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A Mutational Mimic Analysis of Histone H3 Post-Translational Modifications: Specific Sites Influence the Conformational State of H3/H4, Causing either Positive or Negative Supercoiling of DNA

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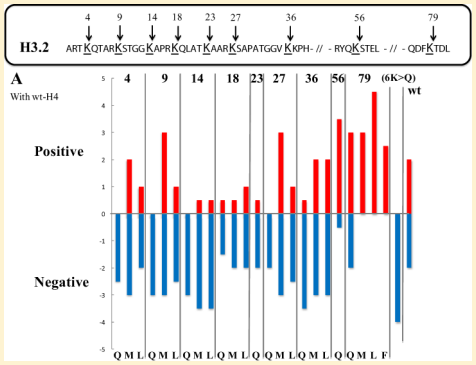
Supporting Information

ABSTRACT: Histone H3 has specific sites of post-translational modifications that serve as epigenetic signals to cellular machinery to direct various processes. Mutational mimics of these modifications (glutamine for acetylation, methionine and leucine for methylation, and glutamic acid for phosphorylation) were constructed at the relevant sites of the major histone variant, H3.2, and their effects on the conformational equilibrium of the H3/H4 tetramer at physiological ionic strength were determined when bound to or free of DNA. The deposition vehicle used for this analysis was NAP1, nucleosome assembly protein 1. Acetylation mimics in the N-terminus preferentially stabilized the left-handed conformer (DNA negatively supercoiled), and mutations within the globular region preferred the right-handed conformer (DNA positively supercoiled). The methylation mimics in the N-terminus tended to maintain characteristics similar to those of wild-type H3/H4; i.e., the conformational equilibrium maintains similar levels of both left- and right-handed conformers. Phosphorylation mimics facilitated a mixed effect, i.e., when at serines, the left-handed conformer, and at threonines, a mixture of both conformers. When double mutations were present, the conformational equilibrium was shifted dramatically, either leftward or rightward depending on the specific sites. In contrast, these mutations tended not to affect the direction and extent of supercoiling for variants H3.1 and H3.3. Variant H3.3 promoted only the left-handed conformer, and H3.1 tended to maintain both conformers. Additional experiments indicate the importance of a propagation mechanism for ensuring the formation of a particular superhelical state over an extended region of the DNA. The potential relevance of these results to the maintenance of epigenetic information on a gene is discussed.



Nucleosomes serve to order and condense eukaryotic DNA into a defined structure by providing a basic "bead" for which the DNA is wound nearly twice in a left-handed superhelix that is approximately 146 bp in length.[1,2] The histones within the nucleosome itself are composed of an octamer formed by two dimers of H2A/H2B and two dimers of H3/H4. The globular domains in the histones are responsible for the histone−histone interactions that stabilize these dimers. The two dimers of H3/H4 can also form a fairly stable tetramer prior to deposition on the DNA through the C-terminal portion of each histone H3, residues 100−135, which forms a four-helix bundle between them.[3,4] Nucleosomes are dynamic structures in which the conformational state of these interactions can be altered to allow cellular processes such as replication, DNA repair, and transcription to occur.[5] Proteins such as RNA polymerase, chromatin remodeling factors, including histone chaperones and histone-modifying enzymes, have been shown to facilitate this conformational dynamic.[6] For example, the nucleosome can be disrupted by disassembly of the octamer during transcription, when processivity of the RNA polymerase forces topological changes by forming positive stress on the DNA in front of the polymerase and negative stress in its wake.[7,8] In in vitro transcription studies, it was found that the radiolabeled H3/H4 tetramer remained bound to this positively stressed DNA, and the radiolabeled H2A/H2B dimers were displaced to a competitor that consisted of a negatively coiled DNA that also contained unlabeled H3/H4.[9] Histones H3/H4 have a strong affinity for positively supercoiled DNA.[10] The negatively supercoiled competitor simulated the state of the DNA in the wake of the polymerase in which H3/H4 would efficiently rebind the transiently released H2A/H2B. From these studies, it was proposed that a chiral transition at the H3−H3 interface (right-handed conformation), originally observed and described by the Prunell group,[11,12] would facilitate the binding to positively supercoiled DNA and the subsequent displacement of H2A/H2B. It was also observed that acetylation would promote this chiral transition,[13,14] particularly if NAP1 were involved in the deposition.[15] NAP1 is a histone chaperone that is generally thought to facilitate displacement of H2A/H2B[16] and the sliding of H3/H4[17] as a part of chromatin remodeling complexes.[18−21] NAPI forms a stable dimer[22,23] and deposits

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an H3/H4 tetramer onto DNA to form a left-handed supercoil.[24,25] However, when NAPI is in excess, the dimer−tetramer equilibrium of H3/H4 is shifted toward the dimer[26] and the NAP1 dimer will deposit an H3/H4 dimer onto the DNA, at which time the H3−H3 interface between the two dimers is established. Either a left- or right-handed conformation will form at the interface.[27] It remains to be determined what specific post-translational sites on H3 were responsible for this potential regulation of this chiral transition in the earlier studies.[15] These two different forms of the tetramer will be termed the L-tetrasome (forms negative supercoils) and the R-tetrasome (forms positive supercoils).[5]

All histones have specific sites of post-translational modifications (PTMs) and can be methylated, acetylated, or phosphorylated.[28−30] Histone H3 can be modified at residues 3, 4, 6, 8−11, 14, 18, 23, 27, 28, 36, 56, and 79, while histone H4 can be modified in its N-terminal tail at residues 1, 3, 5, 8, 12, 16, 18, and 20.[29] Many of these PTMs were discovered using mass spectrometry and are present physiologically in various combinations.[31] The multiple modification sites may serve to amplify a particular signal and result in greater chromatin structural changes. This occurs during marking for transcriptional activation or repression as well as during other cellular events.[32−34] For example, methylation at lysine 4 is indicative of transcriptional activation when acetylation at either K9, -14, -18, or -23 is also present.[35,36] In addition, methylation of residues in the globular domain, K36 and -79, has been correlated with active transcription.[37,38] Histone H3 possesses five phosphorylation sites within its N-terminal tail at serines 10 and 28 and threonines 3, 6, and 11.[39] Phosphorylation of serine 10 occurs in a temporal fashion during mitosis. More recently, phosphorylation of serine 10 and 28 and all the modifiable threonine residues have been implicated in the regulation of transcription.[40−43] It is thought that these modifications serve as markers that define the interaction of proteins that assist in establishing a particular epigenetic state. It is less established whether these modifications also prepare the nucleosome for that particular state.

The PTMs are not exclusively present on one particular H3 variant. All three H3 variants, H3.1, H3.2, and H3.3, are subject to these modifications.[34] Variants H3.1 and H3.2 are associated exclusively with DNA replication and are not deposited outside of S phase, except in instances of repair following DNA damage. Histone H3.3 is deposited in a replication-independent fashion and has been found at transcriptionally active loci.[44] A study that characterizes the role of PTMs must consider the variant context in which they are observed.

To study the effects of the PTMs on the H3/H4 chiral transition, mutational mimics were placed at the relevant H3 sites. In previous studies, glutamine substitutions have been used to simulate acetylation[45] and glutamic acid substitutions for serines and threonines to mimic phosphorylation.[46] Methionine substitutions have been used to simulate methylation,[47] and we would propose that leucine would better describe the hydrophobic character of di- and trimethylation. These latter mimics are unable to maintain the positive charge of methylated lysines and therefore are less effective as mimics. We focused specifically on the topological effect of these mutations on DNA supercoiling by H3/H4 in the absence of H2A/H2B. The histones were deposited on the DNA by NAP1 under conditions that favor either direct tetrameric deposition or deposition of the dimeric form of H3/H4. The results indicate that these site-specific mutations affect the conformational state of H3/H4 and ultimately the topological state of the DNA. The stability of a nucleosome would be expected to be greatly influenced by this preference for either the L- or R-tetrasomal conformation and subsequently facilitate either transcriptional activation or repression by the appropriate accessory proteins.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The DNA sequences from *Xenopus laevis* H3 and H4 were used to obtain mutants using the QuikChange protocol from Stratagene. The primers were obtained from Operon (see Table 1 of the Supporting Information). Sequence verification was by Retrogen, and protein expression was conducted in BL21 and in some instances with C41 or C43 cells from Lucigen. The H3.3 sequence and the ΔN-H3.2 sequence (lacking amino acids 1−26) were obtained from GenScript.

Expression and Purification of Histone Modification Mimics. Bacterial cultures were seeded into 2 L of LB, grown to log phase, and induced with 0.7 mM IPTG overnight at 37 °C while being shaken. Cells were harvested, sonicated, and repelleted (25000*g* for 20 min) in a series of four washes containing 50 mM Tris, 100 mM NaCl, 1 mM 2-ME, 1 mM benzamidine, and 1% Triton. A final wash was conducted in the absence of Triton; this pellet was homogenized in DMSO, incubated at 4 °C for 1 h, and subjected to centrifugation, and the resulting pellet was suspended in 6 M guanidinium hydrochloride, 20 mM sodium acetate, and 5 mM 2-ME and rotated overnight at 4 °C. Following centrifugation (180000*g* for 14 h) to remove debri and remaining DNA, the supernatant was applied to a Sephadex G100 column and eluted in 7 M urea, 20 mM KP_i, 50 mM NaCl, 1 mM EDTA, and 5 mM 2-ME (buffer A). When further purification was needed, H3 was concentrated and applied to a MonoS column with a gradient from 0.2 to 0.6 M NaCl in 6 M urea, 20 mM Tris, and 5 mM 2-ME. Fractions were collected, concentrated, and directly used for reconstitution with purified histone H4.

Reconstitution of Histones. Individual histone modification mimics were mixed with histone H4 in buffer A and 20 mM DTT to form a 3.5 mg/mL solution, incubated for 60 min at 23 °C, and dialyzed against 600 mM NaCl, 40 mM Tris, and 5 mM 2-ME for 16 h at 4 °C. Samples were then centrifuged on a microfuge (6000*g*) for 4 min to remove aggregated material, and the supernatants were collected. When H3 was mixed with H4, a 5% excess of H4 was included to ensure that all H3 is complexed with H4. When not bound to H3, histone H4 is insoluble in 600 mM NaCl. The excess is removed during this centrifugation.

Preparation of DNA. Preparation of plasmid 2T7/T3-19 (2255 bp) was performed by purification on successive CsCl−EtBr density gradients. The ccc plasmid is negatively coiled at −0.05 SD and was relaxed by topoisomerase I prior to use for the depositional assays. Two topological standards were used. The negatively supercoiled marker was created by relaxation of DNA with topoisomerase I in 100 mM tryptophan and 40 mM Tris (pH 8.0) at 23 °C, and the positively supercoiled marker was created by relaxation in 10 mM $MgCl_2$ and 10 mM Tris (pH 8.0) at 4 °C. Each DNA was extracted with a phenol/chloroform mixture and found to be −0.01 SD for the negative marker and 0.01 SD for the positive marker.

Preparation of Topoisomerase I and NAP1. Topoisomerase I was prepared from MSB cells as previously described.[48] One unit is defined as the quantity that achieves

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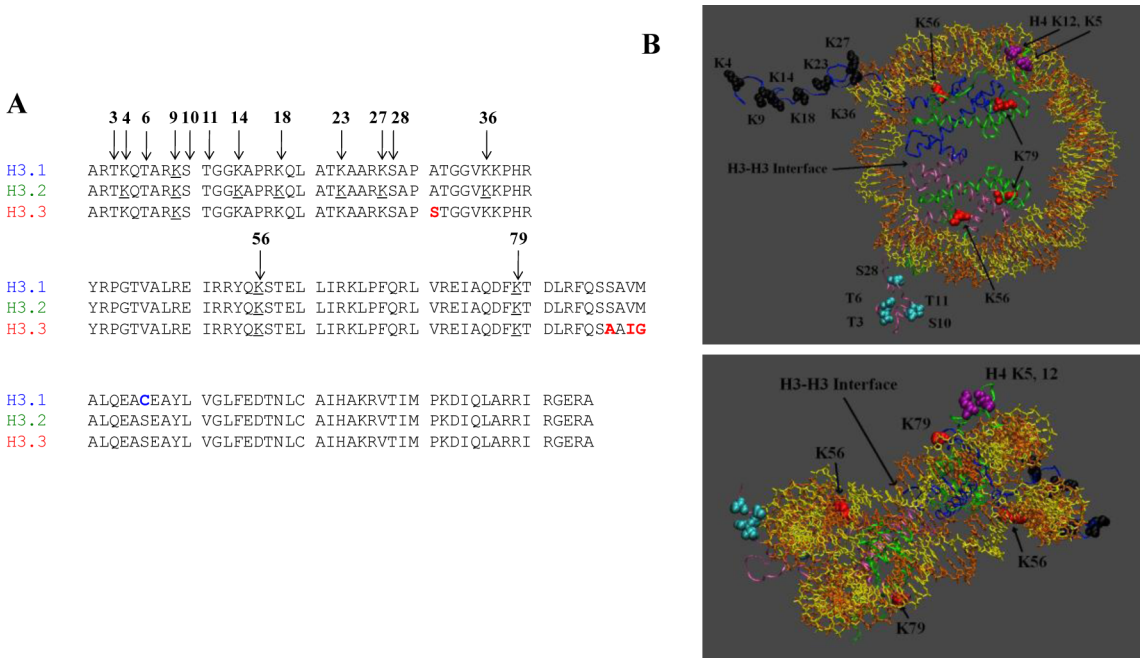

Figure 1. Amino acid sequence and structural location of the mutational sites in histone H3. (A) The sequences of H3.1, H3.2, and H3.3 are shown. The sequence variation between H3.2 and H3.3 is indicated in red and that between H3.2 and H3.1 in blue. The mutational sites are indicated by the arrows. (B) Structural locations of these sites in H3/H4 when the species is bound to DNA in the nucleosome (Protein Data Bank entry 1KX5 using VMD modeling). H3 is colored blue and pink and H4 green. An arrow indicates the site of the four-helix bundle in which the H3−H3 contacts are made to establish the tetrameric state and the subsequent superhelical pitch. The lysines of the N-terminus of H3 are colored black and the serines and threonines cyan. Lysines 56 and 79 of H3 are colored red. Two of the lysines in the N-terminus of H4, K5 and K12, are colored purple.

100% relaxation of 0.5 μg of DNA in 30 min at 37 °C. Yeast NAP1 was prepared from *Escherichia coli* strain BL21 that was transformed by plasmid TN2. The isolation procedure was a modification[49] of the procedure of Fujii-Nakata et al.[50] NAP1 concentrations were determined by absorbance using a calculated molar extinction coefficient of 36100 M^{-1} cm^{-1} at 276 nm.[23,51]

Topological Assay for Examining Histone−DNA Interactions. Each reconstituted histone sample was adjusted to 200 μg/mL in 100 mM NaCl, 40 mM Tris, 1 mM EDTA, and 5 mM DTT and incubated for 10 min at 23 °C. NAP1 was then added to two 35 μL aliquots, yielding 2:1 and 0.5:1 H3/H4 dimer:NAP1 dimer molar ratios. The 2:1 ratio facilitates deposition of H3/H4 as a tetramer, and the 0.5:1 ratio facilitates deposition of H3/H4 as a dimer.[27] A third 35 μL was a control to which no NAP1 was added. The samples were incubated for 30 min at 23 °C, and three aliquots (11 μL each) were added to DNA that had previously been relaxed by being exposed to topoisomerase I in a 5 min preincubation. The amount of DNA that was added establishes weight ratios of 0.8:1, 1.2:1, and 1.6:1 (histone:DNA). Samples were incubated for 90 min at 35 °C, and the reaction was terminated by the addition of 2× STOP buffer [1.25% SDS, 20% glycerol, 125 mM Tris, and 25 mM EDTA (pH 8.0)]. Deposition is generally complete within 30 min, but the incubation is extended to 90 min to ensure full completion. The final samples were then subjected to electrophoretic analysis on 1.2% agarose (Calbiochem) in TAE buffer [50 mM Tris, 45 mM acetic acid, and 1.25 mM EDTA (pH 8.0)] at 90 V for approximately 3 h at 4 °C. These conditions facilitate the separation between negatively and positively supercoiled DNA. To confirm the results, two-dimensional agarose gel electrophoresis was performed by running the second dimension of the gel in the presence of 15 μM chloroquin[27] (see Figure S1 of the Supporting Information for further explanation).

Analysis of the Histone Dimer−Tetramer Equilibrium. The buffer for the H3/H4 sample was adjusted to include 100 mM NaCl, 40 mM KP_i, and 5 mM DTT (pH 7.2) (buffer B) in the presence of BSA, carbonic anhydrase, and H2A/H2B (chick erythrocyte) in a total volume of 180 μL. Samples were then applied to a 5 to 20% glycerol gradient in buffer B (4 mL total volume). Ultracentrifugation was conducted using a SW60Ti rotor at 320000*g* for 24 h at 4 °C. Fractions from each gradient were collected and diluted using 5× STOP buffer and 50 μL aliquots subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis. Glycerol gradients were used rather than size-exclusion columns because H3/H4 tends to interact with column matrices when characterized under this lower-salt condition.

RESULTS

Assay Method Used To Characterize the Leftward and Rightward Conformers of H3/H4. In our previous studies, we have observed that acetylation of the N-terminus of H3 and H4 greatly influenced the level of positive or negative supercoiling that was observed after deposition of H3/H4 by NAP1.[15] To assess the site-specific dependence of this effect, mutational mimics of acetylation, methylation, and phosphorylation modifications were constructed at the relevant sites in the N-terminus of H3 as shown in Figure 1A. The structural locations of the sites that were modified are shown in Figure 1B. An initial comprehensive analysis was done using canonical H3.2, and an example of the assay is shown in Figure 2A. In this example, wild-type H3.2 (wt-H3.2) and modification mimics K79M and K23Q were deposited on DNA at physiological ionic strength with the histone chaperone NAP1. The following

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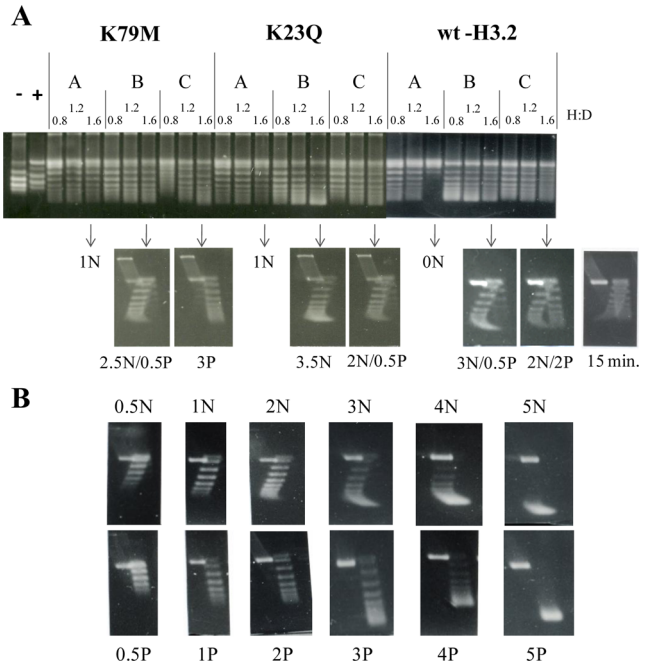

Figure 2. Representative supercoiling assays of H3.2/H4 using NAP1 as the deposition vehicle. (A) Assay of H3.2 mutants K79M and K23Q and wt-H3.2 when reconstituted with wt-H4. The following conditions were used for the deposition of H3/H4: (A) no NAPI, (B) 2:1 H3/H4:NAP1 molar ratio, and (C) 0.5:1 H3/H4:NAP1 molar ratio. After incubation for 30 min in the presence or absence of NAP1, H3/H4 was added to the DNA at weight ratios of 0.8:1, 1.2:1, and 1.6 (H:D). The incubation with the DNA lasted for 90 min at 35 °C. A 15 min time point is also shown for wt-H3.2 under condition C. (B) Two-dimensional analysis and scoring of the DNA topology. These standards are used to numerically score the two-dimensional gels in panel A and the subsequent data of this report.

histone:chaperone ratios were used: no NAP1 (A condition), 2:1 H3/H4:NAP1 (B condition), and 0.5:1 H3/H4:NAP1 (C condition). The B condition includes two H3/H4 dimers (a tetramer) per NAP1 dimer. The C condition includes one H3/H4 dimer per NAP1 dimer and one excess NAP1 dimer. Under the B condition, it has been shown in previous studies that deposition of the native wild-type tetramer primarily forms left-handed supercoils.[24,25] When the histones are deposited as dimers (the C condition), the H3−H3 interactions are established after deposition on the DNA, thus allowing for the formation of either a L- or R-tetrasome.[27] For each of those conditions, the histone:DNA weight ratio was 0.8:1, 1.2:1, or 1.6:1. The latter condition is designed to fully saturate the DNA with H3/H4, promoting histone−histone interactions between the tetramers.[27] All of the varying conditions for each sample were subjected to electrophoresis on a 1.2% agarose gel. As shown in Figure 2A, a laddering pattern is observed that shows a slightly different mobility for negatively supercoiled DNA versus positively supercoiled DNA. When NAP1 is absent (condition A), H3/H4 deposition is inefficient. Those supercoils that are formed are negative for all three forms of H3.2. When NAP1 is present, the deposition is more efficient. There are multiple supercoils, but the type of supercoil does vary, particularly under condition C. Because of the spatial proximity of the different coils, it is difficult to fully assess this variation. We, therefore, conducted a two-dimensional analysis in which after an initial electrophoresis in the first dimension, each lane was electrophoresed in a second dimension while in the presence of chloroquin (see Figure S1 of the Supporting Information for further explanation). These data are shown in Figure 2A for the three forms of H3.2 when assayed at a histone:DNA ratio of 1.6:1. As shown in these data, the K23Q modification formed primarily negative supercoils on the DNA, irrespective of whether the H3/H4 species were deposited as a dimer (condition C) or tetramer (condition B). In contrast, the K79M mutation formed primarily negative supercoils when H3/H4 was deposited as a tetramer, but positive supercoils when H3/H4 was deposited as a dimer. When deposited as a tetramer, wt-H3.2 formed negative supercoils, but as a dimer, both negative and positive supercoils were observed on separate plasmid molecules. We have attempted to quantitate these results by developing a numerical system for characterizing the level of supercoiling activity. The scoring was done by comparison with the standards of Figure 2B, and those values are given at the bottom of each of the two-dimensional panels in Figure 2A. For example, the second-dimension analysis of wt-H3.2 in Figure 2A for the C condition is assessed at 2N/2P using those standards. We interpret this result as an indication that after the initial deposition of the first two H3/H4 dimers on a plasmid molecule, a tetramer is formed with an H3−H3 interface that is either left- or right-handed. Once this superhelical pitch has been established, the subsequent deposition of H3/H4 dimers tends to propagate that same pitch because of the interaction with the initial H3/H4 tetramer. Therefore, plasmids in the same sample will have either negative or positive supercoils. The 15 min time point in Figure 2A shows that equivalent levels of both negative and positive supercoils are observed rather quickly, which is an indication that the formation of both types of supercoils is an early process and does not involve an interconversion between the two forms that changes as a function of time. The type of supercoil is reflective of the initial deposition event. The 90 min time point is more than sufficient to complete the deposition process. The efficiency of this propagation process can vary significantly with the various H3 constructs. As the efficiency decreases, multiple tetramers on a plasmid molecule will tend to randomly form either L- or R-tetrasomes. The equal numbers of negative and positive supercoils offset each other, and very few supercoils will be observed on the plasmid. This lack of propagation is reflected in an overall lower level of supercoils. It should be noted that the presence of the excess NAP1 ensures that when working with these saturating conditions of histone to DNA, the DNA remains soluble and any excess histones that are unable to bind the DNA remain bound to NAP1.[27] Topoisomerase I is therefore able to efficiently relax the DNA despite the high level of H3/H4 content on the DNA. For condition A in which no NAP1 is present, this saturating condition limits the solubility and topoisomerase I activity. That data will not be reported for the H3 constructs. The data from conditions B and C will be reported using bar graphs that describe the relative quantity and type of supercoil.

Two Different H3/H4 Conformations Are Observed When the Acetylation Mimics Are Located on the N-Terminus versus the Central Core Helices of H3. A summary of the single mutations for acetylation (glutamine) and methylation (methionine and leucine) is graphically represented in Figure 3A using condition C and a histone:DNA ratio of 1.6. In the N-terminus of H3, glutamine substitutions at positions 4, 9, 14, and 27 promoted the exclusive formation of negative supercoils. Substitutions at positions 18, 23, and 36

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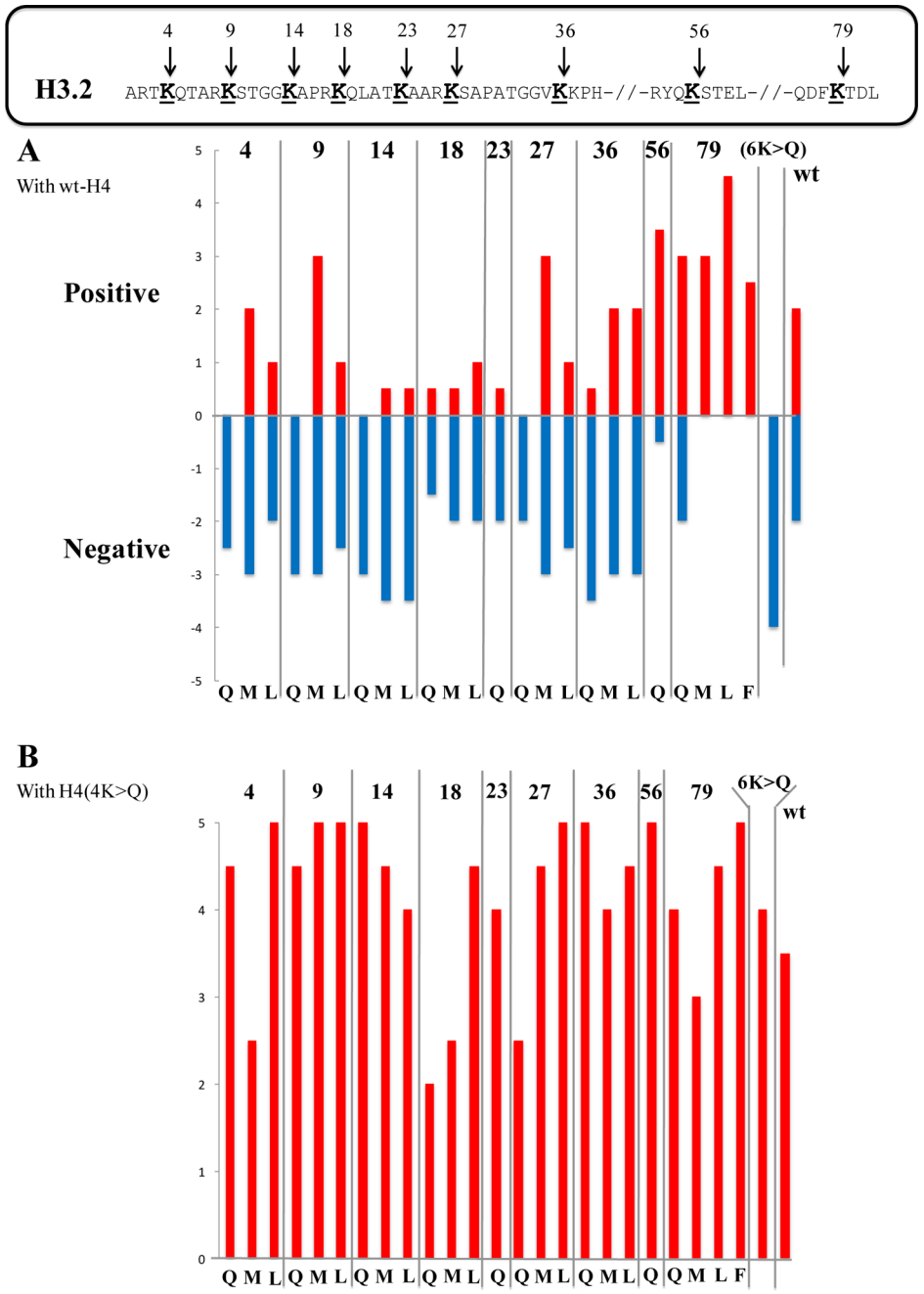

Figure 3. Deposition profile for mutational sites that involve lysines. (A) Bar graph summarizing results for the single mutants of H3.2 when reconstituted with wt-H4. (B) Summary of results when the histones are reconstituted with an H4 in which K5, -8, -12, and -16 were mutated to glutamine and is termed H4(4K>Q). The deposition was conducted under condition C. The mutations are glutamine (Q), methionine (M), and leucine (L). The (6K>Q) is a construct of H3.2 in which K4, -9, -14, -18, -23, and -27 were all mutated to glutamines, which is termed H3.2(6K>Q) in the text. The red bars indicate positive supercoils and the blue bars negative supercoils. Repetitive analysis and subsequent evaluation by the coauthors of these data produced numbers that have an average variation of ±0.5. Figure S2 of the Supporting Information shows the results for the tetrameric deposition (condition B) with both wt-H4 (A) and H4(4K>Q) (B).

promoted primarily negative supercoils, with a small percentage of positive supercoils present. The major sites of known acetylation in histone H3 occur at lysines 9, 14, and 23.[52−56] Sites in H3 that are less often acetylated are positions 4, 18, 27, 36, and 79. These studies indicate that in general, acetylation of H3 at the major sites would tend to stabilize the L-tetrasome. We tested whether multiple acetylations might further enhance this effect by characterizing an H3 in which six of the sites (K4, -9, -14, -18, -23, and -27) were mutated to glutamines. As shown in Figure 3A, this construct promoted a nearly fully negatively supercoiled DNA. There appears to be an additive effect in that an increase in the number of glutamines in the N-terminus increases the number of L-tetrasomes on the DNA. Figure S2A of the Supporting Information shows that this preference for the negative supercoils is also a characteristic for deposition of the tetramer (condition B).

Acetylation does not appear to uniformly stabilize this left-handed conformer. The mutational mimics, K56Q and K79Q, promoted the formation of positive supercoils. Both sites are located in the central body of the H3 on the outer face of the

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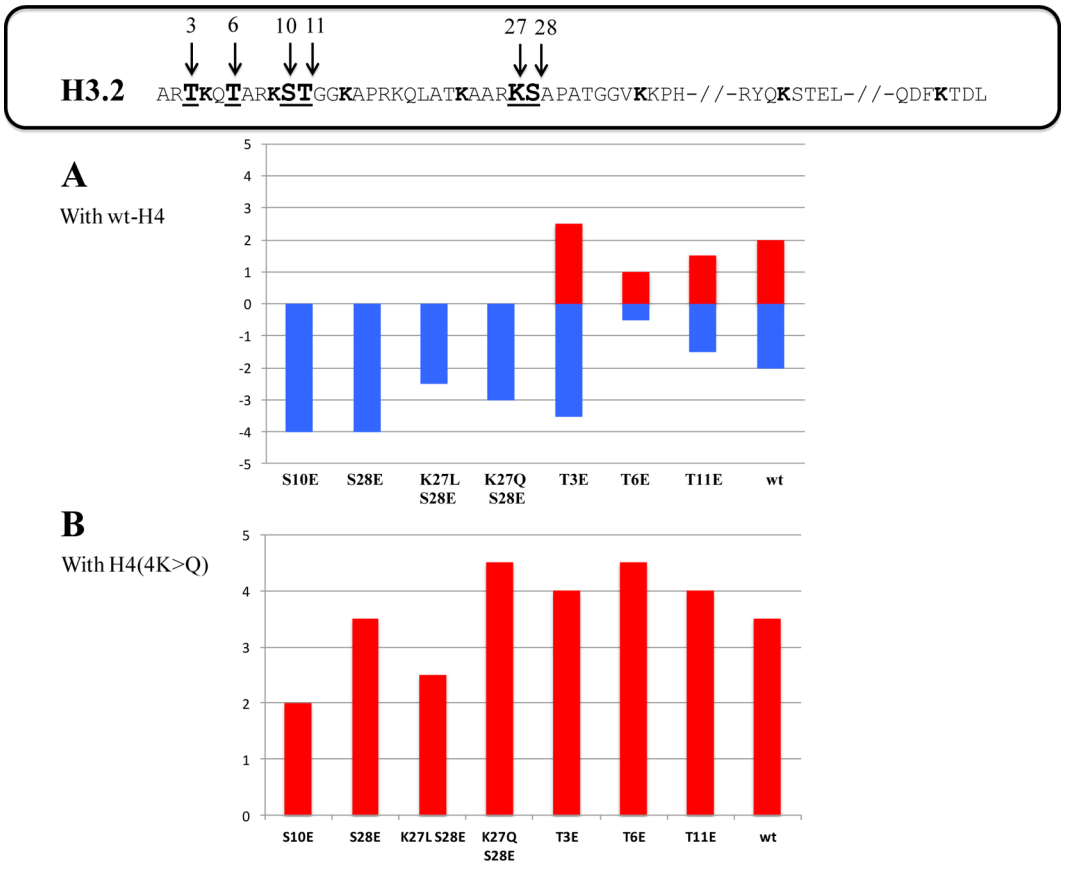

Figure 4. Deposition profile for mutational sites that involve serines and threonines. (A) The mutant H3.2 forms were reconstituted with wt-H4. (B) The mutant H3.2 forms were reconstituted with H4(4K>Q).

nucleosome (Figure 1B). Acetylation of K56 is known to be involved in histone deposition during DNA replication in yeast.[57] It is also involved in facilitating transcription and DNA repair by chromatin assembly and disassembly in that same organism.[58,59] It is involved in transcriptional regulation in higher eukaryotes.[60,61] Methylated K79 has been correlated with transcriptional activity.[37,38] These results are an indication that this particular characteristic of the H3/H4 tetramer, forming positive supercoils, is likely to be found in nuclear functions that require more "dynamic" nucleosomes.

The Methylation Mimics in the N-Terminus of H3 Have Subtle Effects on the Conformational State of H3/H4. Methylation was also studied by substitution of methionine or leucine at the same N-terminal sites (Figure 3A). As shown in Figure 3A, all substitutions except for those of K79 showed a mixture of positive and negative supercoils for both mimics. The extent to which the supercoiling was propagated did vary. The methionine substitutions tended to propagate to a higher level than the leucine substitutions, except for those of K79. Methylation mimics at positions 4, 9, 27, and 36 were similar to wt-H3.2 in that a nearly equal distribution of positive and negative supercoils was observed. In contrast, mimics at positions 14, 18, and 79 were divergent. Sites K14 and K18 tended to prefer negative supercoils, and K79 formed exclusively positive supercoils. Primary sites of methylation in H3 can be found at residues 4, 9, 27, and 79, and at residue 36 to a lesser extent.[28,62] For all but one of these sites, the left- and right-handed H3/H4 conformation is permitted. The K79 site is unique in that any type of methylation mimic promotes exclusively positive supercoils. This exclusivity is not observed for the acetylation mimic at K79. The presence of significant levels of negative supercoiling in this mutant is an indication that perhaps the more hydrophilic the modification, the more likely the observation of negative supercoils. We further tested this possibility with a substitution of phenylalanine at K79. As shown in Figure 3A, only positive supercoils were formed. There appears to be a general trend in which the more hydrophilic modification (glutamine) promotes negative supercoils. The more hydrophobic methylation mutants tend to be more like the wild type, except for K79. The ability to form the R-tetrasome in these mutants is very dependent on the deposition of H3/H4 as a dimer. Figure S2A of the Supporting Information shows that when the histone is deposited as a tetramer (condition B), negative supercoils predominate. Because the methylation mimics in the N-terminus tend not to substantially alter the conformational equilibrium, it is likely that single-site methylation would not result in significant potentiation of the nucleosome for activation or repression.

The same set of histone H3 modification mimics were also reconstituted with histone H4(4K>Q). This construct contains glutamine substitutions at K5, -8, -12, and -16 in the N-terminus and is designed to mimic a highly acetylated H4 for instances in which the epigenetically modified H3s would be present on DNA of actively transcribed regions.[45] As shown in Figure 3B, only positive supercoils were observed for all of the various H3 mutations. There was some variation in the quantity of these supercoils, which may indicate variable efficiency in the propagation of the supercoils through tetramer−tetramer interactions on the DNA. There does not appear to be any pattern with respect to whether a hydrophobic or hydrophilic

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substitution changes the propagation. Glutamine substitutions at positions 4, 9, 14, 36, 56, and 79 propagated efficiently, but those at K18 and K27 did not. There was also variation in the methylation mimics that did not follow any recognizable pattern. The overall conclusion from this analysis is that glutamine mutations in the N-terminus of H4 are dominant over the modifications in the N-terminus of H3. This dominance was further demonstrated in the high levels of positive supercoils that were observed even in the H3.2(6K>Q) construct. In a previous study, we showed that when acetylated H3 was reconstituted with unacetylated H4, the L-tetrasome was favored. When acetylated H4 was used, the R-tetrasome was favored.[15] The glutamine substitutions in the N-terminus of both H3 and H4 are essentially showing the same results, which is an indication that glutamine is an effective acetylation mimic for these studies. These results indicate that epigenetic marks of H3 tend not to influence the conformational state of H3/H4 in the context of an active gene in which a highly acetylated H4 may be present. Those tetramers would strongly prefer the right-handed conformation.

This preference for the right-handed state was even observed in tetramer deposition. Figure S2B of the Supporting Information shows that except for a few mutations, the majority of the tetramers that were deposited formed positive supercoils. It is difficult to assess whether this effect is due to deposition of tetramers that are already in the R-tetrasomal conformation or that after the subsequent deposition of these tetramers, they are prone to change from the L-tetrasome to the R-tetrasome. In either case, the presence of this modified H4 has substantially altered the equilibrium for these two conformational states.

The H3/H4 Conformation in the Phosphorylation Mimics Is Dependent on Whether the Substitution Is Introduced at Serines or Threonines. Besides the PTMs that are found at the lysines in the N-terminus of H3, there are also two serines and three threonines that are known to be phosphorylated.[39] Glutamic acid was substituted at serine 10 or serine 28 and threonine 3, 6, or 11. When reconstituted with wt-H4, both the substitutions at serine 10 and 28 promoted exclusively negative supercoils (Figure 4A). A different result was observed with the threonine substitutions. For all three sites, a mixture of positive and negative supercoils was observed. Considerably less overall supercoiling was observed with T6E than with T3E, which is an indication that propagation may be inhibited because of the T6E mutation. This intermixing of positive and negative supercoils is somewhat similar to the case for wt-H3.2/H4 and is an indication that phosphorylation at the threonines is not likely to significantly affect the H3/H4 conformational equilibrium. Phosphorylation at these sites has been implicated in the regulation of transcription, and it may be that the primary function of these sites is to facilitate the interaction of accessory proteins involved in that regulation, which is similar to what was observed with the methylated sites (Figure 3A). In contrast, the exclusive formation of negative supercoils that is observed for serines 10 and 28 would indicate a condition in which the nucleosome is stabilized into a more closed structure, a condition that would favor chromatin condensation during mitosis.

Because of the spatial proximity of K27 to S28 in the sequence of H3, we considered whether the phosphorylation mimic would alter the level of supercoils that were observed with K27Q and K27L. The K27Q single mutant normally promotes negative supercoils, and the K27L single mutant promotes both negative and positive supercoils (Figure 3A). As shown in Figure 4A, the inclusion of the S28E mutation did not significantly alter the number of negative supercoils that were promoted by the K27Q mutant. However, when the S28E mutation was combined with the K27L mutation, the positive supercoils that were normally present in the K27L single mutant were no longer observed. Thus, placing a negative charge at S28 promotes the conformation of the L-tetrasome despite the adjacent epigenetic mark. These phosphorylation mimics were also tested when reconstituted with H4(4K>Q). As shown in Figure 4B, only positive supercoils were observed. The state of modification in the N-terminus of H4 continues to be the dominant factor. We have not found a mutation in the N-terminus of H3.2 that can block this effect.

Double Mutations with the Acetylation and Methylation Mimics Significantly Alter the Conformational Equilibrium of H3/H4. Mass spectrometric analysis of the N-terminus of H3 has indicated that multiple PTMs are frequently present on the same N-terminus.[63] Therefore, it was important to elucidate whether various combinations would affect the conformational equilibrium of H3/H4. When the K4L mutation was combined with either the K9L mutation or the K27L mutation, a mixture of negative and positive supercoils was observed (Figure 5A). This result is similar to that of the single mutants (Figure 3A). There is a weak preference for positive supercoils, but generally, there is not a substantial additive effect. A different result was observed when the K4L mutation was combined with the K36L mutation. Normally, single mutations at both sites promote a mixture of positive and negative supercoils, but when the mutations are combined, only positive supercoils are formed. In contrast, when the K9L mutation was combined with the K36L mutation, the mixture of positive and negative supercoils persisted. The K4L mutation is preferentially facilitating the right-handed conformation in the double mutant, while the K9L mutation does not. Similarly, the K27L mutation facilitated the formation of the right-handed conformation when combined with the K36L mutation (Figure 5A). Thus, it appears that the K36L mutation, while not preferentially promoting positive coils on its own, does so in the presence of the K4L and K27L mutations. The K79 site is even more dominant. When the K79L mutation was combined with the K9L mutation, only positive supercoils were observed. The K79 site is dominant over any tendency of the other sites to maintain negative supercoils. In general, these results suggest that sites distal to the end of the N-terminus tend to dominate with regard to the type of supercoiling that is observed.

In Figure 3A, it is shown that acetylation mimics at positions 4, 9, and 14 preferentially formed negative supercoils. To determine whether this preference would be maintained in the presence of mutations that were more inclined to form positive supercoils, several double mutations were made. When the K4L, K27L, and K79L mutations were combined with the glutamine mutations, negative supercoils dominated (Figure 5B). Only the K4Q/K79L double mutant showed a mixture of both negative and positive supercoils, indicating that the K79 site is able to partially resist the strong influence of the acetylation mimics at the N-terminus.

In Figure 3A, it is shown that the K56Q mutant demonstrated a strong preference for positive supercoils. Because this site is also distal to the N-terminus, similar to K79, we tested whether it could show a similar resistance to the strong dominance of the acetylation mimics in the N-terminus.

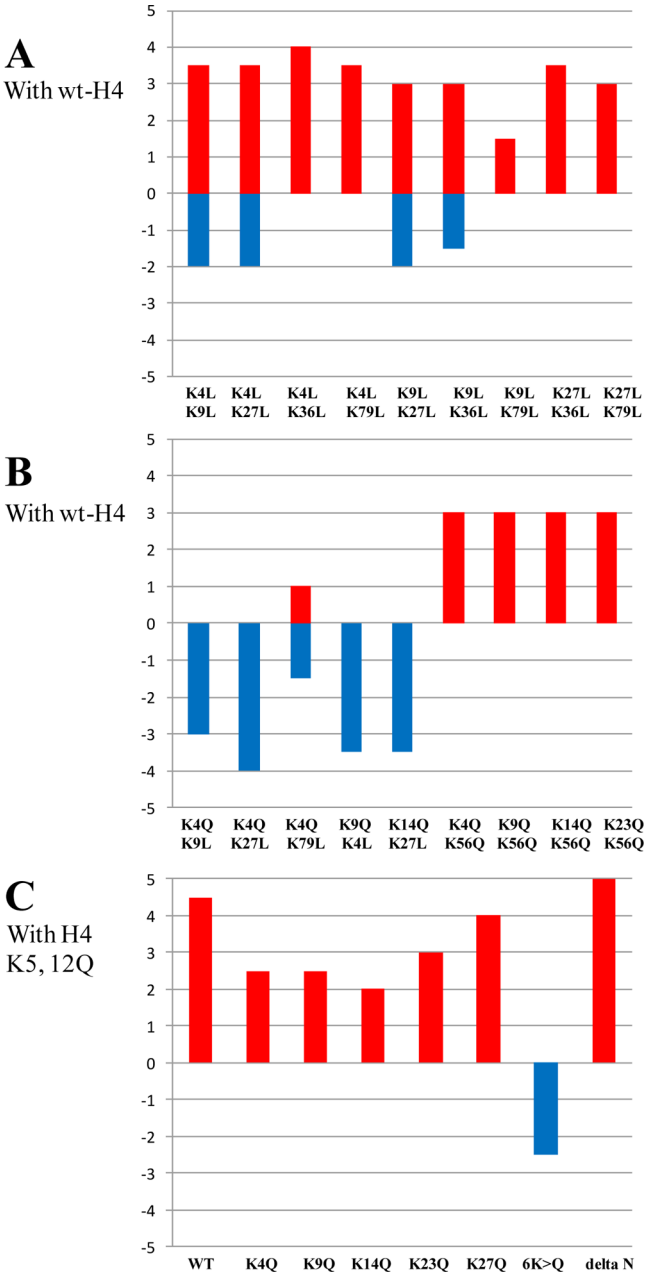

Figure 5. Deposition profile of multiple mutations that are designed to mimic the interplay between methylation and acetylation. (A) Double leucine mutations in H3.2 that were reconstituted with wt-H4. (B) Selected combinations of glutamine and methionine mutations in H3.2 that were reconstituted with wt-H4. (C) Selected H3.2 mutations are reconstituted with H4 in which K5 and K12 are mutated to glutamine, H4K5,12Q.

Double mutations including either the K4Q, K9Q, K14Q, or K23Q mutation were combined with the K56Q mutation. As shown in Figure 5B, a uniform level of positive supercoils was present for all of the constructs. In this case, the K56Q site was dominant. It is the only site that we have found in H3.2 that shows dominance over the acetylation mimics in the N-terminus.

In Figure 3B, it is shown that when the acetylation mimics in H3.2 were reconstituted with H4(4K>Q), only positive supercoils were present. Because we observed that the K56Q mutant also exhibited this similar dominance, and because this modification is present on newly synthesized H3 of yeast,[57] perhaps the acetylation level that is common to newly synthesized H4 would also show this dominance. It is known that K5 and K12 are selectively acetylated in newly synthesized H4.[64] Glutamine mutations were placed at those sites, and this H4 was reconstituted with wt-H3.2 or with an H3.2 containing either the K4Q, K9Q, K14Q, K23Q, or K27Q mutation. As shown in Figure 5C, wt-H3.2 has very high levels of positive supercoils. The glutamine mutations in H3.2 have various levels of positive supercoils that are lower than that of wt-H3.2. This result begs the question of whether combining all the glutamine mutations into the N-terminus of one H3 would be able to overcome the dominance of H4K5,12Q. As shown in Figure 5C, H3.2(6K>Q) did indeed overcome this dominance as only negative supercoils were observed. This result was not the case when four glutamines were present, the H4(4K>Q) construct. Only positive supercoils were present (Figure 3B). Reducing the number of glutamines in H4 has allowed H3(6K>Q) to shift the conformational equilibrium toward the L-tetrasome. These results indicate that this equilibrium is very dependent on the quantity of glutamines that are present in the N-terminus of H3 versus those that are in the N-terminus of H4.

This tendency to promote negative supercoils may be due to the removal of positive charges and the subsequent reduction in the level of binding of DNA. If this assessment is correct, the removal of the N-terminus of H3.2 (amino acids 1−26) should result in a similar level of negative coils when reconstituted with H4K5,12Q. As shown in Figure 5C, only a high level of positive supercoils was observed. These data indicate that the N-terminus must be present to facilitate the stabilization of the L-tetrasome and would imply that interaction with the DNA continues to persist, except that the mutational glutamines have now altered the nature of that interaction.

H3.3 Prefers the L-Tetrasome and Is Refractory to Mutations That Would Inhibit It. Histone H3 variants can be either replication-dependent, H3.1 and H3.2, or replication-independent, H3.3. Histone H3.3 varies from H3.2 in only four amino acids, while H3.1 differs from H3.2 in only one amino acid (Figure 1A). These differences may influence the conformational equilibrium of the H3/H4 tetramer. As shown in Figure 6A, H3.1 demonstrated a mixture of positive and negative supercoils, which is similar to the case for H3.2. The level of negative supercoils is substantially lower, which is an indication of a slight preference for the R-tetrasome. In contrast, H3.3 promoted the opposite result. Only negative supercoils were observed (Figure 6C). To evaluate the effects of PTMs on these two variants, we focused specifically on lysines 9, 56, and 79, because these three lysines represent sites in which either negative supercoils, positive supercoils, or a mixture of both was observed in H3.2 (Figure 3A). As shown in Figure 6A for H3.1, a mixture of positive and negative supercoils was observed for all sites except for K56Q, which was exclusively positively supercoiled. This exclusivity was similar to what was observed for H3.2 (Figure 3A). The mixture of supercoiling observed for K9Q and K79Q is quite different. For H3.2, K9Q was exclusively negatively supercoiled and K79M was exclusively positively supercoiled (Figure 3A). The presence of this mixture (mirroring wt-H3.1/H4) is an indication that this variant is more resistant to the effects of those mutational sites. When these mutations were applied to H3.3, only negative supercoils were observed for all the sites (Figure 6C). Even the K56Q mutation would not alter this characteristic. This variant is quite refractory to these

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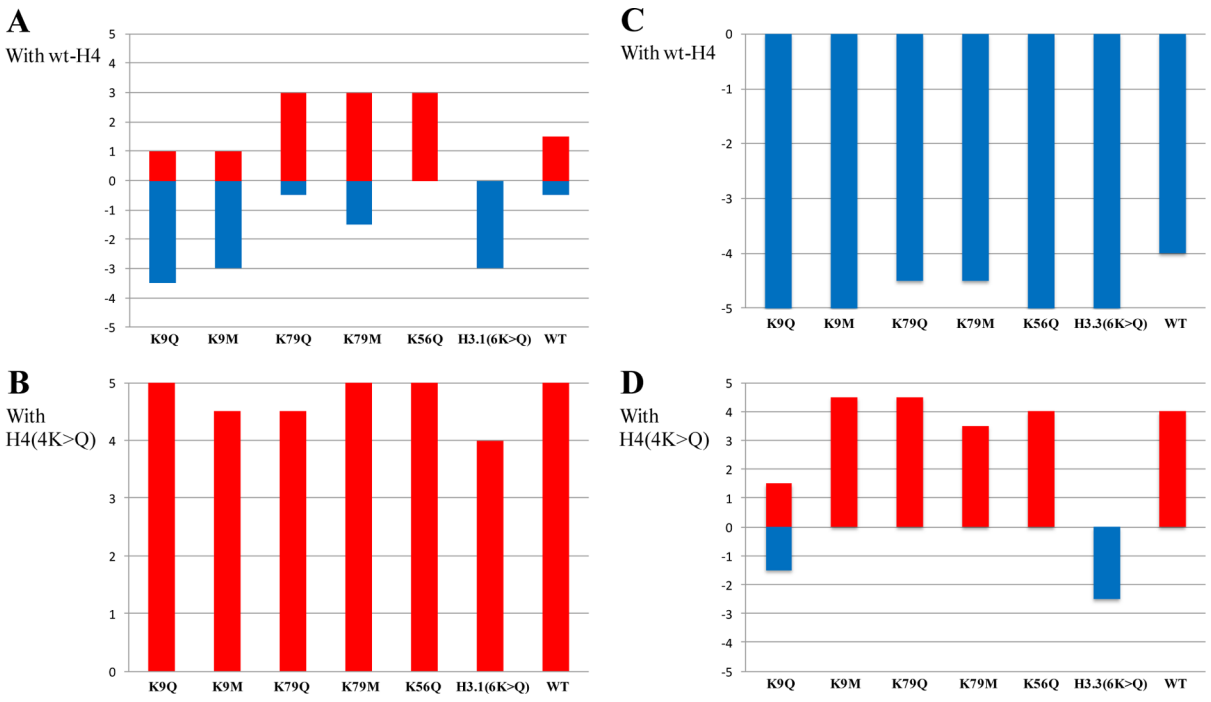

Figure 6. Deposition profile for selected mutations in the H3 variants, H3.1 and H3.3. H3.1 (A) and H3.3 (C) when reconstituted with wt-H4 or H3.1 (B) and H3.3 (D) when reconstituted with H4(4K>Q).

mutations. Because H3.3 is expressed in a manner independent of replication, acetylation at K56 would not likely be prominent on this variant as it would be for both H3.1 and H3.2. There is a substantial difference in preferred conformational states between replication-dependent and -independent H3 variants.

Previously it was shown that when any histone H3 modification mimic was reconstituted with histone H4(4K>Q), H4 was dominant in its characteristics of deposition. High levels of positive supercoils were present (Figure 3B). We evaluated whether this characteristic would also be observed when the histones were reconstituted with H3.1 and H3.3. As shown in Figure 6B, wt-H3.1 and the modification mutants promoted exclusively positive supercoils. The results were very similar to those for H3.2. As shown in Figure 6D, H3.3 also formed exclusively positive supercoils, except for one mimic, K9Q. In this instance, K9Q demonstrated a strong tendency to stabilize the nucleosome and counteract the effects of the K>Q mutations in the N-terminus of H4. Overall, the K>Q mutation in the N-terminus of H4 is dominant with respect to the PTMs on H3 variants. An exception to this conclusion was when the multiple lysines in the N-terminus of H3.3 were mutated to glutamines. The H3.3(6K>Q) construct formed exclusively negative supercoils when reconstituted with H4(4K>Q) (Figure 6D). It is the only construct we have found that was able to do this.

The R-Tetrasome Will Dominate over the L-Tetrasome Because of Its Greater Proclivity To Propagate. In a previous study, we interpreted that the increase in the level of positive supercoils that was observed in H3/H4 as the histone:DNA ratio was increased was due to interactions between adjacent H4 species in each tetramer that were aligned on the same DNA.[27] Figure 7A shows the two-dimensional analysis of a condition in which 0.8:1 and 1.6:1 ratios (H:D) were used in the deposition of wt-H3/H4 by NAP1. The lower ratio formed exclusively negative supercoils, whereas the higher ratio formed a mixture of both positive and negative supercoils. This higher ratio is the condition that we have used in our analysis thus far. Figure 7A also includes additional two-dimensional data for representative mutants at the two different ratios. Note that for those mutations that we have previously shown selectively form negative supercoils at the 1.6:1 ratio, i.e., H3.2K9Q/H4, H3.2(6K>Q)/H4, and H3.3/H4, when the ratio is decreased 2-fold to 0.8:1, there is a proportional decrease in the number of negative supercoils. Each tetramer stably maintains negative supercoils in a manner largely independent of the adjacent tetramer. In contrast, for those mutations that we have previously shown selectively form high levels of positive supercoils at the 1.6:1 ratio, i.e., H3.2K56Q/H4, H3.2K79L/H4, and H3.2/H4K5,12Q, the 0.8:1 ratio has a mixture of negative and positive supercoils. The 2-fold decrease in the histone:DNA ratio greatly limits the tendency to form positive supercoils for those mutants. The R-tetrasome is being stabilized by interaction with adjacent R-tetrasomes at the higher ratio. This type of stabilization was not needed for H3.2/H4(4K>Q) (Figure 7A). The 0.8:1 ratio contains a more proportional decrease in the number of positive supercoils when compared to the 1.6:1 ratio. The R-tetrasome is highly stable when there are multiple glutamines in the N-terminus of H4.

It was of interest to determine whether the mixing of mutants that prefer negative supercoils with those that prefer positive supercoils would result in a dominance similar to that observed for the double mutants (Figure 5B), namely that those that prefer the "L" conformation would dominate over those that prefer the "R" conformation. We tested this potential scenario by equally mixing the two versions together in two different approaches. In the first instance, there is a 30 min preincubation with one of the mutant H3/H4 forms followed by the addition of the second mutant for an additional 90 min incubation (lane a). After the second addition, the histone:DNA ratio is at a saturated level (1.6:1). This approach is then applied in the reverse order (lane b). The second

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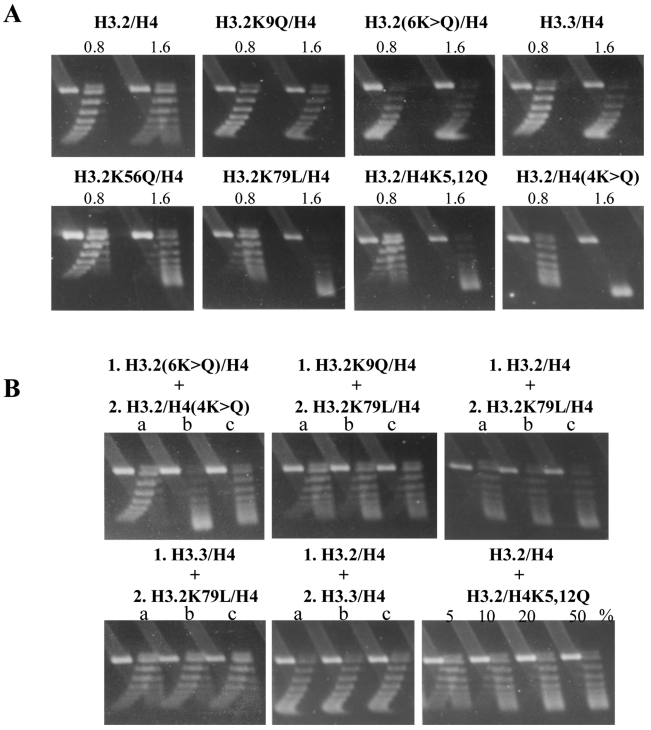

Figure 7. Two-dimensional analysis of the competitive propagation potential between mutant H3/H4 forms that preferentially form either positively or negatively supercoiled DNA. (A) Level and type of supercoiling that are observed with selected mutant constructs at histone:DNA ratios of 0.8:1 and 1.6:1. The deposition was done using condition C. (B) Mutant constructs in panel A are mixed in various combinations following three different protocols. The first protocol involves a preincubation of construct (1.) with DNA for 30 min followed by addition of the second construct (2.) for an additional 90 min incubation (lane a). The second protocol involves an incubation in which the reverse order is used (lane b). The third protocol involves a premixing of both the first (1.) and second (2.) constructs before addition to the DNA (lane c). For the panel in which the two constructs, H3.2/H4 and H3.2/H4K5,12Q, are shown, these two constructs were premixed such that 5, 10, 20, and 50% of the total H3/H4 came from H3.2/H4K5,12Q. This sample was then added to the DNA for a 2 h incubation.

approach is to premix the two different NAP1:H3/H4 complexes and then immediately add them to the DNA (lane c). As shown in Figure 7B, when this procedure was applied to H3.2(K>Q)/H4 and H3.2/H4(K>Q), the order of addition defined the type of supercoiling. If H3.2(K>Q)/H4 were added first, then only negative supercoils were formed (lane a). If H3.2/H4(K>Q) were added first, only positive supercoils were formed (lane b). We interpret these results as indicating that in the first instance, the H3.2(6K>Q)/H4 tetramer is establishing a L-tetrasome, and once that is completed, those tetramers are resistant to the ability of H3.2/H4(K>Q) to subsequently propagate positive supercoils after it had been added. If all of the tetramers of H3.2/H4(K>Q) were to form positive supercoils, the level of positive supercoils would be equal to the number of negative supercoils that were formed by the H3.2(6K>Q)/H4 tetramers. These supercoils would neutralize one another, and no coils would be observed. This latter scenario is clearly not observed. Not only was the H3.2-(6K>Q)/H4 tetramer kept in the left-handed conformation, but also a significant quantity of the H3.2/H4(4K>Q) tetramer would have had to have also assumed the left-handed conformer. The tetramers that were deposited first are controlling the conformational state of those that were added later. When the reverse order is considered (lane b), after the addition of H3.2/H4(4K>Q) in the first step, the R-tetrasome would have formed at a reduced level because of the 0.8:1 histone:DNA ratio (see Figure 7A), but with the addition of H3.2(6K>Q)/H4 to yield the 1.6:1 histone:DNA ratio, these latter tetramers were now also forced to form positive supercoils. The tetramers that were deposited first are again controlling the conformation state of those that were added later. On the basis of the level of positive coils that are observed, nearly all of the H3.2(6K>Q)/H4 tetramers would have had to have formed positive supercoils. The R-tetrasome appears to show a greater dominance. This dominance was even more evident when both sets of histones were added simultaneously to the DNA (lane c). High levels of positive supercoils were observed. Thus, when both forms of the tetramer have equal opportunity to establish a topological state, the R-tetrasome is the one that establishes the DNA topology. This effect is likely due to a more efficient interaction between tetramers that facilitate that particular conformation.

The H3/H4 species that were used in the previous analysis represented the extremes of positive and negative supercoiling. The physiologically relevant mutations are more likely to be those that represent lower levels of PTMs. We repeated this protocol with H3.2K9Q/H4, which forms exclusively negative supercoils, and H3.2K79L/H4, which forms exclusively positive supercoils (Figure 7A). As shown in Figure 7B, both negative and positive supercoils were present, but positive is clearly preferred. The preincubation with H3.2K9Q/H4 (lane a) could not completely block the ability of H3.2K79L/H4 to subsequently propagate the positive supercoils. This means that even though negative supercoils were initially formed on the DNA by H3.2K9Q/H4, the subsequent addition of H3.2K79L/H4 has forced many of these tetramers to change from the L-tetrasome to the R-tetrasome. The conformation of the tetramer is changing while it is still bound to the DNA. As expected, when these two mutants were premixed and then added to DNA (lane c), the positive supercoils remained dominant. This dominance is not as extensive as that observed with H4(4K>Q). If it had a similar dominance, we would have observed the very high levels of positive supercoils that were observed in Figure 7A for H3.2K79L/H4 at the 1.6:1 ratio. Therefore, some of the H3.2K9Q/H4 tetramers still maintained the left-handed conformation and negated the positive ones. Nevertheless, the rather high levels of positive coils in the mixed samples are an indication that the majority of the H3.2K9Q/H4 tetramers had also shifted to the right-handed conformation. The conformational change can occur even when they are bound to DNA.

This dominance of H3.2K79L/H4 could potentially be present in a physiological context with both H3.2 and H3.3. We applied this protocol with these histones, and as shown in Figure 7B, H3.2K79L was strongly dominant with wt-H3.2 in all three incubation conditions. Because wt-H3.2/H4 has the unique property of forming either the L-tetrasome or R-tetrasome equally well, the equilibrium between these two forms can be easily shifted by the K79L mutation, even if H3.2/H4 is already bound to the DNA. However, the K79L mutation was unable to do this with wt-H3.3. Both negative and positive supercoils were observed in all three incubation conditions, with the negative supercoils being more extensive. H3.3 is particularly effective in blocking the propagation of the R-

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tetrasome. This dominance of wt-H3.3 was also observed when it was mixed with wt-H3.2/H4 (Figure 7B). High levels of negative supercoils were observed for all three incubation conditions, and the level was very similar to what was observed if only H3.3 were present at the high histone:DNA ratio (compare to the 1.6:1 ratio of Figure 7A). The conformational equilibrium of H3.2/H4 can be readily altered by interaction with types of H3/H4 tetramers that are much more restricted in their conformational states.

Another physiologically relevant condition in which the interconversion of H3.2/H4 may be observed is under conditions of DNA replication. It is known that H2A/H2B is displaced from the parental H3/H4 during replication of the parental DNA[65] and therefore could potentially interact with the newly synthesized H3.2/H4K5,12Q that is located on the nascent DNA. This interaction might possibly result in the formation of an R-tetrasome for this tetramer. Figure 7B shows an analysis in which the NAP1 complexes of both H3.2/H4 and H3.2/H4K5,12Q were premixed in amounts in which H3.2/H4K5,12Q was 5, 10, 20, and 50% of the total H3/H4 and then added to the DNA for a 2 h incubation. At a 5% level, there was no appreciable effect on the type of supercoiling that was observed for the remaining 95% of the H3.2/H4. Both positive and negative supercoils were present. However, at the 10% level, there was an appreciable shift toward positive supercoils that continued to increase as the percentage was increased to 50%. If such an interaction were to occur during DNA replication, the R-tetrasome would be the preferred conformation for both parental and newly synthesized H3/H4.

Glycerol Gradient Analysis of Tetramer Stability: Those H3/H4 Mutations That Are Prone to Forming the R-Tetrasome Have Tetramers That Are as Stable as Those That Prefer the L-Tetrasome. A substantial variation has been observed in the type of supercoiling that is promoted by the PTM mimics and the H3 variants. One might predict that those mimics and variants that promote the L-tetrasome would have a more stable H3−H3 interface than those that promote the R-tetrasome. The four-helix bundle at this interface is the stabilizing factor, and those interactions would be expected to be fully present in the L-tetrasome and perhaps less so in the R-tetrasome. The mimics that promote the R-tetrasome might tend to shift the dimer−tetramer equilibrium toward the dimer. An analysis of this equilibrium was done using glycerol gradients in 100 mM NaCl. Histone H2A/H2B and carbonic anhydrase were included with the sample and served as markers for the locations of the dimer and tetramer in the gradient, respectively. Our initial analysis was conducted with variants H3.1, H3.2, and H3.3. As shown in Figure 8, there was a considerable variation in stability among the three variants. Variant H3.1/H4 (panel C) formed a very stable tetramer and H3.2/H4 (panel B) and H3.3/H4 (panel A) less so. On the basis of the markers, we have numerically assigned the relative stability, and these data are graphically shown in Figure 9A. Under these conditions, approximately 35% of wt-H3.2 and wt-H3.3 were in the tetrameric form as compared to 100% for wt-H3.1. The S96C substitution (see Figure 1A) that differentiates between H3.1 and H3.2 has significantly increased the stability of the tetramer. Figure 9B shows that when these three variants are reconstituted with wt-H4 and deposited as a tetramer (condition B), only negative supercoils are observed. Therefore, when not associated with DNA, the tetramer conformation that is likely present in the glycerol gradients is the L-tetrasome. It appears that the factor that defines tetramer

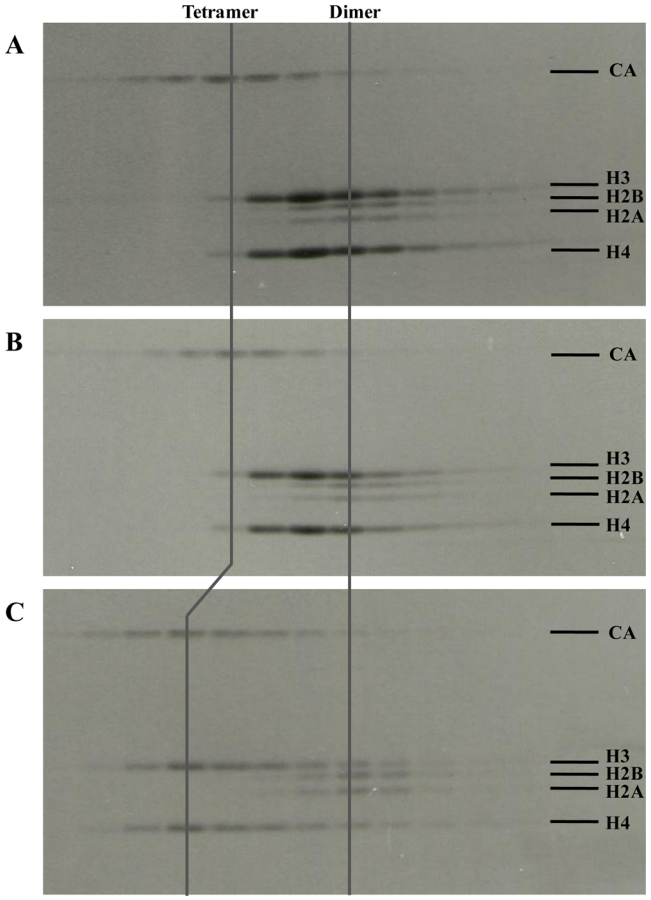

Figure 8. Analysis of the stability of the dimer−tetramer interface in H3 variants (A) H3.3, (B) H3.2, and (C) H3.1. The analysis was conducted on 5 to 20% glycerol gradients in 100 mM NaCl, 20 mM KP_i (pH 7.4), and 5 mM DTT. SDS−PAGE analysis was conducted with the fractions from the gradients. Carbonic anhydrase (CA) and histones H2A/H2B were included in the sample to serve as standards for the gradients. The tetramer and dimer positions are marked on the panels.

stability is the type of variant in which it is present. To evaluate the R-tetrasome, we reconstituted the wild-type versions of these variants with H4(4K>Q). As shown in Figure 9B, when the histones were deposited as a tetramer, positive supercoils were exclusively formed for H3.1 and H3.2. Those tetramers would be more inclined to form the R-tetrasome, even when they are free of DNA. This inclination is less so for the tetramers of H3.3/H4(4K>Q). Both positive and negative supercoils were observed, which is an indication of this variant's resistance to the R-conformation. We examined their relative stabilities on the glycerol gradients, and as shown in Figure 9A, the stabilities increased by a small percentage for H3.2, but not for H3.3. The enhanced stability that was observed for H3.1 remained unchanged. The tetramers prone to forming the R-tetrasome would appear to be as stable as those that form the L-tetrasome. We next examined these variants under conditions in which the 6K>Q mutations were introduced into the N-terminus of each H3 variant. As shown in Figure 9B, when the histones were deposited as a tetramer, only negative supercoils were formed for all three variants when they were reconstituted with wt-H4. When the histones were examined on glycerol gradients, the apparent stability decreased slightly, implying that the 6(K>Q) mutations have partially reduced the stability of

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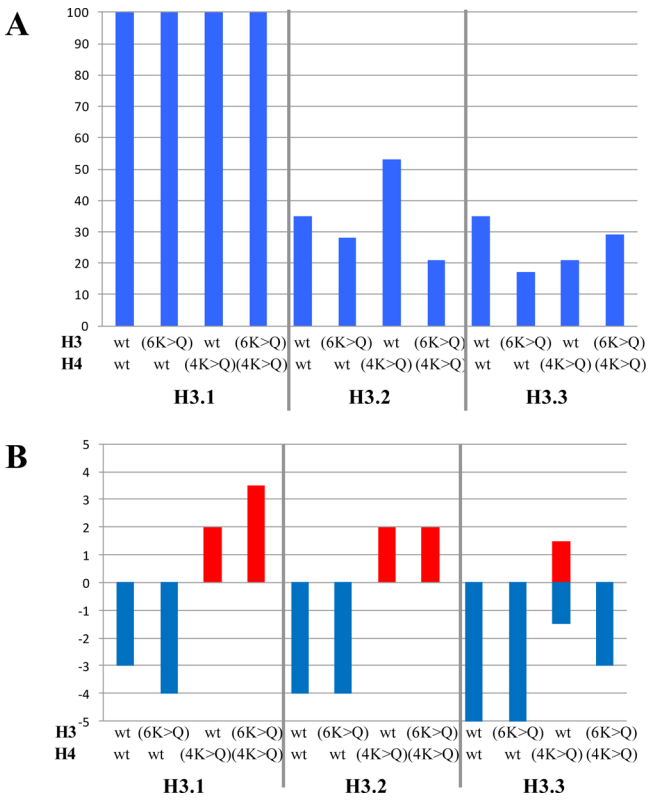

Figure 9. Relative stabilities of the dimer−tetramer interface for the variants and various H3 and H4 mutations. (A) Estimated percentages of the tetramer that was present when reconstitution of the H3 variants and their H3(6K>Q) counterparts was conducted with either H4 or H4(4K>Q). The percentage is based on the relative position of H3/H4 on the 5 to 20% glycerol gradients as shown in Figure 8. (B) Deposition profile for these same constructs when they are deposited as tetramers (condition B).

the tetramer. Yet again, the enhanced stability of H3.1 remained unaffected. The overall conclusion from this analysis is that the stability of the tetramer is not defined by whether a L- or R-tetrasome is present. The stability is an inherent characteristic of the variant. Whatever that stability is, the L-or R tetrasome will generally be equally stable within it. The R-tetrasome must be able to maintain binding energies similar to that of the L-tetrasome within the H3−H3 interface. As a result, the PTMs can readily shift the equilibrium between them.

DISCUSSION

Many PTMs map to the N-terminus of histone H3. These modifications have been shown to be involved in cellular processes through the alteration of nucleosomal organization and subsequent overall chromosomal structure. This effect is often thought to occur primarily through the interaction of accessory proteins that utilize these modifications as a code for establishing a particular epigenetic state. The results of this report suggest that sites of modification may also potentiate the nucleosome for that state. Acetylation and methylation mimics were applied to the modification sites of H3. The sites at which acetylation mimics were placed caused H3/H4 to deviate from its normal condition in which both the leftward and rightward conformations of the tetramer were equally present to a condition in which the conformation is exclusively leftward or rightward. The sites in the N-terminus prefer leftward, and the sites in the regions of the H3/H4 histone fold prefer rightward. Of particular interest are K56 and K79. They are located on the outer face of the nucleosome (Figure 1B). The crystal structures of K56Q and K79Q have been determined.[66] In both cases, there is a reorientation of the glutamines so that they face outward and would be available for interaction with adjacent tetramer−DNA complexes. A similar reorientation was observed for dimethylated K79, which would facilitate interaction with appropriate accessory proteins or with adjacent H3/H4 tetramers.[67] In circumstances in which H2A/H2B would have been displaced, the H3/H4 tetramer might be able to assume this rightward conformation, particularly if the tetramers were able to slide together and establish an interaction. Such a condition could be present because of the concerted action of chromatin remodeling complexes in the initiation and elongation phases of transcription. There is substantial evidence that H2A/H2B is extensively mobile as part of the action of these complexes and/or the inclusion of histone chaperones.[19−21] This depletion of H2A/H2B is also expected to occur during DNA replication, at which time the parental H2A/H2B histones are transiently displaced from the parental H3/H4.[65] The deposition of new histones is a stepwise process that requires an initial deposition of new H3/H4 followed by H2A/H2B, using unique chaperones for each set of proteins.[68−70] It remains to be determined whether this transient absence of H2A/H2B is of sufficient duration to permit substantial R-tetrasome formation for both replication and transcription.

The tendency of the acetylation mimics of the N-terminus to prefer the L-tetrasome may reflect its unique location in the nucleosome. The N-terminus extends through the two supercoils near the central dyad at the entry−exit point (Figure 1B). The loss of charge and the maintenance of hydrophilic character are a combination that may limit the stacking potential of the tetramer. The L-tetrasome tends to be preferred when propagation of the R-tetrasome is limited (Figure 7). This same limitation was observed when serine 10 and serine 28 were mutated to glutamic acid. The loss of overall positive charge and the maintenance of hydrophilic character once again preferred the L-tetrasome (Figure 4A). In contrast, the methylation mimics in the N-terminus still permitted the presence of the right-handed conformer (Figure 3A). Therefore, it is not just sufficient to remove the charge; the hydrophilic nature must also be maintained. There are exceptions to this overall conclusion. The glutamic acid substitutions at the threonines (T3, T6, and T11) still permitted the presence of the R-tetrasome (Figure 4A), indicating that the location of the mutation on the N-terminus also significantly defines how this change in charge ultimately affects the overall structure of the tetramer. The N-terminus of H3 must continue to interact with the DNA and perhaps adjacent H3/H4 species. For example, the need for this interaction to maintain negative supercoils was seen in the experiment in which the N-terminus was removed. Positive supercoils were observed when this construct was reconstituted with H4K5,12Q, yet with the H3.2(6K>Q) construct, negative supercoils were observed (Figure 5C). The H3.2(6K>Q) construct has been previously used to characterize salt- and cation-dependent oligomerization of nucleosomes.[45] These investigators did not observe an extensive reduction of oligomerization, but rather a reduction in the level of nucleosomal wrapping. It was concluded that the neutralization of charge on the N-terminus allowed the entry−exit points of the DNA to more freely unwrap and yet continue to maintain

interaction with the DNA. It is possible that this same general mechanism of action is applicable to conditions in which H2A and H2B are absent. The N-terminus is likely to undergo a structural change, perhaps forming an α-helix, to provide the specificity that is observed in our studies.[15,71] Thus, the N-terminus of H3 is much more than a binding site for accessory proteins.

The use of mutational mimics for characterizing PTMs has its limitations. Whereas the general chemical nature of acetylation and phosphorylation is simulated fairly well with glutamine and glutamic acid mutations, respectively, the methylation mimics are more nuanced. The various levels of methylation that occur on a lysine do not reduce the positive charge, whereas a mutation with methionine or leucine does. These mutations are able to reflect only the general hydrophobicity that is characteristic of methylation events. Because many of the substitutions of methionine and leucine in the N-terminus of H3 tend to maintain supercoiling characteristics similar to those of wt-H3.2, it is likely that this hydrophobic character is substituting for the charge and those sites continue to maintain interaction with DNA. The presence of a positive charge in a methylated lysine would be expected to further enhance this interaction with DNA and therefore maintain the same supercoiling characteristic of wt-H3.2. Therefore, despite the lack of positive charge, the mimics do indicate the potential importance of the hydrophobic character within the methylated lysines in maintaining interaction with DNA.

Very high levels of negative supercoils were observed with the H3.2(6K>Q) construct when it was reconstituted with wt-H4 (Figure 3A). This level was higher than those of any of the constructs containing the single mutations, which suggests that there is an additive effect in the promotion of the L-tetrasome. This additive effect may serve as a means of titrating the opposite effect that is observed when histone H4 is acetylated. For example, it is shown in Figure 3B that the H4(4K>Q) construct could overcome any attempt by the H3(6K>Q) construct to block the formation of positive supercoils, yet when the H4(K5,12Q) construct was used, only negative supercoils were observed (Figure 5C). Therefore, depending on the level of acetylation in H3 versus H4, a condition in which a finely tuned switching between the left- and right-handed states of H3/H4 would be possible. This finely tuned behavior was also observed even within the same protein when considering the double mutants of the N-terminus of H3 (Figure 5A,B). When the K36L or K79L mutation was combined with the K4L or K27L mutation, only the right-handed conformer was promoted, yet as single mutants a mixture of both leftward and rightward were observed for K4L and K27L (Figure 3A). Most of the single methylation mimics in the N-terminus of H3 tended not to appreciably alter the conformational equilibrium of the H3/H4 tetramer. The double mutations have changed that dynamic. When the double mutations involved a combination of glutamine mutations with leucine mutations, the glutamine mutations in the N-terminus tended to be dominant and negative supercoils were selectively formed. It is possible that post-translational methylation at a single site may truly function only as a marker for the binding of accessory proteins. It is the multiple modifications that are likely to potentiate the nucleosome for the ultimate function of those binding proteins.

The archaeal histones have the interesting property in that under conditions in which the histone:DNA ratio is increased, there is a transition in which the supercoiling shifts from negative to positive coils on the DNA.[72] This transition is similar to what is observed with wt-H3/H4, providing sufficient NAP1 is present to facilitate dimer deposition (Figure 3A). The archaeal histones exist as stable dimers.[73] The tetrameric condition exists only when the histones are bound to DNA. These histones are also missing the N-terminus of H3 or H4 and therefore do not require a chaperone for deposition.[74] It has been proposed that the increase in the histone:DNA ratio propagates the positive supercoils through repeated dimer interaction.[74] We would propose that the unique location of K56 and K79 at the outer face of the DNA supercoil facilitates a similar repeat that will also be right-handed, i.e., a repeat of R-tetrasomes. This tandem repeat would continue to place these residues on the outer face for interaction with adjacent tetramers. We have previously shown that with formaldehyde cross-linking, there is extensive H4−H4 interaction in the right-handed H3/H4−DNA complex.[27] Such interaction would be expected to be extensive in this tandem repeat. The N-terminus of H4 (note the location of K5 and K12 in Figure 1B) is also located on the outer face close to the K79 site, and we would suggest that a similar charge neutralization of this region would equally facilitate this right-handed supercoil. Because the N-terminus is missing, the archaeal system does not have the capacity to epigenetically regulate expression at the level of sophistication that is observed in the higher eukaryotic systems. These N-termini provide an important means of modulating the conformational state of H3/H4. It is therefore tempting to speculate that this evolutionarily conserved propagation of right-handed supercoils is important in epigenetic regulation.

The left-handed conformer of H3/H4 might be expected to maintain the more stable tetramer, because it is the conformation that is in the nucleosome and represents conditions that in theory would maximize interactions within the four-helix bundle of the H3−H3 interface. Surprisingly, using glycerol gradients in 100 mM NaCl to analyze stability, those tetramers that had the propensity to form the R-tetrasome were as stable as those mutants that preferred the L-tetrasome (Figure 9), even when they were characterized in H3 variants with substantially different levels of tetramer stability. The minor variant, H3.1, formed a very stable tetramer. The S96C substitution that defines the difference between H3.1 and H3.2 (Figure 1A) is located in helix 2. It is inside a hydrophobic cage consisting of F67, A95, and L100 in H3 and L58, F61, and L62 in helix 2 of H4. It is a major site for stabilization of the H3/H4 dimer. This location is not near the four-helix bundle. If the stability of the dimer is a prerequisite for the stability of the tetramer, the cysteine may well enhance that stability within the cage. It is known that the H3/H4 dimer is less stable than the H2A/H2B dimer.[4] The functional significance of this enhanced stability remains to be determined.

Histone H3.3 deposition is unique among the variants in that it forms exclusively negative supercoils. There is a four-amino acid variation from H3.2 (Figure 1A), three of which are on the end of helix 2, an extreme distance from the H3−H3 interface. These residues are involved in stabilization of helix 2 with helix 1 of H3 and of helix 2 with the C-terminus of H4. Which of these sites play a major role in this unique property of H3.3 remains to be determined. With regard to this property, the preference for the L-tetrasome might be of particular value in a replacement process. It would in theory form a more stable nucleosome than H3.2 and, because of this preference, be more resistant to histone displacement. Biophysical studies do

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indicate that H3.3- and H3.2-containing nucleosomes have similar stabilities.[75] It is unknown whether there is a difference in stability under transcription conditions. It has been reported that H3.3 is enriched in modifications associated with active genes, i.e., K4 and K79 methylation.[76] On the basis of our studies with the mutational mimics, the preference for the L-tetrasome does not appear to be affected by such modifications (Figure 6C). It may be that these sites function solely to facilitate interaction with accessory proteins, without affecting nucleosome stability. This unique dependence on the type of variant to define the H3/H4 conformation has also been described for the centromeric H3. In this instance, depending on whether it is complexed with a full complement of H2A/H2B, the DNA may assume either a negative or a positive superhelical pitch.[77] Therefore, depending on which variant is involved, which PTM is present, and the content of H2A/H2B, it is possible to establish conditions that can prefer a particular H3/H4 conformational state.

The mixing experiments depicted in Figure 7 showed the importance of the higher histone:DNA ratio in facilitating the propagation of positive supercoils. For those mutations that promoted those coils, the 0.8:1 ratio showed a disproportionately lower level of them than the 1.6:1 ratio (Figure 7A). The mutants that promoted negative supercoils did not show this characteristic. Therefore, when H3.2(6K>Q)/H4 was intermixed with H3.2/H4(4K>Q) and added to DNA at this higher ratio, the latter histones were dominant and high levels of positive supercoils were present (Figure 7B, lane c). Even intermixing of H3.2K9Q/H4 with H3.2K79L/H4 resulted in the latter histones dominating, and positive supercoils were preferred (Figure 7B). This preference for positive supercoils was even more evident when H3.2K79L/H4 was mixed with H3.2/H4. Because H3.2/H4 can equally form either the L- or R-tetrasome, the conformation can be easily shifted toward the R-tetrasome, even if it had been previously bound to the DNA as an L-tetrasome. Similarly, when H3.2/H4 was intermixed with H3.3/H4, the preference of H3.3/H4 for the L-tetrasome forced H3.2/H4 to form this same conformation. Perhaps under conditions in which H2A/H2B histones were displaced and the local H3/H4 tetramer concentration was increased due to sliding, the presence of H3.3 on a gene would tend to stabilize that local region even in the presence of multiple H3.2 histones on that same gene. A methylated H3K79 in the presence of multiple H3.2 species would have the opposite effect and tend to open that particular region. This more "open" state would also be present during DNA replication. Only 10% of H3.2/H4K5,12Q was sufficient to shift the equilibrium of H3.2/H4 to the right-handed conformer (Figure 7B). The ability of wt-H3.2/H4 to readily shift between conformers allows the PTMs to regulate far beyond the specific H3/H4 tetramer on which those modifications are located.

■ ASSOCIATED CONTENT

Supporting Information

A table is shown that lists the DNA sequences (primers) used to obtain the mutations and the supercoiling activity for the acetylation and methylation is graphically shown for the conditions under which H3/H4 was deposited by NAP1 as a tetramer. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

NAP1, nucleosome assembly protein 1; 2-ME, 2-mercaptoethanol; CsCl−EtBr, cesium chloride−ethidium bromide; SD, superhelical density; ccc, circular, covalently closed; PTMs, post-translational modifications.

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