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Effect of Histone Lysine Methylation on DNA Lesion Reactivity in Nucleosome Core Particles

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Abstract

Lysine methylation is a common posttranslational histone modification that regulates transcription and gene expression. The lysine residues in the histone tail also react with damaged nucleotides in chromatin, including abasic sites and N7-methyl-2'-deoxyguanosine, the major product of DNA methylating agents. Lysine monomethylation transforms the ϵ -amine into a secondary amine, which could be more nucleophilic and/or basic than the ϵ -amine in lysine, and therefore more reactive with damaged DNA. The effect of lysine methylation on the reactivity with abasic sites and N7-methyl-2'-deoxyguanosine was examined in nucleosome core particles using a lysine analogue derived from cysteine. ϵ -Amine methylation increases the rate constant for abasic site reaction within nucleosome core particles. Reactivity at the two positions examined increased less than two-fold. Mechanistic experiments indicate that faster β -elimination from an intermediate iminium ion account for accelerated abasic reactivity. The rate constants for nucleophilic attack (Schiff base/iminium ion formation) by the lysine and methylated analogues are indistinguishable. Similarly, the rate constants describing nucleophilic attack by the lysine and methylated lysine analogues on β -2'-fluoro-N7-methyl-2'-deoxyguanosine to form DNA-protein cross-links are also within experimental error of one another. These data indicate that abasic site containing DNA will be destabilized by lysine methylation. However, these experiments do not indicate that DNA-protein cross-link formation, a recently discovered form of damage resulting from N7-guanine methylation, will be affected by this posttranslational modification.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. Nano LC-Orbitrap MS experimental procedure, oligonucleotides used to construct nucleosomal DNA, ESI-MS characterization of nonnative histone H4 proteins, representative autoradiograms of time course experiments, NCP structure showing proximity of H4-K20 to C8-dG89.

Keywords

DNA damage; abasic sites; DNA alkylation; histone modification; lysine methylation; mechanism

Introduction.

Histone lysine methylation is a common posttranslational modification that regulates transcription and gene expression.¹⁻⁵ The lysine residues within histone tails also react with some forms of damaged DNA, such as abasic sites (AP)⁶⁻¹⁵ and N7-methyl-2'-deoxyguanosine (MdG),¹⁶ that are produced endogenously and/or by antitumor agents. The histone lysines act as nucleophiles and bases within nucleosome core particles (NCPs). Some histone lysines are post-translationally methylated, resulting in a mixture of unmethylated, as well as mono- di- and trimethylated derivatives. The relative amounts of the four lysine forms are cell cycle dependent and vary from one lysine to another.¹⁷ Lysine monomethylation potentially increases the nucleophilicity and pK_a of the ϵ -nitrogen, which could result in even greater reactivity with the electrophilic, damaged DNA in chromatin.¹⁸ Consequently, we examined the consequences of histone tail lysine methylation on the reactivity of an AP and MdG in a bottom-up experimental approach using NCPs comprised of site specifically chemically methylated histone H4 and synthetic DNA containing either an AP or MdG lesion at a defined position.

Lysine rich histone tails catalyze DNA strand cleavage at AP and oxidized abasic sites (C4-AP, DOB) via a mechanism similar to that of the lyase reactions of DNA repair enzymes (Scheme 1).¹⁹⁻²¹ The lysines activate abasic sites to elimination by forming transient DNA-protein cross-links (DPCs) containing uncleaved DNA (DPC_{un}).⁶⁻⁸ A second lysine or histidine, as well as glutamic or aspartic acid residues present in mutant histones then induce β -elimination to form a DPC with cleaved DNA (DPC_{cl}), which yields a single strand break (SSB) upon hydrolysis.⁹ β -Elimination in NCPs occurs as much as 1,500-times faster than in free DNA.¹² Furthermore, histone catalyzed elimination of C4-AP and DOB results in

concomitant lysine modification.^{11, 12} Formally, the lysine modifications constitute novel posttranslational modifications. Given that they are derived from DNA lesions produced by chemotherapeutic agents (and other oxidants), they may represent a source of downstream biochemical effects that contribute to cytotoxicity.²²

Characterization of these reaction pathways was facilitated by the availability of synthetic NCPs. Using this approach, we recently discovered that monofunctional alkylated DNA (MdG) reacts reversibly with histone tail lysines in NCPs to form DNA-protein cross-links in competition with depurination (Scheme 2).¹⁶ These DPCs are also produced in Chinese Hamster lung fibroblast cells treated with methyl methanesulfonate. DPC formation from MdG may contribute to the cytotoxicity of monofunctional alkylating agents.

The aforementioned studies in NCPs were without exception carried out using histone proteins expressed in *E. coli* that lack any post-translational modifications. Histone lysines, particularly those in the tail regions, are frequently acetylated or methylated.⁴ S-adenosyl methionine dependent methyl transferases add between 1 and 3 methyl groups to the lysine side chains.^{23, 24} Although lysine acetylation prevents the residue from reacting with damaged DNA by masking the nitrogen nucleophilicity, methylation could increase the ϵ -amino group reactivity. Monomethylation produces a secondary amine, which is typically more nucleophilic and basic in water than a primary amine, such as the ϵ -amino group in a lysine side chain. If this reactivity trend holds in the NCP chemistry introduced above, one would expect that methylated lysines would react more rapidly with the DNA lesions.

Experimental Procedures.

Materials.—Oligonucleotides used for site-directed mutagenesis PCR were purchased from Integrated DNA Technologies. Oligonucleotides used for enzymatic ligation to generate 601 DNA were synthesized on an Applied Biosystems Incorporated 394 oligonucleotide synthesizer. Oligonucleotide synthesis reagents were purchased from Glen Research. T4 polynucleotide kinase (PNK), T4 DNA ligase and trypsin were from New England Biolabs. Sequenase version 2.0 DNA polymerase was obtained from Affymetrix. Dimethyl sulfate, D/L-methionine, 2-chloroethyl methylammonium chloride and propionic anhydride were purchased from Sigma. 2-Bromoethyl ammonium bromide was purchased from Toronto Research Chemicals. γ -³²P ATP was purchased from Perkin Elmer. β -2'-F-dGTP was purchased from TriLink BioTechnologies as a 0.1 M solution. Siliconized tubes were purchased from Bio Plas, Inc. Quantification of radiolabeled oligonucleotides was done using a Molecular Dynamics Phosphorimager 840 equipped with ImageQuant TL software. Photolyses were carried out in a Southern New England Rayonet photoreactor equipped with 16 $\times$ 350 nm lamps. Room temperature was maintained by using fans at the top and bottom of the chamber.

Synthesis of β -2'-F-MdGTP.— β -2'-F-dGTP solution (50 μ L, 5 μ mol) was dried in a speed vacuum and resuspended in pH 4.6 citrate buffer (50 μ L, 0.5 M) to a final concentration of 100 mM. DMS (3 μ L, 31.6 mmol) was added and the reaction mixture was vortexed at room temperature for 20 min, at which time additional DMS (3 μ L, 31.6 mmol) was added and the reaction was continued for 70 min. The reaction mixture was diluted with

water (450 μL), injected to a 1 mL Mono-Q column and eluted at 4 °C with 50 mM HEPES buffer (pH 6.8) in a linear gradient of 0-500 mM NaCl over 60 min (1 mL/min). The β -2'-F-MdGTP eluted at ~12 min while the unreacted 2'-F-dGTP eluted at ~28 min. The concentration of β -2'-F-MdGTP was determined by UV-Vis ($\epsilon_{258} = 9.8 \text{ mmol}^{-1}\text{cm}^{-1}$).²⁵ β -2'-F-MdGTP ($\text{C}_{11}\text{H}_{16}\text{FN}_5\text{O}_{13}\text{P}_3$) was characterized by MALDI-TOF mass spectrometer using the negative reflectron mode and anthranilic acid/nicotinic acid (AA/NA) as matrix.²⁶ Calculated m/z 538.20 ($\text{M}-\text{H}^+$), found 538.38. The β -2'-F-MdGTP (~1 μmol , 20% yield) was stored at -20 °C.

Preparation of 145-mer 601 DNA containing site-specific AP precursor or β -2'-F-MdG.

—The 145-mer 601 DNA containing AP precursor at position 89 or 205 was generated as previously described.⁷ The 145-mer 601 DNA containing β -2'-F-MdG at position 89 was prepared by enzymatic ligation of β -2'-F-MdG₈₉ containing oligonucleotide with other chemically synthesized oligonucleotides. The β -2'-F-MdG₈₉ containing oligonucleotide was prepared by enzymatically phosphorylating the 5'-terminus of the chemically synthesized oligonucleotide (Fig. S1A) in a reaction (50 μL) containing 4 nmol DNA, 1 x PNK buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 , 5 mM DTT), 2 mM ATP and 50 units T4 PNK at 37 °C for 4 h. T4 PNK was inactivated by incubating at 65 °C for 30 min. The phosphorylated oligonucleotide was combined with scaffold (6 nmol, Fig. S1A) in 1 x Sequenase buffer (40 mM Tris-HCl, pH 7.5, 20 mM MgCl_2 , 5 mM DTT and 50 mM NaCl) and hybridized by heating at 90 °C for 2 min, followed by slow cooling to room temperature. β -2'-F-MdGTP (64 μL , final concentration of 470 μM) and Sequenase (13 units) were added and the mixture (~150 μL) was incubated at room temperature for 12 h. The reaction mixture was mixed with 30 μL 95% formamide and purified by 20% denaturing PAGE. The gel (40 $\times$ 32 $\times$ 0.1 cm) was run at 4 °C under the limiting power (45 W) until the bromophenol blue migrated to the bottom of the gel. The product band was excised from the gel and the DNA was eluted overnight in elution buffer (3 mL, 0.2 M NaCl and 1 mM EDTA) at 4 °C. The slurry was filtered using a polyprep-column (BioRad) and the DNA was desalted using a 1 mL C18 Sep-pak desalting column. The eluted DNA (~0.7 nmol) was flash frozen in liquid nitrogen, evaporated to dryness in a speed vacuum at room temperature, and finally resuspended in water (20 μL). To generate the 145-mer 601 DNA containing β -2'-F-MdG₈₉, the chemically synthesized oligonucleotides (Fig. S1B) were enzymatically phosphorylated at their 5'-termini, each in a 20 μL reaction containing 0.7 nmol DNA, 1 x T4 DNA ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl_2 , 10 mM DTT, 1 mM ATP) and 50 units T4 PNK at 37 °C for 4 h. T4 PNK was inactivated by incubating at 65 °C for 30 min. The phosphorylated oligonucleotides were combined with the unphosphorylated oligonucleotide (0.7 nmol, Fig. S1B) together with the scaffolds (1 nmol, Fig. S1B) and heated at 95 °C for 2 min, followed by chilling on ice. The 5'-phosphorylated β -2'-F-MdG₈₉ containing oligonucleotide (0.7 nmol) was added and the mixture was incubated at room temperature for 2 h. T4 DNA ligase (3600 units) was added and the mixture (~150 μL) was incubated overnight at 16 °C. The reaction was phenol extracted (equal volume). The aqueous solution was collected, mixed with 30 μL 95% formamide, and purified by 8% denaturing PAGE. The gel (20 $\times$ 16 $\times$ 0.1 cm) was run at 4 °C under the limiting power (7 W) until the xylene cyanol migrated to the bottom of the gel. The product band was excised from the gel and eluted overnight in elution buffer (1 mL,

0.2 M NaCl and 1 mM EDTA) at 4 °C. The slurry was filtered using a polyprep-column (BioRad) and the 145-mer DNA product was concentrated and buffer exchanged extensively with water using a 10 K Amicon Ultra membrane at 4 °C. The 145-mer ligated DNA product (~120 pmol) was stored at -80 °C.

Preparation of H4-K20C-me0 and H4-K20C-me.—pET3a-H4-K20C was generated by site-directed mutagenesis PCR using the following primers: H4-K20C-F: 5'-d(GGTGCTAAACGTCACCGTTGCGTTCTGCGTGACAACATC); H4-K20C-R: 5'-d(GATGTTGTACGCAGAACGCAACGGTGACGTTTAGCACC). The correct mutation (K20C) was confirmed by Sanger sequencing from GENEWIZ. H4-K20C protein was purified as previously described.²⁷ H4-K20C-me0 and H4-K20C-me were prepared following the reported protocol with some minor modifications.²⁸ H4-K20C (5 mg) was resuspended in buffer (500 µL) containing HEPES (1 M, pH 7.8), guanidine (4 M) and D/L-methionine (10 mM), and the mixture was sonicated briefly on ice to facilitate dissolving the protein. DTT was then added to a final concentration of 20 mM, and the mixture was incubated at 37 °C for 1 h to fully reduce the proteins. To generate H4-K20C-me0, 2-bromoethyl ammonium bromide was dissolved in water (1 M) and added to the reaction to achieve a final concentration of 100 mM. The reaction mixture was covered with aluminum foil and incubated at room temperature for 6 h. The reaction was quenched by adding β-mercaptoethanol to a final concentration of 0.7 M and incubating at room temperature for 30 min. To prepare H4-K20C-me, 2-chloroethyl methylammonium chloride was dissolved in water (3 M) and added to a final concentration of 450 mM. The reaction mixture was covered with aluminum foil and incubated at room temperature for 18 h. The reaction was quenched by adding β-mercaptoethanol to a final concentration of 0.7 M and incubating at room temperature for 30 min. Please note that the alkylating agents were handled in a fume hood. The alkylated proteins were purified using desalting columns with H₂O containing 3 mM β-mercaptoethanol on a FPLC at 4 °C. The fractions containing proteins were collected, evaporated to dryness in a speed vacuum and stored at -80 °C. The molecular weights of intact proteins were confirmed by UPLC-MS.²⁹

Trypsin digestion and LC-MS/MS characterization of proteins containing lysine analogues.³⁰—H4-K20C-me0 or H4-K20C-me (~10 µg, 1 nmol) was treated with propionic anhydride and digested with trypsin. The peptides were then treated with propionic anhydride and desalted using C18 resin (Zip Tip, Millipore Sigma). All the above procedures were performed following the reported protocol without modifications. The desalted peptides (~10 ng, 1 pmol) were analyzed by nano-LC-MS/MS (Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer). The data was processed by Xcalibur™. Additional details concerning the LC-Orbitrap MS method can be found in the Supporting Information.

Reconstitution of nucleosome core particles.—Refolding of histone octamers was performed as previously described.²⁷ Salmon sperm DNA (~20 µg, 168 pmol of 145 bp) and 5'-³²P labeled 145 bp DNA (4 pmol) were combined in a siliconized tube to a final volume of 20 µL containing 2 M NaCl and 0.1 mg/mL BSA. Various amounts of histone octamer (1.0 or 1.2 equivalent of total DNA) were added to each DNA sample. The mixture was

incubated at 4 °C for 30 min before a series of dilutions at 4 °C using a buffer containing 10 mM HEPES, pH 7.5, 1 mM EDTA and 0.1 mg/mL BSA. Dilution #: volume of buffer added in μL , incubation time in min-1: 24, 60; 2: 12, 60; 3: 12, 60; 4: 20, 30; 5: 20, 30; 6: 40, 30; 7: 100, 30; 8: 200, 30. After the final dilution (total volume $\sim 448 \mu\text{L}$), any precipitate was pelleted by briefly spinning at 13,000 g at 4 °C. The solution was then transferred to a fresh siliconized tube. A small aliquot ($\sim 5 \mu\text{L}$) was mixed with 40 % sucrose ($3 \mu\text{L}$) and analyzed by 6% native PAGE to determine the reconstitution efficiency. The gel ($10 \times 8 \times 0.15 \text{ cm}$) was run at 4 °C under the limiting power (3 W) until the xylene cyanol migrated to the middle of the gel.

Determining the rate constants of AP disappearance (k_{Dis}).—NCPs ($100 \mu\text{L}$, $\sim 10^6$ cpm) with or without NaBH_3CN (30 mM) were photolyzed at 350 nm for 20 min. A portion of the NCPs ($20 \mu\text{L}$) was removed to determine the fraction of AP precursor converted to AP. The remainder of the NCPs ($80 \mu\text{L}$) was incubated at 37°C. Aliquots ($10 \mu\text{L}$) were removed at appropriate times and stored at -80°C until the final time point. To determine the fraction of AP precursor converted to AP, NCP ($10 \mu\text{L}$) was treated with NaOH (100 mM) at 37°C for 30 min, followed by neutralization with HCl and treatment of proteinase K (8 units) at room temperature for 30 min. The cleaved DNA was analyzed by 10 % denaturing PAGE. The gel ($20 \times 16 \times 0.1 \text{ cm}$) was run at room temperature under the limiting power (15 W) until the xylene cyanol migrated to the middle of the gel. To determine the products from AP, aliquots ($10 \mu\text{L}$) were treated with fresh NaBH_4 (100 mM) at 4°C for 60 min, followed by mixing with 3 x SDS-loading buffer ($5 \mu\text{L}$). The samples were analyzed by 10% SDS PAGE at room temperature, which separates intact DNA, from DPC_{un} , DPC_{cl} and SSB.^{6, 7} The gel ($20 \times 16 \times 0.1 \text{ cm}$) was run at room temperature under the limiting power (7 W) until the bromophenol blue migrated to the bottom of the gel. The total amount of reacted AP (AP_{Dis}) as a function of time was calculated using the equation (1).

$$\text{AP}_{\text{Dis}} = \frac{\text{DPC}_{\text{un}} + \text{DPC}_{\text{cl}} + \text{SSB}}{(\text{DPC}_{\text{un}} + \text{DPC}_{\text{cl}} + \text{SSB} + \text{DNA}_{\text{Intact}}) \times \text{photolysis efficiency} \times \text{reconstitution efficiency}} \quad (1)$$

The rate constant for AP disappearance (k_{Dis}) was calculated by fitting the remaining AP ($1 - \text{AP}_{\text{Dis}}$) to a first-order reaction.

Determining the DPC yields from NCPs containing $\beta\text{-}2'\text{-F-MdG}_{89}$.—NCPs ($100 \mu\text{L}$, $\sim 2 \times 10^6$ cpm) were incubated at 37 °C. Aliquots ($10 \mu\text{L}$) were removed at appropriate times and stored at -80°C until the final time point. To determine the amounts of DPCs, the aliquots were mixed with 3 x SDS-loading buffer ($5 \mu\text{L}$) and analyzed by 10% SDS PAGE. The gel ($20 \times 16 \times 0.1 \text{ cm}$) was run at 4 °C under the limiting power (3 W) until the bromophenol blue migrated to the bottom of the gel.

Results.

Nucleosome core particle design and preparation.

The DNA in the vicinity of superhelical location 1.5 is kinked and this region is a hotspot for DNA damaging agents (Figure 1A).³¹⁻³³ AP and MdG reactivity in this region were examined previously.^{6-8, 16} In this study, we examined AP₈₉ and AP₂₀₅ (Figure 1B), which are present in opposite strands within one gyre of DNA, as well as β -2'-F-MdG₈₉ reactivity in NCPs. β -2'-F-MdG was incorporated at position 89 instead of MdG to enable us to focus on DPC formation by eliminating competing depurination (Scheme 2). Lysine 20 of histone H4 (H4-K20) is methylated in cells and this amino acid has been implicated in AP₈₉ reactivity.³⁴ Monomethylated H4-K20 reaches a peak of almost 40% during the cell cycle.¹⁷ Examination of an NCP crystal structure suggested that β -2'-F-MdG₈₉ would also be accessible to this lysine.²⁹ Consequently, we chose to examine the effect of methylation at this lysine on the reactivity of the aforementioned DNA lesions.

The monomethylated lysine (H4-K20C-me) was installed using the method developed by Shokat in which a cysteine is modified using an appropriate alkyl halide (Scheme 3).²⁸ Although this method gives rise to proteins containing sulfurated lysine analogues, the methylated proteins are recognized by antibodies and other proteins that act on chromatin.^{35, 36} To our knowledge, the effect of replacing a side chain methylene group in lysine with sulfur on reactivity has not been examined. Consequently, we sought to prepare the lysine analogue (H4-K20C-me0) as well to verify that sulfur substitution within the naturally occurring lysine does not significantly affect the amino acid's basicity or nucleophilicity. Histone H4 cysteine 20 (H4-K20C) was prepared by site-directed mutagenesis PCR. H4-K20C alkylation was carried out as previously described.²⁸ However, in our hands when the reaction was pushed to completion monoalkylated protein was accompanied by ~10-15% of dialkylated product, despite experimenting with a variety of reaction conditions. Alkylation at cysteine 20 to generate H4-K20C-me0 or H4-K20-me was verified by nano LC-Orbitrap MS following propionic anhydride treatment and trypsin digestion.^{29, 30} The crude proteins were used to reconstitute NCPs because it was impractical to separate monoalkylated and dialkylated material on a preparative scale by HPLC. We anticipated that dialkylated product was a result of random reaction with lysines in histone H4. As a control, wild type histone H4 was subjected to the same reaction conditions with 2-chloroethyl methylammonium

chloride (**1**), which again resulted in ~10-15% monoalkylated product. AP reactivity in NCPs composed of wild type histone H4 that was or was not treated with alkylating agent was compared to ascertain any effect of random alkylation. Octamer refolding and NCP reconstitution were carried out as previously described.²⁷ NCPs were characterized by DNase I footprinting.

The effect of lysine monomethylation on AP reactivity in NCPs.

AP disappearance was monitored as a function of time using SDS-PAGE, which enables simultaneous detection of starting material, SSB, as well as DPC_{cl} and DPC_{un} . Reactions were also carried out in either the absence or presence of NaBH_3CN (30 mM). The reducing agent does not react with AP but traps Schiff bases (DPC_{un} , DPC_{cl} , Scheme 1) and respective iminium ions, competing with reversion to starting material. Attack by the methylated lysine yields an iminium ion, which is also reduced by NaBH_3CN . Hence, the rate constant for AP disappearance (k_{Dis}) determined from reactions carried out in the presence of NaBH_3CN enable comparison of k_1 (Scheme 1), which reflects amino acid nucleophilicity.

The reactivity of AP_{89} , and AP_{205} in NCPs containing wild type histone H4 that was subjected to **1** (alkylated wild type H4, Table 1) was compared to that in NCPs reconstituted solely from unadulterated, wild type histones. The rate constant for disappearance (k_{Dis}) of AP_{89} and AP_{205} in NCPs containing wild type histone proteins was within experimental error of that previously reported. Moreover, k_{Dis} for each AP lesion was independent (within experimental error) of whether it was generated in NCPs comprised of wild type histone H4 or the same protein that was treated with **1**. This also was true for reactions carried out in the presence of NaBH_3CN . These data indicate that minor amounts of apparent random histone H4 alkylation does not affect AP_{89} and AP_{205} reactivity in NCPs.

With this assurance in hand, we examined the reactivity of AP_{89} and AP_{205} in NCPs containing the histone H4 lysine analogue at position 20 (H4-K20C-me0) or the corresponding *N*-methyl analogue (H4-K20C-me) (Table 2). The average rate constants for AP_{89} or AP_{205} disappearance within the H4-K20C-me0 NCP were slightly greater than in core particles prepared from wild type histone H4 (Table 1). However, the respective k_{Dis} 's are within experimental error of one another. The rate constants are also within experimental error of one another when carried out in the presence of NaBH_3CN . These data indicate that replacement of a methylene group in the lysine side chain with the slightly more electronegative sulfur atom, that forms longer bonds with carbon, and introduces smaller rotational barriers within the side chain, does not alter the amino group reactivity.

Methylation of the lysine analogue ϵ -nitrogen results in a clearly distinguishable increase in k_{Dis} for AP at both positions examined (Table 2). The rate constant for AP_{205} disappearance is not quite double when the histone H4 lysine 20 analogue is methylated ($k_{\text{Rel}} = 1.7$). The effect of methylation on AP_{89} reactivity is smaller $k_{\text{Rel}} = 1.3$, but k_{Dis} in NCPs containing the methylated amino acid is 30% faster than when the unmethylated lysine analogue is present. The enhanced AP reactivity essentially disappears when NCPs containing AP_{89} or AP_{205} are incubated in the presence of NaBH_3CN (Table 2). NCP incubation in the presence of NaBH_3CN simplifies the kinetics for AP disappearance by severely reducing the relative

contribution of Schiff base hydrolysis (k_{-1} , Scheme 1) to the process. The convergence of k_{Dis} for abasic sites in H4-K20C-me0 and H4-K20C-me containing NCPs indicates that methylation does not enhance reactivity by increasing the rate constant for DPC_{un} formation (k_1 , Scheme 1).

Reduction by NaBH_3CN also provides insight into the effect of lysine analogue methylation on β -elimination (k_2 , Scheme 1) by competing with this process. The greater the ratio of DNA-protein cross-links containing uncleaved (DPC_{un} , Scheme 1) versus cleaved (DPC_{cl} , Scheme 1) DNA the slower β -elimination (k_2). The $\text{DPC}_{\text{un}}:\text{DPC}_{\text{cl}}$ ratio from AP_{89} and AP_{205} in the presence of NaBH_3CN (30 mM) is larger in NCPs containing H4-K20C-me0 than H4-K20C-me (Figure 2). This indicates that β -elimination (k_2 , Scheme 1) increases upon lysine methylation.

The effect of lysine monomethylation on DNA-protein cross-link formation with β -2'-F-MdG₈₉ in NCPs.

Depurination of MdG produces abasic sites that form transient DPCs in NCPs.^{6, 16} Utilizing β -2'-F-MdG₈₉ simplifies determining whether lysine methylation affects DPC formation in NCPs by eliminating depurination as a competing pathway. The β -isomer of a 2'-fluoro nucleotide is frequently used as a non-hydrolyzable mechanistic probe that adopts the 2'-endo conformation typically observed in DNA.³⁷ The corresponding β -2'-F-MdGTP was prepared and incorporated enzymatically into nucleosomal DNA using methods reported for MdGTP.^{16, 38} X-Ray crystal structure of a NCP containing native nucleotides suggest that the C8-position of β -2'-F-MdG₈₉ should be accessible to the H4-K20 analogues.²⁹ Although H4-K20C-me was more reactive with AP_{89} than the nonmethylated analogue (Table 2), no difference was observed in the DPC yield from β -2'-F-MdG₈₉ in NCPs containing H4-K20C-me0 or H4-K20C-me (Figure 3).

Discussion.

The goal of this investigation was to determine whether lysine methylation increases histone reactivity with damaged DNA in NCPs. We were motivated to carry out this study by three facts. i) Lysine residues react with abasic sites and N7-methyl-2'-deoxyguanosine in nucleosome core particles.^{6-8, 16} ii) Lysine methylation is an important posttranslational histone modification.^{1, 4} iii) Methylated lysine could be expected to be more reactive than the unmodified amino acid.¹⁸ It was not possible to predict how much more reactive the methylated lysine (H4-K20C-me) should be a priori. Measurements of the nucleophilicities of primary and secondary amines in water suggest that H4-K20C-me could be ~10 to 10,000 times more nucleophilic than H4-K20C-me0.¹⁸ Similarly, differences in basicity were also dependent upon the nitrogen substituents. However, secondary amines are typically less than 10 times more basic than primary amines.

For synthetic expediency, we utilized chemistry developed by the Shokat group to selectively introduce an N-methyl group at a position 20 of histone H4 (H4-K20C-me), a position that is methylated in cells.²⁸ Selectivity is achieved by alkylating a single cysteine introduced via mutagenesis in histone H4 in place of Lys20 (Scheme 3). Control experiments showed that net substitution of a sulfur atom in a lysine side chain (H4-K20C-

me0), prepared in a like manner, did not affect AP₈₉ reactivity in an NCP containing a native lysine (Table 1, 2).

The observed rate constant ($k_1 k_2 / k_{-1}$, Scheme 1) for histone catalyzed cleavage of AP sites in NCPs is dependent on reversible nucleophilic addition (k_1 , k_{-1}) and rate limiting β -elimination (k_2).¹⁰ NCPs comprised of methylated histone H4 (H4-K20C-me) were more reactive with AP₈₉, and AP₂₀₅ than the unmethylated analogue (H4-K20C-me0). The magnitude of the effect of methylating position 20 in histone H4 on AP₈₉ reactivity had the anticipated opposite effect of substituting alanine for lysine, and was of comparable magnitude.⁷ Lysine (analogue) methylation had a smaller effect on AP₈₉ reactivity and this could reflect a smaller contribution of H4-K20 to reactivity at this site (Figure 1B). Mechanistic studies on histone catalyzed abasic site cleavage that employed alanine histone variants revealed that replacing a single lysine resulted in less than a 2-fold decrease in reaction rate constants.⁷ Moderation of the effects of losing a single nucleophilic amino acid was ascribed to compensation by the remaining lysines in the flexible histone tails. Similarly, the enhanced reactivity resulting from methylation of a single lysine in a histone could be suppressed by the other four lysines in the histone H4 tail that compete for reaction with the AP sites.

Information concerning the source of increased AP₈₉ and AP₂₀₅ reactivity due to lysine methylation (H4-K20C-me) was extracted from experiments carried out in the presence of NaBH₃CN, a reducing agent that traps the Schiff base intermediates (DPC_{un}, DPC_{cl}, Scheme 1). These experiments indicated that lysine methylation increased the rate of β -elimination (k_2 , Scheme 1) but not Schiff base formation. At first glance, this was surprising as methylation was expected to have a larger effect on lysine nitrogen nucleophilicity than basicity. However, the absence of a detectable difference in nucleophilicity may be attributed to the multi-step reaction processes (Scheme 4) and the reliance on NaBH₃CN trapping, which is indirect. Reaction between the secondary amine (H4-K20C-me) and AP produces an iminium ion (Im_{Me}); whereas H4-K20C-me0 ultimately yields a Schiff base (DPC_{un}) that undergoes reversible protonation (Im_H) en route to elimination (Scheme 4).¹⁸ Although initial formation of T_{Me} may be more rapid than that of T_H, this may not be reflected in the overall k_{Dis} (Scheme 1) since it is not the rate determining step. In addition, Im_{Me} may hydrolyze more rapidly than Im_H. Finally, committed iminium ion formation from H4-K20C-me (Im_{Me}), as opposed to equilibration between Im_H and DPC_{un}, could explain the faster elimination rate when position 20 is methylated.

Reversible DNA-protein cross-link formation with β -2'-F-MdG₈₉ is simpler mechanistically. For this reason, there are fewer alternate explanations to the possibility that the nucleophilicities of H4-K20C-me and H4-K20C-me0 are more similar than expected to rationalize the absence of a difference in DPC formation with β -2'-F-MdG₈₉. One possible explanation that is consistent with the reversibility of the process is that H4-K20C-me is more nucleophilic but also a better leaving group, much like iodide is more nucleophilic and a better leaving group than other halide ions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AP	Apurinic abasic site
C4-AP	C4'-oxidized abasic site
L	2-deoxyribonolactone
DMS	dimethyl sulfate
DOB	dioxobutane abasic site
DPC	DNA-protein cross-link
MdG	N7-methyl-2'-deoxyguanosine
β-2'-F-MdG	β-2'-fluoro-N7-methyl-2'-deoxyguanosine
NCP	nucleosome core particle
NaBH₃CN	sodium cyanoborohydride
SSB	single strand break

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Figure 1. Nucleosome core particle structure. A) NCP with one DNA gyre hidden for clarity. B) Region of superhelical location 1.5. (Structures from pdb: 1kx5)

Figure 2.
Ratio of DNA-protein cross-links from AP sites trapped by NaBH₃CN (30 mM) upon incubating for 12 h.

Figure 3.
Time dependent DPC yields from β -2'-F-MdG in NCPs.

Scheme 1.
Histone catalyzed cleavage of an abasic site.

Scheme 2.

Reaction of N7-methyl-2'-deoxyguanosine in a NCP.

Scheme 3.
Preparation of histone H4 lysine analogues.

Scheme 4.

Stepwise addition of H4-K20C-me0 and H4-K20C-me to AP.

Chart 1.
Damaged DNA that reacts with histone proteins in NCPs.

Table 1.Effect of treatment of histone H4 with alkylating agent (**1**) on AP reactivity in NCPs.^a

Position	NaBH ₃ CN	Wild type H4		alkylated wild type H4	
		k_{Dis} (10^{-6} s^{-1})	$t_{1/2}$ (h)	k_{Dis} (10^{-6} s^{-1})	$t_{1/2}$ (h)
AP ₈₉	–	28.4 ± 1.8	6.8 ± 0.4	27.9 ± 3.1	7.0 ± 0.8
AP ₈₉	+	40.7 ± 4.2	4.8 ± 0.5	35.4 ± 5.5	5.5 ± 0.8
AP ₂₀₅	–	7.7 ± 1.0	25.3 ± 3.3	7.2 ± 0.7	26.7 ± 2.7
AP ₂₀₅	+	21.3 ± 1.2	9.1 ± 0.5	21.4 ± 1.0	9.0 ± 0.4

^a Results are the average ± std. dev. of 2 experiments, each consisting of 3 replicates.

Table 2.Effect of lysine analogue methylation on AP reactivity in NCPs.^a

Position	NaBH ₃ CN	H4-K20C-me0		H4-K20C-me		<i>k</i> _{Rel} ^b
		<i>k</i> _{Dis} (10 ⁻⁶ s ⁻¹)	<i>t</i> _{1/2} (h)	<i>k</i> _{Dis} (10 ⁻⁶ s ⁻¹)	<i>t</i> _{1/2} (h)	
AP ₈₉	–	31.3 ± 1.1	6.2 ± 0.2	40.2 ± 3.6	4.6 ± 0.4	1.3
AP ₈₉	+	41.0 ± 1.2	4.7 ± 0.1	43.2 ± 0.7	4.5 ± 0.1	1.1
AP ₂₀₅	–	8.1 ± 0.6	24.0 ± 1.9	13.6 ± 0.7	14.2 ± 0.1	1.7
AP ₂₀₅	+	23.8 ± 1.7	8.1 ± 0.6	23.6 ± 0.6	8.2 ± 0.2	1.0

^aResults are the average ± std. dev. of 2 experiments, each consisting of 3 replicates.^b*k*_{Rel} = *k*_{Dis(me)}/*k*_{Dis(me0)}.