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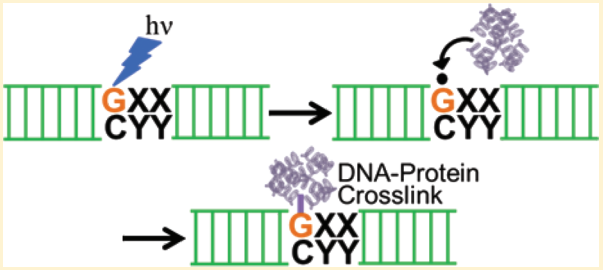
Dependence of DNA–Protein Cross-Linking via Guanine Oxidation upon Local DNA Sequence As Studied by Restriction Endonuclease Inhibition

Amanda L. Madison, Zitadel A. Perez, Phuong To, Tiffany Maisonet, Eunice V. Rios, Yuri Trejo, Carmen Ochoa-Paniagua, Anita Reno, and Eric D. A. Stemp*

Department of Physical Sciences and Mathematics, Mount St. Mary's College, 12001 Chalon Road, Los Angeles, California 90049, United States

ABSTRACT: Oxidative damage plays a causative role in many diseases, and DNA–protein cross-linking is one important consequence of such damage. It is known that GG and GGG sites are particularly prone to one-electron oxidation, and here we examined how the local DNA sequence influences the formation of DNA–protein cross-links induced by guanine oxidation. Oxidative DNA–protein cross-linking was induced between DNA and histone protein via the flash quench technique, a photochemical method that selectively oxidizes the guanine base in double-stranded DNA. An assay based on restriction enzyme cleavage was developed to detect the cross-linking in plasmid DNA. Following oxidation of pBR322 DNA by flash quench, several restriction enzymes (PpuMI, *Bam*HI, *Eco*RI) were then used to probe the plasmid surface for the expected damage at guanine sites. These three endonucleases were strongly inhibited by DNA–protein cross-linking, whereas the AT-recognizing enzyme AseI was unaffected in its cleavage. These experiments also reveal the susceptibility of different guanine sites toward oxidative cross-linking. The percent inhibition observed for the endonucleases, and their pBR322 cleavage sites, decreased in the order: PpuMI (5′-GGGTCCT-3′ and 5′-AGGACCC-3′) > *Bam*HI (5′-GGATCC-3′) > *Eco*RI (5′-GAATTC-3′), a trend consistent with the observed and predicted tendencies for guanine to undergo one-electron oxidation: 5′-GGG-3′ > 5′-GG-3′ > 5′-GA-3′. Thus, it appears that in mixed DNA sequences the guanine sites most vulnerable to oxidative cross-linking are those that are easiest to oxidize. These results further indicate that equilibration of the electron hole in the plasmid DNA occurs on a time scale faster than that of cross-linking.



Oxidative damage to DNA is associated with cancer, neurodegenerative diseases, and the aging process.[1−3] The cell undergoes >10^3 damage events per day,[4] and there are a host of cellular proteins assigned to repairing these damaged sites.[5−7] When repair processes are deficient, cells can either die or become cancerous.[8,9] A clear picture of how DNA lesions form is thus central to understanding the progression of molecular disease.

Since it is the DNA base with the lowest oxidation potential (1.3 V),[10] guanine is particularly susceptible to oxidative damage. The one-electron oxidation of guanine leads to permanent damage products such as 8-oxoguanine, FapyG, and oxazolone.[11] Further oxidation of 8-oxoguanine leads to hyperoxidized guanine products such as spiroiminohydantoin and guanidinohydantoin.[12] It has been known for some time that the local sequence surrounding a guanine base influences its reactivity in redox reactions. In particular, the 5′ G of GG and GGG sequences has been found to be especially susceptible to one-electron oxidation,[13,14] and Saito and co-workers calculated that the ionization potential of guanine should increase in the order GGG < GG < GA < GC < GT.[15]

One important form of DNA damage relatively understudied heretofore is the DNA–protein cross-link.[16] Morin and Cadet showed over a decade ago that type I photooxidation of guanine in solution leads to adducts with hydroxy and amino groups,[17,18] and more recent studies have demonstrated cross-linking between small oligonucleotides and lysine.[19,20] Using the flash quench technique to selectively oxidize guanine, Nguyen et al. demonstrated that the one-electron oxidation of guanine in double-stranded DNA leads to the formation of DNA–histone cross-links.[21] Kurbanyan et al. then showed that this approach could be applied to induce cross-linking with other proteins and with a variety of photosensitizers and that the efficiency of cross-linking depended on the strength of the oxidant used.[22] Exploiting the fact that electron holes are mobile in double-stranded DNA, Bjorklund and Davis showed that a specific DNA–protein cross-link can be formed in a nucleosome core particle at a long molecular distance from the photooxidant.[23] Thus, the formation of DNA–protein cross-links resulting from guanine oxidation is a topic of increasing interest.

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Given that DNA is associated with protein in vivo, we wanted to determine how the local sequence influences oxidative DNA−protein cross-linking induced by guanine oxidation. The sequence dependence of oxidative cross-linking in plasmid DNA was studied using restriction endonucleases. Given our own status as a research group at a small college, we sought to develop a new assay for cross-linking that eschewed the use of radioactivity and that could provide additional details regarding the sites of cross-linking. Here, we show that the inhibition of restriction enzyme cleavage can be used to probe a DNA surface for guanine−protein cross-links. In particular, we find that inhibition occurs only for those restriction enzymes that contain guanines in the recognition sequence (*Eco*RI, *Bam*HI, PpuMI). Moreover the extent of inhibition observed reveals the reactivity of specific sequences toward oxidative cross-linking. Since the percent inhibition decreased in the order PpuMI > *Bam*HI > *Eco*RI, the GG and GGG sites known to be hot spots for oxidative damage in naked DNA also appear to be the most susceptible to DNA−protein cross-linking initiated by the one-electron oxidation of guanine.

METHODS

Materials. Sodium chloride ultrapure, sodium dodecyl sulfate, sodium phosphate monobasic, proteinase K from *Tritirachium album*, and histone III-S from calf thymus were obtained from Sigma. Pentaamminechlorocobalt(III) chloride was obtained from Aldrich. Histone H2A, pBR322 plasmid DNA, restriction enzymes (NdeI, *Bam*HI, *Eco*RI, PpuMI, AseI), and 1 kb DNA ladder were obtained from New England Biolabs. Gel loading dye, ultrapure agarose, and TBE running buffer were obtained from Invitrogen. $[Ru(phen)_2dppz]Cl_2$ was prepared as described previously.[24] Prior to use, pBR322 plasmid DNA was exchanged into a buffer of 10 mM sodium phosphate (NaPi), 20 mM NaCl (pH 7) via multiple rounds of ultrafiltration using Centricon 30 centrifugal filter devices (Millipore). Stock solutions were prepared utilizing the following extinction coefficients: ε_{440} = 21.0 mM^{-1} cm^{-1} for $Ru(phen)_2dppz^{2+}$, ε_{276} = 0.46 mM^{-1} cm^{-1} for $Ru(NH_3)_6Cl_3$, and ε_{260} = 6.6 mM^{-1} cm^{-1} for pBR322 plasmid DNA. Concentrations of pBR322 plasmid DNA are given in nucleotides (nuc). $Co(NH_3)_5Cl_3$ and histone were quantified by weight. Because the cobalt complex slowly decomposes in aqueous solution, fresh cobalt solutions were frozen and kept at −20 °C, and aliquots were thawed just prior to use.

Sample Irradiation. Samples of 10 μL containing $Ru(phen)_2dppz^{2+}$ (10 μM), $Co(NH_3)_5Cl_3$ (100 μM), histone (50−250 μg/mL, type III-S protein), and DNA (pBR322 at 500 μM nucleotides) in a buffer of 10 mM NaPi and 20 mM NaCl (pH 7) were prepared. The samples were kept under reduced light until irradiation with a HeCd laser (Liconix), output 9−13 mW; the 442 nm output predominantly excites the ruthenium intercalator.

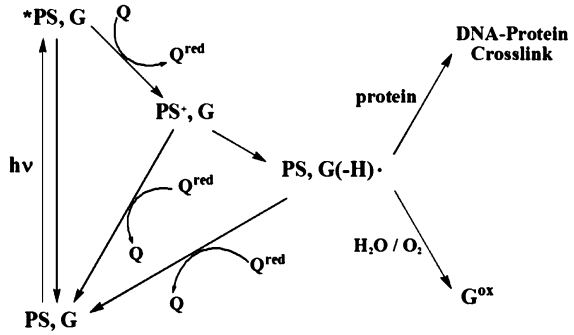
Enzymatic Digestion. To the irradiated samples, restriction enzyme (5−20 units) was added, and incubation was carried out under reduced light at 37 °C for 1 h. After restriction enzyme incubation, 1.3 μg of proteinase K was added, and the samples were incubated under reduced light at 37 °C for 1 h; addition of protease was necessary to ensure that the DNA could enter the gel.

Agarose Gel Electrophoresis of pBR322. Gel loading dye containing SDS and bromphenol blue was added to the irradiated, digested samples. Approximately 1 μg of DNA was added to each well of a 0.8% agarose gel. The gel was run at 80 V for 140 min at room temperature in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3, Invitrogen) using a Hoefer HE99X horizontal submarine electrophoresis unit (Amersham) connected to an Electrophoresis Power Supply EPS 301 (Amersham). The bands were visualized by ultraviolet illumination after staining with ethidium. Gels were imaged using the Gel Doc System (Bio-Rad), and band intensities were obtained using Quantity One 4.1.0 software (Bio-Rad). Care was taken to avoid saturation of band intensities during quantification.

RESULTS

Originally pioneered by Gray and co-workers for studies of protein electron transfer,[25] the flash quench technique offers a simple way to produce the guanine radical by using visible light to create a strong ground state oxidant bound to the DNA.[26,27] The potent oxidant, created in situ by oxidative quenching of an excited photosensitizer bound to DNA, removes an electron from guanine to make the guanine radical. In this method (Scheme 1), a ground state photosensitizer, PS, is excited with

Scheme 1



visible light to form the excited state *PS, which can then transfer an electron to quencher Q. The oxidant thus formed, PS^+, can either undergo back electron transfer with reduced quencher, Q^{red}, or oxidize guanine, G. The resulting guanine radical cation is acidic (pK_a 3.9) and rapidly deprotonates to form the neutral radical, $G(-H)^{\bullet}$.[28] This radical can either be repaired by electron transfer from Q^{red} or react further to yield permanent damage in the form of G^{ox} or a DNA−protein cross-link. Here, PS represents the metallointercalator $Ru(phen)_2dppz^{2+}$ and Q is $Co(NH_3)_5Cl^{2+}$; this cobalt complex is the quencher of choice because its reduced form decomposes[29] and thus does not reduce either PS^+ or $G(-H)^{\bullet}$. Lastly, it is important to note that, because of the mobility of electron holes in DNA, the sites of ultimate damage are spread throughout the DNA,[30] i.e., the intercalator need not be bound at the site of oxidation and will not preclude binding of protein to the DNA. Upon one-electron oxidation, guanine can react with nucleophiles (e.g., water) to form permanent lesions such as 8-oxo-G or with protein side chains to form DNA−protein cross-links[17−23] (Figure 1).

Cross-Linking of Histone with pBR322. Figure 2 shows DNA−protein cross-linking as detected by a traditional gel shift assay. pBR322 plasmid was oxidized via the flash quench technique (vide supra) in the presence of histone. As can be seen in lane 1, the linearized plasmid runs as a single band. However, with increasing irradiation time for a sample with both intercalator and quencher (lanes 6−9), the band corresponding to free DNA decreases in intensity. This loss

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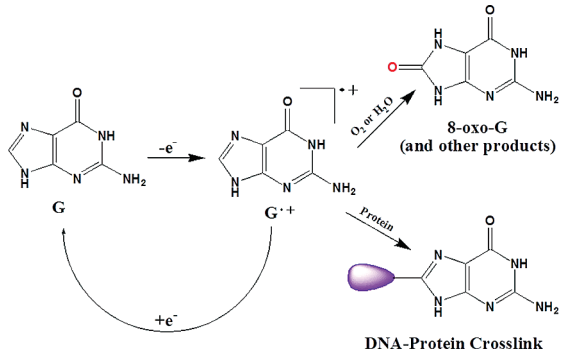

Figure 1. Two possible fates for guanine radical. Upon one-electron oxidation, guanine can react to form DNA–protein cross-links or permanent damage products such as 8-oxo-G (a myriad of other lesions are also formed).

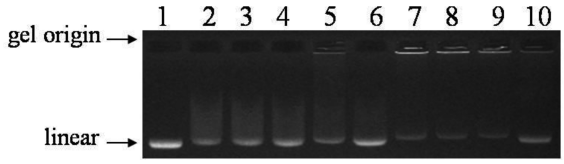

Figure 2. Gel-shift assay of pBR322 DNA cross-linked with histone by the flash quench technique. Lane 1: NdeI-linearized pBR322 DNA. Lane 2: DNA + histone. Lane 3: DNA + histone irradiated for 120 s. Lane 4: DNA + histone + $Co(NH_3)_2Cl^{2+}$ irradiated for 120 s. Lane 5: DNA + $Ru(phen)_2dppz^{2+}$ + histone irradiated for 120 s. Lanes 6–9: DNA + $Ru(phen)_2dppz^{2+}$ + histone + $Co(NH_3)_2Cl^{2+}$ irradiated for 0, 30, 60, and 120 s. Lane 10: same sample as lane 9, but treated with proteinase K (1 mg/mL for 1 h at 37 °C) prior to loading. The samples were run in a 0.8% agarose gel with ~1 μg of DNA per well at 80 V for 140 min then stained with ethidium bromide.

of free DNA intensity is accompanied by the concomitant appearance of a broad smear of retarded DNA along with well-shifted material at the longest irradiation times. Minimal loss of free DNA or appearance of gel shifted material occurs if any of the flash quench components (intercalator, quencher, or light) are omitted (lanes 2–6). If the irradiated sample is treated with proteinase K (lane 10) prior to loading onto the gel, then a single band of greater mobility is restored, suggesting that the retardation of mobility in lanes 6–9 is indeed due to DNA–protein cross-linking rather than DNA–DNA or DNA–intercalator cross-linking.

Restriction Enzyme Inhibition Assay for Detecting Cross-Linking. Although the gel shift assay provides strong evidence that DNA–protein cross-linking is occurring, it does not provide any information regarding the *sites* of DNA damage. We sought to develop an assay to detect DNA–protein cross-links that (i) did not involve the use of radioactivity and (ii) revealed the sites of cross-linking on the double helix. We also hoped to apply this assay to determine the susceptibility of different DNA sequences toward oxidative cross-linking. Restriction endonucleases recognize and cleave specific sequences in DNA. If a protein were to cross-link at these recognition sites, then inhibition of enzymatic cleavage should be observed (Figure 3). Previous studies of DNA damage generated by the flash quench technique have shown that the easily oxidized guanine base is selectively damaged.[22,27,28,30] Thus, several restriction enzymes (*Bam*HI, *Eco*RI, PpuMI) with guanines in their recognition sequences (Table 1) were chosen to probe the DNA surface for damage,

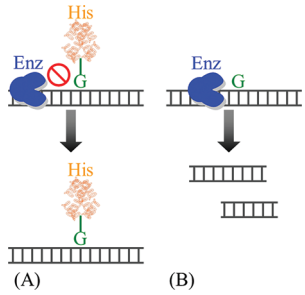

Figure 3. Inhibition of enzymatic cleavage by cross-linking. DNA containing a guanine–protein cross-link will resist endonuclease cleavage at that site (A), whereas DNA sites lacking cross-links at the recognition site will be cleaved (B).

Table 1

Restriction Enzyme	Recognition Site	Cleavage Position in pBR322
AseI	5′ ATTAAT 3′ 3′ TAATTA 5′	3538
BamHI	5′ GGATCC 3′ 3′ CCTAGG 5′	375
EcoRI	5′ GAATTC 3′ 3′ CTTAAG 5′	4359
PpuMI	5′ GGGTCCT 3′ 3′ CCCAGGA 5′	1439,
	5′ AGGACCC 3′ 3′ TCCTGGG 5′	1481

with an AT-recognizing enzyme used as a control. It is also known that the 5′ G of GG or GGG sequences is particularly vulnerable to oxidation,[13,14] and theoretical calculations[15] predict that the ionization potential of the 5′ G should increase in the order GGG < GG < GX (X ≠ G). Thus, the comparison of *Bam*HI, *Eco*RI, and PpuMI should also yield information about how the oxidation potential of guanine, as modulated by local sequence, influences its propensity for cross-linking with proteins.

Figure 4A illustrates this approach, as applied to cleavage of pBR322 by restriction enzyme *Bam*HI. The NdeI-linearized plasmid runs as a single band in the gel. Upon digestion with *Bam*HI, the DNA is fully converted to the cleaved form, displaying two higher mobility bands in the gel. When histone and the flash quench components ($Ru(phen)_2dppz^{2+}$ and $Co(NH_3)_5Cl^{2+}$) are present, the cleavage is still quite efficient, but the bands are slightly shifted toward lower mobility. Upon irradiation of the samples, there is a diminution in intensity for the cleaved bands with a concomitant appearance of a lower mobility band for the uncleaved plasmid. As shown in Figure 2, DNA that is cross-linked to histone runs slowly in the gel but can recover its mobility upon treatment with proteinase K. Thus, all samples containing histone were treated with proteinase K after restriction so as to allow the DNA and its cleavage products to enter the gel. Omission of either the intercalator (Figure 4B) or quencher (Figure 4C) needed to carry out oxidation and induce cross-linking results in a cleavage pattern that is much less dependent on irradiation

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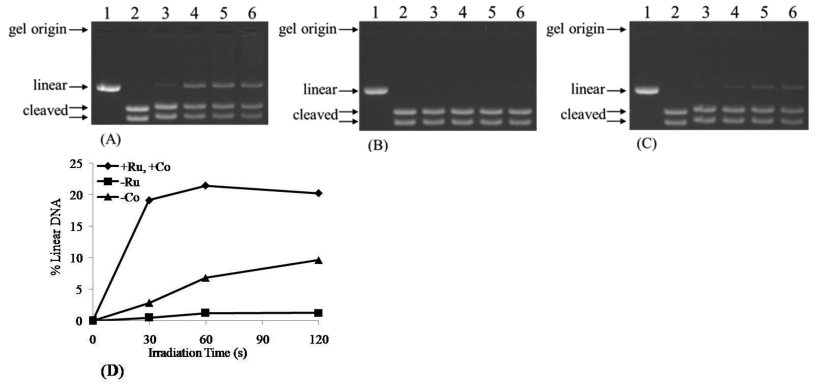

Figure 4. Agarose gel electrophoresis of linear pBR322 DNA and its *Bam*HI cleavage products as a function of irradiation time. Lane 1: linear pBR322 DNA. Lane 2: DNA cleaved with *Bam*HI (20 units for 1 h at 37 °C). Lanes 3–6: DNA incubated with *Bam*HI after being irradiated for 0, 30, 60, and 120 s in the presence of $Ru(phen)_2dppz^{2+}$ + histone + $Co(NH_3)_2Cl^{2+}$ (A), histone + $Co(NH_3)_2Cl^{2+}$ (B), or $Ru(phen)_2dppz^{2+}$ + histone (C). Samples were irradiated at 442 nm (9–12 mW), then cleaved with *Bam*HI (20 units for 1 h at 37 °C), and treated with proteinase K (1 mg/mL for 1 h at 37 °C). The samples were run in a 0.8% agarose gel with ~1 μg of DNA/well at 80 V for 140 min and then stained with ethidium bromide.

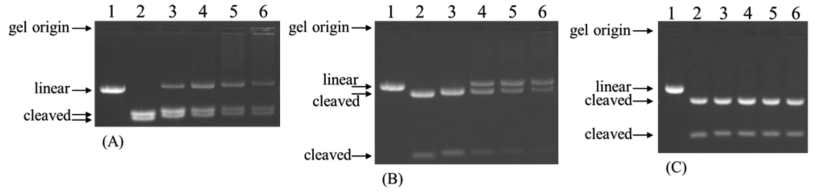

Figure 5. Agarose gel electrophoresis of linear pBR322 DNA and its cleavage products with *Eco*RI (A), PpuMI (B), or AseI (C) as a function of irradiation time. Lane 1: linear pBR322 DNA. Lane 2: DNA cleaved with restriction enzyme. Lanes 3–6: DNA + $Ru(phen)_2dppz^{2+}$ + histone + $Co(NH_3)_2Cl^{2+}$ irradiated for 0, 30, 60, and 120 s and then cleaved with either *Eco*RI (20 units), PpuMI (5 units), or AseI (10 units) for 1 h at 37 °C. Other conditions the same as in Figure 4.

time; quantitation of the gel data clearly indicates a much greater level of inhibition when all three flash quench components are present (Figure 4D). A slight restoration of uncleaved DNA at the longest irradiation times in Figures 4B and 4C may be attributed to the production of singlet oxygen by the ruthenium intercalator[27] and a low-yield redox photodecomposition process of the cobalt complex[31] (vide infra), respectively.

To investigate the effect of DNA sequence on the assay, the experiments were repeated with three additional endonucleases. Figure 5A shows the cleavage inhibition assay for *Eco*RI. Here the plasmid runs as one band and, upon *Eco*RI digestion, produces two bands that run very close to each other; a small amount of DNA remains uncleaved even in the absence of histone (lane 1) or light (lane 2). Upon irradiation, the overall intensity decreases slightly, but the relative intensity of the cleavage products diminishes while the intensity of uncleaved DNA increases. For PpuMI, the heavier restriction fragment runs just below the uncleaved linear plasmid, but the results (Figure 5B) are otherwise similar to those obtained with *Bam*HI and *Eco*RI, showing a clear increase in uncleaved DNA at the expense of cleaved DNA. Like *Bam*HI, neither *Eco*RI nor PpuMI experiences any inhibition of cleavage if either intercalator or quencher is omitted from the sample (data not shown). The restriction enzyme AseI was also examined as a control (Figure 5C); since the flash quench technique oxidizes only the guanine base, AseI should not experience inhibition, owing to the fact that its recognition sequence contains only AT base pairs (Table 1). For AseI, the linear plasmid is efficiently cleaved into two fragments, and there is no restoration of uncleaved DNA upon irradiation.

To compare the degree of inhibition for the different enzymes, the band intensities in each gel were quantified. Figure 6A shows a graphical representation of the *Bam*HI inhibition experiment, showing a decrease in height for the two peaks at R = 0.4 for cleaved product and a rise in the single peak for uncleaved DNA near R = 0.3. Integration under these peaks allows for the calculation of the % increase in uncleaved DNA caused by irradiation, i.e., the amount of inhibition due to DNA–protein cross-linking. The results of these integrations are shown in Figure 6B, and the % of uncleaved DNA decreases as follows: PpuMI > *Bam*HI > *Eco*RI > AseI.

Given the highly specific binding of restriction enzymes to their recognition sites, it seemed prudent to determine whether other types of oxidative damage, more subtle than cross-linking, might also result in inhibition of restriction enzyme cleavage. Guanine is known to form a number of permanent damage products upon oxidation, including 8-oxo guanine, Fapy-G, imidazolone, and spiroiminohydantoin.[12] The assay was therefore applied to a plasmid oxidized by flash quench in the absence of the histone protein. Indeed, some inhibition of cleavage by *Bam*HI (Figure 7) is observed, but the effect is much less pronounced than with histone present; similar results are observed with *Eco*RI and PpuMI.

DISCUSSION

DNA–protein cross-links are one consequence of DNA damage and are receiving increasing interest. In order to induce DNA–protein cross-links, a photochemical method called the flash quench technique was employed. This technique results in the selective one-electron oxidation of guanine bases in DNA,[26,27] which in turn can produce cross-links with bound protein.[21,22,32]

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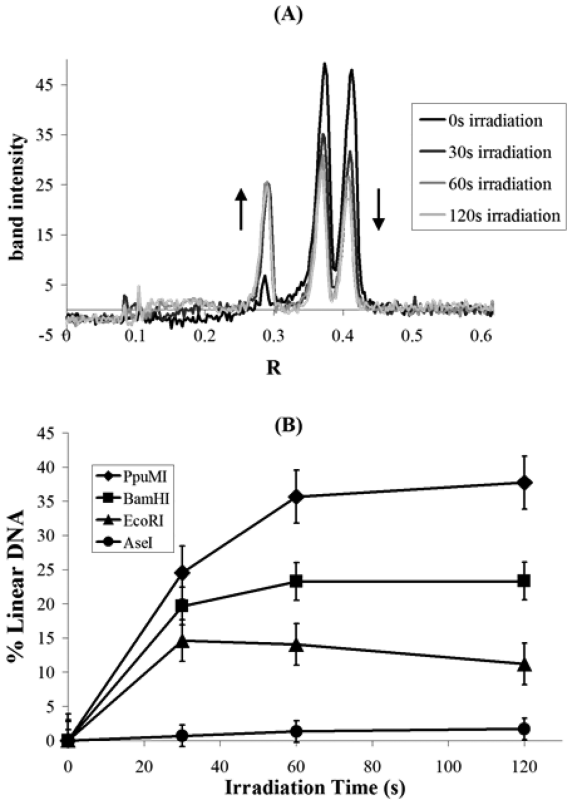

Figure 6. Dependence of % cleavage inhibition upon restriction enzyme. (A) Band intensity plot for gel of pBR322 DNA cleaved by *Bam*HI. The intensities of lanes 3–6 from Figure 5 were obtained using Quantity One 4.1.0 software (Bio-Rad). Band intensities were obtained from the peak areas and plotted in (B). (B) Dependence of % cleavage inhibition upon restriction enzyme. The % increase in linear DNA is plotted as a function of irradiation time; the t = 0 intensity was subtracted from each data set. Shown are data for PpuMI (diamonds), *Bam*HI (squares), *Eco*RI (triangles), and AseI (circles).

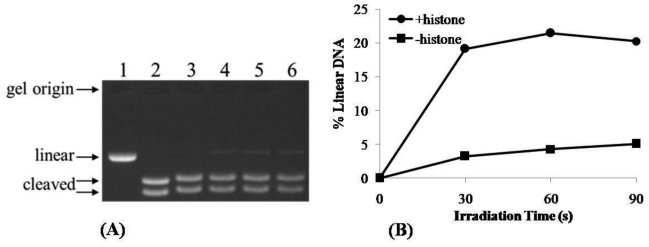

Figure 7. (A) Agarose gel electrophoresis of DNA irradiated in the absence of histone and treated with *Bam*HI. Lane 1: linear pBR322 DNA. Lane 2: DNA cleaved with *Bam*HI (20 units for 1 h at 37 °C). Lanes 3–6: linear DNA + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_2Cl^{2+}$ irradiated for 0, 30, 60, and 120 s at 442 nm (9–12 mW). The DNA was then cleaved with *Bam*HI (20 units for 1 h at 37 °C) and treated with proteinase K (1 mg/mL for 1 h at 37 °C). Other conditions same as in Figure 4. (B) The % increase in linear DNA is plotted as a function of irradiation time; the t = 0 intensity was subtracted from each data set.

Numerous studies of guanine damage have shown that the reactivity of a guanine base toward oxidation depends strongly on the flanking bases, with the 5′ G of a GG doublet or GGG site particularly prone to one-electron oxidation. This observation has a theoretical underpinning: Saito and co-workers have calculated an uncommonly high HOMO energy for the 5′G of the guanine multiplets[15] and have predicted that the ionization potential of guanine increases in the order GGG < GG < GA < GC < GT. Given that DNA in vivo is often associated intimately with protein and that oxidation of guanine leads to DNA–protein cross-links, we sought to elucidate how local DNA sequence might influence DNA–protein cross-linking induced by guanine oxidation.

Development of a Nonradioactive Assay for Detecting DNA–Protein Cross-Links. There are many ways to detect DNA–protein cross-linking, but most involve the use of radioactivity. To probe the plasmid DNA, we first needed to develop an appropriate assay. Our approach was based in part on the experiments of Winkle and Mallamaci, who utilized restriction enzyme mapping to detect binding of carcinogens to pBR322.[33,34] Here, our goal was to develop a nonradioactive assay involving the inhibition of restriction enzyme cleavage and to determine if such an assay could be used to probe whether specific sites on the DNA had undergone cross-linking. Figure 2 illustrates that cross-linking of histone with linear pBR322 leads to a decreased mobility that can be mostly restored upon treatment with protease, as seen previously for supercoiled DNA,[21] consistent with oxidative cross-linking.

While the electrophoretic mobility shift assay (EMSA) in Figure 2 provides excellent evidence for cross-linking, it does not reveal information regarding the location of the cross-links. Since the flash quench technique selectively oxidizes guanine bases in double-stranded DNA,[26,27] cross-linking is expected at guanine sites. Moreover, the presence of a cross-linked protein should interfere with restriction endonuclease cleavage if the enzyme contains guanine in its recognition sequence. Indeed, for several enzymes containing guanine in their recognition sequences (*Eco*RI, *Bam*HI, PpuMI), induction of cross-links by flash quench results in a diminution of cleavage (Figures 4–6). Importantly, this inhibition of cleavage is not observed for AseI, a restriction endonuclease that contains only AT base pairs in its recognition sequence. Presumably, some inhibition could occur if histone protein were cross-linked immediately adjacent to the AseI recognition site or perhaps on either side, as might be expected at very high protein concentrations. However, at concentrations up to 250 μg/mL of histone, minimal inhibition of AseI enzyme was observed.

Control experiments also indicate that considerably less inhibition of cleavage results if any of the flash quench components (metal, quencher or light) are omitted (Figure 4). Some reaction is visible in the absence of quencher; this is expected, as $Ru(phen)_2dppz^{2+}$ can induce DNA damage at longer irradiation times, owing to singlet oxygen formation. However, the amount of damage observed in the control is actually an overestimation of the amount of singlet oxygen damage in this system, since (i) the $Ru(phen)_2dppz^{2+}$ excited state lifetime is shortened by electron transfer to the oxidative quencher ($Co(NH_3)_5Cl^{2+}$) and (ii) this redox quenching is more efficient than energy transfer to molecular oxygen when these two processes compete.[35] Typically, the amount of damage incurred by visible irradiation of DNA and histone in the presence of the cobalt quencher is minimal, as seen in lane 4 of Figure 2 and also in previous studies.[21,22] Excitation of Co(III) complexes in the UV charge transfer band leads to redox decomposition and photoaquation.[31] For $Co(NH_3)_5Cl^{2+}$, 488 nm excitation of the ligand field band leads primarily to photoaquation but is accompanied by a small amount of photoredox decomposition in which homolytic cleavage forms a Co(II) species and a ligand radical,[36] the latter of which could potentially cause some cross-linking. In either the intercalator

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or quencher control samples, however, the amount of reaction is small. The majority of DNA–protein cross-linking, and subsequent inhibition of cleavage, is thus attributable to the one-electron oxidation of guanine effected by the flash quench technique.

Interestingly, flash quench treatment of pBR322 in the absence of histone produced a relatively small amount of inhibition of restriction enzyme cleavage compared to samples containing histone (Figure 7). It is known that oligonucleotide duplexes subjected to flash quench produce 8-oxoguanine[30] and that the presence of guanine lesions interferes with cleavage of restriction enzymes.[37] Cadet et al. have suggested that nucleophilic attack on the electrophilic guanine radical is a major route to permanent guanine lesions such as 8-oxoguanine.[38] If so, then one plausible explanation for the difference seen in Figure 7 is that the trapping of the guanine radical as a permanent damage product may simply be more efficient for histones; being rich in lysine, histones should provide stronger nucleophiles than water. Moreover, the DNA–protein cross-link, being considerably larger than a base lesion such as 8-oxoguanine, presents both a steric problem and a recognition problem for the endonuclease. Regardless of the reason, it is apparent that the assay described herein is quite adept at detecting DNA–protein cross-linking at guanine sites. Thus, this method is a highly selective, if not specific, way to detect DNA–protein cross-linking in DNAs of known sequence.

There are many methods for detecting DNA–protein interactions, including gel shift assays, DNA footprinting, and a number of fluorescence-based assays. Presumably, the restriction enzyme approach described here could be applied to detection of strong noncovalent interactions between DNA and protein as well. In a recent related experiment, Xu et al. developed a fluorescence recovery assay where they utilized exonuclease digestion of DNAs containing fluorescent dyes and quenchers to obtain relative binding affinities of sequence-specific DNA binding proteins.[39] Their assay, while quick to carry out, requires the synthesis of two covalently modified DNA strands. In contrast, while the restriction enzyme assay described here requires multiple electrophoresis experiments, commercial DNA can be used without modification.

Dependence of Oxidative DNA–Protein Cross-Linking upon Local DNA Sequence. Having demonstrated that inhibition of restriction enzyme cleavage can be used to detect DNA–protein cross-linking, we then sought to apply this new method toward elucidating the propensity of different DNA sequences in pBR322 DNA toward oxidative cross-linking with protein. Guanine is the most easily oxidized base, and it has been known for some time that the local environment of the guanine base influences its ease of oxidation. The 5′ G of GG and GGG sequences is known to be particularly susceptible to photooxidation, and theoretical studies have attributed this trend to the high HOMO energy of the 5′ G, with the calculated ionization potentials of guanine increasing in the following order: GGG < GG < GA < GC < GT.[15]

Here, % inhibition values were obtained for three restriction enzymes containing guanines in their recognition sequences. The results (Figure 6B) show that the % inhibition decreases in the order PpuMI > *Bam*HI > *Eco*RI, which corresponds to a sequence order of GGG > GG > GA; the actual sites in pBR322 cleaved by PpuMI (Table 1) are 5′-GGGTCCT-3′ and 5′-AGGACCC-3′, so both GGG and GG sites are being probed by this enzyme. Though it binds to all sites in double-stranded DNA, the ruthenium intercalator does have a slight sequence preference, but it favors binding to AT sites,[40] so the binding preference of the metallointercalator cannot explain the current results since it would predict the most inhibition for *Eco*RI.

An alternative explanation for the trend observed in Figure 6B could be selective binding by histone based on GC content. The histone III-S employed here is enriched in linker histone H1.[41] Histone H1 is typically thought to prefer AT-rich regions[42−44] or to bind nonspecifically.[45] However, if H1 bound preferentially to GC-rich sequences, the trend observed in Figure 6B could simply be a reflection of the greater binding affinity of linker histone H1 for these GC-rich sequences. Despite the lack of precedent for this, there are additional arguments against this possibility. First, Nguyen et al. have shown that upon flash quench treatment histone III-S cross-links more with calf thymus DNA (42% GC) than with poly(dGdC) (100% GC), despite the fact that the quenching process is actually more efficient for the all-GC polymer.[21] Moreover, the possibility that GC content determines the endonuclease inhibition level was addressed experimentally. Figure 8 shows restriction enzyme inhibition assays for both *Eco*RI and AseI with histone H2A, which as part of the

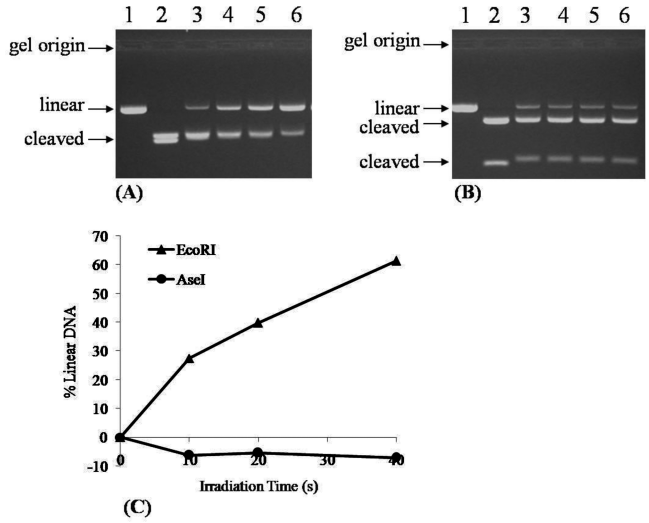

Figure 8. Agarose gel electrophoresis of linear pBR322 DNA and its cleavage products with *Eco*RI (A) or AseI (B) as a function of irradiation time. Lane 1: linear pBR322 DNA. Lane 2: DNA cleaved with restriction enzyme. Lanes 3–6: DNA + $Ru(phen)_2dppz^{2+}$ + histone H2A + $Co(NH_3)_2Cl^{2+}$ irradiated for 0, 10, 20, and 40 s and then cleaved with either *Eco*RI (20 units) or AseI (5 units) for 1 h at 37 °C. Other conditions same as in Figure 7. (C) The % increase in linear DNA is plotted as a function of irradiation time; the t = 0 intensity was subtracted from each data set. Shown are data *Eco*RI (triangles) and AseI (circles).

nucleosome core particle should, if anything, exhibit a preference for binding to AT-rich sequences. As seen in the gels (Figure 8A,B) and the analysis (Figure 8C), H2A exhibits cross-linking only with the guanine containing sequence. Furthermore, more cross-linking is observed for *Eco*RI with histone H2A than with histone III-S (Figure 6B), the opposite of what would be expected were preferential binding of histone III-S to GC-rich regions causing the inhibition trend observed in Figure 6B. Thus, taken together, these results do not support the idea that GC-selective binding by histone III-S is driving the cross-linking trend.

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Instead, the propensity for DNA−protein cross-linking thus appears to track with the ease of oxidation of the guanine base, with the most easily oxidized sites exhibiting the most cross-linking. This result is consistent with other photooxidation experiments of DNA bound with protein. For example, Nuñez et al. examined guanine damage in a nucleosome core particle fragment, showing that oxidative damage can extend out over 100 Å away from the binding site of the photooxidant even when DNA is wrapped with histone.[46] While they did not examine DNA−protein cross-linking directly, the amount of alkali-labile guanine damage was also found to be concentrated in GG and GGG sites. Presumably some of this damage was in the form of DNA−protein cross-links which, when formed by one-electron oxidation of guanine, have been shown to exhibit alkali sensitivity.[22,47] More recently, Bjorklund and Davis have also observed long-range guanine oxidation in a nucleosome core particle.[23] The sites exhibiting the most damage in their experiments were still sites of multiple guanines, as observed by Nuñez et al.[46] Interestingly, they find that a tyrosine−thymine cross-link presents as the ultimate product, and they attribute this finding to oxidation of tyrosine by guanine cation radical, as shown earlier in studies of guanine oxidation in the presence of Lys-Tyr-Lys peptides;[48] other model systems have also observed tyrosine−thymine adducts.[49] Thus, while our experiments with restriction enzymes represent only a beginning, it does appear that in mixed DNA sequences the guanine sites most vulnerable to cross-linking are those that are easiest to oxidize, i.e., that the guanine cluster hotspots observed in photooxidation experiments with either naked or protein-bound DNAs can also be extended to oxidative cross-linking. These results also have implications for the kinetics of the cross-linking reaction. Given that $Ru(phen)_2dppz^{3+}$ is capable of oxidizing guanine in any sequence context, the trend observed here, more cross-linking at the more easily oxidizable guanine sites, requires that that equilibration of the electron hole in the plasmid DNA occur on a time scale faster than that of adduct formation.

CONCLUSION

In this paper, we describe the development of a new method for detecting DNA−protein cross-linking based on restriction enzyme inhibition and apply this method to demonstrate that in pBR322 plasmid the sites with the highest yield of cross-linking are those which are known to be the most readily oxidized, as the amount of cross-linking decreases in the order GGG > GG > GA.

AUTHOR INFORMATION

Corresponding Author

*E-mail: estemp@msmc.la.edu. Tel: 310-954-4102. Fax: 310-954-1745.

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ABBREVIATIONS

EMSA, electrophoretic mobility shift assay; FMOC, fluorenylmethyloxycarbonyl; PS, photosensitizer; Q, quencher.

REFERENCES

(1) Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J. 17*, 1195−1214.

(2) Barnes, D. E., and Lindahl, T. (2004) Repair and genetic consequences of endogenous DNA base damage in mammalian cells. *Annu. Rev. Genet. 38*, 445−476.

(3) Storz, G., Tartaglia, L. A., and Ames, B. N. (1990) Transcriptional regulator of oxidative stress-inducible genes: Direct activation by oxidation. *Science 248*, 189−194.

(4) Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J., and Telser, J. (2004) Role of oxygen radicals in DNA damage and cancer incidence. *Molec. Cellul. Biochem. 266*, 37−56.

(5) Scharer, O. D. (2003) Chemistry and biology of DNA repair. *Angew. Chem., Int. Ed. 42*, 2946−2974.

(6) Imlay, J. A. (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu. Rev. Biochem. 77*, 755−776.

(7) Hoeijmakers, J. H. J. (2001) Genome maintenance mechanisms for preventing cancer. *Nature 411*, 366−374.

(8) Jiricny, J. (2006) The multifaceted mismatch-repair system. *Nat. Rev. Mol. Cell Biol. 7*, 335−346.

(9) Hsieh, P., and Yamane, K. (2008) DNA mismatch repair: Molecular mechanism, cancer, and ageing. *Mech. Ageing Dev. 129*, 391−407.

(10) Steenken, S., and Jovanovic, S. (1997) How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. *J. Am. Chem. Soc. 119*, 617−618.

(11) Burrows, C. J., and Muller, J. G. (1998) Oxidative nucleobase modifications leading to strand scission. *Chem. Rev. 98*, 1109−1151.

(12) Luo, W., Muller, J. G., Rachlin, E. M., and Burrows, C. J. (2001) Characterization of hydantoin products from one-electron oxidation of 8-Oxo-7,8-dihydroguanosine in a nucleoside model. *Chem. Res. Toxicol. 14*, 927−938.

(13) Saito, I., Takayama, M., Sugiyama, H., and Nakatani, K. (1995) Photoinduced DNA cleavage via electron transfer; demonstration that guanosine residues located 5′ to guanine are the most electron-donating sites. *J. Am. Chem. Soc. 117*, 6406−6407.

(14) Hall, D. B., Holmlin, R. E., and Barton, J. K. (1996) Oxidative DNA-damage through long-range electron-transfer. *Nature 382*, 731−735.

(15) Sugiyama, H., and Saito, I. (1996) Theoretical studies of GC-specific photocleavage via electron transfer: significant lowering of ionization potential and 5′-localization of HOMO of stacked GG bases in B-form DNA. *J. Am. Chem. Soc. 118*, 7063−7068.

(16) Barker, S., Weinfeld, M., and Murray, D. (2005) DNA − protein cross-links: Their induction, repair, and biological consequences. *Mutat. Res. 589*, 111−135.

(17) Morin, B., and Cadet, J. (1994) Benzophone photosensitization of 2′-deoxyguanosine: Characterization of the 2R and 2S diastereoisomers of 1-(2′-deoxy-β-D-erythro-pentofuranosyl)-2-methoxy-4,5-imidazolidinedione. A model system for the investigation of photosensitized formation of DNA-protein cross-links. *Photochem. Photobiol. 60*, 102−109.

(18) Morin, B., and Cadet, J. (1995) Type I benzophone-mediated nucleophilic reaction of 5′-Amino-2′,5′-dideoxyguanosine. A model system for the investigation of photosensitized formation of DNA-protein cross-links. *Chem. Res. Toxicol. 8*, 192−199.

(19) Perrier, S., Hau, J., Gasparutto, D., Cadet, J., Favier, A., and Ravanat, J. (2006) Characterization of lysine − guanine cross-links upon one-electron oxidation of a guanine-containing oligonucleotide in the presence of a trilysine peptide. *J. Am. Chem. Soc. 128*, 5703−5710.
(20) Solivio, M. J., Joy, T. J., Sallans, L., and Merino, E. J. (2010) Copper generated reactive oxygen leads to formation of lysine − DNA adducts. *J. Inorg. Biochem. 104*, 1000−1005.
(21) Nguyen, K. L., Steryo, M., Kurbanyan, K., Nowitzki, K. M., Butterfield, S. M., Ward, S. R., and Stemp, E. D. A. (2000) DNA-protein cross-linking from oxidation of guanine via the flash-quench technique. *J. Am. Chem. Soc. 122*, 3585−3594.
(22) Kurbanyan, K., Nguyen, K. L., To, P., Rivas, E. V., Lueras, A. M .K., Kosinski, C., Steryo, M., Gonzalez, A., Mah, D. A., and Stemp, E. D. A. (2003) DNA-protein cross-linking via guanine oxidation: Dependence upon protein and photosensitizer. *Biochemistry 42*, 10269−10281.
(23) Bjorklund, C. C., and Davis, W. B. (2007) Stable DNA − protein cross-links are products of DNA charge transport in a nucleosome core particle. *Biochemistry 46*, 10745−10755.
(24) Amouyal, E., Homsi, A., Chambron, J.-C., and Sauvage, J.-P. (1990) Synthesis and study of a mixed-ligand ruthenium(II) complex in its ground and excited-states − bis(2,2′-bipyridine)(dipyrido[3,2-a:2′,3′-c]phenazine-N^4N^5)ruthenium(II). *J. Chem. Soc., Dalton Trans.*, 1841−1845.
(25) Chang, I. J., Gray, H. B., and Winkler, J. R. (1991) High-driving force electron transfer in metalloproteins - intramolecular oxidation of ferrocytochrome c by $Ru(2,2'\text{-bpy})_2(Im)(His\text{-}33)^{3+}$. *J. Am. Chem. Soc. 113*, 7056−7057.
(26) Dunn, D. A., Lin, V. H., and Kochevar, I. E. (1992) Base-selective oxidation and cleavage by photochemical cosensitized electron transfer. *Biochemistry 31*, 11620−11625.
(27) Stemp, E. D. A., Arkin, M. R., and Barton, J. K. (1997) Oxidation of guanine in DNA by $Ru(phen)_2(dppz)^{3+}$ using the flash-quench technique. *J. Am. Chem. Soc. 119*, 2921−2925.
(28) Candeias, L. P., and Steenken, S. (1989) Structure and acid-base properties of one-electron-oxidized deoxyguanosine, guanosine, and 1-methylguanosine. *J. Am. Chem. Soc. 111*, 1094−1099.
(29) Simic, M., and Lilie, J. (1974) Kinetics of ammonia detachment from reduced cobalt(III) complexes based on conductometric pulse radiolysis. *J. Am. Chem. Soc. 96*, 291−292.
(30) Arkin, M. R., Stemp, E. D. A., Pulver, S. C., and Barton, J. K. (1997) Long-range oxidation of guanine by Ru(III) in duplex DNA. *Chem. Biol. 4*, 389−400.
(31) Adamson, A. W., and Sporer, A. H. (1958) Photochemistry of Complex Ions. I. Some Photochemical Reactions of Aqueous $PtBr_6^{-2}$, $Mo(CN)_8^{-4}$ and Various Co(III) and Cr(III) Complex Ions. *J. Am. Chem. Soc. 80*, 3865−3870.
(32) Copeland, K. D., Lueras, A. M. K., Stemp, E. D. A., and Barton, J. K. (2002) DNA cross-linking with metallointercalator − peptide conjugates. *Biochemistry 41*, 12785−12797.
(33) Mallamaci, M. A., Bascoy, M. L., Brown, J., Combates, N. J., and Winkle, S. A. (1992) Locating binding-sites for the carcinogen n-acetoxy-n-acetyl-2-aminofluorene using restriction enzyme-inhibition assays. *J. Biomol. Struct. Dyn. 10*, 83−96.
(34) Mallamaci, M. A., Reed, D. P., and Winkle, S. A. (1992) Restriction enzyme mapping of carcinogen binding regions on pBR322. *J. Biomol. Struct. Dyn. 10*, 73−82.
(35) Wessels, J. M., Foote, C. S., Ford, W. E., and Rodgers, M. A. J. (1997) Photooxidation of tryptophan: $O_2(^1\Delta_g)$ versus electron-transfer pathway. *Photochem. Photobiol. 65*, 96−102.
(36) Pribush, R. A., Poon, C. K., Bruce, C. M., and Adamson, A. W. (1974) Photochemistry of Complex Ions. XII. Photochemistry of Cobalt(III) Acidoammines. *J. Am. Chem. Soc. 96*, 3027−3032.
(37) Wood, M. L., Dizdaroglu, M., Gajewski, E., and Essigmann, J. M. (1990) Mechanistic studies of ionizing radiation and oxidative mutagenesis: Genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. *Biochemistry 29*, 7024−7032.
(38) Cadet, J., Douki, T., Gasparutto, D., Gromova, M., Pouget, J. P., Ravanat, J. L., Romieu, A., and Sauvaigo, S. (1999) Radiation-induced damage to DNA: mechanistic aspects and measurement of base lesions. *Nucl. Instrum. Methods Phys. Res., Sect. B 151*, 1−7.
(39) Xu, X., Zhao, Z., Qin, L., Wei, W., Levine, J. E., and Mirkin, C. A. (2008) Fluorescence recovery assay for the detection of protein − DNA binding. *Anal. Chem. 80*, 5616−5621.
(40) Holmlin, R. E., Stemp, E. D. A., and Barton, J. K. (1998) $Ru(phen)_2dppz^{2+}$ luminescence: Dependence on DNA sequences and groove-binding agents. *Inorg. Chem. 37*, 29−34.
(41) Glynn, B., Colliton, J., McDermott, J., and Witters, L. A. (1985) Assay of protein kinase C with an N-bromosuccinimide-cleavage fragment of histone H1. *Biochem. J. 231*, 489−492.
(42) Cui, F., and Zhurkin, V. B. (2009) Distinctive sequence patterns in metazoan and yeast nucleosomes: Implications for linker histone binding to AT-rich and methylated DNA. *Nucleic Acids Res. 37*, 2818−2829.
(43) Zlatanova, J., and Yaneva, J. (1991) DNA sequence specific interactions of histone H1. *Mol. Biol. Rep. 15*, 53−56.
(44) Roque, A., Orrego, M., Ponte, I., and Suau, P. (2004) The preferential binding of histone H1 to DNA scaffold-associated regions is determined by its C-terminal domain. *Nucleic Acids Res. 32*, 6111−6119.
(45) Yaneva, J., and Zlatanova, J. (1992) Histone H1 interacts specifically with certain regions of the mouse alpha-globin gene. *DNA Cell Biol. 11*, 91−99.
(46) Núñez, M. E., Noyes, K. T., and Barton, J. K. (2002) Oxidative charge transport through DNA in nucleosome core particles. *Chem. Biol. 9*, 403−415.
(47) Merino, E. J., and Barton, J. K. (2008) DNA oxidation by charge transport in mitochondria. *Biochemistry 47*, 1511−1517.
(48) Wagenknecht, H.-A., Stemp, E. D. A., and Barton, J. K. (2000) DNA-bound peptide radicals generated through DNA-mediated electron transport. *Biochemistry 39*, 5483−5491.
(49) Nackerdien, Z., Rao, G., Cacciuttolo, M. A., Gajewski, E., and Dizdaroglu, M. (1991) Chemical Nature of DNA-Protein cross-links produced in mammalian chromatin by hydrogen peroxide in the presence of iron or copper ions. *Biochemistry 30*, 4873−4879.