Oxidized Guanine Lesions as Modulators of Gene Transcription. Altered p50 Binding Affinity and Repair Shielding by 7,8-Dihydro-8-oxo-2′-deoxyguanosine Lesions in the NF-κB Promoter Element†

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ABSTRACT: A number of common promoter elements that drive transcription of redox sensitive genes have runs of guanines in their transcription factor recognition sequence. A paradox exists insomuch that the same guanine runs necessary for transcription factor recognition are thermodynamically prone to oxidative modification, potentially altering the binding affinity of transcription factors. 7,8-Dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) is a common oxidative modification of guanine that is generated by a variety of metals and reactive oxygen species. We have used the p50 subunit of the NF-κB transcription factor to show that oxidation of guanine to 8-oxo-dG at sites critical for protein recognition impacts transcription factor binding affinity differently depending upon the site of oxidation. It can be argued that the impact of such oxidation will be minimal in repair proficient cells. Therefore, we have developed an assay to assess the ability of these lesions to be shielded by transcription factor binding from recognition and repair by base excision repair (BER) enzymes. In this study, 8-oxo-dG was substituted for guanine at sites G_1-G_4 in the NF-κB sequence 5′-d(AGTTGAG$_1$G$_2$G$_3$G$_4$ACTTTCCCAGCC)-3′. We have observed that substitution of 8-oxo-dG at the G_1 site increases p50 binding affinity by ∼2.5-fold compared to that of the unmodified DNA sequence, while substitution at G_3 reduces the binding affinity by ∼4-fold. Substitution of 8-oxo-dG at the G_2 and G_4 sites had a minimal impact on p50 binding affinity. Both *Escherichia coli* fapy glycosylase (Fpg) and human 8-oxo-DNA glycosylase (hOGG1) recognized and cleaved 8-oxo-dG at all four sites within the promoter element. The addition of the p50 transcription factor shielded these lesions from cleavage by the glycosylase in a manner that correlated with the binding affinities of p50 for the different modified sites. These data imply that lesion formation in DNA response elements can modulate gene transcription during oxidative events and that protein binding to these modified sites may allow these lesions to persist on a time scale that impacts global cellular gene transcription.

Oxidation of guanine to 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG)[1] is a common DNA lesion produced by endogenous metabolic processes and redox-active xenobiotics (*1*). This type of lesion has been shown to cause a number of aberrant cellular effects, including mutation, cell transformation, and changes in gene transcription (*1*−*4*). It has been estimated that oxidative lesions such as 8-oxo-dG occur at a frequency of ∼10000 bp per cell per day (*2*). This oxidative DNA damage has been implicated in the etiology of a number of disease states, including cancer and aging (*4*). The high frequency of oxidative guanine modification within DNA arises from its oxidation potential being lower than those of the other nucleic acid bases (*5*). This lower oxidation potential is exacerbated in consecutive runs of guanines within a DNA sequence causing an increase in oxidative reactivity at the 5′-guanine (*6*). On the basis of the oxidation potentials, DNA sequences that contain a high guanine content or consecutive runs of G's should be particularly prone to oxidative modification. Given this tendency toward oxidative modification of high-guanine content DNA, it is surprising to note that a number of transcription factor regulatory elements driving redox sensitive gene expression contain such consecutive runs of guanines within their consensus transcription factor binding sites. One of these redox sensitive transcription factors with high guanine content in its recognition sequence is NF-κB (*7*). NF-κB regulation of gene transcription is redox sensitive, but its modulation is usually studied in terms of cytoplasmic activation followed by translocation into the nucleus (*8*). The crystal structure of the NF-κB p50 homodimer bound to its consensus DNA sequence has been determined (*9*, *10*). Figure 1 shows the multiple contacts via hydrogen bonding between N-7 and O-6 of guanines within the consensus binding sequence and specific amino acids of the p50 protein that confer binding specificity. Formation of oxidized guanine lesions within the DNA consensus binding site is predicted to change hydrogen

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[1] Abbreviations: BER, base excision repair; 8-oxo-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; EMSA, electromobility shift assay; OD, optical density; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBE, Tris-borate-EDTA; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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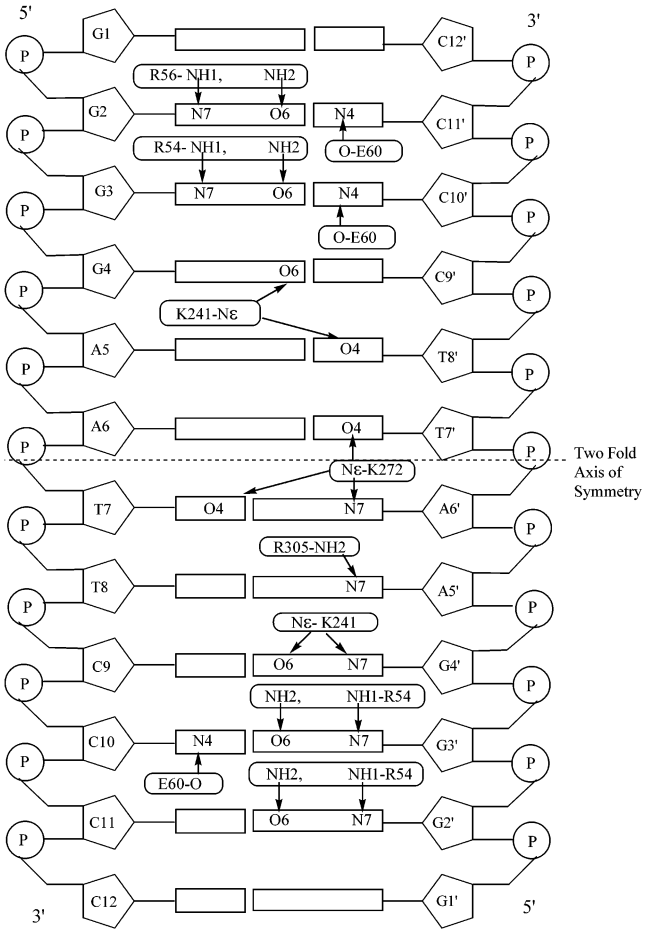

FIGURE 1: Sequence-specific DNA−protein interactions identified for the p50 NF-κB subunit binding with its consensus oligonucleotide sequence. From PDB entry 1NFK (*10*).

bond donor−acceptor patterns that would alter recognition by the transcription factor protein (*11*). These oxidative modifications may also induce structural changes within the DNA such as base flipping, bending, or kinking (*12*).

For oxidized guanine lesions to have a significant impact upon gene transcription, they must be resistant to recognition and repair from endogenous repair enzymes on a reasonable cellular time scale. These oxidized lesions are usually recognized and excised within DNA by the base excision repair enzymes (BER), Fapy glycosylase, Fpg, or MutM in *Escherichia coli* or the DNA glycosylase (hOGG1) in humans (*13*, *14*). If oxidized guanine lesions are present in promoter elements and affecting gene transcription, such repair would serve to switch the response element, along with any transcriptional effects, back to their "normal" levels.

A 22 bp duplex oligonucleotide containing the NF-κB p50 consensus binding site was chosen for this study. An 8-oxo-dG lesion was placed at each of the guanine positions, G_1−G_4, in the NF-κB consensus recognition sequence 5′-d(AGTTGAG$_1$G$_2$G$_3$G$_4$ACTTTCCCAGCC)-3′ on a single strand of DNA within the consensus 22 bp duplex. We have observed that the binding affinity of p50 for its DNA cognate sequence was either increased, unchanged, or diminished in the response elements containing an 8-oxo-dG lesion and that the nature and magnitude of this effect were dependent upon the site of the lesion within the DNA cognate sequence. These results indicated that oxidative damage in the response element can modulate gene expression on the basis of changes in the binding affinity of transcription factors for modified guanine lesions within the DNA cognate recognition sequence.

Table 1: Control and Modified Oligonucleotide Sequences Incorporating the NF-κB Promoter Site

oligo no.	sequence (top strand only)[a]
1	5′-AGT TGA G$_1$***G$_2$G$_3$ G$_4$AC TTT CCC*** AGC C-3′
2	5′-AGT TGA G$_1$°***G$_2$G$_3$ G$_4$AC TTT CCC*** AGC C-3′
3	5′-AGT TGA G$_1$***G$_2$***°***G$_3$ G$_4$AC TTT CCC*** AGC C-3′
4	5′-AGT TGA G$_1$***G$_2$G$_3$***° ***G$_4$AC TTT CCC*** AGC C-3′
5	5′-AGT TGA G$_1$***G$_2$G$_3$ G$_4$***°***AC TTT CCC*** AGC C-3′

[a] Bold and italic type denotes the p50 consensus recognition sequence.

We have further shown that shielding of repair from BER enzymes does indeed occur by the binding of the p50 transcription factor to the modified DNA sequences. This shielding effect directly correlated with the differing binding affinities afforded by site-specific oxidized DNA lesions. This type of DNA damage shielding by a protein can be compared to the high-mobility group protein shielding of platinated DNA adducts and would be expected to significantly increase the lifetime and impact of these lesions on gene expression. The existence of such a signaling mechanism proposed herein may account for some of the discrepancies between cellular responses to redox-active carcinogens and describes a novel mechanism by which oxidized guanine lesions may control cellular behavior and induce cell transformation.

MATERIALS AND METHODS

Deoxyribonucleotides. The unmodified oligonucleotides used in this study were synthesized using standard automated solid-state methods. The 8-oxo-dG-containing oligonucleotides (G° indicates 8-oxo-dG) and complementary strands were purchased from TriLink BioTechnologies. Table 1 shows the sequence and site of modification of the top strand of the oligonucleotides used in this study. Purification of the oligonucleotides prior to use was accomplished by HPLC using a Dionex Nucleopac PA-100, 4 mm × 250 mm anion exchange column employing a linear gradient from 90% mobile phase A (10% aqueous acetonitrile) and 10% mobile phase B [1.5 M ammonium acetate (pH 7.0) in 10% acetonitrile] to 100% mobile phase B over the course of 31 min. Eluting oligonucleotides were monitored with a diode array at 268 nm. The fraction containing the oligomer was eluted as a single peak, EtOH precipitated, and evaporated to dryness. Pure oligonucleotides were stored at −20 °C until they were needed. The 5′-^{32}P-end-labeled oligomers were prepared by standard methods.

Electromobility Shift Assay (EMSA). Purified recombinant human (p50) protein was purchased from Promega Inc. DNA−p50 binding reactions were carried out using 6.4 pmol (640 nM) of the appropriate 5′-^{32}P-end-labeled oligonucleotide that had been annealed to its complementary sequence to generate the double-stranded oligonucleotide necessary for p50 recognition. The annealing was carried out using an MJ Research DNA thermal cycler in 10 mM Tris-HCl (pH 7.4) at 95 °C for 5 min followed by a slow cooling to room temperature (RT) over the course of 2 h. Identical concentrations were assured by measuring the OD at A_{260} of the stock DNA solutions. The reactions for p50 binding were carried out in binding buffer containing 1 mM $MgCl_2$, 0.5 mM

EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 μg/μL poly(dI·dC) in 4% glycerol. The p50 protein, 2.4−20 pmol (240−2000 nM), was added at increasing concentrations to the reaction mixtures and allowed to bind for 30 min at RT. An additional 1 μL of 40% glycerol was added to all reaction mixtures to facilitate loading. Gels were run on a 6% Novex DNA retardation gel at 300 V for ~15 min. The gel shifts were analyzed by autoradiography and quantified by densitometry using a Bio-Rad GS-800 calibrated densitometer using QuantityOne software.

Data Analysis. The EMSA analysis of binding affinity for the control and 8-oxo-dG-modified oligonucleotides was carried out by integrating the area for each band and dividing the area of the protein-bound DNA by the total area of the bound and free DNA bands to give a percent bound. The apparent dissociation constant (K_{app}) was determined graphically as the point where the fraction bound equals 50%. Analyses of the gel shifts were carried out on two to four gels for each of the different modifications.

Oxidative Lesion Shielding Assay. Purified human 8-oxoguanine DNA glycosylase (hOGG1) and *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) were purchased from Trevigen. Reactions were carried out using 10 pmol (1000 nM) of the appropriate 5′-^{32}P-end-labeled oligonucleotide that had been annealed to its complementary sequence to generate the double-stranded oligonucleotide necessary for p50 or glycosylase recognition. Identical concentrations of DNA were assured by measuring the OD at A_{260} of the stock DNA solutions.

DNA Cleavage Assays with Fpg. The reactions for p50 shielding with Fpg were carried out at 25 °C in 10 mM HEPES-KOH (pH 7.4), 10 mM KCl, 1 mM EDTA, and 0.01 mg/mL BSA with a total reaction volume of 60 μL in a manner similar to that described previously (*15*). The p50 transcription factor protein was added at increasing concentrations to the 8-oxo-dG-modified oligonucleotide and allowed to incubate for 30 min at 25 °C before addition of 900 nM Fpg. The p50:Fpg enzyme ratios tested for lesion shielding were 0, 0.22, 0.35, and 0.61. Aliquots (5 μL) were removed at 0, 0.25, 0.5, 1, 2, 4, 8, 15, 30, and 60 min for analysis of DNA cleavage.

DNA Cleavage Assays with hOGG1. The reactions for p50 shielding with hOGG1 were carried out in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA. p50 was preincubated with the modified oligonucleotides at 25 °C for 30 min before addition of 3000 nM hOGG1 to give p50:hOGG1 enzyme ratios of 0, 0.07, 0.11, and 0.19. Reactions were allowed to proceed at 37 °C, and 5 μL aliquots were removed at 0, 1, 2, 4, 8, 15, 30, 60, 90, and 120 min for analysis of DNA cleavage.

Gel Electrophoresis Conditions and Data Analysis of Fpg and hOGG1 Cleavage of 8-Oxo-dG-Modified Oligonucleotides. At each time point for the respective glycosylase, the samples were quenched by the addition of an equal volume of formamide denaturing loading dye (10 mL of formamide, 10 mg of xylene cyanol FF, and 10 mg of bromophenol blue) that was preheated to 95 °C. The sample mixtures were kept at 95 °C for 4 min before being loaded on a 15% TBE, 7 M urea precast mini gel. Gels were run in TB buffer for ≈40 min at 180 V. The faster migrating bands resulting from glycosylase recognition and excision were analyzed by autoradiography and quantified by densitometry using a Bio-Rad GS-800 calibrated densitometer with QuantityOne software. Two to four gels were used for quantification of each modified oligonucleotide at each time point.

Table 2: Binding Affinity of 8-Oxo-dG-Modified Oligonucleotides with p50

oligonucleotide	K_{app}, binding affinity (nM)
control	672 ± 22
8-oxo-dG at G_1	283 ± 7
8-oxo-dG at G_2	644 ± 35
8-oxo-dG at G_3	2340 ± 40
8-oxo-dG at G_4	550 ± 35

RESULTS

The impact of oxidized guanine lesions on transcription factor binding was tested using the p50 subunit of NF-κB with its DNA cognate recognition sequence, 5′-d(AGTTGAG$_1$G$_2$G$_3$G$_4$ACTTTCCCAGCC)-3′. An electromobility shift assay (EMSA) was carried out with increasing concentrations of the p50 transcription factor to characterize the impact that single 8-oxo-dG sites at positions G_1−G_4 have on the affinity of p50 for its cognate sequence. Representative EMSA autoradiograms in Figure 2A−E show the binding affinity of the p50 transcription factor with the ^{32}P-labeled unmodified DNA cognate recognition sequence and each of the ^{32}P-labeled oligonucleotides containing the four sites of 8-oxo-dG modification (Table 1). The autoradiograms show the concentration-dependent formation of the gel-shifted band corresponding to the DNA−protein complex (DNA−P) with increasing p50 concentrations between 0 and 20 pmol (0−2000 nM). Densitometric analysis was performed on a minimum of two autoradiograms for the unmodified DNA sequence and the four 8-oxo-dG-modified DNA sequences to determine the percent of DNA that was in the form of the DNA−protein complex (Figure 3). The apparent dissociation constants (K_{app}) under these conditions for each of the 8-oxo-dG-modified sites and the unmodified control were determined graphically as the point where the percent bound was equal to 50%. The calculated K_{app} values for the unmodified and the four 8-oxo-dG-modified oligonucleotides are given in Table 2. The relative K_{app} for the unmodified control under these conditions was found to be 672 nM with little change in the 50% binding affinity observed with the 8-oxo-dG modifications at G_2 and G_4. However, the oligonucleotide modified with 8-oxo-dG at G_2 showed a significant dropoff of p50 binding affinity beyond the 50% level (Figure 3), which suggests that modification at this site negatively impacts p50 binding affinity. The 8-oxo-dG modification, when present at the G_3 site, showed an approximate 4-fold decrease in K_{app} for binding of the p50 subunit to this modified DNA sequence versus the unmodified DNA sequence. This 4-fold decrease in K_{app} is in agreement with that seen previously when p50 binds as a monomer to a single DNA half-site (*16*). This lowered p50 binding affinity suggests that 8-oxo-dG modification at G_3 causes a loss in recognition of one of the DNA half-sites of the target oligonucleotide. This lower affinity at a single half-site may account for the appearance of higher-molecular weight bands observed in Figure 2D due to structural isoforms of the p50 homodimer and DNA. Conversely, the G_1 8-oxo-dG modification showed a nearly 2.5-fold increase in binding affinity over that of the unmodified cognate DNA

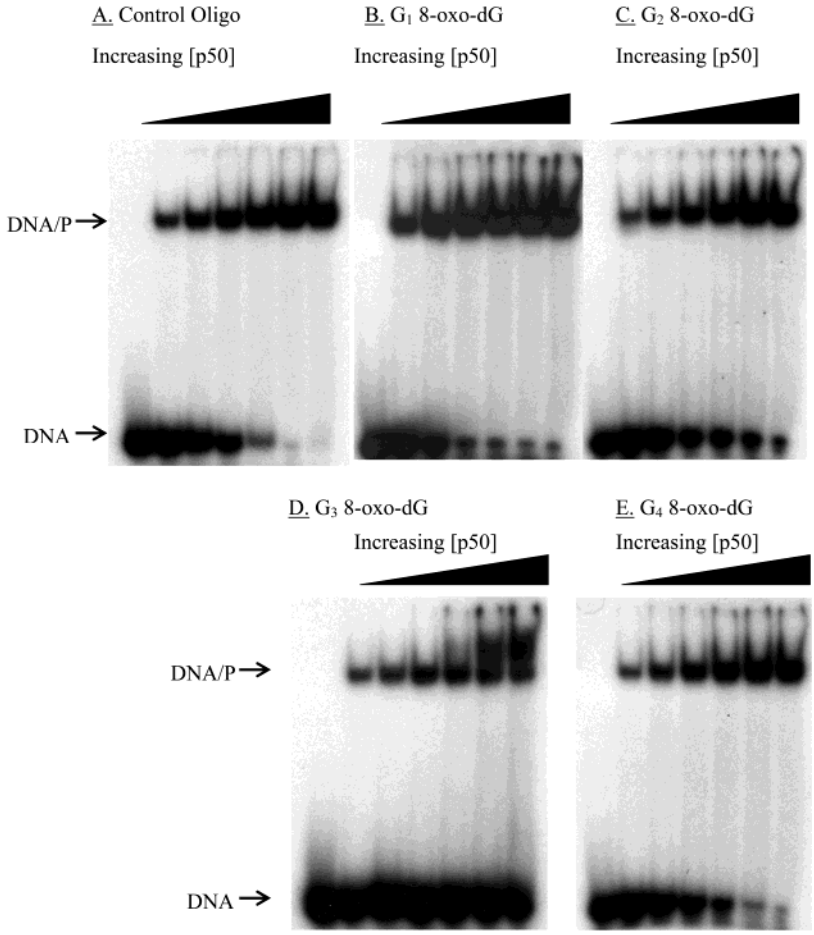

FIGURE 2: Representative autoradiograms showing the gel shift induced by binding of the p50 subunit of NF-κB to the control and 8-oxo-dG-modified 5′-^{32}P-labeled DNA recognition sequence. All five panels demonstrate the concentration dependence of the DNA−p50 complex (from left to right) with the addition of 0, 2.4, 4.8, 7.5, 10, 15, and 20 pmol of p50 (0−2000 nM p50). The bands labeled DNA correspond to the free oligonucleotide, while the bands labeled DNA/P correspond to the gel-shifted DNA−p50 complex.

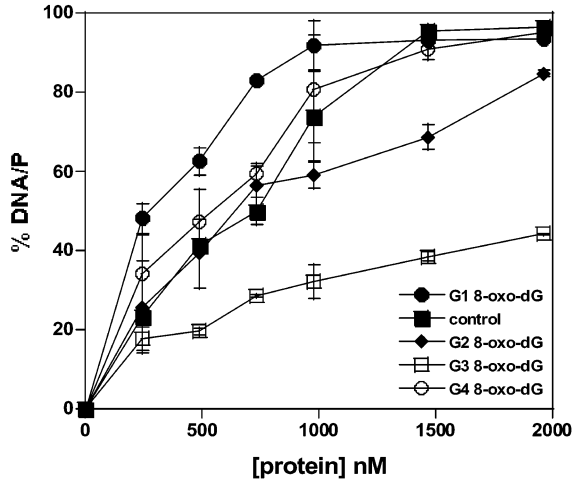

FIGURE 3: Graphical representation of the densitometric data obtained from the autoradiograms in Figure 2. Data are reported as a percent of the DNA complexed with protein vs the concentration of p50 needed to induce the corresponding gel shift. An average of a minimum of two gels (maximum of four gels) for each data point was plotted.

sequence. These data demonstrate that, depending upon the site of modification within the DNA cognate sequence, an increase or decrease in p50 binding affinity occurs upon the formation of oxidized lesions. In turn, these lesions would be expected to modulate gene transcription based on their ability to change this transcription factor binding affinity.

8-Oxo-dG lesions are recognized and repaired by endogenous base excision repair enzymes. For oxidized guanine lesions in regulatory elements to impact gene transcription by changing transcription factor binding affinity, their cellular lifetime (resistance to repair) must be on a time scale that could lead to significant changes in cellular protein synthesis. Our hypothesis was that binding of transcription factors to oxidatively modified promoter sites affords protection from recognition and repair by BER and results in a significant expansion of the lifetime in the cell which dramatically impacts gene transcription. We have developed a competition cleavage assay based on the work of Leipold et al. (*15*) using the *E. coli* BER enzyme (Fpg) or the human BER enzyme (hOGG1). This assay relies on the ability of these repair enzymes to recognize and cleave oxidized guanine lesions on DNA. The ability of p50 bound to the modified NF-kB promoter site to shield the lesions from BER recognition was determined by loss of DNA cleavage. Panels A−D of Figure 4 are representative autoradiograms showing that increasing p50:Fpg ratios markedly affect the time-dependent ability of the BER enzyme to recognize and cleave 8-oxo-dG at the G_1 site in the DNA recognition sequence. Panels A−D of Figure 4 correspond to increasing p50:Fpg ratios of 0, 0.22, 0.35, and 0.61, respectively. The time dependence of the cleavage reaction using Fpg was determined between 0 and 60 min. Under conditions where no p50 is present

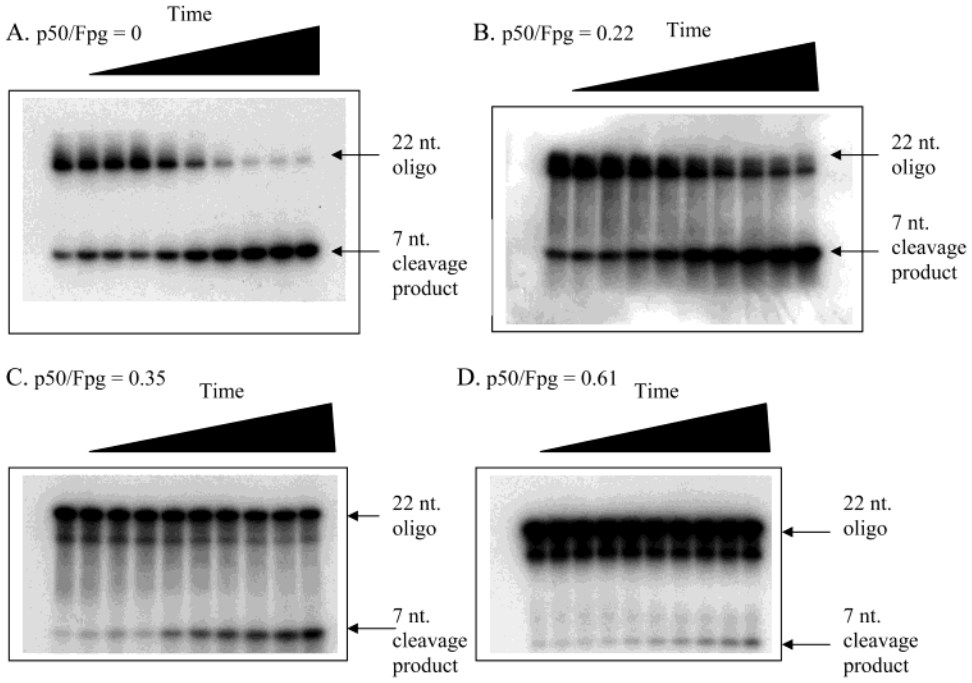

FIGURE 4: Representative autoradiograms from the Fpg cleavage assay for determining the time-dependent and p50 concentration-dependent shielding of the G_1 8-oxo-dG in the 22 bp NF-κB recognition element. The time points for each cleavage analysis for all gels were (from left to right) 0, 0.25, 0.5, 1, 2, 4, 8, 15, 30, and 60 min. Panel A shows the time-dependent cleavage with no p50 added. Panel B shows the time-dependent cleavage with a p50:Fpg ratio of 0.22. Panel C shows the time-dependent cleavage with a p50:Fpg ratio of 0.35. Panel D shows the time-dependent cleavage at a p50:Fpg ration of 0.61. The bands labeled 22 nt. oligo are the uncleaved DNA, while the bands labeled 7 nt. cleavage product are the cleavage products generated from base excision by the Fpg enzyme.

Table 3: Percent Inhibition of Fpg Cleavage of 8-Oxo-dG-Modified Oligonucleotides at 60 min with Different p50 Molar Ratios

oligonucleotide	0.22 p50:Fpg molar ratio	0.35 p50:Fpg molar ratio	0.61 p50:Fpg molar ratio
8-oxo-dG at G_1	17.1 ± 11.4	48.7 ± 10.7	92.4 ± 5.2
8-oxo-dG at G_2	8.5 ± 8.7	30.4 ± 5.9	62 ± 3.2
8-oxo-dG at G_3	−27.9 ± 7.8	−22.6 ± 5.9	−17.4 ± 20.1
8-oxo-dG at G_4	42.3 ± 2.1	68.9 ± 11.7	91.9 ± 2.1

(Figure 4A), the cleavage reaction at the 8-oxo-dG site is complete by 30 min. With increasing amounts of p50, the 8-oxo-dG site is shielded from recognition and cleavage by Fpg with nearly 100% shielding afforded at a p50:Fpg ratio of 0.61 for up to 60 min. The smearing of the upper bands observed in the autoradiograms containing p50 (Figure 4B−D) was a consequence of the binding of p50 to the modified oligonucleotide. The time-dependent cleavage shieldings for all four 8-oxo-dG modifications at the four different p50:Fpg ratios are shown in panels A−D of Figure 5. As would be expected, the cleavage shielding of the 8-oxo-dG modification afforded by p50 directly correlates with the p50 transcription factor's binding affinity for these modified sites. The relative levels of shielding of the lesion by p50 for the different modifications were as follows: $G_1 > G_4 > G_2 \gg G_3$. The G_3-modified site showed no lesion shielding effect (or even an enhancement of cleavage) for p50 (Figure 5C) which corresponded with the significantly lower binding affinity in comparison to that of the G_1-modified site. A list of the percent shielding for the different sites of modifications at the 60 min time point and at the different p50:Fpg ratios is given in Table 3.

A similar experiment for p50 lesion shielding was carried out using the human base excision repair enzyme (hOGG1). A representative set of autoradiograms for the same oligonucleotide modified with 8-oxo-dG at G_1 is shown in panels A−D of Figure 6. Due to the lower reactivity of hOGG1 toward the 8-oxo-dG lesion, an increased enzyme concentration was used and the experiment was carried out for 120 min at 37 °C. The hOGG1 enzyme also demonstrated nearly 100% cleavage at 30 min when no p50 was present (Figures 6A and 7A). With the addition of p50 and hOGG1 at ratios of 0.07, 0.11, and 0.19, a significant degree of shielding of the 8-oxo-dG-modified lesion from cleavage by hOGG1 was again observed. All four sites of 8-oxo-dG modification were analyzed using identical p50:hOGG1 ratios over the 120 min time course (Figure 7A−D). Once again, the shielding of the lesions from BER recognition roughly followed the p50 transcription factor binding affinity changes for the different lesions. The G_3 modification showed the weakest ability to shield the 8-oxo-dG modification from recognition and cleavage, although not as well as with the Fpg. The G_1, G_2, and G_4 modifications all exhibited similar levels of shielding ability at the different p50 concentrations. The shielding data for hOGG1 are listed for the 60 min time point in Table 4. The p50:hOGG1 ratio that was necessary to induce nearly 100% shielding of the lesions in modifications at G_1, G_2, and G_4 is ~3 times lower than that needed for the Fpg enzyme. Taken together, these results have shown that the

Table 4: Percent Inhibition of hOGG1 Cleavage of 8-Oxo-dG-Modified Oligonucleotides at 120 min with Different p50 Molar Ratios

oligonucleotide	0.07 p50:hOGG1 molar ratio	0.11 p50:hOGG1 molar ratio	0.19 p50:hOGG1 molar ratio
8-oxo-dG at G_1	42.9 ± 1.7	62.3 ± 13.6	83.1 ± 17.2
8-oxo-dG at G_2	45.5 ± 3.7	60.3 ± 11.7	85.2 ± 13.5
8-oxo-dG at G_3	20.6 ± 2.0	33.6 ± 13.9	46.8 ± 8.4
8-oxo-dG at G_4	38.7 ± 3.2	54.4 ± 5.0	85.5 ± 5.9

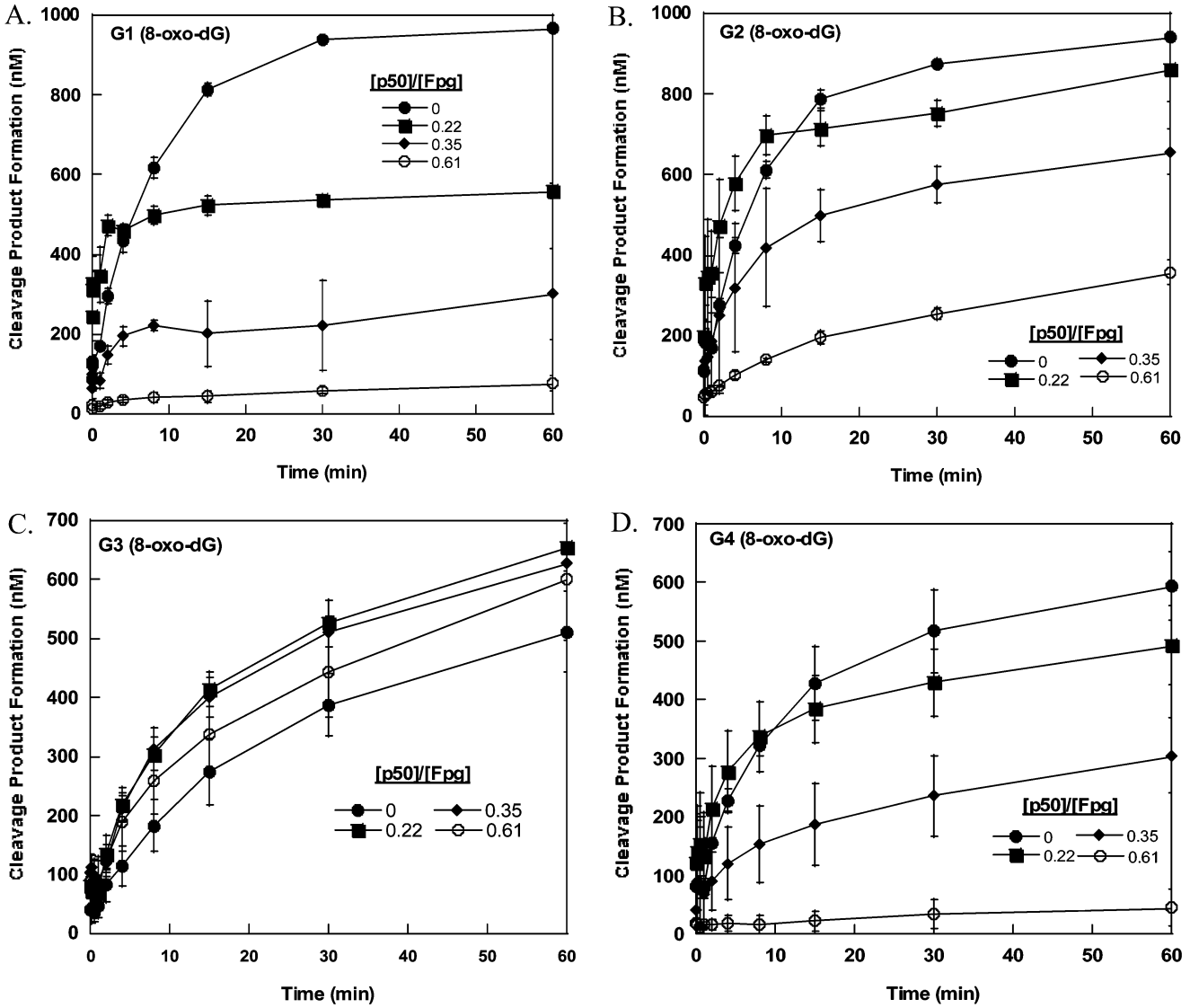

FIGURE 5: Graphical representation of the time-dependent cleavage of the 8-oxo-dG-modified oligonucleotides (G_1-G_4) at different p50:Fpg ratios. An average of a minimum of two gels (maximum of four gels) for each data point was plotted.

p50 transcription factor, when bound to an 8-oxo-dG-modified promoter element, can effectively shield these lesions from glycosylase recognition and repair which could extend their cellular lifetimes and exacerbate their effect on gene transcription.

DISCUSSION

Nucleic Acid and Protein Structure. The formation of the oxidized guanine lesion 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA is one of the most common types of oxidative base lesions produced by ROS and redox-active metal compounds (*1*). A guanine positioned 5′ to a contiguous run of guanines is more prone to oxidation to the 8-oxo-dG lesion (*6*). The NF-κB transcription factor binds a DNA upstream regulatory element with a consensus GGGRNNYYCC sequence that includes such a guanine run, with the first five bases more highly conserved that the second five (*8*, *17*). The base immediately preceding this sequence could be any one of the four bases, although this base is predominately a guanine in the stress response genes (*18*−*23*). While this preceding guanine base is outside of the consensus recognition sequence, X-ray crystallography has identified a histidine residue within the p50 protein that can make contact with this base (*9*). The NF-κB protein binds DNA as a heterodimer consisting of p50 and p65 (RelA) subunits, although the p50 subunit can also bind and activate transcription as a homodimer (*9*, *10*). The NF-κB transcription factor recognizes its consensus binding site through hydrogen bonding interactions between the p50 subunit and nucleic acid bases in the major groove of double-stranded DNA. The structural consequence of the oxidation of guanine to 8-oxo-dG is the conversion of N-7 of guanine from a hydrogen bond acceptor ("A") to a hydrogen bond donor ("D") (Figure 8). Several of the bases, such as G_2 and G_3 (see Table 1 in Materials and Methods), have two stabilizing hydrogen bonds per base. This oxidative modification does not affect the hydrogen bond accepting ability of O-6 of guanine. Further sequence binding specificity involving guanine in this promoter element is conferred through the formation of a bifurcated hydrogen bond between O-6 of G_4 (upper strand) and O-4 of $T_{8'}$ (lower strand). In normal B-form DNA, an 8-oxo-dG modification has not been shown to induce a significant structural change in base stacking but does form the basis for recognition by DNA repair enzymes (*11*).

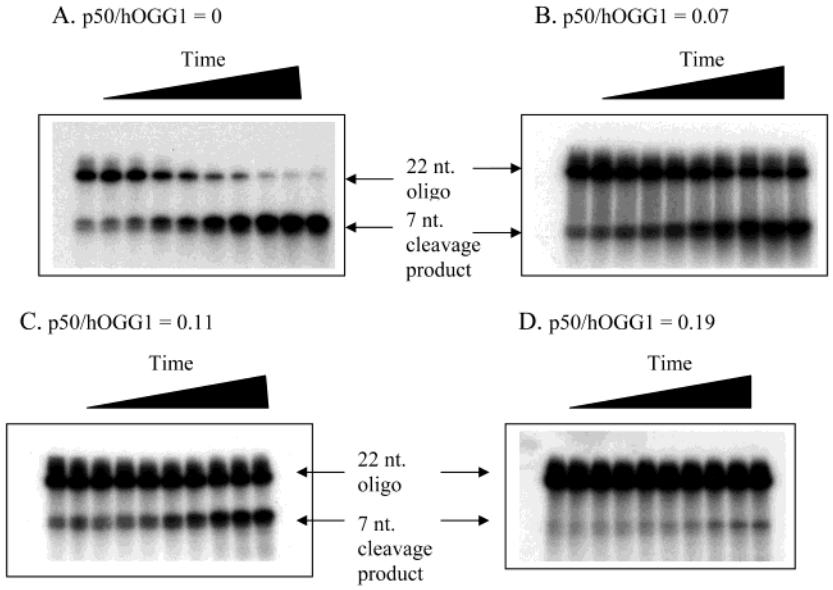

FIGURE 6: Representative autoradiograms from the hOGG1 cleavage assay for determining the time-dependent and p50 concentration-dependent shielding of the G_1 8-oxo-dG site in the 22 bp NF-κB recognition element. The time points for each cleavage analysis for all gels were (from left to right) 0, 1, 2, 4, 8, 15, 30, 60, 90, and 120 min. Panel A shows the time-dependent cleavage with no p50 added. Panel B shows the time-dependent cleavage with a p50:hOGG1 ratio of 0.07. Panel C shows the time-dependent cleavage with a p50:hOGG1 ratio of 0.11. Panel D shows the time-dependent cleavage at a p50:hOGG1 ratio of 0.19. The bands labeled 22 nt. oligo are the uncleaved DNA, while the bands labeled 7 nt. cleavage product are the cleavage products generated from base excision by the hOGG1 enzyme.

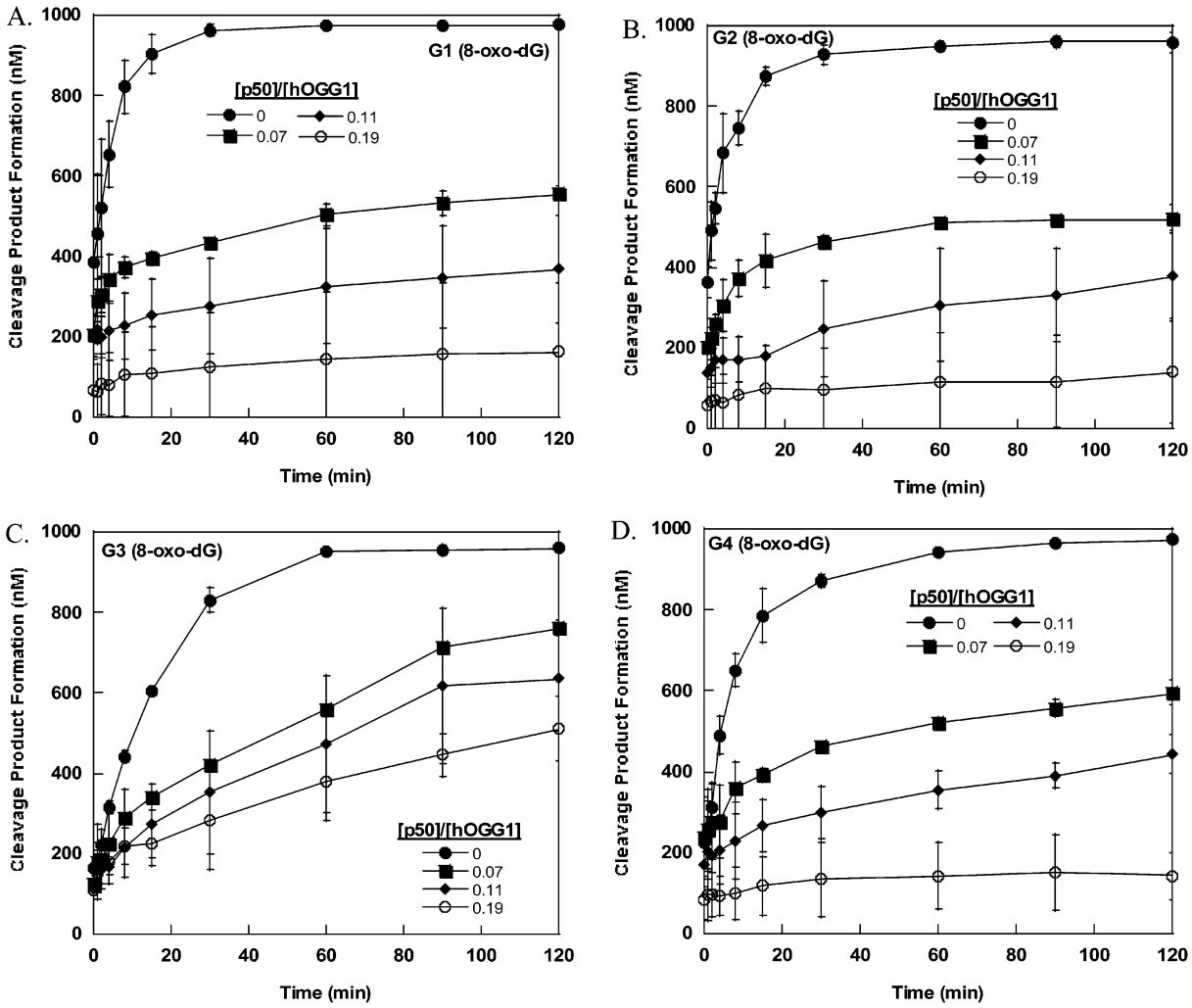

FIGURE 7: Graphical representation of the time-dependent cleavage for hOGG1 cleavage of the 8-oxo-dG-modified oligonucleotides (G_1-G_4) at different p50:hOGG1 ratios. An average of a minimum of two gels (maximum of four gels) for each data point was plotted.

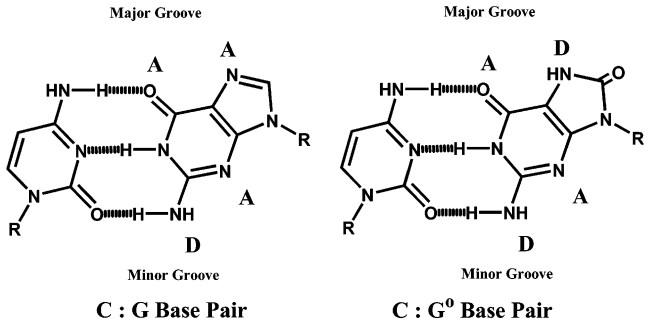

FIGURE 8: Hydrogen bond donor−acceptor pattern for guanine and 8-oxo-dG (G°) in double-stranded DNA.

We have studied the effect of 8-oxo-dG at each guanine site within the DNA binding sequence for the p50 subunit of the NF-κB transcription factor. The synthesis of oligonucleotides containing the NF-κB regulatory element with single 8-oxo-dG modifications at sites G_1-G_4 was designed to study the effect of this common DNA lesion on the binding affinity of the p50 transcription factor. Our hypothesis was that a single p50 subunit would have a disrupted binding site (loss of a stabilizing H-bond) but the other subunit would have a "normal" binding site across the 2-fold axis of symmetry. A loss of a stabilizing hydrogen bond should result in the overall lowering of binding affinity of p50 for this modified sequence with a concomitant decrease in the degree of gene transcription. This manner of transcriptional control in the nucleus by ROS modification of promoter/enhancer sites would be profoundly different than the commonly accepted role of ROS on transcription factors. This method of transcriptional control would constitute a "direct functional role" for reactive oxygen species in the NF-κB transcriptional response (*24*). Currently, the accepted role for ROS in relation to gene transcription changes involves oxidation of critical cysteine residues that act as a sensor of redox status to modulate DNA binding or transactivating activity of the transcription factor protein (*25*).

p50 Binding Affinity Changes. Our studies have shown that incorporation of 8-oxo-dG within the NF-κB regulatory element can affect binding of the p50 subunit of this transcription factor, but that the effect is dependent upon the position of the modified lesion. Since the initial binding to DNA by transcription activating proteins is required for recruitment of the remaining transcription factor complex proteins, these effects should lead to a change in the rate of gene expression. An up- or downregulation of gene transcription could be envisioned, depending upon whether this modification results in higher or lower binding affinity of transcription factors. We note that at this time we are not measuring gene transcription changes *per se*, but only transcription factor binding affinities.

In the case of the GGGG quadruplet within the NF-κB promoter sequence that we have studied, oxidative modification at guanine residue G_4 showed no change in the affinity of p50 for the modified DNA sequence. This finding was not unexpected since the site-specific hydrogen bond formed between a lysine on p50 and G_4 is centered at the O-6 position, which would not have altered hydrogen bond donating character at this site upon oxidation to the 8-oxo-dG lesion. The modifications of sites G_2 and G_3 both showed lowered p50 binding affinity, although the modification at G_2 exhibited this lower affinity only at higher p50 concentrations. The loss of one of the hydrogen bond donor−acceptor pairs between an arginine on p50 and the modified N-7 of 8-oxo-dG can account for this loss of p50 binding affinity as described above. This loss of transcription factor recognition for its consensus binding site with an inserted 8-oxo-dG modification is consistent with that seen previously with the Sp1 and AP-1 transcription factors (*26*, *27*). However, it contrasts with previous work with the NF-κB consensus binding site, where no change in binding affinity with incorporation of 8-oxo-dG was observed (*28*). The difference between the current study using the NF-κB regulatory element and the previous study by Ghosh and Mitchell (*28*) may be their failure to purify the 8-oxo-dG-modified DNA and the use of a single p50 concentration to determine binding affinity instead of generation of binding curves with a broad range of p50:DNA ratios. Differences in the 5′ and 3′ DNA flanking sequence of the cognate recognition site may also account for the discrepancy between this study and that of Ghosh and Mitchell (*28*).

An unexpected result was the observation that the 5′-guanine, G_1, in the GGGG quadruplet, which is outside the established DNA consensus site, resulted in higher p50 binding affinity upon oxidation. We had originally hypothesized that, as this position is most sensitive to oxidation (*6*), a guanine at this site would act as a sacrificial oxidant to maintain the integrity of the G_2-G_4 bases necessary for p50 and p65 site-specific recognition and would not affect the ability of the transcription factor to bind to the recognition sequence. That the oxidation of this guanine to 8-oxo-dG increased the binding affinity of the p50 protein for the DNA response element suggests that it may also play an active role in gene regulation. This raises the intriguing possibility that the promoter element may be designed to use this site to sense oxidative conditions and regulate gene transcription.

We have hypothesized that changes in the hydrogen bond donor−acceptor patterns between the protein and DNA form the basis of the altered binding affinity based upon crystal structures of 8-oxo-dG in DNA that have shown little perturbation in DNA structure (*11*). It is also possible that under conditions where p50 is bound, a base flip, kink, or bend is generated at the site of the lesion within a double-stranded oligonucleotide. These structural perturbations at the site of the lesion could enhance p50 binding affinity by the formation of additional hydrogen donor−acceptor pairs with adjacent amino acids or through structural mimicry. Such structural mimicry has been characterized for high-mobility group proteins binding to cisplatin-bent oligonucleotides (*29*) or for repair enzyme recognition of base-flipped structures (*12*). Runs of guanines such as those in the current NF-κB regulatory element and in telomeric repeats have been previously shown to form quadruplex DNA (*30*). Insertion of 8-oxo-dG at the 5′-position in a GGG triplet of a human telomeric sequence can induce quadruplex formation and inhibit telomerase activity (*31*). We cannot rule out the possibility that this same structural change could account for the high-affinity p50 recognition site generated at the G_1 site observed in this study.

Lesion Shielding Assays. The impact of base lesions on transcription factor binding and the ensuing gene transcription would depend on the cellular lifetime of the lesion. Repair of base lesions in regulatory elements would quickly return gene transcription to normal levels with little overall effect.

We have, however, shown that the binding of p50 to the 8-oxo-dG-modified NF-κB regulatory element can effectively shield these lesions from recognition and repair by base excision repair enzymes. The shielding effect afforded by p50 directly correlated with the binding affinity that was induced by the 8-oxo-dG modifications. The 5′-G, G_1, in the GGGG quadruplet sequence showed the highest binding affinity and the greatest lesion shielding. If present in cellular systems, this combination of effects would serve to exacerbate the change in gene transcription by both enhancing gene transcription and increasing the cellular longevity of the lesion. Conversely, the modification at G_3 in the quadruplet sequence showed the greatest loss in binding affinity and a negligible repair shielding effect. Modification at this site may not impact gene transcription as dramatically since the lesion would be readily recognized and excised from the sequence. Precedent for this type of repair shielding has been shown for the cisplatin antitumor agent where binding of high-mobility group proteins to cisplatin-modified DNA has been shown to shield this lesion from nucleotide excision repair enzymes, leading to an exacerbation of its toxic effect (*29*). This study suggests that modulation of gene transcription via guanine oxidation in promoter elements is a dynamic process that balances transcription factor binding affinity with DNA repair of the modified bases in a concerted manner.

Biological Implications. We have determined that when the base immediately preceding the NF-κB response element is a guanine residue, its oxidation to 8-oxoguanine increases the affinity of the p50 protein for the DNA response element. This suggests that a guanine in this position not only may be acting as a sacrificial oxidant to maintain the fidelity of the regulatory element but also may play an active role in gene regulation. As a eukaryotic transcription factor, NF-κB has been attributed to the regulation of more than 150 genes and is activated by more than 150 different stimuli (*32*). NF-κB regulates the expression of a large number of genes, including those involved in the response to stress and infection (*18*, *24*). NF-κB is a central mediator of the immune and of the stress response, and of ER overload, and an adventitious virulence mediator of some adapted pathogenic viruses such as HIV. The question of how all genes in all cells are not turned on in response to any stimuli remains. The numerous cytosolic regulatory and inhibitory mechanisms known for this transcription factor clearly play a substantial role. Perhaps because of the sheer numbers of genes involved, it appears that further control mechanisms may exist for the regulation of the DNA binding and transactivation properties of this transcription factor following its translocation to the nucleus.

There is renewed discussion of the heterogeneity that exists among the response elements themselves and of a "sequence context" that influences the strength of the transcription response of the activated nuclear transcription factor. Most genes contain numerous transcription factor response elements in their 5′-untranslated region. A "combinatorial regulation" model describes a synergistic activation that requires the interaction of NF-κB with additional transcription factors for a "full" transcriptional response (*32*). It has also been determined that different sequences have different binding affinities for p50 homodimers and p65 homodimers and the more common p50−p65 heterodimer (*33*). A further confounding factor is that p50 homodimers may also serve as transcriptional repressors (*16*). In this scenario, the effect on gene transcription would be dependent upon both the dimer that is formed and the gene regulatory element.

We propose that a component of this "sequence context" is the presence of a guanine residue immediately preceding the consensus site that is capable of forming additional hydrogen bonding contacts with the p50 protein upon oxidation. In the NF-κB promoter element studied herein, G_1 is the thermodynamically most labile toward oxidation and also produces the highest transcription factor binding affinity and the greatest repair shielding. This suggests a concerted mechanism whereby certain DNA promoter elements are thermodynamically "tuned" by their sequence to use site-specific oxidative DNA damage either as a sacrificial oxidant to maintain promoter integrity or as a switch to up- or downregulate gene transcription. The consequences of such a mechanism of control may give rise to beneficial or aberrant gene expression, depending upon the gene product that is regulated. The stress response genes that contain four or more runs of guanines in their NF-κB enhancer elements (*19*−*23*, *28*) may exhibit enhanced expression through this mechanism under oxidizing conditions. Aberrant downregulation of genes through oxidation of the G_2 and G_3 positions would have a detrimental effect in the regulation of, for example, tumor suppressor genes. A classical example would be the p53 tumor suppressor gene that does contain an NF-κB binding site in its promoter region (*34*) and whose detrimental effects due to ineffective expression are well-documented.

The potentially transient nature of this guanine modification with any ensuing changes in gene transcription could be seen as a new form of an epigenetic mechanism for transcriptional control. Epigenetic mechanisms alter gene expression without changing the genetic sequence to cause either permanent or reversible transformations in cellular behavior. The interplay of genetic and epigenetic factors that give rise to changes in gene expression upon cellular exposure to a toxicant is central to understanding the process of transcriptional regulation and cellular transformation, leading to carcinogenesis. DNA methylation has been a widely studied model of this interplay between genetic and epigenetic modulation of gene expression, while modulation of gene transcription by redox-active carcinogens has been more commonly interpreted only in terms of activation or deactivation of cytoplasmic transcription factors, genetic changes (mutations) in the gene encoding a DNA binding protein, or mutations in the response element. Little attention has focused on the effect of transient nucleic acid base lesions formed within DNA regulatory elements on transcription factor binding affinity and, ultimately, control of gene expression. Many DNA lesions are readily repaired and do not lead to a permanent change in the DNA sequence. This makes their impact on gene transcription in cellular systems problematic to assess outside of an epigenetic model. Discrepancies in the rates of cellular transformation have been noted for some carcinogens that do not correlate with their observed mutational frequency (*35*). These data are, however, more consistent with epigenetic events that may include transient nucleic acid base lesions in targeted guanines in promoter sites that may serve to alter transcription factor binding affinity.

Taken together, these preliminary results suggest an intriguing mechanism by which oxidative modification of promoter regions in the genome could result in up- or downregulation of gene transcription. It is our hypothesis that this type of mechanism may be used as a secondary form of transcriptional regulation within the genome. The ubiquity of critical guanine residues in transcription factor response elements suggests that these promoter regions may be thermodynamically tuned to the redox status of the cell such that transient oxidation of these guanines serves to up- or downregulate gene transcription. Since these types of base lesions are readily repaired, this up- or downregulation of gene transcription could be considered an "epigenetic signaling mechanism" since they would not give rise to a permanent change in sequence. This epigenetic signaling mechanism would be similar to the changes in gene transcription observed upon cytosine methylation (*36*). These data imply a potentially new regulatory mechanism by which redox-active metals and endogenous ROS may serve to cause aberrant shifts in the oxidation status of promoter regions to cause changes in the regulation of gene transcription.

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