylation (Tóth et al., 2006). Again, this technique has the drawback that the samples produced are always heterogeneous.

Using alternative residues as acetyl-lysine analogs (cysteine derivatives and glutamine)

Another advantage of using e-coli is that the DNA given to the e-coli may be easily modified using standard E-coli cloning and mutagenesis. Acetylated lysine is quite similar in terms of its properties to glutamine (Figure 3). Indeed, in the literature, there are examples where this glutamine mutagenic strategy does not appear to do good job of recapitulating the activity of acetylated lysine (VanDemark et al., 2007).

Figure 3 Comparison of Acetyllysine and a canonical amino acid analog, L-Glutamine. They both contain an acetyl group on the gama linked chain of the amino acid. Acetyl-lysine is however 2 carbons longer, and the primary amide of l-glutamine will be slightly more reactive than the secondary amide of acetyl-lysine.

Incorporating cysteine residues to replace choice lysine residues allows subsequent chemical steps to generate acetyl lysine analogs (Chalker et al., 2012; Guo et al., 2008; Huang et al., 2010; Li et al., 2011; Simon et al., 2007). This technique is useful because the cysteine residue is the most nucleophilic of all 20 amino acid residues. In this way, organic chemists can easily use unique cysteine side-chains on a protein to perform organic reactions. Cysteine acetyl-lysine analogs have disadvantages of not being exactly the same, chemically, as acetyl-lysine and are costlier and more time consuming than other methods available, including native chemical ligation. Cysteine analogs of specifically methylated lysines on H3 have been successful however (Lu et al., 2008; Simon et al., 2007).

In-vitro enzyme mediated acetylation

PCAF and CBP/P300 are histone acetyltransferases which are known to generate specific acetylated histone products (Das et al., 2009). The primary disadvantage of this approach is that very few PTMs have a known enzyme that generates only one acetylated histone product. Additionally, products will be heterogeneous, as it is difficult to drive an enzymatic reaction to completion.

Native chemical ligation and expressed protein ligation

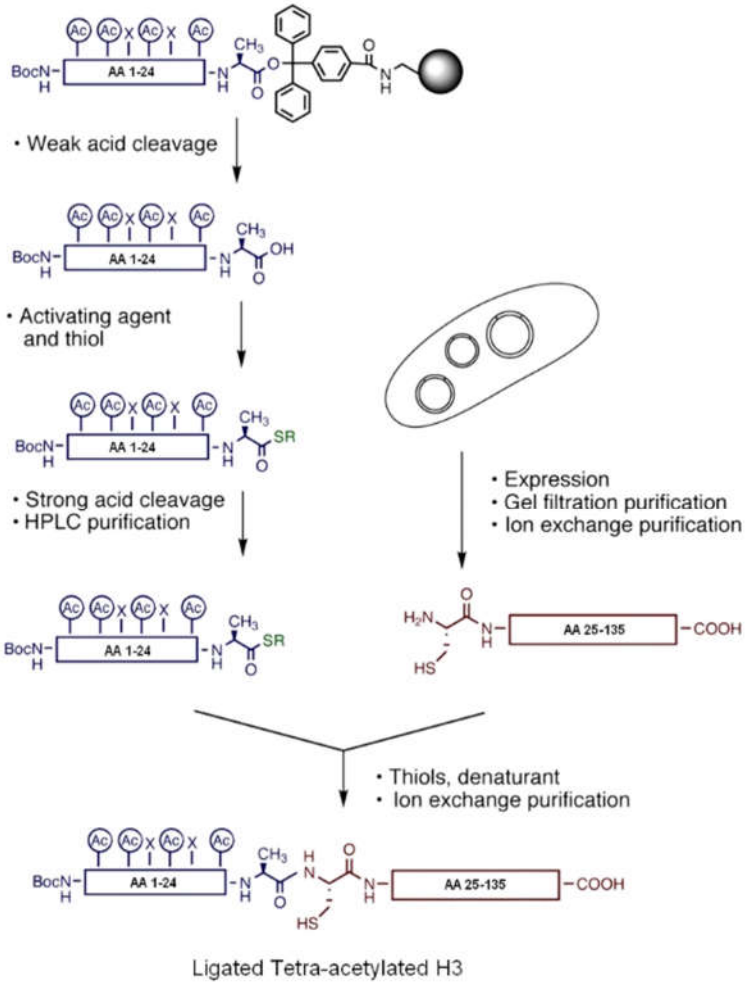

Figure 4 Diagram of Acetylated histone produced via native Chemical ligation. Weak acid cleavage removes acetylated N-terminal tail peptide produced via solid phase peptide synthesis without deprotecting the side chains. Thioesterification reaction followed by strong acid cleavage of protecting groups activates the c terminal of the peptide for ligation to a globular domain histone protein recombinantly expressed in e-coli.

In native chemical ligation, an N-terminal tail of a histone is ligated to the globular domain of the histone protein (Shogren-Knaak et al., 2006; Shogren-Knaak and Peterson, 2003). The C-terminal globular domain is mutated to cysteine at the N-terminal residue and the N-terminal tail is synthesized using solid phase peptide synthesis standard Fmoc chemistry. The C terminal residue is then thioesterified. The resulting thioesterified N-terminal tail must be purified via reversed-phase high pressure liquid chromatography (HPLC) purification which proves costly in terms of yield and is a time intensive process (Figure 4). Additionally, a cysteine mutation in introduced by the process which may add complications if further chemistry is required to modify the histone product.

Enzyme mediated ligation of peptides

Recent work introduced an enzyme-mediated approach to ligate a synthesized N-terminal tail histone peptide to the globular domain of histone H3 (Ringel et al., 2015). In this process, the F40 sortase requires a recognition motif on the C-terminal of the peptide in order to ligate to a di-glycine N-terminal globular histone h3 protein. This strategy requires the production of peptide, similar to native chemical ligation but uses less peptide. This is an improvement in that the cost is reduced, since less peptide needs to be purchased, and the peptide product needs to be purified less extensively due to the selectivity of the enzyme. However, this approach is still potentially difficult to scale-up, and the introduced ligase recognition sequence incorporated into the histone could alter the properties of the histone.

Nonsense suppression: a technique to generate acetylated histone H3

To address some of the shortcomings of the available approaches for generating multiply-acetylated H3 histones, this thesis explores their generation and application via nonsense suppression mutagenesis. This technique, developed initially by the Schultz lab (Ellman et al., 1992), utilizes a unique tRNACUA-Synthetase pair in E. coli to specifically incorporate a non-coding amino acid at an introduced nonsense codon site in a gene of interest (Figure 5). Recently, this technique was re-engineered to generate histone H3 acetylated at K56 (Neumann et al., 2009). It was not, however, shown to be useful to generate histones with more than a single lysine acetylation. Investigating whether more acetyl-lysine residues could be incorporated using this strategy is worthwhile, as it would expand the number and combination of acetyl-lysine sites that could be investigated, and potentially reduce the time and cost of generating such species. However, uncertainty remained as to whether multiple nonsense suppression events could be supported. At the time of this research, there was no clear precedent for multiple nonsense suppression events, but recently it was shown that two unnatural amino acids could be incorporated to generate an intra-protein FRET pair(Lee et al., 2016).

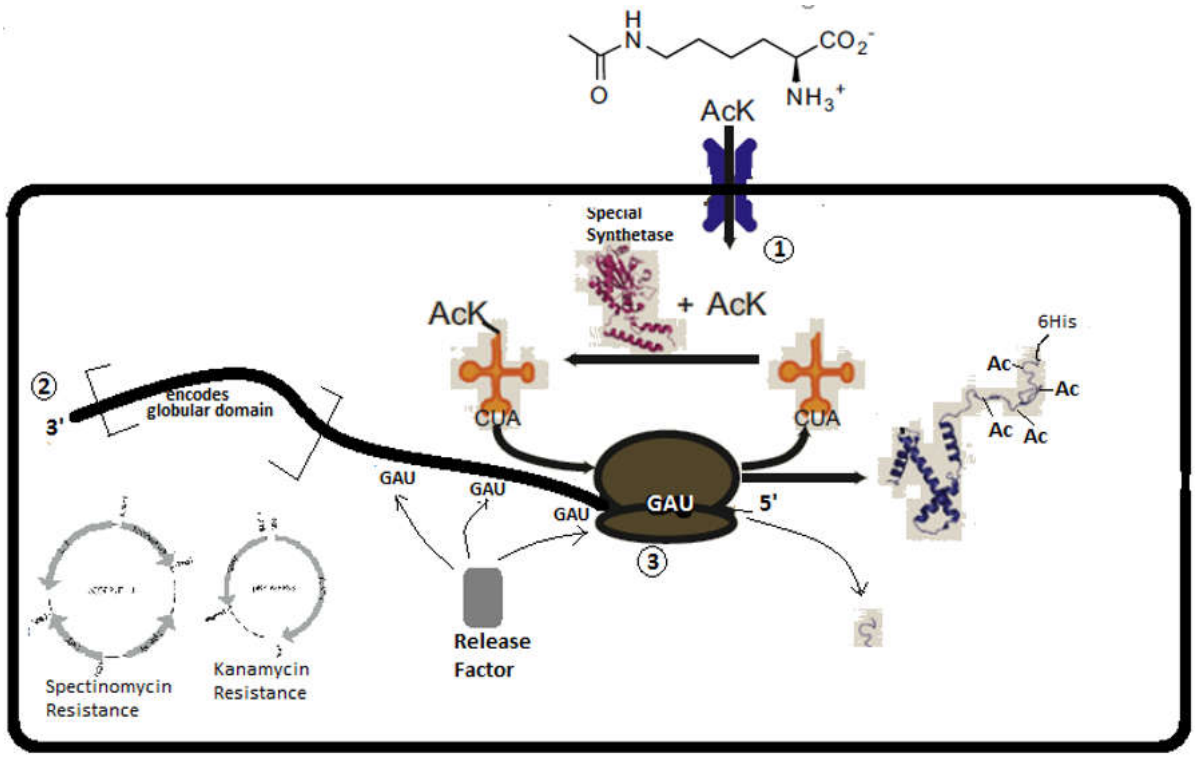

Figure 5 General mechanism of nonsense suppression to produce acetylated histones. Adaptation of (Neumann et al., 2009; Neumann et al., 2008). Rounded large black box represents an E-Coli cell. Inside the cell, two plasmids are inserted which confer the cell with resistance to Spectinomycin and Kanamycin. The cell produces a special t-RNA$_{CUA}$ and a special acetyl-lysine synthetase. (1): The t-RNA$_{CUA}$ is recognized by the Synthetase which has been engineered to accept and install acetyl-lysine (ACK: which is made available in the media) on the t-RNA$_{CUA}$. (2): mRNA containing amber stop codons (portrayed as 3'-GAU-5') transcribed from the plasmid under an inducible LAC promoter by the cell. (3): A fully assembled ribosomal complex with mRNA attached which is actively translating the mRNA into protein containing both full length and truncated versions of acetylated histone. The truncated histone (lower right) is an undesirable side product produced due to Release factor which competes for binding to the amber stop codon and prematurely terminates translation.

CHAPTER 2

EXPRESSION AND PURIFICATION OF HISTONE H3 PROTEINS CONTAINING MULTIPLE SITES OF LYSINE ACETYLATION USING NONSENSE SUPPRESSION

Isaac A Young[1], Chitvan Mittal[2], Michael Shogren-Knaak[3]

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]Primary author, all research except [2]
[2]Contributed figure and method for histone acetyl-transferase assay
[3]Principle investigator and grantsmanship

Introduction

Lysine acetylation is a pervasive post-translational protein modification, where approximately 3,600 sites of lysine acetylation in over 1,700 different proteins have been identified through proteomic studies of human cells (Choudhary et al., 2009). This modification helps to regulate proteins with a wide range of functions, including those involved in metabolism (Xiong and Guan, 2012), cell structure (Perdiz et al., 2011), and cell signaling (Mowen and David, 2014).

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 (Luger et al., 1997), 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 (Zhang et al., 2003). 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 (Robinson et al., 2008). 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 (Li and Shogren-Knaak, 2008; Shogren-Knaak et al., 2006). 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 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 (Young and Schultz, 2010), 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 M. barkeri can efficiently charge a tRNA with acetyl-lysine, and that this residue can be incorporated into histone H3 with good yields (Neumann et al., 2009). 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 (Young et al., 2009), 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 (Ladurner et al., 2003). 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 (Gansen et al., 2009; Tse et al., 1998). 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.

Materials and Methods

Plasmid production

Xenopus laevis H3 histone in a pET3c expression plasmid (Luger et al., 1999a) 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 (Neumann et al., 2009).

Expression of acetylated histone H3

Expression methods were adapted from those previously published (Neumann et al., 2009). 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 OD600 of 0.08 at 37°C. The dilute 2xYT-KS culture was grown to 0.7 OD600. 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 minutes, protein expression was induced with the addition of 0.5 mM IPTG. After 4 hours, 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.

Purification of acetylated histone H3

Inclusion bodies from 250 ml of culture were isolated as previously described (Luger et al., 1999b). The insoluble final pellet containing histone was macerated with 0.25 mL DMSO and then thoroughly resuspended in 15 mL unfolding buffer (UB: 6M guanidine hydrochloride, 20 mM Tris hydrochloride, 5 mM β mercaptoethanol, pH 8.0) and stirred at room temp for 1 hour. The mixture was clarified via centrifugation (12,000 RCF, 10’). The supernatant was added to

2.5 mL of pre-equilibrated and drained Ni2+-NTA Bead Resin (Qiagen) in a 30-ml disposable column and stirred at room temperature for 1 hour. The column was drained and the resin washed two times with 30-ml Guanidine Wash Buffer (GWB; 6M 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 hours at room temperature. The column was then drained and resuspended in 15 mL UB and stirred at RT for 1 hour. UB eluent containing histones was dialyzed into SAU buffer (7M 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.

Assembly of mononucleosomes

Wild type histones and the tetra-alanine H3 histone were expressed, purified, and quantified according to literature protocols (Luger et al., 1999b). 177 bp DNA templates containing the 601 strong positioning sequence (Lowary and Widom, 1998) and BglI non-

palindromic sticky ends were prepared as previously described (Mittal et al., 2014). Octamers were assembled from the appropriate recombinant histones and purified via size exclusion chromatography as published previously (Luger et al., 1999b). Mononucleosomes were reconstituted from the DNA template and histone octamer via rapid dilution methods (Widlund et al., 1997), 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 (Mittal et al., 2014). Briefly, 4.66 pmol of the biotinylated adapter were bound to 200 □g of paramagnetic streptavidin beads (NEB) at RT for 20 minutes in 1X binding buffer (10 mM Tris-HCl, pH 7m.4, 100 mM NaCl) to get a final reaction volume of 100 □l. The supernatant was removed and beads were washed three times with 50 □l of 1X ligation buffer (50 mM Tris, 10 mM MgCl2, 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 □l. 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 hours. Excess unbound nucleosomes were then washed off. The beads were resuspended in 31 □l 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 hours. The liberated nucleosomes were analyzed on 4% native PAGE.

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 (Mittal et al., 2014). 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 MgCl2 , 1 mM DTT, 0.1% triton X-100) and for additional 5 times at 37°C with 15 µl 1X WB2 (50 mM NH2OH , pH 7.5, 0.1% triton X-100), with 25 minutes 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%.

Results

We have been interested in how histone H3 tail acetylation is established by the SAGA family of transcriptional co-activitors (Li and Shogren-Knaak, 2008, 2009; Mittal et al., 2014), and how this acetylation affects chromatin structure and function, such as subsequent histone

acetylation (Li and Shogren-Knaak, 2009). 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 (Figure 1) (Grant et al., 1999). Nonsense suppression has been successfully employed to generate H3 histones containing lysine 14 acetylation (Neumann et al., 2009), 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 E. coli (Luger et al., 1999a), and H3 K14 acetylated histone was previously generated from an overexpression plasmid containing an amber codon at amino acid residue 14 (Figure 1) (Neumann et al., 2009). 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

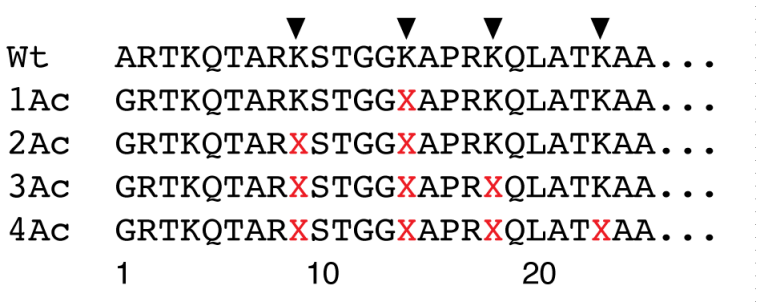

Figure 1. Sequences of acetylated histone H3 proteins. Top row shows the sequence of the N-terminal tail of histone H3. Triangles point to the major sites of SAGA-mediated lysine acetylation. Subsequent rows show the acetylated histones generated, where 'X' denotes a site of amber codon replacement in 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.

To test the efficiency of acetyl-lysine incorporation, we first over-expressed singly acetylated H3 K14 acetylated histone according to literature protocols (Neumann et al., 2009). 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 (Figure 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 (Figure 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 (Figure 2B). Further, expression of histones with fewer than four acetyl-lysines was also feasible, with greater expression occurring when fewer amber codons are suppressed (Figure 2C).

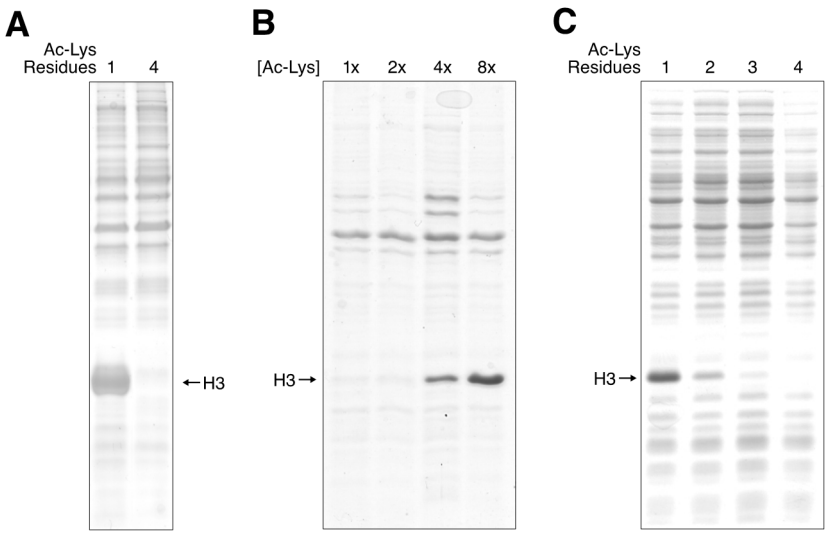

Figure 2. Expression of acetylated histone H3 proteins. Proteins were resolved on an 18% SDS-PAGE gel and stained with Coomassie Blue. A) Comparison of amounts of full-length mono- and tetra-acetylated histone protein in inclusion bodies under published nonsense suppression conditions. B) Comparison 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.

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 acetylated histone (Neumann et al., 2009), 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 (Figure 3A, lanes 1-3). Histone could then be unfolded and captured on nickel-NTA beads under denaturing conditions to further enrich purity (Figure 3A, lane 4). After solvent exchange into a non-denaturing buffer compatible with TEV protease, H3 histone was liberated from the nickel beads (Figure 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 (Figure 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.

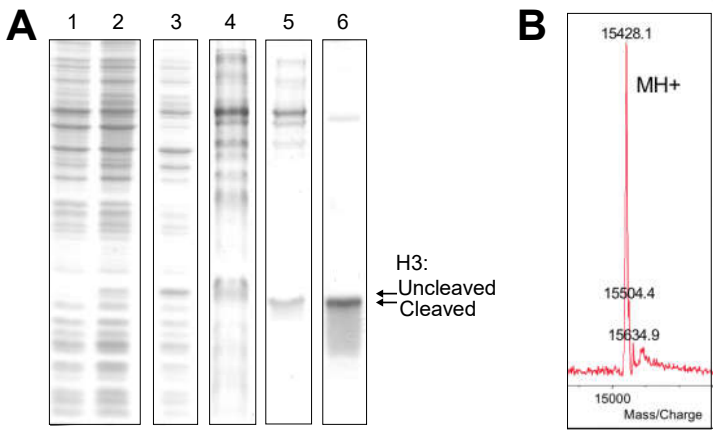

Figure 3. A) Analysis of tetra-acetylated H3 histone purity at various steps of the modified purification protocol. Proteins were resolved on an 18% SDS-PAGE gel and stained with Coomassie Blue. Pre- and post-induction of whole cell extracts are shown in lanes 1 and 2, respectively. Lane 3 shows protein 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. C) MALDI-TOF mass spectrometry analysis of purified tetra-acetylated H3 protein.

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 (Figure 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 (Neumann et al., 2009). 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 (2M NaCl) solution (Luger et al., 1999a). 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 (Figure 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 (Widlund et al., 1997), and all three mononucleosomes appear largely similar in their extent of assembly and their electrophoretic mobility (Figure 4B). Each mononucleosome was immobilized onto a paramagnetic bead, and then used as a substrate for a SAGA-mediated acetyltransferase assay (Figure 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 a manner similar to loss of the lysine residue.

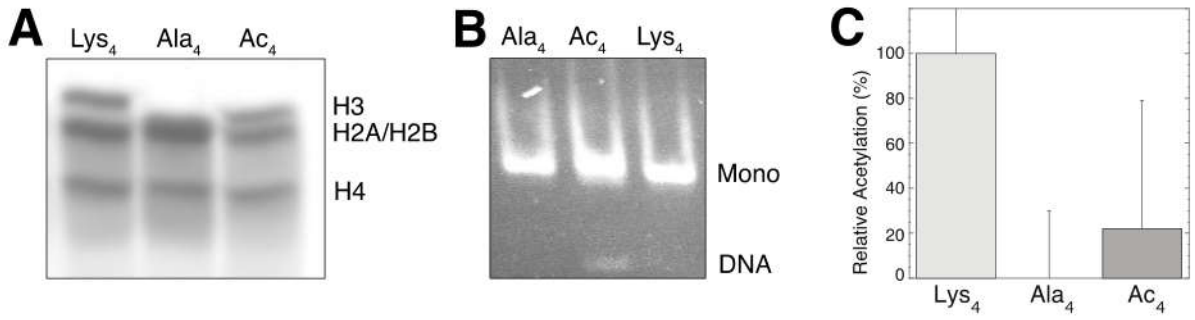

Figure 4. Utilization of tetra-acetylated histone H3 protein. A) Denaturing protein gel analysis of histone octamers containing unacetylated, tetra-alanine, or tetra-acetylated H3 histones. Histones were resolved on an 18% SDS PAGE gel with Coomassie Blue staining. B) Native gel analysis of mononucleosomes 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.

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 (Figure 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 (Figure 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 (Figure 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.

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.

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CHAPTER 3

FUTURE DIRECTION: USE OF ACETYLATED HISTONES TO PROBE THE ESTABLISHMENT AND FUNCTION OF NUCLEOSOME ACETYLATION

Our work with nonsense suppression mutagenesis has provided us with an effective means to readily access H3 histones with a wide range of acetylation states. These reagents offer us the means to probe to an unprecedented degree how acetylated lysine residues individually and in combinations modulate nucleosome structure and function. Future work in the Shogren-Knaak lab will initially focus on two questions – 1) How acetylation of specific H3 tail lysine residues influences the subsequent acetylation of the histone lysines within and between nucleomes, and 2) How specific H3 tail acetylations promote the progression of nucleosome disassembly.

Investigating SAGA's Postulated Acetylation Spreading Mechanism

In vivo studies of nucleosome acetylation, such as those using Chromatin IP, have revealed that a basal level of histone acetylation is present ubiquitously throughout the genome, and that when further acetylation is introduced, it tends to rise significantly within a nucleosome, and to spread outward to neighboring nucleosomes (Bulger, 2005). These results have led to models in which pre-existing nucleosome acetylation can promote subsequent acetylation, and that this can lead to a processive spreading of acetylation between nucleosomes. This model is further bolstered by the fact that histone acetyl-transferase enzymes, such as the SAGA complex, contains domains that bind specificially to acetylated histone tails (Hassan et al., 2002). Nonetheless, mechanistic details are largely absent, and can

be studies with our ability to readily install specific patterns of lysine acetylation in the H3 histone tail.

Within a nucleosome, recent results from our group and others have indicated that nucleosome acetylation is processive, and that pre-existing acetylation can potentially facilitate this processivity. Pre-existing acetylation on only one of the two N-terminal H3 histone tails within a nucleosome results in increased levels of acetylation on the unacetylated tail (Li and Shogren-Knaak, 2008). Trimethylation of lysine residue 4 of histone H3 has recently been proven to increase processivity of the SAGA complex (Ringel et al., 2015). Recent preliminary evidence suggests the pre-steady state kinetics of SAGA contain a burst-phase of multiple acetylations. Work is ongoing to determine the number of acetylations measured (Mittal and Shogren-Knaak, Unpublished results). Building on these results, we seek to better understand how specific individual and combinations of lysine acetylations and their recognition by the bromodomain contributes to this processivity. To that end, we will first continue studying pre-steady state kinetics of SAGA on nucleosome substrates to fully characterize the burst-phase. Then secondly, incorporate the use of nucleosomes containing pre-existing acetylation in measuring the burst-phase of SAGA and compare those results to SAGA mutants where one or more of the bromodomains have been deleted. We expect these experiments may tell us something about the factors which dictate the parameters of the burst-phase pre-steady state kinetics of nucleosomes.

Processivity between nucleosomes has not been previously demonstrated, but access to acetylated histone and tools developed from our lab will provide a means to probe to what extent this processivity occurs. Chitvan Mittal has developed a technique in our lab which we

call nucleosome acetylation sequencing (NAS) to measure levels of catalytically installed acetylation of individual nucleosomes within nucleosomal arrays(Mittal et al., 2014). NAS follows a strategy of first ligating individual nucleosomes sequentially onto a solid substrate, catalytically acetylating using a tritium labeled acetyl group, and finally cleaving individual nucleosomes from the bead to read the levels of radioactivity in a scintillation counter. Scintillation counts may then be correlated to an average number of acetylations per nucleosome.

NAS and nonsense suppression give us unique tools to investigate a possible answer to the aforementioned question of SAGA's ability to navigate and acetylate chromatin in a selective manner. In our future investigations, we want to examine a nucleosomal array by NAS which contains non-radioactive acetylation (generated via nonsense suppression) on 1 out of many positioned nucleosomes in the array. Higher levels of acetylation at those nucleosomes which are neighboring the pre-acetylated nucleosome when compared to those nucleosomes at the outskirts of the array would be sufficient to indicate that SAGA is, at least in part, preferentially acetylating nucleosomes that have acetylated neighboring nucleosomes.

Characterizing how Specifically Acetylated Lysines Disrupt Nucleosome Stability

Studies have shown that nonspecific bulk acetylation of histones destabilizes nucleosomes (Gansen et al., 2009; Tóth et al., 2006; Tse et al., 1998; Widom, 1999). However, it is not clear which sites or combination of sites contribute most significantly. Utilizing multiply acetylated histones generated via nonsense suppression, we can study effects of acetylation at a number of different sites. Single molecule techniques suggest the first step of nucleosome

dissociation is a peeling away of the DNA at the entry exit domain with an H2A/H2B dimer adhering to the DNA and not the rest of the histone octamer (Andrews and Luger, 2011; Bohm et al., 2011; Koopmans et al., 2009; Li et al., 2005). Histone H3 tail DNA is located near the entry/exit sites of the DNA that wraps around the histone core and potentially interacts with DNA that becomes unpeeled from the H2A/H2B dimers. Thus, by systematically varying the number and position of lysine acetylation, we should be able to test whether all H3 tail lysines are equally involved in stabilizing the nucleosomes. If no destabilization is observed, changes in electrostatic interactions with the nucleosome entry-exit DNA might be measured by neutralizing lysine residues in various patterns to see if they facilitate the peeling away of the nucleosomal DNA from the histones.

We will detect the potentially small changes in structure brought on by acetylation of H3 using single molecule total internal reflection fluorescence microscopy (smTIRFM). In this technique, fluorescently labeled nucleosomes will be assembled with internal FRET pairs. Using smTIRFM, live changes in FRET of single nucleosomes can be observed in real time (Luo et al., 2014). A graph is generated which plots nucleosome dissociation events against time. Because dissociation is a stochastic event, the curve may be fit to exponential decay and generates a decay constant.

The decay constant is the inverse of the average lifetime of molecules and is analogous to the kinetic off rate. Because nucleosomes are stable and off rates are immeasurably slow in low salt buffers using smTIRFM, salt buffer will be added at a variety of salt concentrations. It has been previously observed using single molecule techniques that the salt destabilizes the mononucleosomes which make the off-rate easier to measure (Koopmans et al., 2009).

Single molecule techniques make it possible to see changes with very little noise because the user selects only molecules which are free of artifacts. A histogram can be generated which shows changes in populations of high FRET and low FRET efficiency species. The effect of acetylation at lysine 56 on histone H3 was shown to reduce counts of individual nucleosomes with high FRET between dye pairs at the entry-exit region but not at dye pairs located on an internal position within the nucleosome (Neumann et al., 2009).

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