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Effect of oxidative DNA damage in promoter elements on transcription factor binding

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ABSTRACT

Reactive oxygen species produced by endogenous metabolic activity and exposure to a multitude of exogenous agents impact cells in a variety of ways. The DNA base damage 8-oxodeoxyguanosine (8-oxodG) is a prominent indicator of oxidative stress and has been well-characterized as a premutagenic lesion in mammalian cells and putative initiator of the carcinogenic process. Commensurate with the recent interest in epigenetic pathways of cancer causation we investigated how 8-oxodG alters the interaction between *cis* elements located on gene promoters and sequence-specific DNA binding proteins associated with these promoters. Consensus binding sequences for the transcription factors AP-1, NF-κB and Sp1 were modified site-specifically at guanine residues and electrophoretic mobility shift assays were performed to assess DNA–protein interactions. Our results indicate that whereas a single 8-oxodG was sufficient to inhibit transcription factor binding to AP-1 and Sp1 sequences it had no effect on binding to NF-κB, regardless of its position. We conclude from these data that minor alterations in base composition at a crucial position within some, but not all, promoter elements have the ability to disrupt transcription factor binding. The lack of inhibition by damaged NF-κB sequences suggests that DNA–protein contact sites may not be as determinative for stable p50 binding to this promoter as other, as yet undefined, structural parameters.

INTRODUCTION

Various endogenous metabolic processes as well as external ultraviolet radiation (UVR) produce reactive oxygen species (ROS) including hydroxyl radicals (OH•), superoxide anion and singlet oxygen. Pathways for the formation of oxidation and photooxidation products are complex and lead to various structural modifications in DNA including base damage, deoxyribose damage and cross-links (1,2). Cellular damage caused by ROS may lead to apoptosis (3,4) and contribute to the initiation and promotion of carcinogenesis (5,6).

A common and extensively studied DNA modification caused by oxidation occurs by the addition of OH• to the C-8 position of guanine leading to the formation of 8-oxodeoxyguanosine (8-oxodG) (7,8). Since 8-oxodG does not effectively block the progression of DNA replication it has a high probability of read-through and mutation fixation. Mutations produced by 8-oxodG can arise from either mispairing with adenine leading to guanine to thymine transversions or misincorporation of 8-oxodGTP damaged in the nucleotide pool opposite adenine or cytosine producing thymine to guanine transversions (9). Cells have a variety of mechanisms to repair oxidative DNA damage including base and nucleotide excision repair, although the latter appears to play a secondary role (7). Formamidopyrimidine glycosylase (Fpg) is a bacterial DNA repair enzyme that removes ring-opened purines and 8-oxodG very efficiently from duplex DNA. A β,δ elimination by the Fpg-associated glycosylase leaves a single nucleotide gap and the phosphodiester bond at the abasic site is subsequently cleaved by the Fpg-associated lyase activity (10,11).

In transcription regulation, promoter recognition is mediated through general transcription factors and the levels of expression are regulated by the binding activity of site-specific DNA binding proteins (12). For example, transcription factor Sp1 is a member of a multigene family that binds GC/GT boxes and regulates the expression of several viral and cellular genes (13). In addition, AP-1 and NF-κB are two well-studied transcription factors that are regulated by intracellular oxidation and reduction states (14); AP-1 binds to gene promoters as homo- or heterodimers of jun and fos through the basic region upstream of the leucine zipper domain (15) and NF-κB regulates the expression of nuclear genes after disassociating from an inhibitory protein, I-κB (16). The consensus binding sites for each of these transcription factors are moderately to heavily GC-rich making them particularly susceptible to 8-oxodG formation. Moderate concentrations of intracellular ROS have been shown to influence gene expression through transcriptional or post-translational pathways (17). We hypothesize that DNA damage in the consensus binding sequence of a promoter element may be a mechanism for modulating gene expression.

Several recent studies have led to a better understanding of how DNA damage may effect regulatory mechanisms and how these effects may impinge upon normal growth controls and

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potentially contribute to the etiology of human diseases such as sunlight-induced skin cancer. Discrepancies between mutation and transformation frequencies induced by a given carcinogen were the first indications that factors other than mutation induction may operate in the carcinogenic process (18–20). Altered gene expression associated with different tumor models led to the hypothesis that epigenetic mechanisms such as perturbations in gene expression (21,22), methylation patterns (23) and membrane structure (24) may also be involved.

At first glance, the long latency between carcinogen exposure and the appearance of malignant cells appears to argue against epigenetic mechanisms in carcinogenesis; however, examples supporting the fixation of transient, carcinogen-induced alterations in gene expression have been published. A small change in the expression of one gene can have a significant impact on cellular development. For instance, many developmentally important genes, such as selector genes, have been described that are autoregulatory. Expression of cyclin E enhances expression from the promoter of the E2F1 gene, and E2F1 expression enhances transcription from the cyclin E promoter (25,26). It has been suggested that an autoregulatory circuit exhibiting bistable behavior could be switched from one stable state to another by a transient change in the concentration of a gene product (27). If the gene product also controls the transcription of another gene with pleiotropic effects (e.g., selector genes) stable, inherited genetic alterations in cellular phenotype could result.

In light of these observations, we examined the effects of oxidative DNA damage on gene expression. Here we present data on how a ubiquitous product of oxidation in general (and photooxidation in particular) may influence transcription factor binding. Specifically, we quantified the effects of site-specific 8-oxodG modifications in the AP-1, NF-κB and Sp1 consensus binding sequences on the recognition and binding of their respective transcription factors.

MATERIALS AND METHODS

Oligonucleotide substrates

Consensus oligonucleotides containing the binding sites for transcription factors AP-1, NF-κB and Sp1 were synthesized at Genosys Biotechnology (The Woodlands, TX). Consensus sequences for the binding sites were derived from data compiled by Promega Corporation (Madison, WI). During synthesis a single site-specific guanine residue within each of the binding site was replaced with an 8-oxodG procured from Glen Research (Sterling, VA) (Table 1). All of the substrates used were 22mers each with a single 8-oxodG modification. For the NF-κB sequence four different substrates containing 8-oxodG at four different sites were synthesized to examine putative position effects of 8-oxodG.

Recombinant proteins

Human recombinant c-jun homodimers were used for AP-1 binding experiments; the recombinant human p50 subunit was used for NF-κB and human Sp1 was used for binding to the GC-box. All of the recombinant proteins were purchased from Promega in footprint units (f.p.u.) (Madison, WI). One f.p.u. equals the amount of protein required to give full protection against DNase I digestion on SV40 Early Promoter DNA (Promega).

Formamidopyrimidine DNA glycosylase (Fpg) assay

Fpg assays were performed to verify the presence and location of 8-oxodG modified bases in the synthetic oligonucleotide substrates. The Fpg protein was provided by Dr Wah Kow (Emory University, Atlanta, GA) and reactions were performed according to Tchou and co-workers (28). Complementary strands were annealed to damaged strands and 20 µl reactions consisting of 8 fmol 5' ^{32}P-end-labeled 8-oxodG substrate, 25 mM HEPES–KOH (pH 7.6), 50 mM KCl, 100 mM NaCl, 0.5 mM DTT, 10% glycerol (v/v) and 10 and 20 fmol of Fpg were incubated at 15°C for 30 min. Digested products were resolved by electrophoresis on a 7% denaturing polyacrylamide gel.

Electrophoretic mobility shift assay (EMSA)

EMSA was used to examine the effect of 8-oxodG modification on transcription factor binding. Complementary strands were annealed to each of the oligonucleotides and ~2 ng of each oligomer was end-labeled with [γ-^{32}P]ATP (specific activity 3000 Ci/mol) and EMSA was carried out according to Ghosh and co-workers (29). Recombinant proteins were added in a total volume of 20 µl containing 20 mM Tris (pH 8.0), 50 mM HEPES, 0.2 mM KCl, 2.5 mM EDTA, 25 mM $MgCl_2$, 5 mM DTT and 60% glycerol. The reaction was allowed to proceed for 20 min at 23°C and the bound DNA–protein complexes were separated from free DNA by electrophoresis on a 5% native polyacrylamide gel. Gels were exposed to X-OMAT AR autoradiography film (Kodak) and quantified using a Kodak Digital Science Image Station 440CF and 1D Image Analysis Software (Eastman-Kodak, Rochester, NY).

Methylation interference assay

Methylation interference assays were used to identify points of contact between transcription factors and consensus binding sequences. The assays were carried out according to standard protocols (30). Substrates were ^{32}P-labeled at the 5'-end and methylated at adenine and guanine residues with dimethyl sulfate for 5 min at 23°C. After two rounds of precipitation with 0.3 M sodium acetate, 1 mM EDTA and 100% ethanol the methylated probes were incubated with the respective recombinant proteins. Subsequent to EMSA, free and bound oligomers were isolated from the gel by electroelution and cleaved with 1 M piperidine at 90°C for 30 min. Cleaved fragments were lyophilized repeatedly to remove residual piperidine and analyzed on 20% denaturing polyacrylamide gels.

RESULTS

Consensus binding sequences containing 8-oxodeoxyguanosine

Table 1 shows the sequence of the transcription factor binding sites used in our studies. The consensus binding site is indicated in bold and the 8-oxodG modified base is represented as Z. Sequences of the four NF-κB oligonucleotides used were the same as shown in Table 1 except that the 8-oxodG modification was at a different guanine in each substrate. In order to verify the presence of 8-oxodG within the binding site of NF-κB we digested the modified substrates with Fpg (31) and

Table 1. Oligonucleotide sequences of the consensus binding sites of transcription factors

Consensus sequence	Transcription factor	Protein
5'-CGC TAC A**TZ ACT CA**C GCG CGA C-3'	AP-1	c-jun
3'-GCG ATG T**AC TGA GT**G CGC GCT G-5'		
5'-TGT GCA **Z**[1]**Z**[2]**Z** [3]**Z**[4]**AC TTT CCC** ACG C-3'	NF-κB	p50
3'-ACA CGT **CCC CTG AAA GGG** TGC G-5'		
5'-ATA CGT AC**G GZG CGG GGC** GTG C-3'	Sp-1	Sp1
3'-TAT GCA TG**C CCC GCC CCG** CAC G-5'		

The binding site is in bold. The 8-oxodG modification is represented as Z and Z^1, Z^2, Z^3 and Z^4 represent the four NF-κB oligonucleotides used. Recombinant proteins used in the binding reaction are indicated on the right.

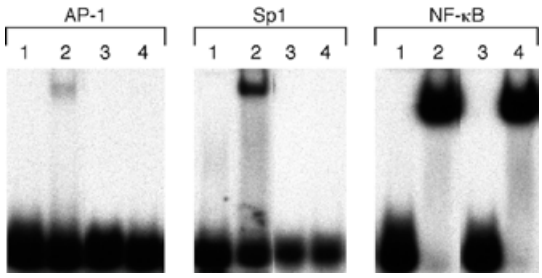

Figure 1. Effect of oxidative damage in DNA on transcription factor binding. Unmodified AP-1, Sp1 and NF-κB oligomers are shown in lanes 1 and 2; oligomers containing a single 8-oxodG residue are shown in lanes 3 and 4. The NF-κB substrate was modified at the Z^3 position. Lanes 1 and 3 of each set were incubated without transcription factor (free probe); lanes 2 and 4 of each set were incubated with 1 f.p.u. of recombinant c-jun for AP-1, Sp1 for Sp1 and p50 for NF-κB as described in Materials and Methods.

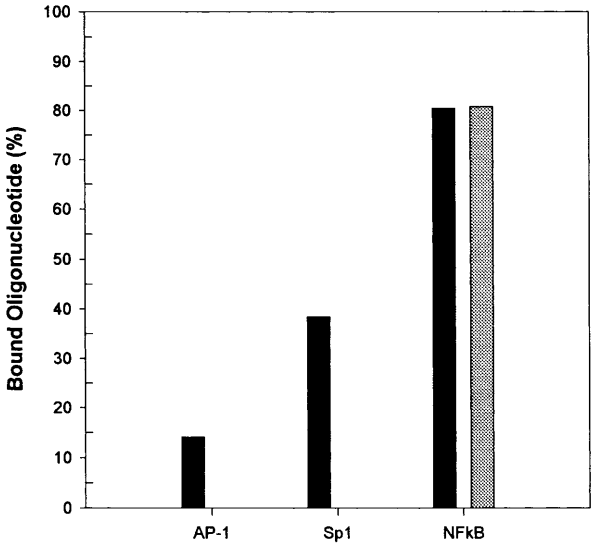

Figure 2. Histogram of gel data shown in Figure 1.

separated the products using denaturing polyacrylamide gel electrophoresis. Fpg recognized and cleaved lesions in all four oligomers; the sizes of the released fragments corresponded with the positions of the 8-oxodG in each of the substrates. Technical limitations precluded fpg saturation in digests containing these small oligonucleotides. In lieu of these data, the manufacturer (Genosys Biotechnology) provided quality assurance in the form of polyacrylamide gels showing a minor gel shift (single band) in the modified substrates compared to the undamaged substrate (data not shown).

Effects of 8-oxo-deoxyguanosine on transcription factor binding

End-labeled duplex consensus binding sequences for AP-1, NF-κB and Sp1 with or without 8-oxodG residues were incubated with 1.0 f.p.u. of the respective transcription factors and binding was determined using EMSA. In Figure 1, binding of transcription factors to undamaged promoter elements and corresponding sequences containing a single 8-oxodG in the unique G position in the AP-1 consensus sequence and at the third G residue in the Sp1 and NF-κB binding sites are shown (Table 1). One f.p.u. of transcription factor resulted in 14, 38 and 81% retardation of undamaged AP-1, Sp1 and NF-κB oligomers, respectively (Figs 1 and 2). Partial binding of 1 f.p.u. to undamaged oligomers was consistently observed and suggests that proteins have less affinity for consensus sequences in small oligonucleotides than for those in larger fragments of DNA (i.e., the SV40 used to determine f.p.u.). In addition, binding (specific activity of protein) varied significantly between batches and was dependent on the freezer age of proteins and buffers (e.g., compare p50 binding to NF-κB in Figs 1 and 3). Although data from multiple experiments could not be combined for this reason the results were consistent and quantitative regarding the effects of 8-oxodG on transcription factor binding: that is, modification at the single G in the AP-1 binding site and at the third G in the Sp1 GC box completely inhibited c-jun and Sp1 binding, respectively; modification at the third G in the NF-κB consensus oligomer had no effect on transcription factor binding (Fig. 2).

To determine if the site of the 8-oxodG residue was determinative for inhibition of transcription factor binding NF-κB

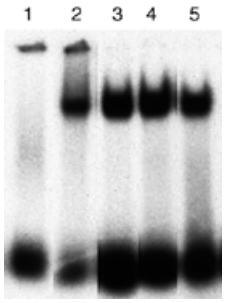

Figure 3. Effect of site-specific oxidative damage on transcription factor binding. Free probe is shown in lane 1; lanes 2–5 show oligomers incubated with 1 f.p.u. of p50 protein as described in Materials and Methods. The NF-κB binding sequence was either undamaged (lane 2) or damaged at Z^1, Z^2 or Z^4 (lanes 3, 4 and 5, respectively) as described in Table 1.

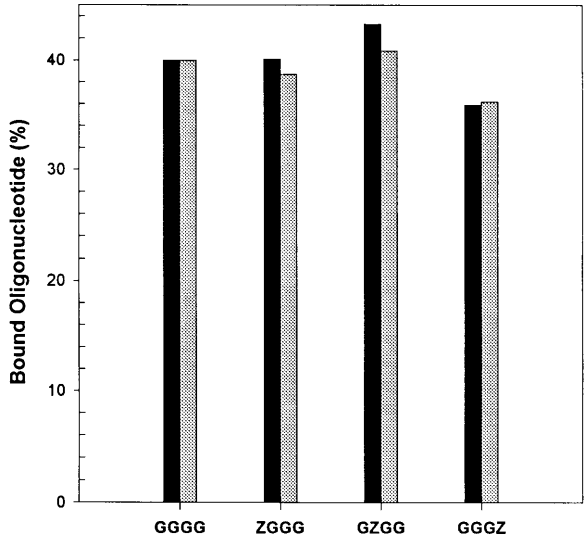

Figure 4. Histogram of gel data shown in Figure 3.

oligomers were synthesized to contain 8-oxodG at each of the other three G residues within the binding sequence (Fig. 3). Each of these consensus oligonucleotides was end-labeled with polynucleotide kinase after the complementary strand was annealed and tested for transcription factor binding using EMSA. In Figure 4 it is evident that binding of the p50 transcription factor to the modified NF-κB substrates was not significantly different from binding to the undamaged oligomer.

Determination of DNA–protein contact sites in consensus binding sequences

Methylation interference experiments are used to identify guanines and adenines in protein binding sites that, when methylated, interfered with protein binding. Unmodified AP-1, NF-κB and Sp1 consensus oligonucleotides were methylated with dimethyl sulfate prior to incubation with transcription factor. Bound and free oligomers were resolved and purified

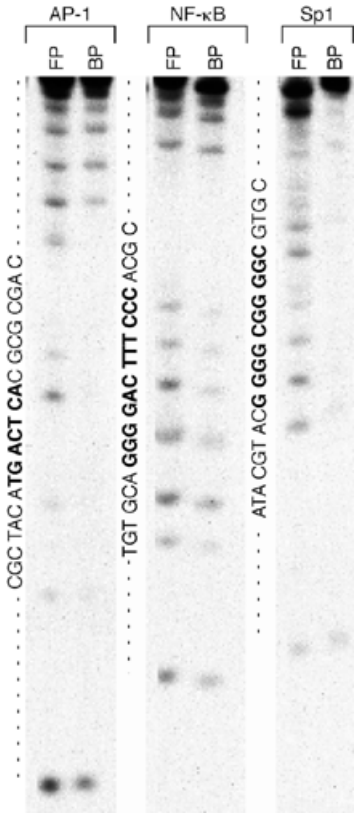

Figure 5. Identification of DNA–protein contact sites using a methylation interference assay. Unmodified oligonucleotides containing consensus binding sites for AP-1, NF-κB and Sp1 were methylated with DMS. Bound and free oligomers were separated by EMSA, digested with piperidine and resolved on 20% polyacrylamide gels. Digestion patterns for bound and free probes are indicated as are oligonucleotide sequences.

using EMSA followed by piperidine cleavage and resolution on 20% sequencing gels (Fig. 5). By comparing free and bound lanes it is evident that methylation of the single guanine and both adenine residues in the AP-1 consensus binding sequence produced a distinct footprint. Likewise, methylation footprints were also observed at guanine residues in the Sp1 consensus binding sequence bound to Sp1 protein. These results strongly implicate these sites as contact points between these two promoter elements and their corresponding transcription factors and suggest that 8-oxodG modification at contact points may disrupt stable interaction. Although the methylation interference footprint was not as well-defined in the NF-κB consensus sequence it is evident in Figure 4 that the quanine residues within the consensus binding sequence take part in DNA–protein binding. The methylation interference patterns of the complementary strand as well as NF-κB oligomers into which 8-oxodG had been inserted during synthesis gave similar results (data not shown). These data are not consistent with our conclusion that modifications at DNA–protein contact sites always disrupt transcription factor binding and suggest that the structural determinants of p50 binding to NF-κB are different from those that direct stable binding at the AP-1 and Sp1 promoters.

DISCUSSION

Because DNA is constantly exposed to genotoxic agents both from internal and external sources, mutation induction and its contribution to genetic instability have been primary foci of studies on the etiology of cancer. The precise relationship between the different types of DNA modifications and cancer is not fully understood. However, there is strong evidence suggesting that irreversible (permanent) changes in DNA are primarily responsible for the changes in cell growth leading to the initiation and promotion of tumorigenesis. Although DNA damage-directed mutagenesis is considered to be the predominant player in the progressive loss of cell growth controls, altered gene expression associated with different tumor models suggests that epigenetic mechanisms such as perturbations in gene expression, methylation patterns and membrane structure may also be involved. Dramatic changes in gene expression are clearly observed in several tumorigenesis models. For example, the cyclin D1 gene is highly overexpressed in mouse skin tumors (32) and in human lung tumors (33) induced by dimethylbenzanthracene and a variety of oncogenes, including cyclin D1, c-myc and c-erbB2 are overexpressed in human breast tumors (34,35).

One mechanism of how DNA damage might alter gene expression in the absence of permanent genetic change has been called 'molecular hijacking' (36). For example, heterologous DNA sequences modified by benzo[a]pyrene-diol-epoxide bind the Sp1 transcription factor (37) and cisplatinum adducts in DNA 'hijack' the high mobility group protein, HMG1 as well as the human upstream binding factor (36,38,39). In the cellular environment this could translate into the appropriation of a particular gene product at a time when it is required to regulate an essential gene function. Such an essential gene could have tumor supressor functions or regulate the expression of an oncogene or a gene involved in a cell-cycle checkpoint. We performed heterologous competitive binding assays to determine if 8-oxodG was capable of 'hijacking' the transcription factors for AP-1, Sp1 and NF-κB with negative results (data not shown).

Over the past few years it has become evident that the genetic instability resulting from the breakdown of gene regulatory systems could assume great importance in the carcinogenic process. By disrupting the function of the *cis*- and *trans*-acting elements that control the initiation and progression of transcription, unrepaired damage at promoters and enhancers may have a significant impact on gene expression. Our data show that the ubiquitous oxidative product 8-oxodG can completely inhibit transcription factor binding to AP-1 and Sp1. These data are consistent with previous reports showing that promoter regions containing UV photoproducts or alkylation damage inhibit transcription factor binding (40–42) and that TATA box binding proteins are blocked by drugs that bind to the minor groove of DNA (43).

AP-1 is known to play a crucial role in the regulation of a wide variety of genes, especially growth factor-inducible genes. Expression of this gene is controlled by homo- or heterodimers of c-fos and c-jun which provide the initial transcription trigger and maintain expression levels, respectively. Several studies have demonstrated that superoxide produced by a xanthine/xanthine oxidase system and hydrogen peroxide in tissue culture cells can alter c-fos and c-jun expression. It is somewhat paradoxical that endogenous ROS have the capacity to induce gene expression while at the same time may inhibit transcription through a damaged promoter as we have shown here. Along similar lines, Sp1 regulates the basal expression of many genes but can also induce many genes in response to specific signals (44). Considering the observed inhibition of Sp1 binding to promoters containing 8-oxodG, it is probable that oxidative damage may have significant impact on the *trans*-activation potential of this transcription factor.

In contrast to AP-1 and Sp1 in which 8-oxodG inhibits transcription factor binding, substituting this lesion for the third guanine in the NF-κB promoter sequence showed no such inhibition. Many of the genes implicated in the pathogenesis of such prolific diseases as AIDS and cancer are regulated by the binding of transcription factors that are in turn regulated by the redox state of the cell. NF-κB is one such example; ROS serve as messengers in its dissociation from IκB prior to transport to the nucleus. It is perhaps not unexpected then that the NF-κB binding sequence is refractory to inhibition by oxidative damage.

The palindromic sequence of the NF-κB binding site has the ability to form a stem–loop structure with the third guanine located within the loop. We hypothesized that whereas base damage within the loop may not effect transcription factor recognition, such damage in the stem may disrupt base pairing and cause disintegration (or instability) of the stem–loop structure; i.e., that loss of base pairing within the stem may disrupt secondary structure and eliminate transcription factor recognition. To test this hypothesis, we synthesized three additional NF-κB oligonucleotides each containing a single 8-oxodG located proximal to the loop and within the hypothetical stem. Because we saw no inhibition of transcription factor binding to these modified sequences, we conclude that either base damage within such secondary structures has no effect on the integrity of these structures or that a stem–loop conformation at the NF-κB binding sequence does not exist or does not signal transcription factor binding.

To further investigate the structural parameters associated with effective transcription factor binding to a promoter element we performed methylation interference assays to see if the damaged guanines were located at contact sites between the p50 protein and the NF-κB consensus binding sequence. We found that whereas base damage at sites of DNA–protein contact in AP-1 and Sp1 inhibited binding to these sequences it had no effect on p50 binding to the NF-κB sequence. Although these results are not conclusive, they suggest that in the case of NF-κB, DNA–protein contact sites may not be as determinative for stable p50 binding as are other, as yet undefined, structural factors. However, we do show that minor alterations in base composition at a crucial position within a promoter element can disrupt transcription factor binding and potentially modify gene expression. As shown for other types of DNA damage, oxidative damage may be an important factor in epigenetic processes that may lead to the aberrant cell growth and differentiation frequently observed in carcinogenesis.

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