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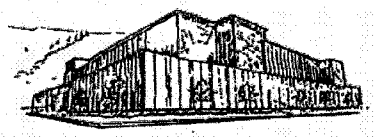

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DAMAGE, RECOGNITION, AND REPAIR OF OXIDIZED GUANINE LESIONS INDUCED BY CHROMIUM EXPOSURE

By

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B.A. Chemistry, West Virginia University, 2001

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Damage, Recognition, and Repair of Oxidized Guanine Lesions Induced by Chromium Exposure

Chairperson: Kent Sugden

Hexavalent chromium is a known human respiratory carcinogen, but the induction of cancer by Cr(VI) is not fully understood. Cellular exposure to Cr(VI) has been shown to cause a wide array of damage including DNA crosslinks, abasic sites and oxidized nucleic acid bases. Our group focuses on the oxidation of DNA at the nucleobase guanine. One well studied oxidized guanine lesions is 7,8–dihydro-8-oxoguanine (8-oxoG). This oxidized base is thought to be a major lesion formed in DNA by oxidative attack. This work will illustrate the potential for disruption in protein-DNA interactions with the insertion of an 8-oxoG lesion in the DNA consensus binding site for the transcription factor, NF-κB. Additionally, data shows that changing the DNA-protein interactions can lead to shielding of the 8-oxoG lesion from repair. Also, studies have shown that 8-oxoG is prone to further oxidation. High valent metals, such as Cr(V), have been shown in vitro to readily oxidize 8-oxoG to form guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp). Previous literature shows that these oxidative lesions are repaired by the base excision repair (BER) system that involves the *E. coli* DNA glycosylases Fpg, Nei, and Nth. Previous to this work, no known mammalian homologues showed affinity to the Sp and Gh lesions. This dissertation will demonstrate that the mammalian glycoslyases, NEIL1 and NEIL2, display a high affinity for the recognition and cleavage of DNA containing the Gh and Sp lesions. The Sp formation in DNA will further be proven by reacting dsDNA with Cr(VI) and ascorbate and determining Sp lesion formation by PAGE sequencing and LC-ESI-MS detection. These findings were supported by studying growth inhibition and oxidized guanine lesion formation in BER deficient *E. coli* strains following chromate exposure. The only BER deficient strain to show growth inhibition by chromate exposure was the Nei mutant strain. HPLC/MS analysis showed the Nei mutant strain accumulated the Sp lesion in genomic DNA at levels 20-fold greater than its wild type counterpart. This work has mechanistic and toxicological implications for how chromate serves as an initiator of carcinogenesis and suggests a role for repair enzymes that may combat the carcinogenic potential of chromate.

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Terminology

8-oxoG	7,8,-dihydro-8-oxoguanine
AP	Apurinic-apyrimidinic
BER	Base Excision Repair
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECD	Electrochemical Detection
EDTA	Ethylenediaminetetraacetic acid
EHBA	2-ethyl-2-hydroxybutanoic acid
EMSA	Electromobility Shift Assay
ESI-MS	Electrospray Ionization Mass Spectrometry
Fpg (MutM)	Formamidopyrimidine-DNA glycosylase
Gh	Guanidinohydantoin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hOGG1	Human 8-oxoguanine DNA glycosylase
Ia	Iminoallantoin
MALDI	Matrix Assisted Laser Desorption/Ionization
MYH	MutY Homologue
Nei	Endonuclease VIII; gene name of glycosylase
NEIL1 and 2	Nei-like 1 and 2
NER	Nucleotide Excision Repair
NF-κB	Nuclear Factor kappa B

Nth	Endonuclease III; gene name corresponding to glycosylase
NTH1	Human Nth Homologue 1
OGG1	8-oxoguanine DNA glycosylase 1
PAGE	Polyacrylamide Gel Electrophoresis
ROS	Reactive Oxygen Species
Salen	N,N-ethylenebis(salicylideneanimato) acid
SDS	Sodium Dodecyl Sulfate
Sp	Spiroiminodihydantoin
TBE	Tris Borate EDTA
Tris	Tris(hydroxymethyl)Aminomethane

Chapter 1: The Biological Role of Chromate as a Human Carcinogen

1.1 Introduction to the Chemistry of Chromium.

The metal chromium exists in a paradoxical state. Chromium has been identified as an essential micronutrient[1] and as a chemical carcinogen.[2] Its role, either positive or negative, depends upon oxidation state and solubility of the compound. A trivalent chromium complex has been reported to be essential for glucose metabolism [3], and bronchogenic carcinomas have been associated with the inhalation of slightly soluble and insoluble hexavalent chromium compounds.[4] Tissue damage, lesions of the skin and respiratory tract, as well as cell mediated allergic reactions caused by exposure to hexavalent chromium compounds, are well documented.[5] In general, compounds of trivalent chromium are less toxic than those of hexavalent chromium.

Chromium is ubiquitous in the environment as it is the seventh most abundant element in the earth's crust. Weathering is responsible for the natural concentrations of chromium in the ground and surface waters, soils, and air. In addition to this natural release of chromium into the environment, a somewhat larger contribution to the total concentration of chromium in the environment comes from the release by anthropogenic sources. Annual domestic processing of chromium is in excess of 500,000 tons, approximately 60% of which is used in the production of a variety of stainless steels and other alloys.[6] In addition to stainless steel production, chromium is used in the production of refractory materials, as a rust inhibitor in factory cooling towers, and a number of end-use consumer products, such as replacement hip joints and applied cosmetics.

Epidemiological studies in the United States[7,8] and in the United Kingdom[9,10] from half a century ago confirmed even earlier German[11] findings on the excess risk of lung cancer associated with exposure to some hexavalent chromium compounds in a variety of occupational and industrial settings, such as chromate producing industries, the manufacture and use of chromium pigments, chromium plating, and stainless steel welding. Hexavalent chromium compounds have also been identified as powerful dermal irritants, with ulceration and perforation of the nasal septum[12] and allergic contact dermatitis occurring in workers exposed to chromate compounds in industrial settings. The International Agency for Research in Cancer (IARC) has classified hexavalent chromium compounds as carcinogenic to humans (group 1)[13], and daily time-weighted exposure limits to chromium in the occupation environment have been set by the U.S. Occupational Safety and Health Administration (OSHA).[14] These exposure limits changed in 2004 from 52 micrograms per cubic meter of air to 1 microgram/m^3.[15]

Due to the high level of occupational and industrial use, environmental and economical disposal of chromium waste has become an ever increasing issue. Kilau and Shah[16] estimated that nearly 100,000 tons of chromium is discarded annually in the slag from stainless steel and chrome alloy production. Land-fill and industrial discharges into municipal sewers has lead to a high level of environmental exposure of chromium compounds to the general population. Due to the ubiquitous nature of chromium in the environment, understanding the biological fate of this metal is of particular importance and concern to the human population. This dissertation will work to illustrate some of the novel findings that may help to explain the carcinogenic nature of this metal in biological systems.

1.2 Chromium Toxicity

Chromate, Cr(VI), has been studied for over 100 years and there is strong epidemiological evidence that it is a human respiratory carcinogen.[17] Although there is good evidence for the induction of cancer, little is known about the mechanism of how chromium damages DNA. When cellular systems are treated with chromate, a large number of different biological lesions have been observed. Cr(VI) has been shown to produce lipid peroxidation products, DNA inter- and intra-strand adducts, DNA-protein crosslinks, DNA strand breaks, abasic sites, and oxidized nucleic acid bases. This has made it difficult to develop rational mechanisms to explain the induction of cancer.

Another factor that makes the development of a mechanism difficult is the multiple species generated during intracellular metabolism of the metal complex. The chromate ion $[CrO_4]^{2-}$, the dominant form of Cr(VI) in neutral aqueous solutions (at physiological pH) can cross cellular membranes via the surface anion transport system (SO_4^{2-} and HPO_4^{2-} channels) and is biologically active (Fig. 1.1).[18-20] Once internalized, Cr(VI) is reduced to the trivalent oxidation state, Cr(III). During this reduction process, high valent metastable oxidation states of +5 and +4 are formed.[21] Also, radicals of carbon, sulfur, and oxygen have been shown to form in vitro when Cr(VI) is reduced.[22] These radical species are confounding secondary DNA damaging agents. Both the high-valent chromium intermediates and free radicals have the potential to cause oxidative DNA damage promoted by Cr(VI).

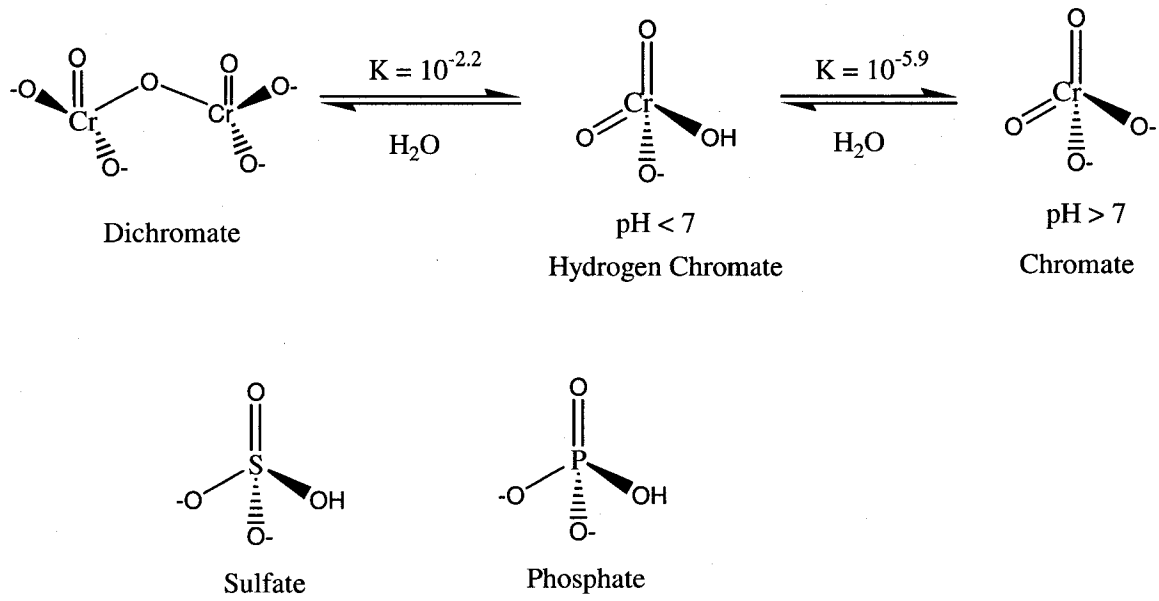
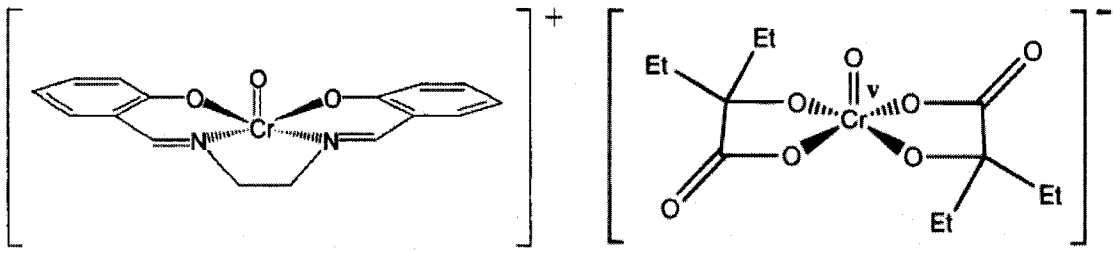


Figure 1.1: Cellular transport of chromate is due to the structural similarity to sulfate and phosphate.

The reduction of Cr(VI) to Cr(III) intracellularly is primarily nonenzymatic and promoted by endogenous reductants such as ascorbate (vitamin C) and the nonprotein thiols of cysteine and glutathione.[23,24] Ascorbate has been shown to be a kinetically efficient reductant of Cr(VI) [24] and has been identified as the major reducing component of Cr(VI) in the rat liver and kidney.[25] The nature of the oxidizing intermediates from Cr(VI) reduction by ascorbate is dependent on the Cr(VI)/ascorbate ratios with formation of Cr(IV) predominating in reactions with excess ascorbate.[22] It has been shown that many types of DNA damage and markers of oxidative stress can also be formed through a direct oxidation mechanism involving the high-valent intermediates of chromium such as Cr(V) and Cr(IV). The DNA damage that occurs during this reduction process

remains in debate, specifically with regard to the type of lesion(s) that is being formed and the mechanism of its formation.

High-valent chromium complexes designed to model intracellular chromate reduction products have primarily used oxygen-ligated anionic complexes such as the readily synthesized Cr(V) complex, 2-ethyl-2-hydroxybutanoic acid or Cr(V)-EHBA. This complex was first shown to cause oxidative damage by relaxation of supercoiled plasmid DNA.[26] Subsequently, a specific oxidative mechanism involving abstraction of the the C-4' hydrogen atom of the deoxyribose moiety of nucleotides and DNA has been identified for this complex.[27-29] Other model Cr(V) complexes have also shown a preference for oxidation occurring at the deoxyribose sugar.[30] However, the first high-valent chromium complex to show convincing interactions with the DNA nucleobase guanine was Cr(V)-Salen (Fig.1.2).[31] The Cr(V) complex, N,N-ethylenebis(salicylideneanimato)oxochromium(V), or Cr(V)-Salen, was chosen as a model complex for study with DNA since its cationic charge should allow good electrostatic interactions between the metal complex and the anionic DNA substrate. This transition metal complex has been used to mimic chromium-peptide complexes that may form upon intracellular reduction of Cr(VI) by virtue of the mixed nitrogen and oxygen ligand chelation.



Cr(V)-Salen Cr(V)-EHBA

Figure 1.2: Structure of model Cr(V) complexes used in DNA oxidation.

1.3 The Formation of Oxidized Guanine Products:

The nucleobase guanine is considered to be the most readily oxidized base (Table 1.1). Significant evidence exists for the interaction of Cr(VI) with guanine nucleotides in DNA, including enhanced chromium association with high-G-content DNA [32], and oxidative damage at guanine sites.[33,34] This G-specific interaction is interesting since it has been shown that guanine modifications represent the major mutations leading to p53-linked cancers.[35] There is also evidence that guanines are the sites for reaction of chromium through both oxidative and binding processes.[32]

Table 1.1: Reduction potentials of nucleosides, DNA sequences, and chromium (VI) to chromium (III)

Species (pH = 7)	E° (V vs. NHE)[a,b]	Sequence (pH = 7)	E° (V vs. NHE)[c]	Cr(VI)/Cr(III)	E° (V vs. NHE)[d]
G	1.29	GGG	0.64	pH = 0	1.33
A	1.42	GG	0.82	pH = 7	0.4
C	1.6	GA	1	pH = 14	-0.12
T	1.7	(8-oxoG)G	0.08		
8-oxoG	0.74	G(8-oxoG)	0.18		

[a] Stevenken et al., J. Am. Chem. Soc. 1997, **119**, 617-618.
[b] Steenken et. al., J. Am. Chem. Soc. 2000, **122**, 2373-2374
[c] Saito et. al., J. Am. Chem. Soc. 1995, **117**, 6406
[d] Connett et. al., Structure and Binding, 1983, **54**, 93-124

Guanine, having the lowest redox potential of the four DNA bases, is the most easily oxidized base. When Cr(VI) is reacted with DNA, the oxidized guanine base 8-oxoguanine (8-oxoG) has been postulated to be the major product.[36] 8-oxoG has been used previously as a biomarker in a number of disease states, including cancer and aging.[37] This type of lesion has been shown to cause a number of aberrant cellular effects, including mutation, cell transformation, and changes in gene transcription.[38-41] It has been estimated that oxidative lesions such as 8-oxoG occur at a frequency of ~1000 base pairs per cell per day.[39] On the basis of oxidation potentials, DNA sequences that contain a high guanine content or consecutive runs of G's should be particularly prone to oxidative modification (Table 1.1). 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. The initial binding of DNA by transcription factors is required for recruitment of the protein complex needed to activate transcription. The assembly of a transcription factor complex is the last point where control can be exerted over gene transcription so, it is evident that the binding affinity of transcription factor proteins to their cognate sequences must play an important role in the regulation of all non-constitutively expressed genes.

Table 1.2 shows the DNA consensus binding sites for several transcription factors as well as the p53 tumor suppressor gene. All of the DNA binding sites contain a run of at least three purines in specific for protein binding. In the case of Sp-1 and NF-κB, a run of four guanines are present within, or overlapping, their binding sequence. A fundamental question is why a series of transcription factors, many of which are sensitive

to oxidative stress, should have regulatory elements that are prone towards oxidation. It is our hypothesis that there exists a level of transcriptional control within the genome whereby formation of oxidative base lesions can modulate gene expression. We are particularly interested in the oxidative modification of guanines within regulatory elements since guanine's tendency towards oxidation is sequence dependent, it occurs with high frequency in regulatory elements, and its oxidative products are well defined in regards to reaction with redox metals. Formation of oxidized guanine lesions within the DNA consensus binding site is predicted to change hydrogen bond donor-acceptor patterns that would alter recognition by the transcription factor protein.[42] These oxidative modifications may also induce structural changes within the DNA such as base flipping [43], bending, or kinking.

Table 1.2: DNA Consensus Binding Sequence for Select Transcription Factors.

Protein	Oligonucleotide Sequence (Top Strand Only)[a]
Sp-1	5'-ATTCGATC***GGGGCG***GGCGAG-3'
AP1	5'-CGCTTGA***TGAGTCA***GCCGGAA-3'
AP2	5'-GATCGAACTGACC***GCCCGCGG***CCCGT-3'
NF-κB	5'-AGTTGAG***GGGACTTTC***CCAGGC-3'
p53	5'-ATAATT***GGGCAAGTCT***AGGAA-3'

[a] = ***bold and italics sequence*** indicates the transcription factor binding sequence

However, 8-oxoG is thermodynamically labile to further oxidation and is seldom observed in in-vitro DNA systems reacted with chromium. While 8-oxoG is considered an intermediate in the overall oxidation process and may serve as a genomic "hot spot" for further oxidative damage, the relevant lesions are thought to be further oxidized products derived from 8-oxoG. Over 50 different base products resulting from the further oxidation of 8-oxoG have recently been identified. Included in this group are imidazolone (Iz) [44], oxazalone (Oa) [45], guanidinohydantoin, (Gh) [46], and spiroiminodihydantoin, (Sp) [47] (Figure 1.3). Based on this observation, modified DNA containing an 8-oxoG nucleotide at a single point within the sequence will be the starting point for many of our reactions.

The Cr(V)-Salen complex shows specificity of oxidation toward the nucleic acid base guanine. The two oxidative products identified from Cr(V)-Salen oxidation of 8-oxoG both in the nucleoside and the oligonucleotide were assigned as guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) on the basis of characteristic mass changes[31]. These data suggest that oxidation of nucleic acid bases may occur from reaction with high-valent chromium intermediates generated from intracellular reduction of Cr(VI). Also, this work demonstrates that biomarkers such as Sp and Gh, and not just 8-oxoG, should be analyzed for the assessment of Cr(VI) exposure and carcinogenicity.

Work on Gh and Sp has shown them to be highly mutagenic. Both lesions promote misincorporation of adenine opposite the oxidized base leading to G:C→ T:A transversions at a rate greater than that with 8-oxoG alone[48]. Also, Sp and Gh have been observed to produce significant levels of G:C → C:G transversion mutations and polymerase arrest.[31,46,48-50] Importantly, these G:C→ T:A and G:C → C:G transversion

mutations are the primary mutations observed in human lung tumors from chromate-exposed workers and in shuttle vector replication assays in Cr(VI)-treated mammalian cells.[51,52]

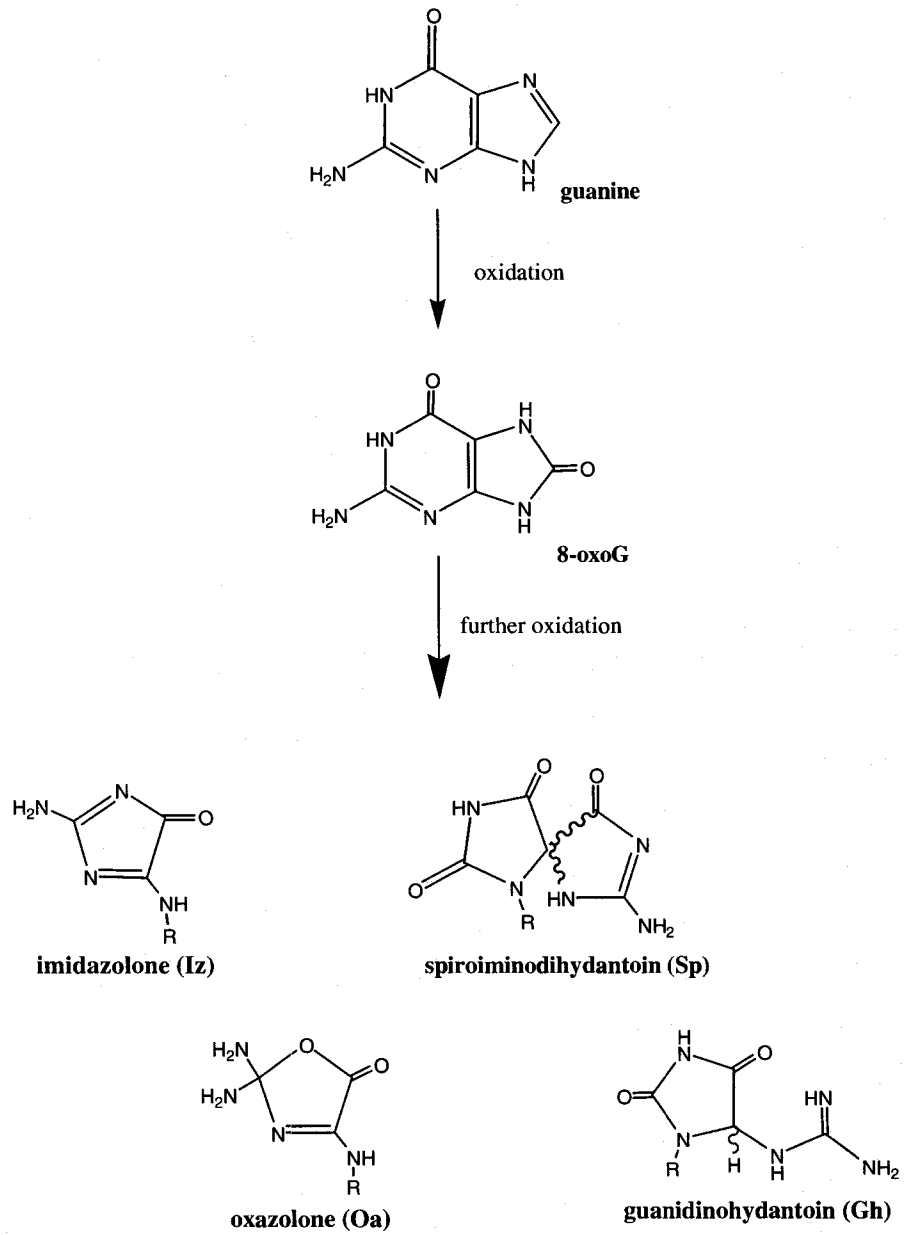


Figure 1.3: Examples of some of the further oxidized lesions of guanine. R = the connection to the deoxyribose sugar backbone.

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. A variety of enzymes are given the daunting task of maintaining the integrity of DNA and many DNA repair mechanisms have been extensively characterized[53]. Although a number of different repair pathways have been uncovered, these pathways are often highly conserved between bacteria and humans, which illustrates the importance in maintaining the integrity of DNA. There are increasing numbers of examples linking the importance of DNA repair fidelity with the prevention of cancer.

Damage to individual DNA bases are usually repaired by the base-excision repair (BER) pathway.[54,55] The initial enzymes involved in the base-excision pathway are DNA glycosylase enzymes, which recognize a variety of modified or mismatched bases and catalyze cleavage of the N-glycosidic bond to release the damaged or incorrect base from the deoxyribose ring.[53] If oxidized guanine lesions are present in promoter elements and affect gene transcription, such repair would serve to switch the response element, along with any transcriptional effects, back to their "normal" levels.

On the basis of these results, we have proposed that the further oxidized products of guanine, Sp and Gh, are equally if not more common in Cr(VI) oxidation reactions with DNA than 8-oxoG itself and, being more mutagenic, may play a primary role in chromium-induced carcinogenesis. Until this point, these lesions had yet to be observed by the direct oxidation of guanine in duplex DNA with any carcinogenic metal. Also, these lesions will be studied for their biological relevance by looking at the recognition and repair of these oxidized guanine lesions by the base excision repair glycosylases.

1.4 The Recognition and Repair of Oxidized Guanine Lesions

Aerobic organisms are constantly bombarded by reactive oxygen species (ROS). Oxidizing agents can be a normal product of a cellular system, as in the case of metabolic intermediates involved in the electron cascade in mitochondria, but they can also be endogenously induced in cells experiencing oxidative stress or exogenously introduced by environmental exposure to transition metals, oxidants, and free radicals. Oxidative damage to DNA has been shown to be a relevant causative agent in aging, carcinogenesis, and neurological disorders.[38,56] Oxidative DNA damage results in strand breaks, DNA-protein cross-links, and base lesions. All of these examples of damage require the action of DNA repair pathways to maintain the integrity of the genome.[57]

A variety of enzymes are given the task of maintaining the integrity of DNA and many DNA repair mechanisms have been extensively characterized. Although a number of different repair pathways have been uncovered, these pathways are often highly conserved between bacteria and humans. There are increasing numbers of examples linking the importance of DNA repair in the prevention of cancer. For example, the relationship between faulty nucleotide excision repair (NER) and the cancer-prone disorder, Xeroderma pigmentosum, has been well-documented.[54,58] In another repair pathway, the base excision repair pathway, a possible link to cancer has been suggested by the observation that the human gene *hOGG1*, which encodes for a repair enzyme involved in the repair of oxidatively damaged guanine residues, is located in a region of the chromosome often deleted in lung cancers.[55,59]

Damage to individual DNA bases is usually repaired by the base-excision repair pathway.[54,55] The initial enzymes involved in the base-excision pathway are DNA

glycosylase enzymes, which are specific for a variety of modified or mismatched bases.[53] Many glycosylases also have lyase activity, which is a β-elimination reaction to cause strand scission after base removal. Subsequent action of apurinic-apyrimidinic (AP) endonucleases and 3'- and 5'-phosphodiesterases remove the remaining sugar fragments to produce a single nucleotide gap. This gap is filled by a DNA polymerase which adds the correct nucleotide. In the last step, the phosphodiester backbone is sealed by a DNA ligase (Figure 1.4).[60]

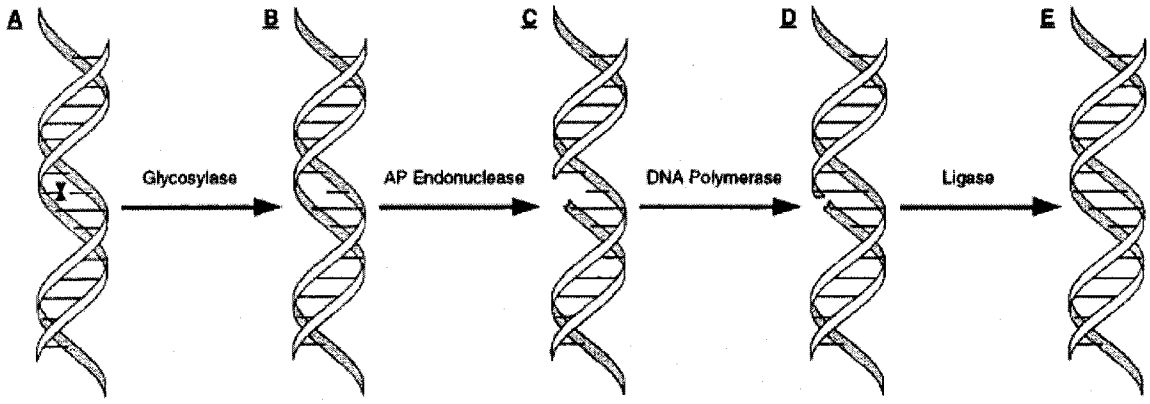


Figure 1.4: Schematic showing the steps in the base excision repair pathway. From: David, S.S. and Williams, S.D. **1998**, *Chemical Reviews* 98, 1221-1261.

The BER pathway is known to be critical for the health of a cellular system and is highly conserved from bacteria to humans. DNA glycosylases have been isolated from numerous sources including bacteria, yeast, and mammals.[53] Much of the initial work in identifying and characterizing members of the BER pathway was performed with bacteria and this work has been critical for the identification of eukaryotic homologues of these enzymes.

There have been over 50 base lesions identified resulting from DNA oxidation.[61] The most common guanine oxidative lesion focused on has been 8-oxoG, which is commonly used as a biomarker of oxidative DNA damage in the cell.[38,61] When 8-oxoG is present in DNA during replication, insertion of A or C opposite the 8-oxoG occurs, depending on the specific polymerase that is involved.[62,63] This insertion of an adenine during replication has been shown to lead to high levels of G:C → T:A transversion mutations in bacterial and mammalian cells.[63-65] However, several repair enzymes have been identified that are specific for recognizing and cleaving the 8-oxoG lesion. In *Escherichia coli (E. coli)*, two base excision repair glycosylases [53], Fpg (MutM) and MutY, catalyze the repair of 8-oxoG lesions. Fpg is responsible for catalyzing the removal of 8-oxoG when paired opposite a cytosine. MutY is considered a second line of defense by removing the mismatched adenine from an 8-oxoG:A pair, giving the cell another chance to remove the aberrant 8-oxoG lesion by Fpg. MutT is the third enzyme that is specific for 8-oxoG removal. While MutT is not a glycosylase, but rather a phosphatase, this enzyme prevents the incorporation of 8-oxoG into DNA by cleansing the dNTP pool of 8-oxoG. It does this by catalyzing the hydrolysis of d(8-oxoGTP) to d(8-oxoGMP) which effectively removes 8-oxoG from the precursor pool.[66]

An important and interesting feature of 8-oxoG is its low reduction potential and ability to act as a "hot spot" towards further oxidation [67] and susceptiblity to oxidation by a variety of cellular oxidants. The formation of further oxidized lesions from 8-oxoG such as Sp and Gh, is an important aspect of cellular toxicity and mutagenicity that needs to be studied in respect to the recognition and repair of these lesions by base excision repair enzymes. The oxidized lesions Sp and Gh present a number of hydrogen-bonding opportunities for base (mis)pairing. The most mutagenic lesion will be the one whose high frequency of formation, low fidelity during replication, and high error rate from DNA repair activity combine to give the greatest mutation rate.[68] In this sense, Sp and Gh are of interest in order to characterize the biochemistry of oxidized lesions in DNA. Not only have the Sp and Gh lesions been shown to induce G → T mutations (Figure 1.5), but they also produce significant levels of G → C transversion mutations as well.[69]

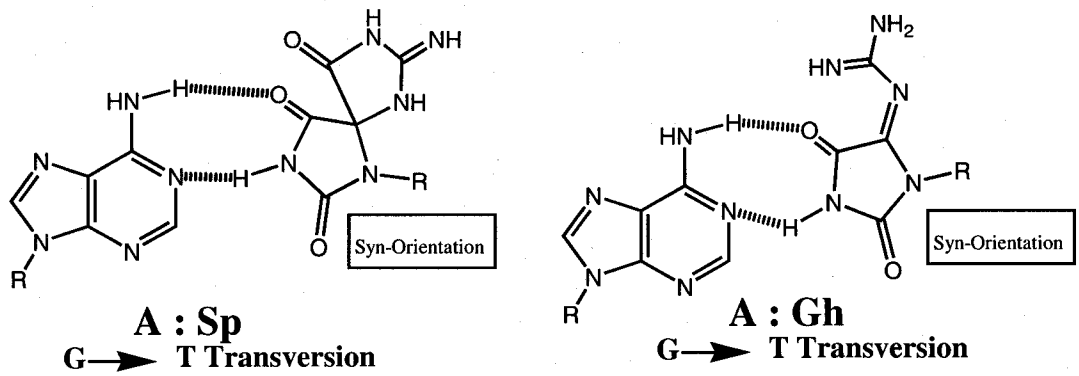


Figure 1.5: Example of Sp and Gh mispairing with adenine in duplex DNA and potentially leading to a transversion mutation.

These further oxidized lesions of guanine, Sp and Gh, are both recognized and cleaved by the base excision glycosylase MutM (Fpg).[68] Besides MutM, two other DNA glycosylases/AP lyases exist in bacterial systems that are specific for oxidative damage, namely Nth and Nei. Nth, discovered on the basis of it endonucleolytic activity on X-ray and heavily UV-irradiated DNA [70,71], removes primarily oxidized pyrimidines. Nei, which is structurally homologous to MutM but not to Nth, was identified as a second pyrimidine-specific glycosylase/AP lysase.[72] More recently it has been shown to possess significant oxidative guanine glycosylase activity [73], with specific recognition and cleavage of not only 8-oxoG but also Sp and Gh, when opposite adenine, cytosine or guanine.[74]

Table 1.3: *Escherichia coli* BER enzymes and substrate specificity

BER Glycosylase	Lesions Excised from DNA
MutM (Fpg)	8oxoG:C, Sp:C, Gh:C, 8oxoG:G, Sp:G, Gh:G.
MutY	8oxoG:A, G:A.
Nei	Sp:A, Gh:A, 8oxoG:C, Sp:C, Gh:C, 8oxoG:G, Sp:G, Gh:G.
Nth	Sp:G, low affinity for Sp:C, Gh:G, Gh:C.

The evolutionary conservation of BER enzymes from bacteria to mammals [75,76] suggests the importance of BER for life and has enabled the identification of various mammalian homologs through database searches using the nucleotide sequences encoding well characterized *E. coli* and yeast enzymes. Three mammalian genes for oxidative DNA repair glycosylases, OGG1, MYH, and NTH1, have been identified in

this way.[77] While these three mammalian genes are specific for some oxidative DNA lesions, none of these glycosylases have shown any specificity for the further oxidized lesions of guanine, Sp and Gh. This lack of glycosylase specificity lead us to study a set of newly identified mammalian glycosylases, NEIL1 and NEIL2, that are homologous to the *E. coli* BER enzyme Nei.

There were many unanswered questions relating to chromate carcinogenicity previous to this research. While there is still much to discover, this body of work will illustrate a number of important findings. First, the ability to potentially modify gene transcription will be illustrated by studying the binding affinity of the p50 subunit of the NF-κB transcription factor when guanine has been oxidized to 8-oxoG at sites critical for protein recognition. The impact of this change in binding affinity will also be assessed by determining if these lesions are shielded from repair by BER enzymes due to the changes in transcription factor binding. Also, evidence will be presented to show that the formation of the further oxidized lesions Sp and Gh can occur by the direct oxidation of Cr(VI) and the reductant ascorbate in duplex DNA. Not only can these lesions be formed in duplex DNA by chromate exposure, but these lesions are recognized and cleaved by the newly identified mammalian BER glycosylases, NEIL1 and NEIL2, in single and double stranded DNA. To further support these findings, growth inhibition studies were conducted in a variety of BER deficient *E. coli* strains undergoing chromate exposure. The genomic DNA was extracted and tested for oxidative lesion formation in 8-oxoG and Nei mutant *E. coli* strains after this chromate exposure. This work demonstrates the significant formation of the Sp lesion in a cellular system after chromate exposure which is the first study to identify this further oxidized lesion in a cellular system. Taken

together, this work has significant mechanistic and toxicological implications for the carcinogenicity of this metal in cellular systems and suggests a role for specific repair enzymes in the process of working to prevent the induction of cancer.

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Chapter 2: An Oxidized Guanine Lesion Modulating Gene Transcription and Base Excision Repair

2.1: Modulation of Gene Transcription

Oxidation of guanine to 7,8-dihydro-8-oxo-2'-deoyguanosine (8-oxoG) is a common DNA lesion produced by endogenous metabolic processes and redox-active xenobiotics.[2] The high frequency of oxidative guanine modification within DNA arises from its oxidation potential being lower than those of the other nucleic acid bases.[3] 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.[4] 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.[5] 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.[6] Like most transcription factors, dimers of NF-κB proteins modulate transcription by directly binding to enhancer sequences located in the regulatory regions of numerous genes. These DNA sequences are collectively known as the κB enhancer element. In mammals, the NF-κB dimers arise from five polypeptides, p50, p52, p65, RelB, and c-Rel. The most abundant

of these dimers are the p50/p65 heterodimer and the p50 homodimer.[7] The crystal structure of the NF-κB p50 homodimer bound to its consensus DNA sequence has been determined.[1,8] Figure 2.1 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 bond donor-acceptor patterns that would alter recognition by the transcription factor protein.[9] These oxidative modifications may also induce structural changes within the DNA such as base flipping, bending, or kinking.[10]

The impact of such oxidation was also tested by assessing the ability of these lesions to be shielded by transcription factor binding from recognition and repair by base excision repair (BER) enzymes. For an oxidized guanine lesion to have significant impact upon gene transcription, it must be resistant to recognition and repair from endogenous repair enzymes on a reasonable cellular time scale. The oxidized lesion, 8-oxoG, is usually recognized and excised within DNA by the base excision repair enzymes, Fapy glycosylase, Fpg (MutM), in *Escherichia coli* or the DNA glycosylase (hOGG1) in humans.[11,12] 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 transcription effects, back to their "normal" levels.

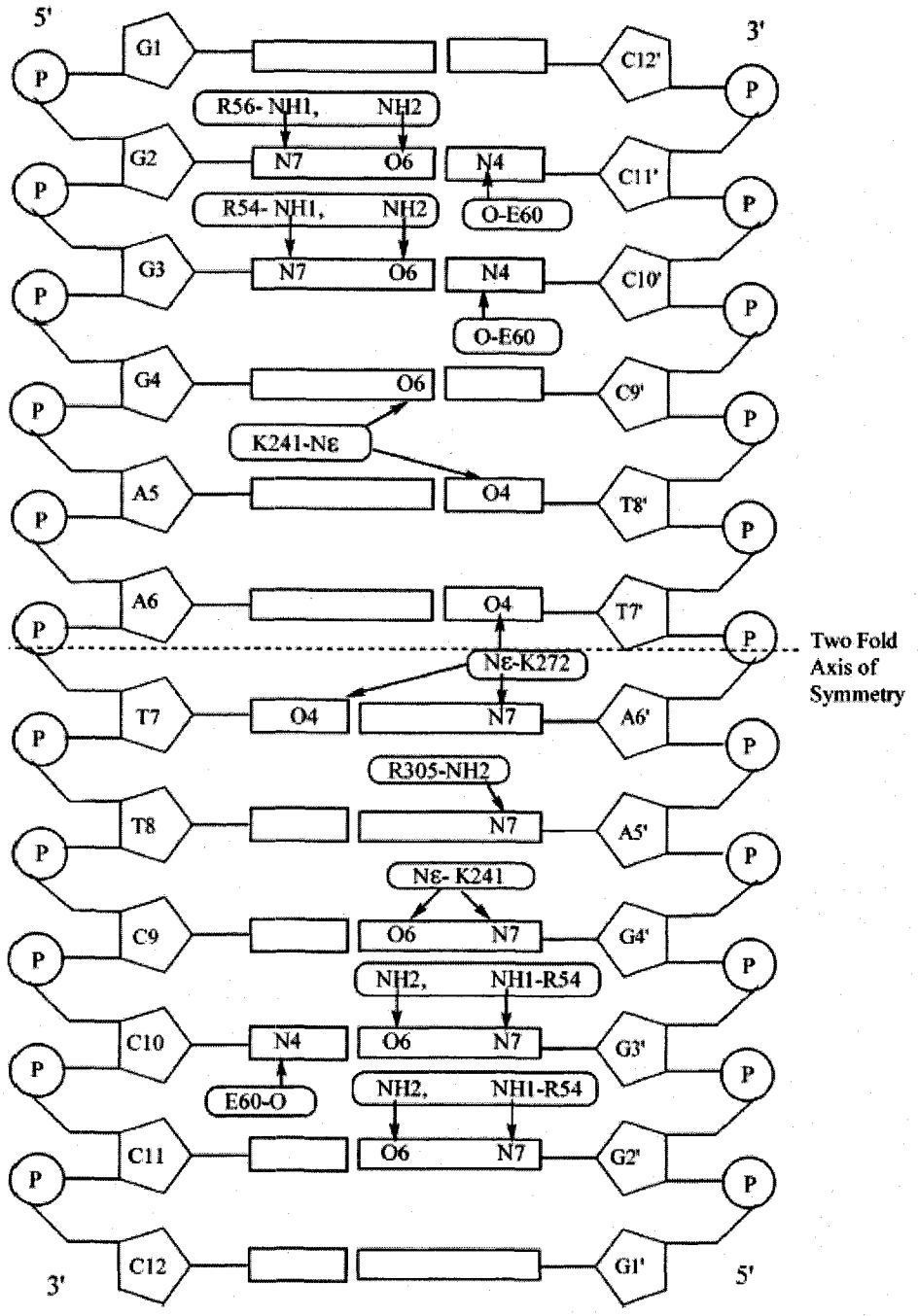

Figure 2.1: Sequence-specific DNA-protein interactions identified for the p50 NF-κB subunit binding with its consensus oligonucleotide sequence. From the PDB entry 1NFK.[1]

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(AGTTGA$G_1G_2G_3G_4$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-oxoG lesion and that the nature and magnitude of this effect was 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 2.1: Control and Modified Oligonucleotide Sequences Incorporating the NF-κB Promoter Site.

oligo no.	sequence (top strand only)[a]
1	5'-AGT TGA G_1***$G_2G_3G_4$AC TTT CCC*** AGC C-3'
2	5'-AGT TGA $G_1{}^{o}$***$G_2G_3G_4$AC TTT CCC*** AGC C-3'
3	5'-AGT TGA G_1***$G_2{}^{o}G_3G_4$AC TTT CCC*** AGC C-3'
4	5'-AGT TGA G_1***$G_2G_3{}^{o}G_4$AC TTT CCC*** AGC C-3'
5	5'-AGT TGA G_1***$G_2G_3G_4{}^{o}$AC TTT CCC*** AGC C-3'

[a]Bold and italic type denotes the p50 consensus recognition sequence.
G^{o} denotes the site of 8-oxoG placement.

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.

In addition to studying the effect of cellular lifetime of a lesion by shielding, structural complications from lesion formation have been investigated. DNA bending, kinking, and base flips are established structural motifs that recruit both transcription factors and repair proteins. Lesion formation in DNA is thought to "hijack" non-target transcription factors and act as promiscuous protein binding sites. The structural impact of 8-oxoG on transcription factors binding to response elements has not been previously investigated. DNA-baiting experiments with 8-oxoG causing defined structural perturbations has been carried out using a biotinylated DNA "bait" bound to a streptavidin column. Elution of HeLa protein extract (normal and heat-shocked) was used to probe for "hijacked" proteins that are bound to the modified sites of the NF-κB regulatory element. Analysis of the impact that these structures have on transcription factor binding will give us an understanding of how these lesions act as oxidative signaling events to cause up- or down-regulation of 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.

2.2 Lesion Impact on Transcription Factor Binding Affinity

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-oxoG sites at position G_1-G_4 have on the affinity of p50 for its cognate sequence. Representative EMSA autoradiograms in Figure 2.2A-E show the binding affinity of the p50 transcription factor with the binding affinity of the ^{32}P-labeled unmodified DNA cognate recognition sequence and each of the ^{32}P-labeled oligonucleotides containing the four sites of 8-oxoG modification (Table 2.1, denoted as G^o). These 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-oxoG-modified DNA sequences to determine the percent of DNA that was in the form of the DNA-protein complex (Figure 2.3). The apparent dissociation constants (K_{app}) under these conditions for each of the 8-oxoG-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-oxoG-modified oligonucleotides are given in Table 2.2. The relative K_{app} for the unmodified control was found to be 672 nM with little change in the 50% binding affinity observed with the 8-oxoG modifications at G_2 and G_4. The 8-oxoG 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 previously observed when p50 binds as a monomer to a single DNA half-site.[7] This lowered p50 binding affinity suggests that 8-oxoG modification at G_3 causes a loss in recognition of one of the DNA half-sites of the target oligonucleotide. Conversely, the G_1 8-oxoG modification showed a nearly 2.5-fold increase in binding affinity over that of the unmodified cognate DNA 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.

Table 2.2: Binding Affinity of 8-oxoG-Modified Oligonucleotides with p50

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

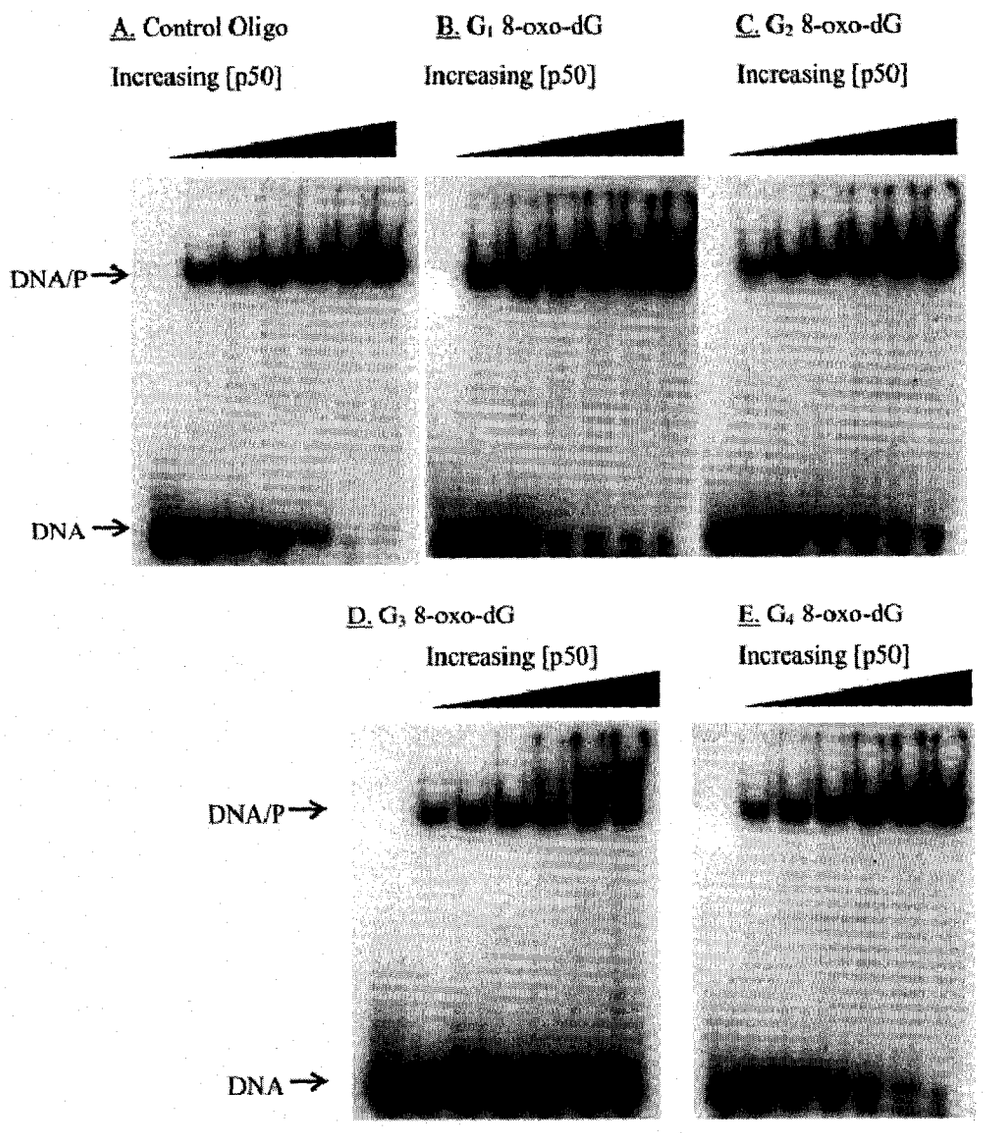

Figure 2.2: Representative autoradiograms showing the gel shift induced by binding of the p50 subunit of NF-κB to the control and 8-oxoG-modified 5'-^{32}P-labeled DNA recognition sequence. All five panels demonstrate the concentration dependence of the DNA-p50 complex (from left to right) with the addition of 0, 2.4, 4.8, 7.5, 10, 15, and 20 pmol of p50. From: Hailer-Morrison, et. al. (2003) *Biochemistry* **42**, 9761-9770.

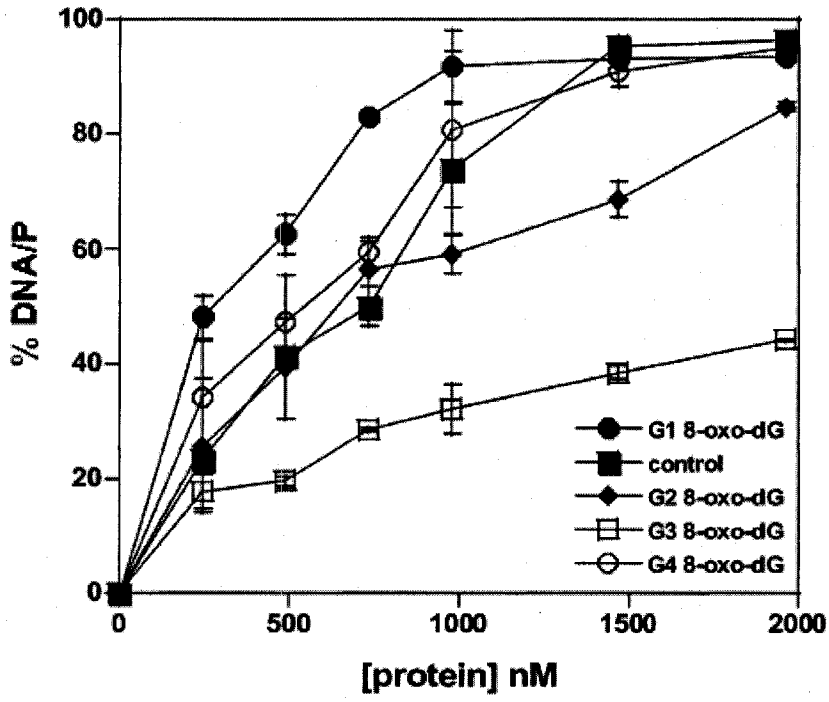

Figure 2.3: Graphical representation of the densitometric data obtained from the autoradiograms in Figure 2.2. Data are reported as a percent of the DNA complexed with protein vs the concentration of p50 needed to induce the corresponding gel shift. From: Hailer-Morrison, *et. al.* (2003) *Biochemistry* **42**, 9761-9770.

2.3 Lesion Shielding from Base Excision Repair by p50 Binding

8-oxoG 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 lesion lifetime in the cell, which dramatically impacts gene transcription. We have developed a competition cleavage

assay based on the work of Leipold *et al.* [13] 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-κB promoter site to shield the lesions from BER recognition was determined by loss of DNA cleavage. Panels A-D of Figure 2.4 are representative autoradiograms showing that increasing p50:Fpg ratios markedly affect the time-dependent ability of the BER enzyme to recognize and cleave 8-oxoG at the G_1 site in the DNA recognition sequence. Panels A-D of Figure 2.4 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 2.4A), the cleavage reaction at the 8-oxoG site is complete by 30 min. With increasing amounts of p50, the 8-oxoG 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 2.4B-D) was a consequence of the binding of p50 to the modified oligonucleotide. The time-dependent cleavage shielding patterns for all four 8-oxoG modifications at the four different p50:Fpg ratios are shown in panels A-D of Figure 2.5. As would be expected, the cleavage shielding of the 8-oxoG 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: $G_1 > G_4 > G_2 >> G_3$. The G_3-modified site showed no lesion shielding effect (or even an enhancement of cleavage) for p50 (Figure 2.5C), which corresponded with the significantly lower binding affinity in comparison to that of

the G_1-modified site. The percent shielding for the different sites of modifications at the 60 minute time point and at the different p50:Fpg ratios is given in Table 2.3.

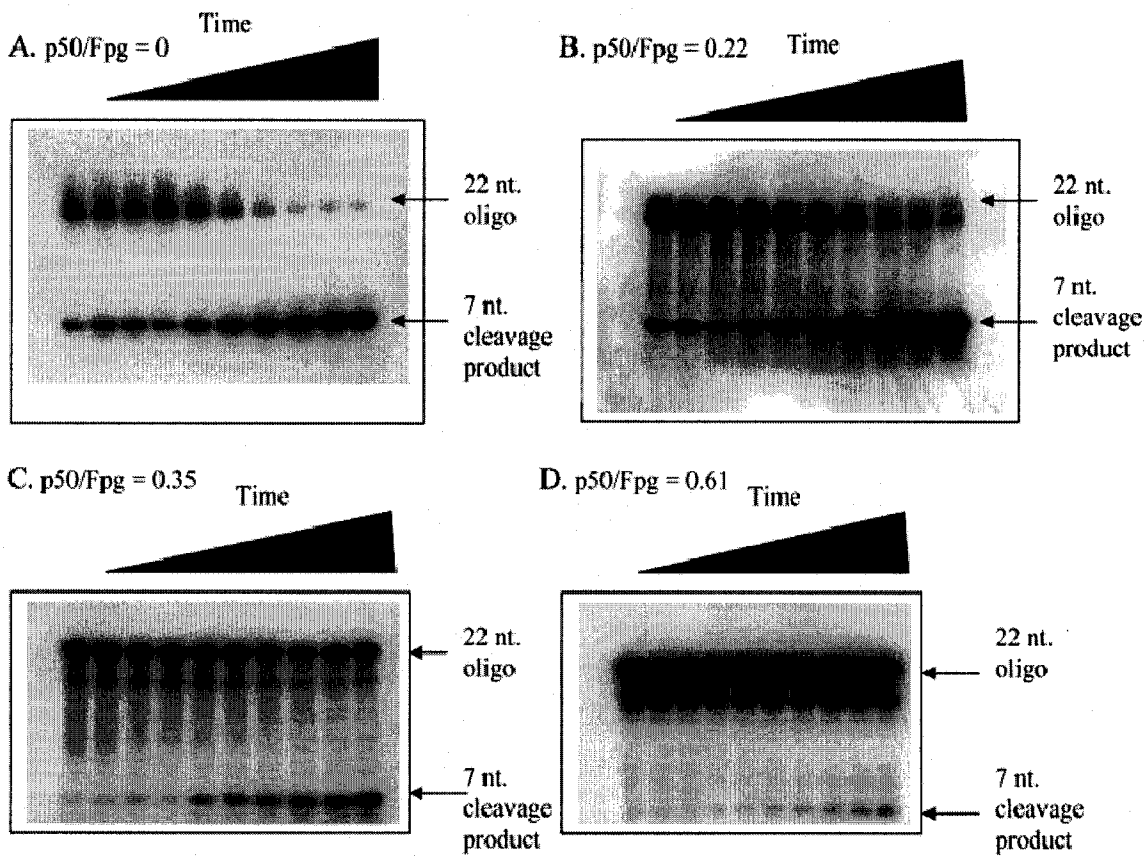

Figure 2.4: Representative autoradiograms from the Fpg cleavage assay for determining the time-dependent and p50 concentration-dependent shielding of the G_1 8-oxoG in the 22 bp NF-κB recognition element. The time points for each cleavage analysis for all gels were (from left to right) 0, 0.25, 0.5, 1, 2, 4, 8, 15, 30, and 60 min. Panel A shows the time-dependent cleavage with no p50 added. Panels B, C, and D show the time-dependent cleavage with a p50:Fpg ratio of 0.22, 0.35, and 0.61 respectively. 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 2.3: Percent Inhibition of Fpg Cleavage of 8-OxoG-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-oxoG at G_1	17.1 ± 11.4	48.7 ± 10.7	92.4 ± 5.2
8-oxoG at G_2	8.5 ± 8.7	30.4 ± 5.9	62 ± 3.2
8-oxoG at G_3	-27.9 ± 7.8	-22.6 ± 5.9	-17.4 ± 20.1
8-oxoG at G_4	42.3 ± 2.1	68.9 ± 11.7	91.9 ± 2.1

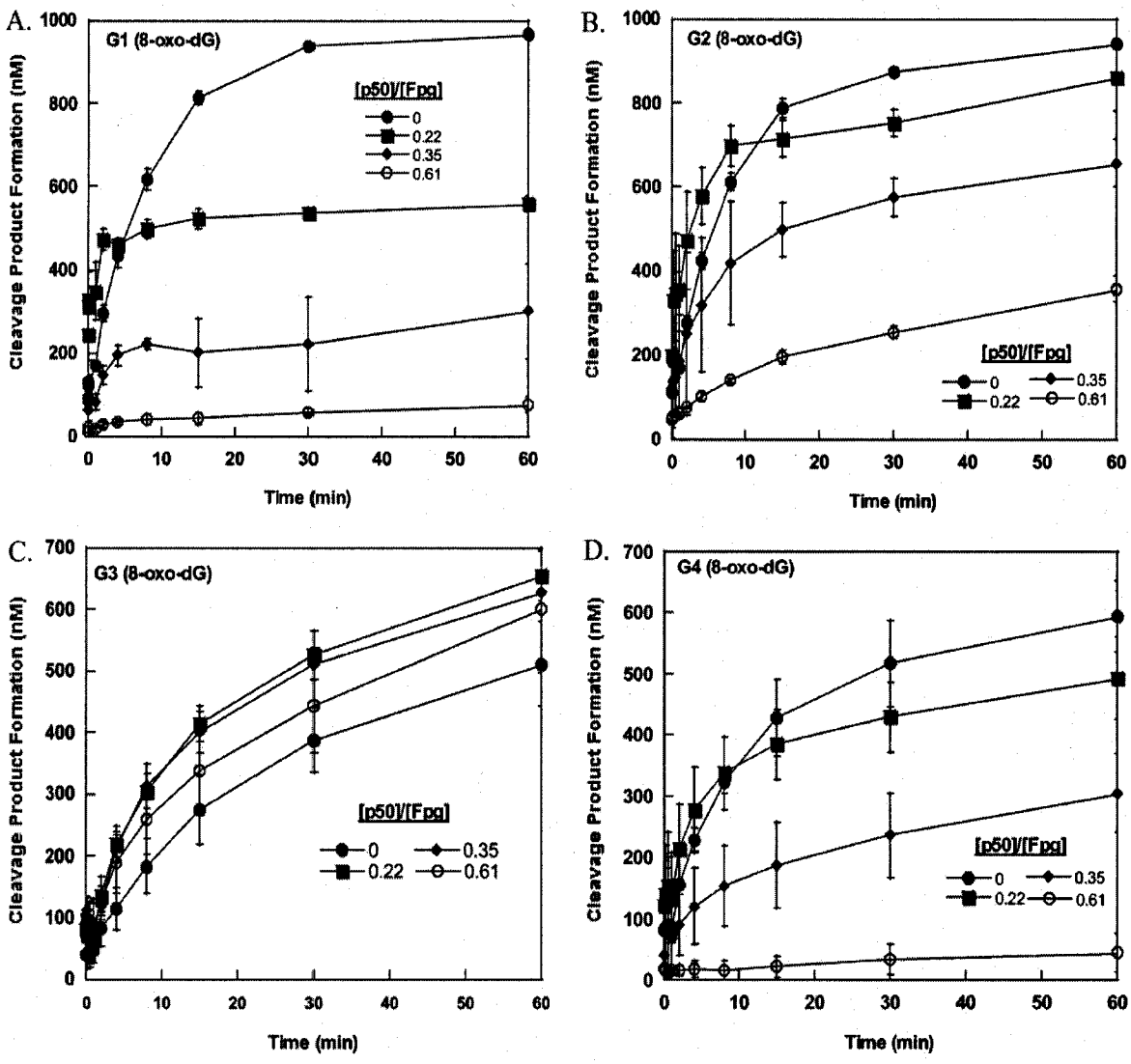

Figure 2.5: Graphical representation of the time-dependent cleavage of the 8-oxoG-modified oligonucleotides (G_1-G_4) at different p50:Fpg ratios. An average of a minimum of two gels (maximum of four gels) for each data point was plotted.

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-oxoG at G_1 is shown in panels A-D of Figure 2.6. Due to the lower reactivity of hOGG1 towards the 8-oxoG 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 2.6A and 2.7A). With the addition of p50 and hOGG1 ratios of 0.07, 0.11, and 0.19, a significant degree of shielding of the 8-oxoG-modified lesion from cleavage by hOGG1 was again observed. All four sites of 8-oxoG modification were analyzed using identical p50:hOGG1 ratios over the 120 min time course (Figure 2.7A-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-oxoG 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 2.4. The p50:hOGG1 ratio that was necessary to induce nearly 100% shielding of the lesions 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 p50 transcription factor, when bound to an 8-oxoG 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.

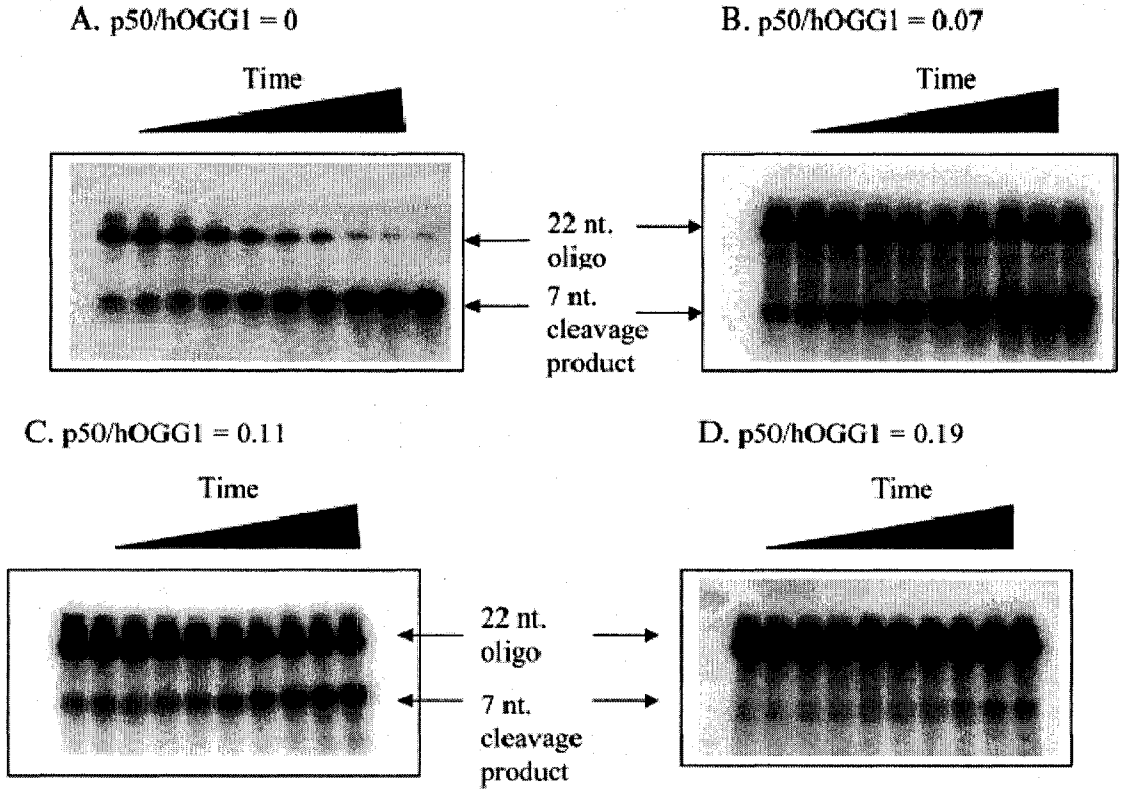

Figure 2.6: Representative autoradiograms from the hOGG1 cleavage assay for determining the time-dependent and p50 concentration-dependent shielding of the G_1 8-oxoG site in the NF-κB recognition element. The time points for each cleavage analysis for all gels were (from left to right) 0, 1, 2, 4, 8, 15, 30, 60, 90, and 120 min. Panel A shows the time-dependent cleavage with no p50 added. Panels B, C, and D show the time-dependent cleavage with a p50:hOGG1 of 0.07, 0.11, and 0.19 respectively. 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.

Table 2.4: Percent Inhibition of hOGG1 Cleavage of 8-oxoG-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-oxoG at G_1	42.9 ± 1.7	62.3 ± 13.6	83.1 ± 17.2
8-oxoG at G_2	45.5 ± 3.7	60.3 ± 11.7	85.2 ± 13.5
8-oxoG at G_3	20.6 ± 2.0	33.6 ± 13.9	46.8 ± 8.4
8-oxoG at G_4	38.7 ± 3.2	54.4 ± 5.0	85.5 ± 5.9

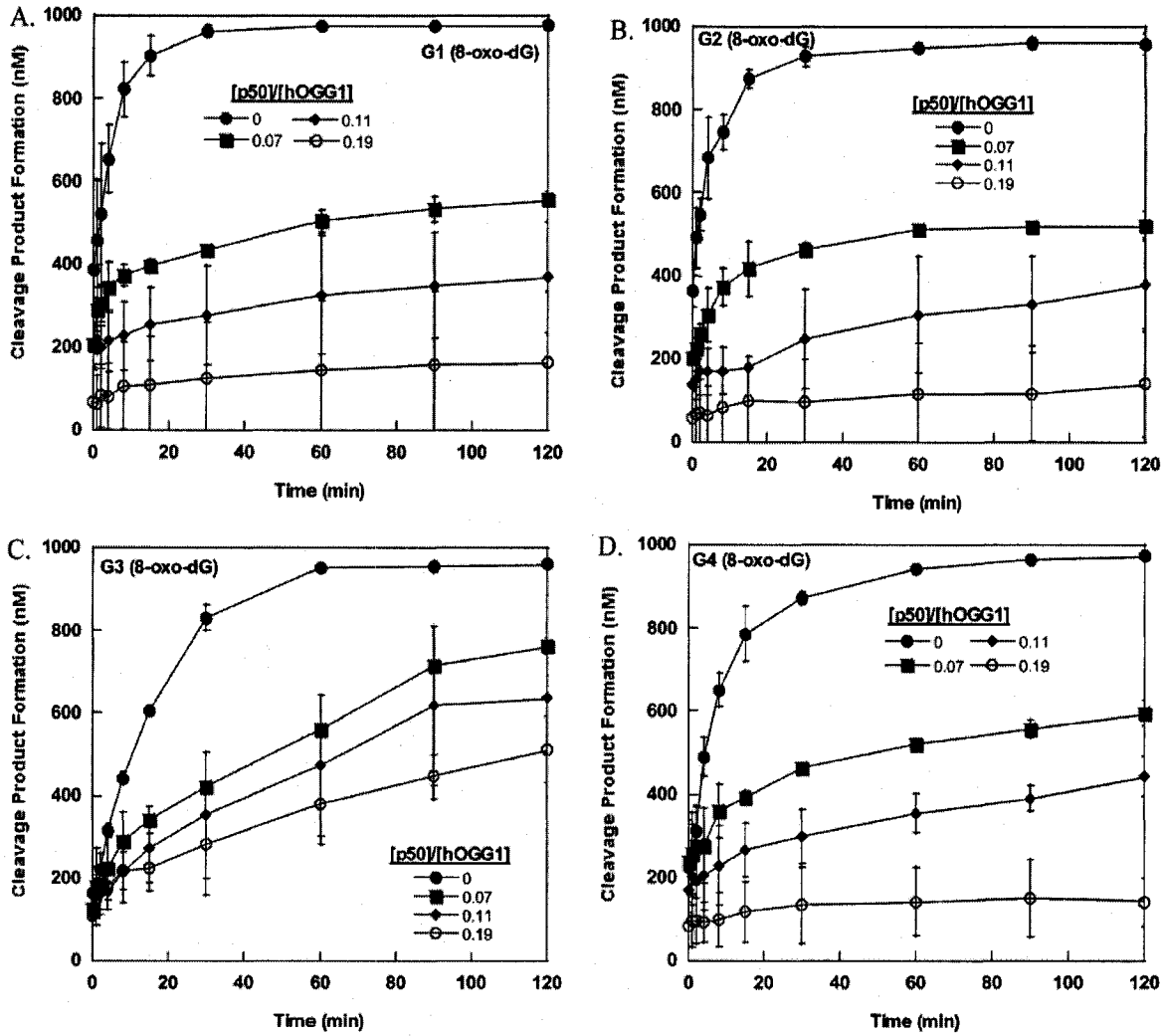

Figure 2.7: Graphical representation of the time-dependent cleavage for hOGG1 cleavage of the 8-oxoG-modified oligonucleotides (G_1-G_4) at different p50:hOGG1 ratios. An average of a minimum of two gels (maximum of four gels) for each data point was plotted.

2.4 Streptavidin/Biotin Pull Down Assay

The biotin/streptividin affinity column assay, or "pull down" assay was used to determine which proteins are recruited to certain DNA sequences with and without oxidative modifications present. A biotinylated double stranded DNA fragment containing a position specific 8-oxoG in the NF-κB response element was bound to a streptavidin column with binding buffer (12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 4 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, 1 mM DTT). The tethered biotin label was placed on the 5'-end of the complementary sequence to the DNA strand containing the lesion in order to minimize interference of the solid matrix (streptavidin-coated beads). Protein extract from HeLa cells were allowed to bind to the DNA on the column and the double-stranded oligonucleotide was washed with increasingly stringent wash conditions (1-2 M KCl). High affinity proteins were not removed by the elution conditions (12% glycerol, 20 mM Tris-HCl (pH 6.8), 1 M KCl up to 2 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1mM DTT). These proteins were removed by boiling the streptavidin beads for 10 min in SDS-PAGE buffer. The eluted proteins were run on a 10-20% gradient gel and visualized by silver staining (Figure 2.8). Initial studies on the control oligonucleotide show bands corresponding to the p50 protein weight. Studies of the modified NF-κB oligonucleotide (G_1 position) were quite specific with a relatively small number of proteins being isolated after increasingly stringent washing of the beads. In addition to a band corresponding to p50, other specific and yet unidentified bands were also isolated. Identification of the specific bands can be accomplished by Western blot (i.e. p50, p65 proteins). This assay needs further development to more conclusively determine the expected and unnatural binding of proteins to a lesion containing DNA promoter site.

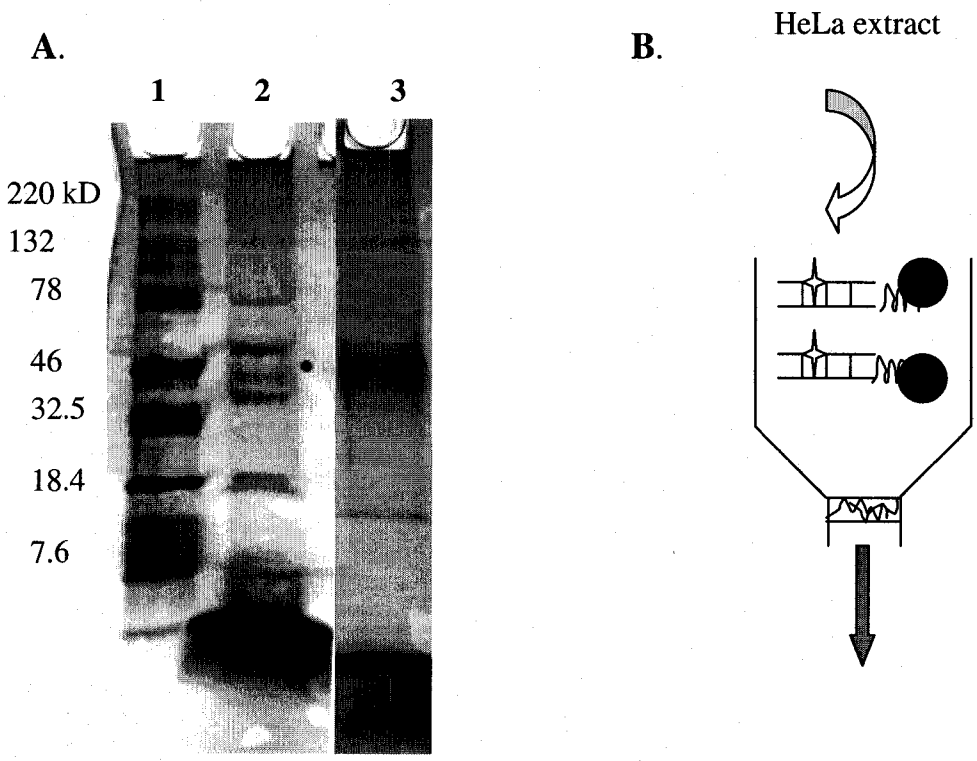

Figure 2.8: A. Silver stain SDS-PAGE gel illustrating the specific proteins attached to an 8-oxoG NF-κB oligonucleotide when reacted with HeLa cellular extract. Lane 1, molecular weight markers. Lane 2, biotinylated oligonucleotide with an 8-oxoG modification at the G_3 position of the NF-κB binding sequence reacted with HeLa cellular extract. Lane 3, biotinylated unmodified oligonucleotide reacted with HeLa celluar extract. Samples 2 and 3 were collected by boiling the streptavidin bead slurry in SDS-PAGE loading buffer before loading onto the gel. B. Schematic of the pull-down assay used to isolate proteins bound to the control and modified oligonucleotides under high affinity conditions.

2.5 Discussion and Conclusions

A guanine positioned 5' to a contiguous run of guanines is more prone to oxidation to the 8-oxoG lesion.[4] 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.[6,14] The

base immediately proceeding this sequence could be any one of the four bases, although this base is predominately a guanine in the stress response genes, i.e. NF-κB.[15-20] 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.[8] 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.[1,8] 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-oxoG is the conversion of N-7 of guanine from a hydrogen bond acceptor ("A") to a hydrogen bond donor ("D") (Figure 2.9). Several of the bases, such as G_2 and G_3 (Figure 2.1), 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-oxoG 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.[9]

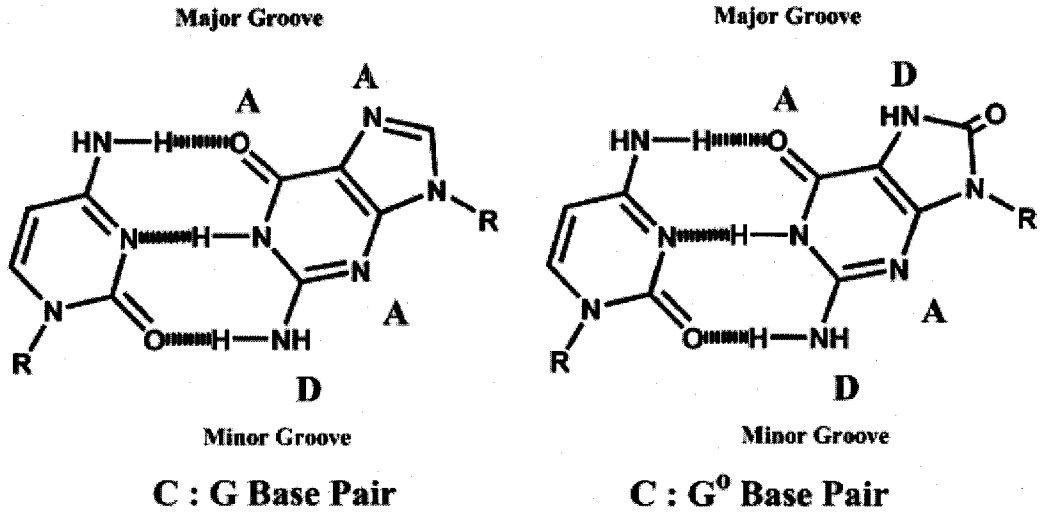

Figure 2.9. Hydrogen bond donor-acceptor pattern for guanine and 8-oxoG (G^o) in double-stranded DNA.

We have studied the effect of 8-oxoG 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-oxoG 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 (see Fig. 2.1). 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 reactive oxygen species (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.[21] 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 the transactivating activity of the transcription factor protein.[22]

p50 Binding Affinity Changes. Our studies have shown that incorporation of 8-oxoG 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 down-regulation 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.

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-oxoG in DNA that have shown little perturbation in DNA structure.[9] 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 [23] or for repair enzyme recognition of base-flipped structures.[10] 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.[24] Insertion of 8-oxoG at the 5'-position in a GGG triplet of a human telomeric sequence can induce quadruplex formation and inhibit telomerase activity.[25] 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. However, this work has shown that the binding of p50 to the 8-oxoG-modified NF-κB regulatory element can effectively shield these lesions from recognition and repair by base excision repair enzymes. The shielding effects afforded by p50 directly correlated with the binding affinity that was induced by the 8-oxoG 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 (tighter binding) and increasing the cellular longevity of the lesion (shielding). 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.[23] 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.

Streptavidin Pull-Down Assay. The biotinylated DNA bound to a streptavidin matrix is a useful way to isolate specific protein-DNA complexes. In our study, the DNA used was specific for the p50 protein of the NF-κB transcription factor complex. We hypothesized that placing a lesion, such as 8-oxoG, into the p50 binding site could possibly change the recruitment patterns of the DNA. The recruitment of specific proteins to DNA containing a lesion was compared to control DNA. Our preliminary data shows that the biotinylated DNA containing the 8-oxoG lesion bound a small number of proteins very tightly and the number and weights of these proteins differed from the proteins bound by the control oligo (see Figure 2.8). Upon probing these proteins by Western Blot with p50 and p65 antibodies (both members of the NF-κB complex), the results were negative. This does not conclusively rule out the recruitment of these two proteins, due to the fact that the positive control for both antibodies was also negative. The potential does exist to identify these proteins (p50, p65) by Western Blot. Other unknown bands can be identified by two dimensional gel analysis, followed by excision of the protein bands and identification either by MALDI-MS or by ESI-MS after an in-gel digest and extraction of the peptide fragments. This assay was necessary to show that these modified promoter elements are indeed recognized by their specific

transcription factors, but can also show whether there is a change in recognition in terms of affinity changes of normal transcription factors or in the unnatural binding of "spectator" proteins.

2.6 Experimental Procedures

2.6.1 Oligonucleotide Preparation

Oligonucleotide substrates: The 8-oxoG containing oligonucleotides were purchased from TriLink BioTechnologies and unmodified strands were purchased from Integrated DNA Technologies. Table 2.1 shows the sequence and site of the 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 x 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 6.0 in 10% acetonitrile) to 100% mobile phase B over 31 minutes. Eluting oligonucleotides were monitored at 268 nm. The fraction containing the oligomer was eluted as a single peak, collected and lyophilized to dryness, and purified into deionized water using a Bio-Rad Micro Bio Spin 6 Column. Pure oligonucleotides were stored at -20 °C until needed. The 5'-^{32}P-end-labeled oligomers were prepared by standard methods.

2.6.2 Electromobility Shift Assay and Data Analysis

Electromobility Shift Assay (EMSA). Purified recombinant human p50 protein was purched from Promega, Inc. DNA-p50 reactions were carried out using 6.4 pmol (640 nM) of the appropriate 5'-^{32}P-labeled oligonucleotide that had been annealed to its

complementary sequence to generate the double-stranded oligonucleotide needed for recognition and binding by the p50 protein. The annealing was carried out by using a 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 hours. Identical concentrations were ensured by measuring the OD at A_{260} of the stock DNA solutions. The reactions for p50 binding were carried out in a 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 of poly(dI·dC) in 4% glycerol. The p50 protein was added at increasing concentrations, 2.4-20 pmol (240-2000 nM), to the reaction mixtures and allowed to bind for 30 min at room temperature. 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 BioRad GS-800 calibrated densitometer using QuantityOne software.

Data Analysis. The EMSA analysis of binding affinity for the control and 8-oxoG 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.[26] The apparent dissociation constant (K_{app}) was determined graphically as the point where the fraction bound equals 50%. Analysis of the gel shifts were carried out on two to four gels for each of the different modifications.

2.6.3 Lesion Shielding Assay and Data Analysis

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.[13] The p50 transcription factor protein was added at increasing concentrations to the 8-oxoG-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. The p50 was preincbated with the modified oligonucleotides at 25 °C for 30 min before the addition of 3000 nM hOGG1 to give p50:hOGG1 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-oxoG-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 BioRad GS-800 calibrated densitometer with QuantityOne software. Two to four gels were used for quantification of each modified oligonucleotide at each time point.

2.6.4 Streptavidin/Biotin Pull Down Assay

Column Format of Streptavidin/Biotin Protein Isolation Assay with 8-oxoG Modified Oligonucleotide. The UltraLink Immobilized Streptavidin kit was purchased from Pierce (Rockford, IL) and the HeLa Cell Lysate was purchased from Stressgen Biotechnologies (Victoria, BC Canada). The streptavidin columns were made following the manufactures instructions for the column format affinity purification procedure. Briefly, 0.35 mL of strepavidin slurry was used to construct the column. The column was first equilibrated with the addition of 5 column volumes of Binding Buffer (12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT). After equilibration, approximately 0.15 mg of a biotinylated unmodified or 8-oxoG containing NF-κB oligonucleotide was added to the column. The biotinylated oligonucleotide was mixed with the streptavidin column for 30 minutes at room temperature with gentle rocking. The biotin/strepavidin column was then washed with 10 column volumes of Binding Buffer. To the column, 10 μL of HeLa cellular extract was added and allowed to react with the column overnight at 4 °C. The following day, the column was again washed with 10 column volumes of Binding Buffer. The un-

bound proteins would be removed during the column washing step. The bound proteins were eluted under increasingly stringent conditions with Elution Buffer (12% glycerol, 20 mM Tris-HCl (pH 6.8), 1 M – 2 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT). Fractions were collected from each elution wash. After eluting the proteins, 20 μL of the column slurry was removed and these beads were boiled for 10 min in SDS-PAGE loading buffer. The elution samples and the boiled bead samples were run on a 10-20% SDS-PAGE gradient gel and Coomassie Blue stained. If no bands were visible by Coomassie staining, the gel was re-stained by Silver staining.

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Chapter 3: The Formation and Repair of Guanidinohydantoin and Spiroiminodihydantoin

3.1 The Further Oxidation of 8-oxoG

Chromate, Cr(VI), has been established as a human carcinogen, although its mechanism of action has not been clearly defined.[1,2] The reaction of chromate with DNA creates a number of putative lesions in cellular systems including inter- and intra-strand cross-linked adducts, DNA-protein cross-links, DNA strand breaks, abasic sites, and oxidized nucleic acid bases.[3-7] The tetrahedral anionic conformation of the +6 oxidation state of chromium facilitates active transport into cell systems through the phosphate and sulfate cellular transport systems.[8] However, Cr(VI) is not the oxidation state that reacts with DNA. The cellular reduction of Cr(VI) to its stable +3 oxidation state forms a wide variety of intermediate high-valent (+4 and +5) oxidation states of chromium, as well as reductant-specific carbon-, oxygen-, and sulfur-based free radicals.[9-12] Both the high-valent chromium intermediates and free radicals have the potential to cause oxidative DNA damage promoted by Cr(VI). The DNA damage that occurs during this reduction process remains in debate, specifically with regard to the type of lesion(s) that is being formed and the mechanism of its formation.

Oxidative damage and the formation of oxidized lesions in DNA is considered one of the critical steps in the induction of carcinogenesis by Cr(VI). Oxidation of DNA can occur at the deoxyribose sugar creating DNA strand breaks or at the nucleic acid bases creating oxidized base lesions.[13] Many oxidative lesions of nucleobases have been

identified and quantified in vitro and in vivo, but to date, only 7,8-dihydro-8-oxoguanine (8-oxoG) has been identified in reactions with Cr(VI).[14,15] The ubiquity of this lesion in many oxidizing systems arises from the lower reduction potential of guanine in comparison to the other nucleic acid bases. Oxidation of guanine has also been observed to be influenced by DNA sequence with the 5' guanine in a run of purines showing enhance levels of oxidation.[16,17] The 8-oxoG lesion is mildly mutagenic and has been shown to mispair with adenine during DNA replication giving rise to G:C → T:A transversion mutations.[18] Recent studies have shown that 8-oxoG has a significantly lower reduction potential than the parent guanine itself, making it highly reactive toward further oxidation.[19,20] Oxidative hole migration can occur over long distances [21], and certain lesions, such as 8-oxoG, can act as sinks for electron hole trapping leading to a hot spot for damage and mutation. Previously, the reaction of Cr(V) complexes with single-stranded DNA containing the 8-oxoG lesion has shown further oxidation exclusively at the 8-oxoG site.[22,23] The further–oxidized lesions formed in this reaction were determined to be spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh).[22]

The Sp and Gh lesions produced the G:C → T:A mutations observed for 8-oxoG but at significantly greater levels than 8-oxoG alone. Also, Sp and Gh have been shown to produce significant levels of G:C → C:G transversion mutations and polymerase arrest.[22,24-27] Significantly, these G:C → T:A and G:C → C:G transversion mutations are the primary mutations observed in human lung tumors from chromate-exposed workers and in shuttle vector replication assays in Cr(VI)-treated mammalian cells.[28,29]

Oxidative damage to individual DNA bases is repaired via the base excision repair (BER) pathway which recognizes and removes damaged bases from DNA.[30]

MutM/Fpg removes the 8-oxoG lesion when it is paired opposite a cytosine while MutY removes the mismatched adenine from an 8-oxoG:A or a G:A pair to provide a second chance for Fpg recognition and repair.[31] Previously only two mammalian repair enzymes, 8-oxoguanine-DNA glycosylase (OGG1) and endonuclease III homolog 1 (NTH1), were known to recognize and cleave oxidized guanine lesions from DNA.[32] Surprisingly, Nth1 knockout mice remain healthy [33] and while Ogg1-/- mice show increased mutation rates in some tissues, they have no associated increase in the incidence of cancer.[34,35] These findings suggested that an additional BER enzyme system exists that can recognize oxidized guanine residues.

A new set of BER enzymes have been identified that are mammalian homologs of the *E. coli* MutM/Nei (endonuclease VIII) family.[36-38] These mammalian homologs of Nei were designated the "Nei-like" or, "NEIL" family of glycosylases. The NEIL1 gene maps to the 15q22 chromosome in humans and loss of heterozygosity at this site is observed in over 70% of small cell lung carcinoma.[39] Initially the identified substrates of NEIL1 and NEIL2 have consisted mainly of oxidized pyrimidines such as 5-hydroxyuracil (5-OHU), the formamido-pyrimidines, (FapyG and FapyA), and thymine glycol.[36-38,40] In addition, the human NEIL enzymes have been shown to recognize 5-OHU and 8-oxoG in single-stranded bubble structures of DNA.[32]

In this chapter, we will report the identification of two additional oxidized lesions that are readily recognized and cleaved by both murine NEIL1 and NEIL2. The lesions guanidinohydantoin (Gh), its isomer iminoallantoin (Ia) (collectively referred to only as Gh), and spiroiminodihydantoin (Sp), are further oxidize products of the common 8-oxoG lesion. Although these further oxidized lesions can be recognized and removed by

the *E. coli* DNA glycosylases Fpg [41] and Nei [42], no mammalian BER counterpart for the recognition of these further oxidized products has been previously determined. This work demonstrates that the murine NEIL1 BER enzyme can remove Gh and Sp when paired opposite all four bases of DNA. NEIL2 can recognize and remove double-stranded Gh opposite all four DNA bases but little cleavage of the Sp lesion was observed irrespective of the base on the complementary strand. However, DNA trapping studies showed that the Sp lesion in double-stranded DNA was recognized by the NEIL2 enzyme. NEIL1 and NEIL2 both showed a high degree of cleavage activity for Gh and Sp lesions in single-stranded DNA. Significant cleavage of the 8-oxoG lesion, in either single- or double-stranded DNA substrates, was not observed for either NEIL enzyme.

In addition to these results, data is shown to help prove the existence of these further oxidized lesions in a mammalian system. The formation of these lesions has been established in vitro and bacterial systems, but they have yet to be extended to mammalian systems. It is known that the OGG1 enzymes repair 8-oxoG lesions and the majority of this chapter will establish that the mammalian NEIL1 and NEIL2 genes cleave the Sp and Gh lesions formed in DNA that has been exposed to chromium. Some preliminary data will be shown that examines the repair kinetics of the 8-oxoG and Sp lesions by mammalian cell extracts, specifically HeLa nuclear extracts. The HeLa cell line was originally derived from epitheloid carcinoma tissue from a human donor. This initial data will illustrate the availability of the mammalian tissues extracts to recognize and cleave these lesions from synthetic oligonucleotides. This study could easily be expanded to study nuclear extracts prepared from mOGG-/- mice and NEIL1 knockdown mice for

their ability to repair the further oxidized lesions, as well as the toxicity and mutagenicity of chromium in a relevant mammalian repair-deficient system.

3.2 Formation and HPLC Separation of Gh and Sp

Reaction of the Cr(V)-Salen complex with the 22-mer 8-oxoG containing oligonucleotide demonstrated the formation of the further oxidized products of 8-oxoG, specifically spiriminodihydantoin (Sp) and guanidinohydantoin (Gh). These lesions can be distinguished from the original 8-oxoG product by separating the different lesions on a HPLC system. As shown in the HPLC chromatogram in Figure 3.1, the formation of oxidized products is observed in the reaction between 100 μM DNA and 800 μM Cr(V)-Salen in 10 mM phosphate buffer at pH 7.0 after a 20 min incubation at room temperature. The elution profile monitored at 260 nm showed two primary oxidation products from the reaction of 8-oxoG and Cr(V)-Salen as well as an unreacted 8-oxoG peak. The five peaks in the reaction were collected, ethanol precipitated, and lyophilized to dryness. Electrospray ionization mass spectrometry, ESI-MS, in negative ion mode was performed and mass differences for the oxidized species were calculated as previously described.[22] Peak 3 was found to be the unoxidized 8-oxoG containing oligonucleotide as expected by their coelution on the HPLC system. The first two peaks (Peaks 1 and 2) were found to give identical M-10 mass changes suggesting that they are structural isomers or tautomers of the same oxidized product. The identity of the oxidized products has been determined to be guanidinohydantoin and its isomer iminoallatoin from the corresponding mass change based on literature precedent [24] for the formation of products observed with Ir(IV) treatment of the 8-oxoG nucleoside and 8-oxoG containing oligomers. Peak 4 in Figure 3.1 corresponds to a M + 16 shift. This

mass shift is characteristic of a Sp containing oligonucleotide and was consistent with that observed for treatment of the 8-oxoG nucleoside with the Cr(V)-Salen complex.[22]

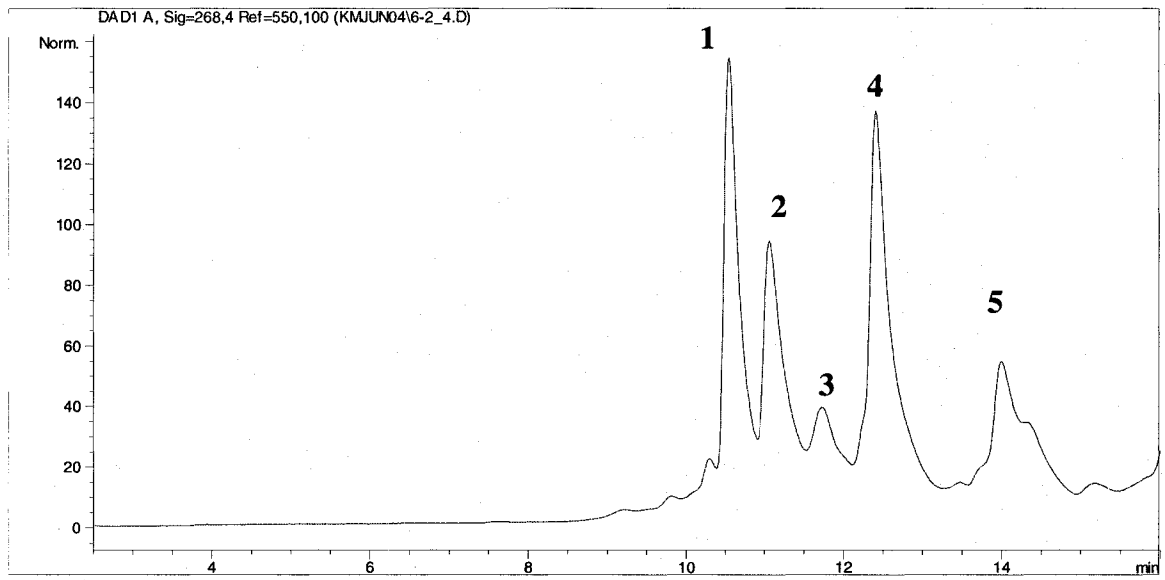

Figure 3.1: HPLC chromatogram of the 8-oxoG-containing oligonucleotide reacted with Cr(V)-Salen. Peaks 1 and 2 show the formation of guanidinohydantoin (Gh), and its isomer iminoallatoin. Peak 3 shows the unreacted 8-oxoG. Peak 4 corresponds to the formation of spiroiminodihydantoin (Sp). Peak 5 is an oxidized product that has yet to be identified.

Cr(V)-Salen at room temperature does not preferentially form either Gh or Sp, but instead gives a mixture of the these further oxidized lesions (Figure 3.2) that are readily separated from one another, and any unreacted 8-oxoG containing oligonucleotide, by HPLC as described above. Gh and Ia are isomers that readily interconvert and are treated as a single lesion. These lesions were placed at nucleotide positions 7 or 9 in the upper DNA strand. No position dependent differences in recognition and cleavage of the modified DNA by the NEIL enzymes were observed.

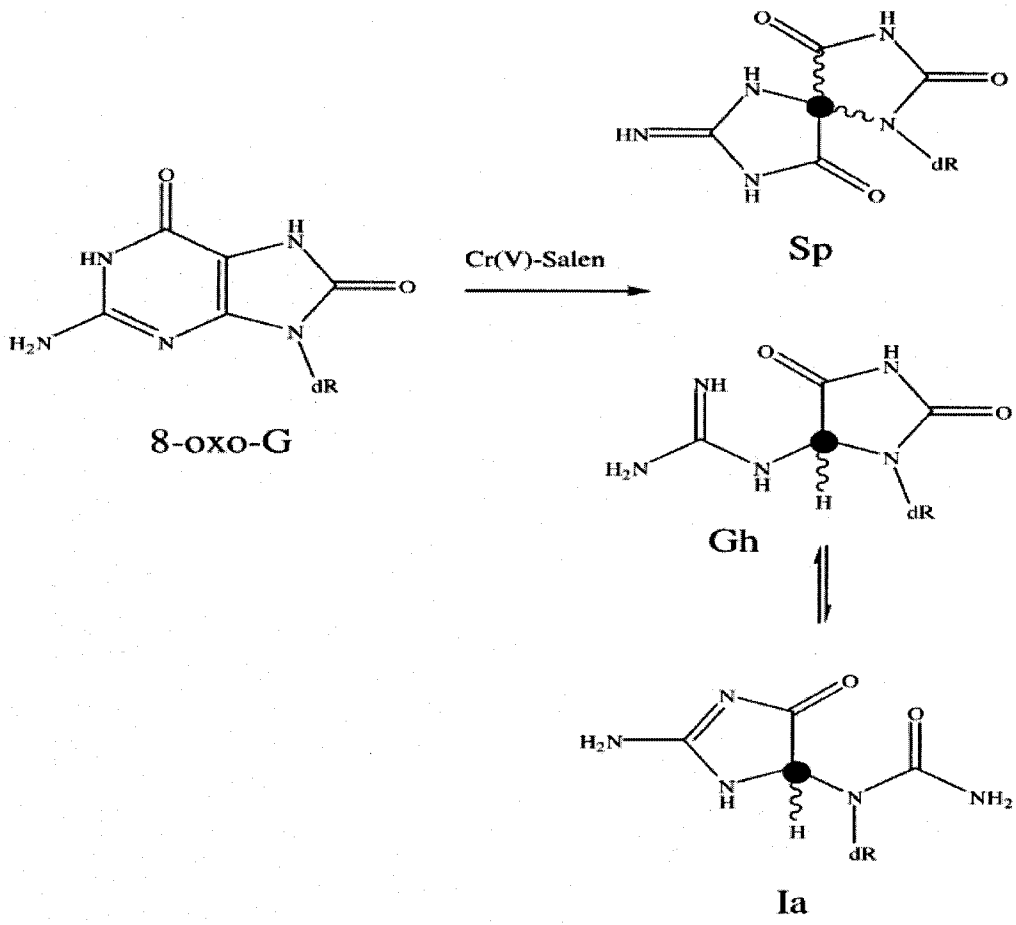

Figure 3.2: Oxidation of 8-oxoG by Cr(V)-Salen produces the lesions guanidinohydantoin (Gh), and spiroiminodihydantoin (Sp). Previous studies have shown that Gh equilibrates with an isomeric form, iminoallantoin (Ia). The highlighted atom in the structure denotes the sp^3 carbon.

3.3 NEIL1 and NEIL2 Affinity for 8-oxoG, Gh, and Sp in ssDNA

NEIL1 and NEIL2 have been shown to catalyze the removal of 5-OHU, TG, FapyG, and FapyA from duplex, single-stranded, and bubble-structure oligonucleotides. Their ability to recognize and excise hydantoin products such as Gh/Ia and Sp, that arise from further oxidation of 8-oxoG, has not previously been examined. NEIL1 and NEIL2 recognition and cleavage assays for Gh/Ia, Sp, and 8-oxoG lesions in single-stranded

DNA were carried out using a 22-nt oligonucleotide containing an oxidized lesion at either position 7 or 9 from the 5'-end as indicated in the figure legend. An unmodified oligonucleotide containing a guanine instead of an oxidized lesion was used as a negative control in these reactions. The four different single-stranded nucleotide substrates (control/unmodified, Gh, Sp, or 8-oxoG) were reacted with NEIL1 or NEIL2 and aliquots were removed for cleavage analysis at 0, 15, 30, and 60 min (Figs. 3.3 and 3.4). The zero time point in these figures refers to the removal of the first aliquot of the reaction mixture for cleavage analysis, which was generally less than 1 min. As expected, the control/unmodified oligonucleotide showed no cleavage with the addition of either NEIL1 or NEIL2 (Figs. 3.3A, 3.4A). The single-stranded 8-oxoG containing oligo showed very slight strand cleavage with NEIL1 (Fig. 3.3B), while NEIL2 showed no recognition and cleavage for the 8-oxoG substrate (Fig. 3.4B) over the 60 min time course. With the Sp and Gh modified 22-nt substrates, two product fragments were resolved by denaturing polyacrylamide gel electrophoresis. The faster migrating product band resulting from cleavage by NEIL1 and NEIL2 was assigned as the δ-elimination product (Figures 3.3C and D, 3.4C and D). The slower migrating cleavage bands in these reactions were assigned as the β-elimination products (Figures 3.3C and D, 3.4C and D). The basis for the assignment of the elimination products are given in Figure 3.5. NEIL2 showed mainly δ-elimination when excising the Sp containing oligonucleotide although a smaller amount of β-elimination product was also observed. The Gh modified oligonucleotide showed all δ-elimination products in this same assay.

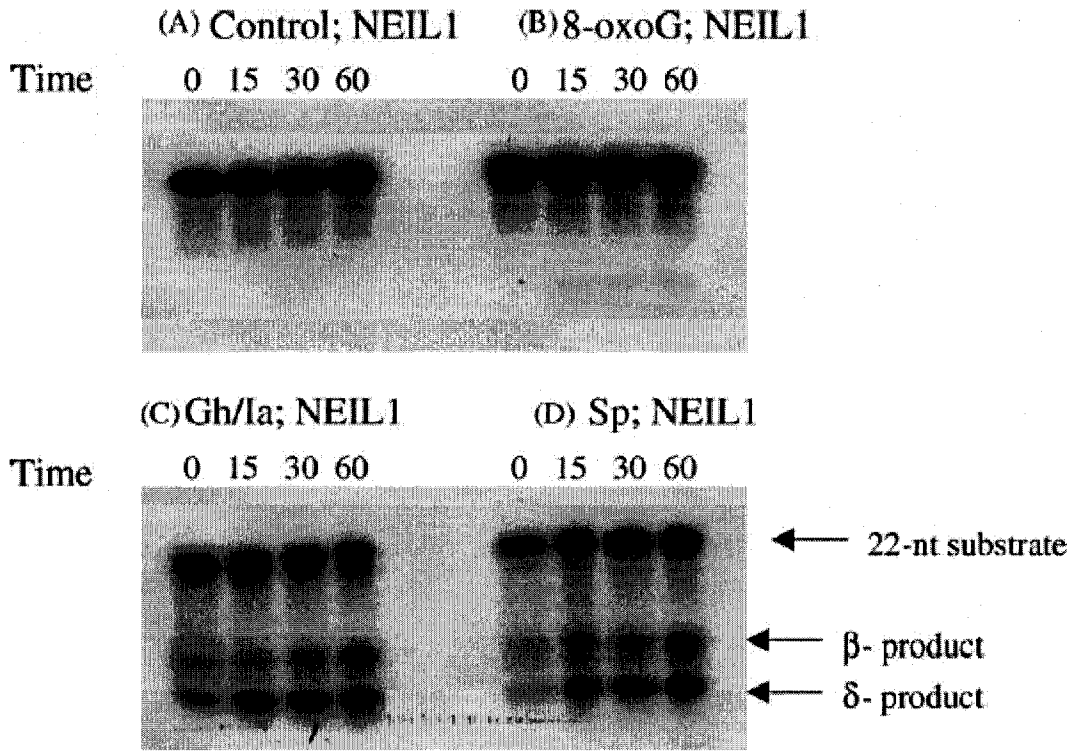

Figure 3.3: Cleavage of oxidatively modified ssDNA by the BER enzyme NEIL1: (A) control/unmodified (no lesion); (B) 8-oxoG modified ss-oligo; (C) Gh modified ss-oligo; (D) Sp modified ss-oligo. In each gel, reactions were performed at 37 °C with 500 nM of DNA, with the lesion positioned at the seventh nucleotide, and 30 nM of NEIL1 with time points of 0, 15, 30, and 60 minutes.

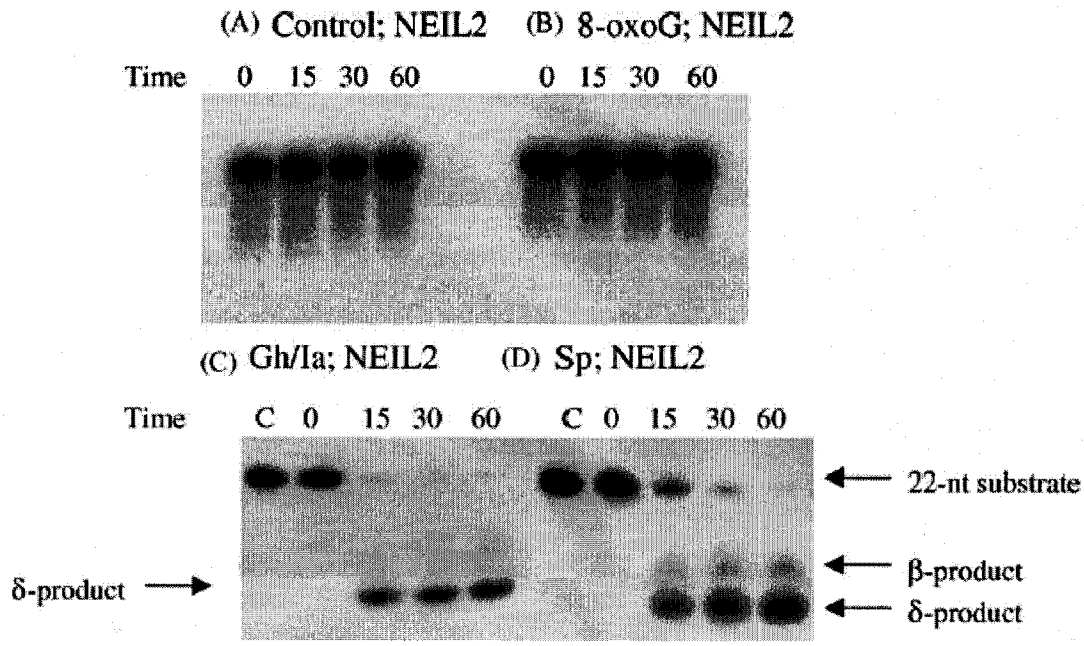

Figure 3.4: Cleavage of oxidatively modified ssDNA by the BER enzyme NEIL2: (A) control/unmodified (no lesion); (B) 8-oxoG modified oligo; (C) Gh/Ia modified oligo, and (D) Sp modified oligonucleotide. In each gel, reactions were performed at 37 °C with 500 nM DNA (with the lesion positioned at the seventh nucleotide for 8-oxoG and Gh and at the ninth nucleotide for Sp), and 30 nM NEIL2 with time points of 0, 15, 30, and 60 minutes.

The amount of cleavage products formed at the 60 min time point was quantified using densitometry. NEIL1 was found to catalyze the removal of approximately 45% of both Gh and Sp lesions from the single-stranded oligonucleotide after 60 min. NEIL2 showed a higher degree of cleavage activity for both the Gh and Sp lesions than NEIL1. Even at the shortest incubation time, 15 min, NEIL2 was able to excise nearly 100% of both Gh/Ia and Sp lesions from the single-stranded oligonucleotide (Figure 3.4C, D).

Assignment of β-elimination and δ-elimination termini from these reactions was carried out by band alignment with BER glycosylases that have established cleavage mechanisms (Figure 3.5). The glycosylase hOGG1 shows only β-lyase activity when

cleaving an 8-oxoG modified double-stranded oligonucleotide (Figure 3.5; Lane 1). This allowed assignment of the slower migrating fragment of cleaved DNA from NEIL1 and NEIL2 reaction with Gh and Sp modified double-stranded substrates as the β-elimination product (Figure 3.5; Lanes 4 and 8). Addition of Fpg, which has β/δ lyase activity, to the reaction mixture after a 2 hour incubation with the appropriate NEIL enzyme showed the formation of the faster migrating δ-elimination product (Figure 3.5; Lanes 3, 5, 7, 9).

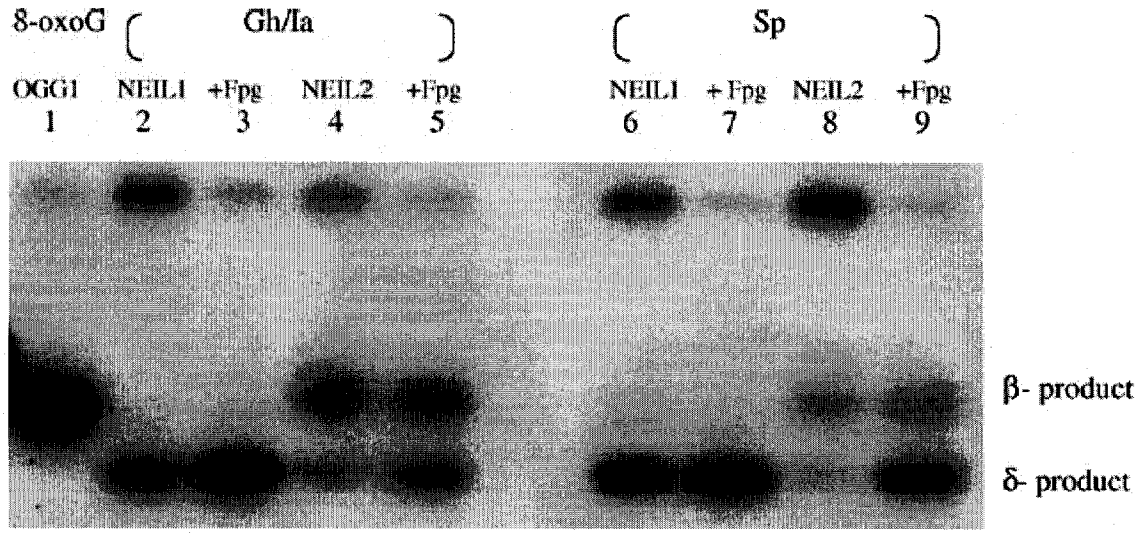

Figure 3.5: Determination of cleavage termini through band alignment with known glycosylases. Lane 1, 8-oxoG modified oligo treated with hOGG1; Lane 2, Gh modified oligo treated with NEIL1; Lane 3, Gh modified oligo treated with NEIL1 + Fpg; Lane 4, Gh modified oligo treated with NEIL2; Lane 5 Gh modified oligo treated with NEIL2 + Fpg; Lane 6, Sp modified oligo treated with NEIL1; Lane 7, Sp modified oligo treated with NEIL1 + Fpg; Lane 8, Sp modified oligo treated with NEIL2; Lane 9, Sp modified oligo treated with NEIL2 + Fpg. All reactions were incubated with the appropriate glycosylase(s) for 3 hr at 37 °C using 500 nM DNA with the lesion positioned at the seventh nucleotide, and 30 nM repair enzyme. The Fpg in lanes 3, 5, 7, and 9 was added after a 2 hr incubation with the NEIL enzymes.

3.4 NEIL1 and NEIL2 Affinity for 8-oxoG, Gh, and Sp in dsDNA

The ability of NEIL1 and NEIL2 to recognize and cleave Gh and Sp in duplex DNA was determined using a 22 bp duplex oligonucleotide containing an X:Y base pair (where X was G, 8-oxoG, Gh, or Sp and Y was C, A, T, or G) at the same sites as that for the single-stranded oligonucleotides used in the studies described above. The X-containing strand was 5'-^{32}P-end-labeled prior to annealing to the complementary strand. The duplexes were incubated with NEIL1 or NEIL2 and aliquots removed for analysis by gel electrophoresis at 0, 15, 30, and 60 minute time points. The control/unmodified oligonucleotide showed no recognition and excision in our assay system by either NEIL1 or NEIL2 and only slight cleavage of the 8-oxoG lesion containing duplex DNA was observed under our conditions.

Reaction of Gh and Sp containing double-stranded oligonucleotides with NEIL1 led to the formation of cleavage bands at the site of the lesion. Once again, the major, faster migrating band was identified as the δ-elimination product and the slower moving band was assigned as the β-elimination product (Figure 3.6A-D) through termini alignment as described in Figure 3.5 previously. NEIL1 was found to efficiently recognize and excise the Gh and Sp lesions opposite all four natural DNA bases. However, differences in cleavage efficiency were noted for both the Gh and Sp, dependent upon the identity of the base opposite the lesion. NEIL1 showed the greatest excision efficiency for both the Gh and Sp lesions when opposite a thymine or adenine (~70% after 60 min), while the lowest amount of excision was observed when the Sp and Gh lesions were paired with a cytosine (~45% at 60 min). Overall, NEIL1 demonstrated better cleavage activity for both lesions in double-stranded versus single-stranded DNA.

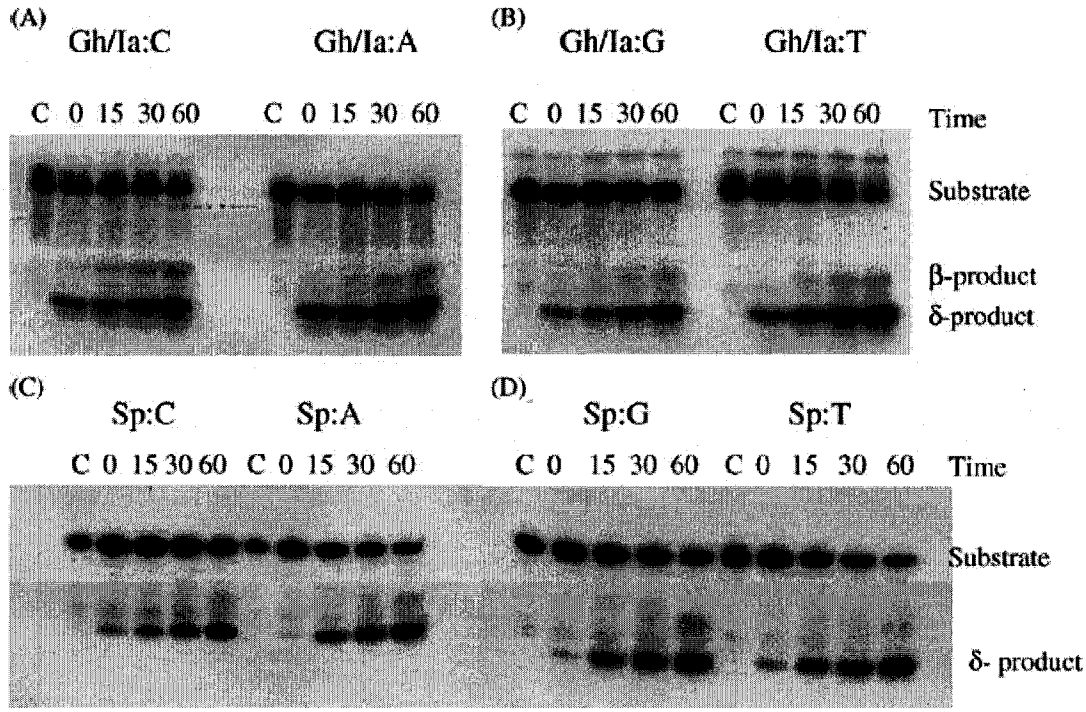

Figure 3.6: Cleavage assay of oxidatively modified duplex DNA with NEIL1 to determine substrate affinity for Gh and Sp opposite the four natural bases, figures A-D. Reactions were carried out at 37 °C using a DNA concentration of 500 nM, with the lesion positioned at the seventh nucleotide for Gh and at the ninth nucleotide for Sp, and a NEIL1 concentration of 30 nM with time points of 0, 15, 30, and 60 min. The C above the first band in each gel represents the control lane when no enzyme is present.

In contrast with the results obtained with NEIL1, NEIL2 was able to recognize and cleave Gh opposite all four DNA bases, but only a minor amount of cleavage was observed when NEIL2 was reacted with the Sp containing strand opposite any of the four bases (Figure 3.7A-D). Cleavage efficiency of Gh by NEIL2 was again dependent upon the base opposite the lesion. The Gh lesion was most efficiently cleaved by NEIL2 when paired opposite thymine and cytosine (~60% at 60 min) while it was least efficiently cleaved when opposite guanine (~45% at 60 min). Interestingly, NEIL2 appeared to

catalyze the removal of the lesions by a β-elimination step in double-stranded DNA under our conditions (Figure 3.7A-D), in contrast with β- and δ-elimination products observed in single-stranded DNA substrates.

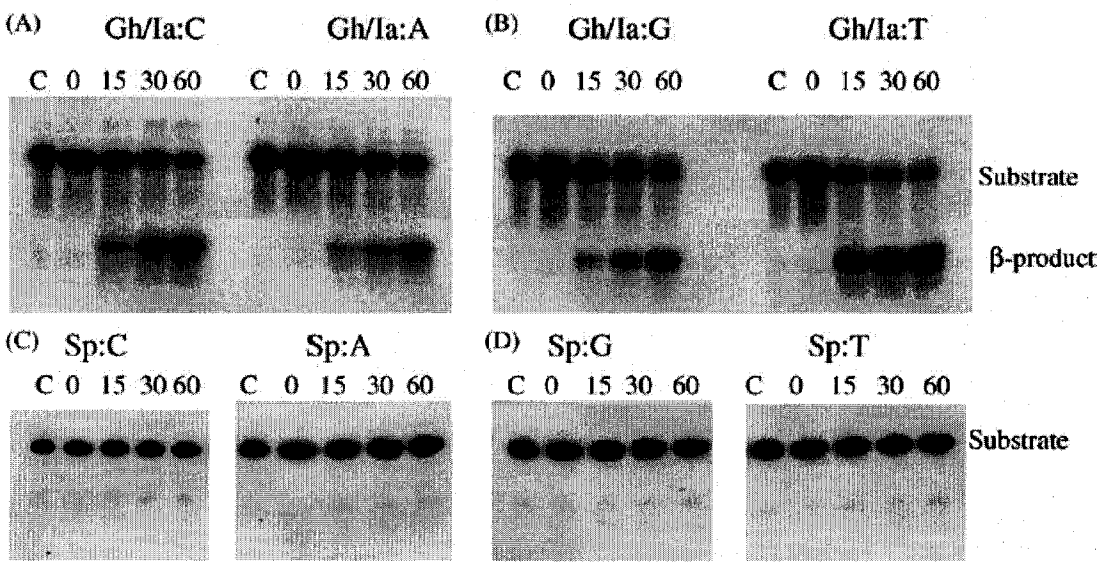

Figure 3.7: Cleavage assay of oxidatively modified duplex DNA with NEIL2 to show substrate affinity for Gh and Sp opposite the four natural bases, figures A-D. Reactions were carried out at 37 °C using a DNA concentration of 500 nM, with the lesion positioned at the seventh nucleotide for Gh and at the ninth nucleotide for Sp, and a NEIL2 concentration of 30 nM with time points of 0, 15, 30, and 60 minutes. The C above the first band in each gel represents the control lane when no enzyme is present.

A trapping assay was used in this study to help identify glycoyslase/AP lyase activities of NEIL1 and NEIL2 for the oxidized lesions, Sp and Gh/Ia. The control (guanine) and lesion containing oligonucleotides were tested for trapping with both enzymes with DNA in single- and double-stranded forms. NEIL1 and NEIL2 formed

trapped complexes with Sp and Gh containing substrates in both single-stranded and duplex DNA, but little trapping of the enzyme was observed with the 8-oxoG and no trapping was observed for the control oligos (Figure 3.8A and B). The low level of trapping of the 8-oxoG lesion is in good agreement with the low level of cleavage observed with NEIL1 and NEIL2 towards this lesion. Interestingly, a significant amount of DNA/enzyme trapping occurred between the duplex Sp containing oligonucleotide and NEIL2 (Figure 3.8B, Lane 6) even though NEIL2 showed negligible cleavage of Sp when in duplex DNA. Cytosine was the only base tested opposite the lesion in the duplex DNA.

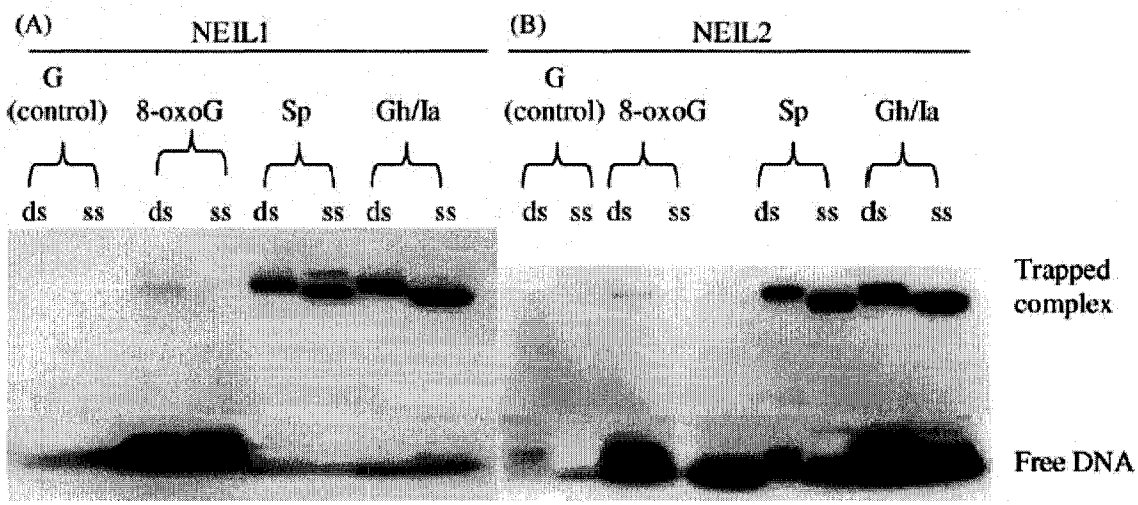

Figure 3.8: Analysis of NEIL1 and NEIL2 trapped complexes with oligonucleotides containing guanine (control), 8-oxoG, Sp, or Gh/Ia. (A) NEIL1 reacted with double- and single-stranded control (Lanes 1 and 2), double- and single-stranded 8-oxoG (Lanes 3 and 4), double- and single-stranded Sp (Lanes 5 and 6), and double- and single-stranded Gh/Ia (Lanes 7 and 8). (B) NEIL2 reacted with double- and single-stranded control (Lanes 1 and 2), double- and single-stranded 8-oxoG (Lanes 3 and 4), double- and single-stranded Sp (Lanes 5 and 6), and double- and single-stranded Gh/Ia (Lanes 7 and 8).

In summary, NEIL1 and NEIL2 were able to efficiently recognize and cleave Gh opposite the four different DNA bases with relatively similar efficiencies but only NEIL1 showed efficient recognition and excision of the Sp lesion in double-stranded DNA. NEIL2 did not significantly cleave the Sp lesion from double-stranded DNA under our reaction conditions regardless of the base opposite the lesion even though this lesion in duplex DNA showed good recognition through our DNA trapping studies. This is in contrast to the almost 100% cleavage observed with NEIL2 when the Sp lesion was present in single-stranded DNA.

3.5 HeLa Nuclear Extract Repair Proficiency

The ability of these lesions to be recognized and removed from an oligonucleotide strand by base excision repair enzymes from a cell lysate solution was also tested. HeLa nuclear extract solutions were used to determine the repair proficiency of the cell line versus the oxidized guanine lesions that were formed in an oligonucleotide strand by chromium exposure. Oligonucleotide strands containing either 8-oxoG, Gh, or Sp were incubated with HeLa nuclear extracts at 37 °C for 3 hrs. Samples were then run on a denaturing 7M urea gel to determine the extent of cleavage of the oxidized guanine lesion from the oliognucleotide strand (Figure 3.9). As shown in Figure 3.9, both the 8-oxoG and Sp containing oligonucleotides were cleaved to appreciable amounts at the 3 hour time point. Since hOGG1 has been shown to have very little affinity for the Sp lesion, it is plausible to credit this cleavage of the Sp lesion to the newly identified NEIL proteins.

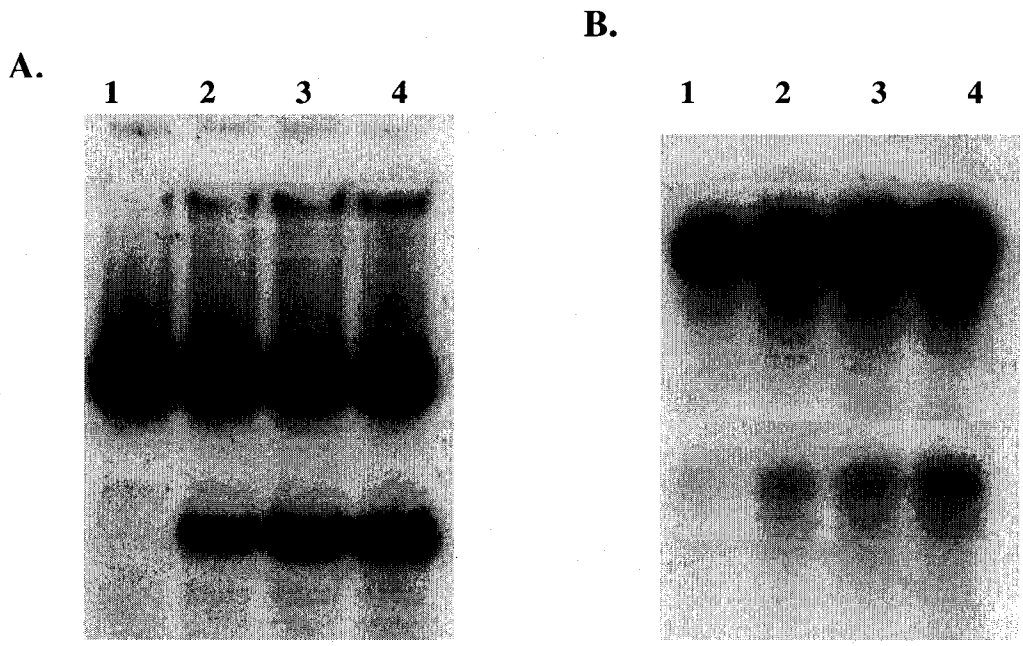

Figure 3.9: Analysis of repair proficiency of HeLa nuclear extracts with double stranded oligonucleotides containing the 8-oxoG and Sp lesions. (A). 8-oxoG containing oligonucleotide reacted with HeLa nuclear extract for 3 hrs at 37 °C. Samples were taken at 4 different time points. Lane 1, 0 time point; Lane 2, 1 hr; Lane 3, 2hrs; Lane 4, 3 hrs. (B). Sp containing oligonucleotide reacted with HeLa nuclear extract for 3 hrs at 37 °C. Lane 1, 0 time point; Lane 2, 1 hr; Lane 3, 2 hrs; Lane 4, 3 hrs. All samples were heated to 95 °C in formamide loading buffer before being run on a 15% 7M urea gel and visualized by autoradiography.

3.6 Discussion and Conclusions

While 8-oxoG is a common biomarker of oxidative damage, its sensitivity towards further oxidation necessitates the need to study the subsequent formation of further oxidized species. The further oxidation of 8-oxoG can be initiated by a number of redox-active compounds including transition metals, ionizing radiation, and photochemical reactions. One redox-active transition metal capable of further oxidizing the 8-oxoG lesion is the cationic Cr(V)-Salen complex which is thought to mimic high-valent chromium intermediates formed during the cellular reductive metabolism of the

human carcinogen chromate. The mechanism by which Cr(VI) induces cancer is unknown although a significant body of evidence suggests that oxidative damage to DNA is a critical step. During the reduction of Cr(VI) to Cr(III), formation of transient and highly oxidizing Cr(V) oxidation states of this metal have been observed.[43] It has been postulated that these transient high-valent states of chromium are responsible for much of the oxidative damage observed in Cr(VI)-treated DNA.

We have identified and characterized the formation of Gh and Sp as the two major products of 8-oxoG oxidation when reacted with Cr(V)-Salen in vitro (Fig. 3.1).[22] The failure to repair these oxidized lesions of guanine, prior to replication, have been shown to lead to mutations. This oxidative damage to single DNA bases is recognized and repaired by the base excision repair (BER) pathway.[31] The ability of oxidized base specific DNA glycosylases to repair Gh and Sp residues in DNA has been thoroughly studied in *E. coli* [42], but to date no mammalian homolog of these glycosylases had been identified that have a substrate specificity for these further oxidized lesions of 8-oxoG. In this chapter, we have described the relative affinity of the newly identified mammalian DNA glycosylases, NEIL1 and NEIL2, to recognize and cleave the further oxidized lesions of Gh and Sp.

The substrate preferences for mammalian NEIL1 and NEIL2 DNA glycosylases are still being determined. NEIL1 and NEIL2 belong to the Fpg/Nei family of enzymes based on their reaction chemistry and homology to the bacterial enzymes. NEIL1 and NEIL2 both recognize the further oxidized lesion Gh in double- and single-stranded DNA. This result is of some interest since many glycosylases are dependent on the complementary strand to help identify and repair the lesion in question. The Sp lesion

gives contrasting results between the two enzymes. NEIL1 efficiently cleaved Sp from single- and double-stranded DNA while NEIL2 was able to cleave the Sp containing DNA only when it was in the single-stranded form.

NEIL2 has β-lyase activity that makes it more like hOGG1 than Fpg and Nei in terms of its backbone cleavage. Examination of the active site of hOGG1 revealed a catalytic lysine poised at the 3'-hydroxyl and the cleaved 8-oxoG nucleobase remains poised in the active site such that it can hydrogen bond to the Nε of the catalytic lysine, and the O4' of the sugar, to act as a catalytic acid/base in the β-lyase cascade.[44] The cleaved residue is held in position by base stacking interactions with a phenylalanine residue and hydrogen bonding between the N1 of 8-oxoG and a glutamine residue. The profound loss of planarity in the Sp lesion would preclude both of these interactions thereby diminishing the capacity of Sp to assist in the catalytic acid/base cascade. It may also be that the comparatively bulky Sp structure occludes the backbone preventing access to this site by the catalytically important amino acid residues required for lyase activity. These conclusions are highly speculative in the absence of detailed structural studies of the NEIL2 protein active site. However, we anticipate that the Sp lesion may prove to be a highly informative substrate with which to probe the NEIL2 active site. The strong preference for this enzyme for single-stranded DNA, however, infers that its biological role may lie more with single-stranded substrates, either in bubble structures or within the transcription coupled repair system.

NEIL1 retains its β-, δ-lyase activity when acting upon double-stranded DNA, which it appears to cleave with slightly higher affinity than single-stranded DNA. In this regard its activity appears more similar to the bacterial Nei enzyme after which it was

named. The trapped DNA-Nei complex reveals an extensive arg-lys hydrogen bonding network to the C1'-carbon and to the phosphate on both sides of the lesion that may contribute to the β-, δ-lyase activity of this enzyme.[45] Interestingly, Zharkov et al could not obtain a structure of the enzyme recognizing the lesion even after soaking crystals with lesion, implying that the active site has little affinity for the lesion upon cleavage and that lyase activity is dependent only upon the acid/base catalysis of the protein amino acids.[45] Doublie et al identified arginine residues within the putative active site of NEIL1 that could potentially be catalytic.[46] However, mutation of these residues to alanine mitigated glycoylase activity without impacting the lyase activity of the enzyme. The basis of the lyase activity of the NEIL1 enzyme remains to be determined.

Structural perturbations of the DNA may play an important role in damage recognition by NEIL1 and NEIL2. The oxidation of 8-oxoG to form the Sp lesion generates a tetrahedral, sp^3 carbon within the normally planar nucleic acid base ring structure (Fig. 3.2). This conformational change would be expected to disrupt normal base pair stacking in duplex DNA and significantly distort the DNA helix. This distortion could play a substantial role in the BER enzymes ability to recognize and cleave Sp lesions formed within DNA. While oxidation of 8-oxoG to form Gh also generates a tetrahedral, sp^3 carbon, free rotation about the tetrahedral atom may allow the modified nucleic acid base to remain relatively planar in duplex DNA and should cause less distortion of the DNA helix than Sp. Interestingly, formation of an 8-oxoG within duplex DNA has shown little or no distortion of the normal B-form DNA helix by X-ray crystallography.[47] These data suggest that a structural basis may exist for the recognition and cleavage of Sp and Gh in DNA.

NEIL1 and NEIL2 have both shown a preference for oxidized pyrimidine bases in DNA.[36-38,40] However, several groups have also shown a weak but most likely significant activity against 8-oxoG:C [36-38], 8-oxoG:A [38] pairs as well as 8-oxoG in single-stranded bubble structures.[32] NEIL1 and NEIL2 possess the ability to recognize and cleave the Sp and Gh lesions in single- and double-stranded DNA, with the ability to act on lesions in single-stranded DNA being reported previously only for uracil-DNA glycosylase.[48] This ability to cleave lesions in ssDNA, specifically the Gh and Sp lesions, could implicate the NEIL glycosylases as being involved in different repair pathways.

The formation of 8-oxoG, Sp and Gh have been established in vitro and bacterial systems, but specifically the formation of Sp and Gh need to be extended into a mammalian system. Oligonucleotides containing 8-oxoG and Sp were incubated with HeLa nuclear extract, which has been shown to be repair proficient. After a 3 hour incubation, significant cleavage was visible in both lesion containing oligoncleotides (Figure 3.9). This preliminary data supports the hypothesis that Sp formation could occur from an oxidative event in a mammalian system. Immunoprecipitation and knockdown studies could be used to further support these preliminary findings. HeLa nuclear extract could be depleted of hOGG1 and NEIL1 by immunoprecipitation to determine if the amount of cleavage of the lesions is diminished with the lack of these two repair proteins. This study could also be expanded to study nuclear extracts prepared from mOGG-/- mice and NEIL1 knockdown mice that have and have not been exposed to an oxidative event, such as chromium exposure, for their ability to repair these further oxidized guanine lesions.

The thermodynamics of base oxidation coupled with the recent discovery of "hole migration" for redox chemistry in DNA strands suggest that 8-oxoG would not be the terminal oxidation product that arises within a cell following an oxidative event. If these further oxidized lesions of 8-oxoG are common in cellular systems, and can be recognized and cleaved by BER enzymes NEIL1 and NEIL2, these results could help to explain the mild phenotypes observed in $Ogg1^{-/-}$ and $Nth1^{-/-}$ mice. These results may also shed light on the mechanism of oxidative DNA damage by the human carcinogen chromate and the resulting pathways for repair of this damage.

3.7 Experimental Procedures

3.7.1 Forming Guanidinohydantoin and Spiroiminodihydantoin

Cr(V)-Salen synthesis: The chromium complex N,N-ethylenebis(salicylidene-animato)oxochromium(V), $[Cr(Salen)(H_2O)_2]PF_6$, containing the N,N-bis(salicylidene)ethylenediamine (Salen) ligand was sysnthesized in the trivalent oxidation state as the hexafluorophosphate salt, using the method of Coggon and McPhail.[49] Reddish brown needles crystallized from water were analyzed by UV/vis spectroscopy in acetonitrile, yielding absorbance spectra, extinction coefficients, and purity comparable to those reported previously for this complex.[49] Stock solutions of the Cr(V)-Salen complex in dry acetonitrile were prepared by dissolving an equimolar amount of the Cr(III)-Salen complex with the oxidant iodosylbenzene.[50] Formation of the Cr(V) oxidation state was confirmed by electron paramagnetic resonance spectroscopy.[51]

Site-specific formation of guanidinohydantoin and spiroiminodihydantoin in DNA: The Gh or Sp lesions were formed at specific sites within the oligonucleotide by reacting Cr(V)-Salen with the 8-oxoG containing oligonucleotide. The 8-oxoG containing oligonucleotides were purchased from TriLink BioTechnologies and the complementary unmodified strands were purchased from Integrated DNA Technologies. The oligonucleotide sequences used were: d(5'-AGTTGAX_1GX_2GACTTTCCCAGCC-3') with complement d(5'-GGCTGGGAAAGTCY_2CY_1TCAACT-3'), where X_1 and X_2 were either 8-oxoG or G, and Y_1 or Y_2 were C, A, G, or T. Reactions between the Cr(V)-Salen complex and the oligonucleotides were carried out in 10mM sodium phosphate buffer (pH 6.0) in 50 μL volumes. A 20 mM stock solution of the Cr(V)-Salen in dry acetonitrile was prepared fresh, and a typical reaction mixture contained 10-100 μM DNA and 800 μM Cr(V)-Salen. Reactions were allowed to proceed at room temperature for 20 minutes prior to HPLC purification.

HPLC analysis of oxidation products: Residual chromium was removed from the reaction mixture using a BioRad Micro Bio-Spin P6 chromatography column. The chromium-treated DNA was reinjected on the HPLC using the same separation conditions as described above. Eluting peaks corresponding to Gh and Sp were collected, evaporated to near dryness, and residuals salts removed using a second BioRad P6 column and eluting the DNA in deionized water. The modified oligonucleotides were resuspended in an aqueous buffer containing 2.5 mM imidazole and 2.5 mM piperidine. Mass spectral characterization of purified nucleoside oxidation products was carried out on these samples with addition of 10% aqueous MeOH. ESI-MS spectra were obtained on a Micromass Quattro II tandem mass spectrometer. The oligomers were introduced

into a QTOF mass spectrometer by direct infusion via a syringe pump at a flow rate of 5 μL/min. The capillary voltage was set to -2200V, and ion signals were detected in the negative ion mode. The initial spectra were charge-state deconvoluted using the transform algorithm featured in the Micromass Mass Lynx version 3.4 software package.

Substrate DNA preparation. A 850 pmol reaction of the modified oligonucleotide was 5'-^{32}P-end labeled with T4 polynuleotide kinase according to the manufacturer's protocol. Excess ^{32}P-γ-ATP was removed with a BioRad Micro Bio-Spin P6 column prior to use. The labeled oligonucleotide was annealed to the complementary strand, which was added in slight excess (15-20%). The annealing step was performed in a 10 mM Tris-HCl (pH 7.4) buffer and was heated to 95 °C and then slowly cooled over a 3 hour period. A 5'-labeled oligonuclotide, either single- or double-stranded, was frozen as a stock solution and the appropriate concentration was removed for the subsequent cleavage assays.

3.7.2 NEIL1 and NEIL2 Cleavage Assay

Purification of NEIL1 and NEIL2. Murine NEIL1 and NEIL2 purified enzymes were a gift from Dr. Thomas Rosenquist of the State University of New York (SUNY), Stoneybrook. The murine NEIL1 and NEIL2 enzymes containing C-terminal his6-tags were cloned, expressed and purified as described previously [40].

DNA recognition and cleavage assays with NEIL1 and NEIL2. Double and single stranded oligonucleotides containing 8-oxoG, Gh, or Sp were analyzed for recognition by the NEIL1 and NEIL2 enzymes. Solutions of oligonucleotides (500 nM) were reacted with 30 nM NEIL1 or NEIL2 in a reaction buffer consisting of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA. Reactions were carried

out at 37 °C and 5 μL aliquots were removed at 0, 15, 30, and 60 min for analysis of DNA cleavage. At each time point the samples were quenched by the addition of an equal volume (5 μL) of formamide denaturing loading dye (10 mL formamide, 10 mg xylene cyanol FF, and 10 mg bromophenol blue) that was preheated to 95 °C. The samples mixtures were placed at 95 °C for 4 min before loading on a 15% TBE, 7M urea pre-cast BioRad mini-gel. Gels were run in Tris-Borate-EDTA buffer for ~40 min at 180V. The bands were analyzed by autoradiography and the faster migrating bands from glycosylase recognition and excision were quantified by densitometry using a BioRad GS-800 Claibrated Densitometer with the QuantityOne Software package.

Analysis of trapped DNA complexes by NEIL1 and NEIL2. ^{32}P-labeled single- and double-stranded oligonucleotides (500 nM) containing guanine (control) or the 8-oxoG, Sp or Gh lesions were incubated with either NEIL1 or NEIL2 enzymes (30 nM) in 60 μL reaction buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA) in the presence of 50 mM $NaCNBH_3$ at 37 °C for 90 min. A 10 μL trapped enzyme/DNA complex was removed and the reaction was topped with the addition of 15 μL of 2X SDS gel loading buffer. Samples were heated to 95 °C for 10 min and flashed cooled. Samples were separated on a 12% SDS-PAGE gel with a 5% stacking layer and the DNA-protein complexes were visualized by autoradiography.

3.7.3 HeLa Nuclear Extract Assay

DNA excision repair catalyzed in vitro by HeLa Nuclear Extract. ^{32}P-labeled double stranded oligonucelotides (1000 nM) containing guanine (control), 8-oxoG, or Sp lesions were incubated with HeLa nuclear extract (18 μg) in a PBS binding buffer (0.1 M phosphate, 0.15 M NaCl (pH 7.2)). The total reaction mixture (60 μL) was allowed to

react at 37 °C for up to 3 hours. 5 μL aliquots were taken at 0, 1, 2, and 3 hour time points. At each time point, samples were quenched with 5 μL of 95 °C formamide loading buffer (10 mL formamide, 10 mg xylene cyanol FF, 10 mg bromophenol blue). All samples were heated to 95 °C for 5 min previous to loading and flash cooled. The samples were run on a 15% TBE 7 M Urea mini gel at 250 V for 40 min and visualized by autoradiography.

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Chapter 4: The Formation and Cellular Accumulation of Spiroiminodihydantoin from Chromium Exposure

4.1 The Oxidation of Guanine by Cr(VI) and Ascorbate Forms Spiroiminodihydantoin

Further oxidation of the ubiquitous 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxoG) lesion in DNA has been a recent topic of considerable interest. An ever increasing number of oxidants including photosensitizing agents [1], peroxynitrite [2], carbonate radicals [3], and high valent metals such as Ir(IV) [4] and Cr(V) [5] have all been observed to specifically react at 8-oxoG sites within duplex DNA to form further oxidized lesions. Previously, the reaction of Cr(V) complexes with single-stranded DNA containing the 8-oxoG lesion has shown further oxidation exclusively at the 8-oxoG site.[5,6] The further-oxidized lesions formed in this reaction were determined to be spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh).[5]

The Sp and Gh lesions show an enhanced polymerase arresting capability in comparison to the parent 8-oxoG lesion as well as significantly increasing levels of transversion mutation in vitro and in cellular systems.[6-9] Significantly, these transversion mutations are the primary mutations observed in human lung tumors from chromate-exposed workers and in shuttle vector replication assays in Cr(VI)-treated mammalian cells.[10,11] Recognition and excision of the Sp and Gh lesions in DNA in vitro have been shown to occur through the bacterial Nei (Endonuclease VIII) base excision repair (BER) enzyme [12] and as shown in chapter 3, through the mammalian NEIL (Nei-like) BER glycosylases.[13] Whereas the NEIL glycosylases have a high affinity for recognition and

cleavage of DNA containing Gh and Sp, they show almost no affinity for the 8-oxoG lesion. While the thermodynamics of 8-oxoG oxidation to the Sp and Gh lesions coupled with the demonstrated recognition of these lesions by endogenous BER enzymes argues for their formation in cellular DNA, the refractory nature of these lesions to detection has previously kept them from being observed in cellular systems.

On the basis of our previous results, we propose that the further oxidized products of guanine, Sp and Gh, are equally if not more common in Cr(VI) oxidation reactions with DNA than 8-oxoG itself and, being more mutagenic, may play a primary role in chromium-induced carcinogenesis. These lesions had yet to be observed by the direct oxidation of guanine in duplex DNA with any carcinogenic metal. In this study, we have reacted a duplex DNA oligonucleotide with sub-toxic cell culture concentrations of Cr(VI) and a molar excess of the reductant ascorbate. The site- and sequence-specificity of DNA oxidation in this reaction was monitored using PAGE sequencing, and lesion formation was determined by a combination of reductive trapping by BER enzymes, HPLC-ESI-MS and HPLC-ECD. Sequencing gels of the Cr(VI)/Asc oxidation showed preferential oxidation of guanine over the other nucleobases, with enhanced oxidation at 5' guanines in purine runs. Reductive trapping of modified DNA with $NaCNBH_3$ and SDS-PAGE using base excision repair glycosylase specific to 8-oxoG and Sp (hOGG1 and mNEIL2, respectively) showed that NEIL2 had a much greater affinity to the lesions being formed than hOGG1, suggesting that 8-oxoG was not the major lesion formed in this reaction.

Also on the basis of our in vitro studies, we hypothesize that Nei^- deficient bacteria should show sensitivity, manifested as differential growth, toward the carcinogen

chromate if lesions such as Sp are formed during the intracellular reduction of Cr(VI) to Cr(III). Furthermore, the Nei deficient cell lines should accumulate the Sp and Gh lesions within their genomic DNA that correlates with the observed growth inhibition. This study analyzed a series of BER deficient *Escherichia coli* (*E. coli*) for differential growth inhibition toward chromate with respect to their wild-type counterparts. The 8-oxoG mutants, ($MutM^-$, $MutY^-$, and the $MutM^-/MutY^-$ double mutant), all demonstrated similar growth curves following chromate treatment to their matched wild-type counterparts. Only the Nei deficient *E. coli* (TK3D11) demonstrated a significant difference in growth with increasing doses of chromate over that of its wild type. The genomic DNA of the Nei^- and the $MutM^-/MutY^-$ double mutant, with respect to their wild-type controls, was assayed for the formation of the Sp lesion and the putative intermediate of this lesion, 8-oxoG. These results are the first to show the formation of the Sp lesion intracellularly and suggest that further-oxidized lesions of guanine, such as Sp, may be the predominant lesion formed intracellularly and may explain the mutagenicity and carcinogenicity of this metal.

4.2 Formation of Oxidized Guanine Products by Cr(VI) and Ascorbate

Previously, this lab has shown that a model Cr(V) complex reacts specifically with 8-oxoG in single-stranded oligonucleotides to produce the further-oxidized lesions of Sp and Gh.[5,6] In this present study, we chose to investigate whether one of these further oxidized lesions, Sp, could be formed directly from oxidation of guanine within normal duplex DNA. We also chose to determine whether these lesions could be formed from established Cr(VI) reduction parameters using the endogenous reductant ascorbate.

We next used our parameters in a cellular system, specifically in various strains of *Escherchia coli*, to determine if the Sp lesion was being formed and to what extent from Cr(VI) exposure. This would be the first identification of a further oxidized guanine lesion in a cellular system.

Identification of guanine specific oxidation sites by Cr(VI)/Ascorbate in duplex DNA. Duplex DNA with the ^{32}P-labeled upper strand sequence of 5'-AGT TGA GGG GAC TTT CCC AGC C-3' was treated with concentrations of Cr(VI) ranging from 3.1 to 50 μM and a 10-fold molar excess of ascorbate (31-500 μM). This range of Cr(VI) concentrations are commonly used in the treatment of mammalian cells in culture and, depending on the cell line, display toxicity profiles ranging from 0 to 100%.[14,15] Unidirectional uptake and reduction of Cr(VI) by A549 cells leads to low steady-state cellular exposure to Cr(VI), but cumulative exposures have been observed to be greater than 1 mM intracellular chromium (presumably as Cr(III)) from a 10 μM treatment of Cr(VI) in media.[14] Reduction of Cr(VI) by ascorbate in the presence of the DNA was allowed to take place for a minimum of 1 hour, at which time absorbance measurements of Cr(VI) at 372 nm showed complete reduction to Cr(III). Piperidine treatment was used to specifically cleave the DNA at the site of the lesion formation. The results in Figure 4.1 show that treatment of DNA with Cr(VI)/ascorbate resulted in the formation of piperidine labile cleavage sites preferentially at guanines. Lanes 1 and 2 are untreated DNA controls, with and without piperidine, respectively, and show little background cleavage. Lane 3 is the Maxam-Glibert A/G lane used for sequence-specific cleavage comparisons with the Cr(VI)/Asc reactions. Lanes 4-8 are piperidine treated duplex DNA samples treated with Cr(VI)/Asc with lane four containing the highest

concentration (50 μM/ 500 μM) of Cr(VI)/Asc and decreasing to the lowest concentration in lane 8 (3.1 μM/ 31 μM). The oxidation of the duplex DNA oligonucleotide by Cr(VI)/Asc indicated that oxidation occurs almost exclusively at guanine and that guanines that are 5' in a run of guanines (or 5' in a run of purines such as G^o) show enhanced oxidation (Figure 4.1). Similar reactions with ascorbate alone showed little or no cleavage of DNA upon piperidine treatment.

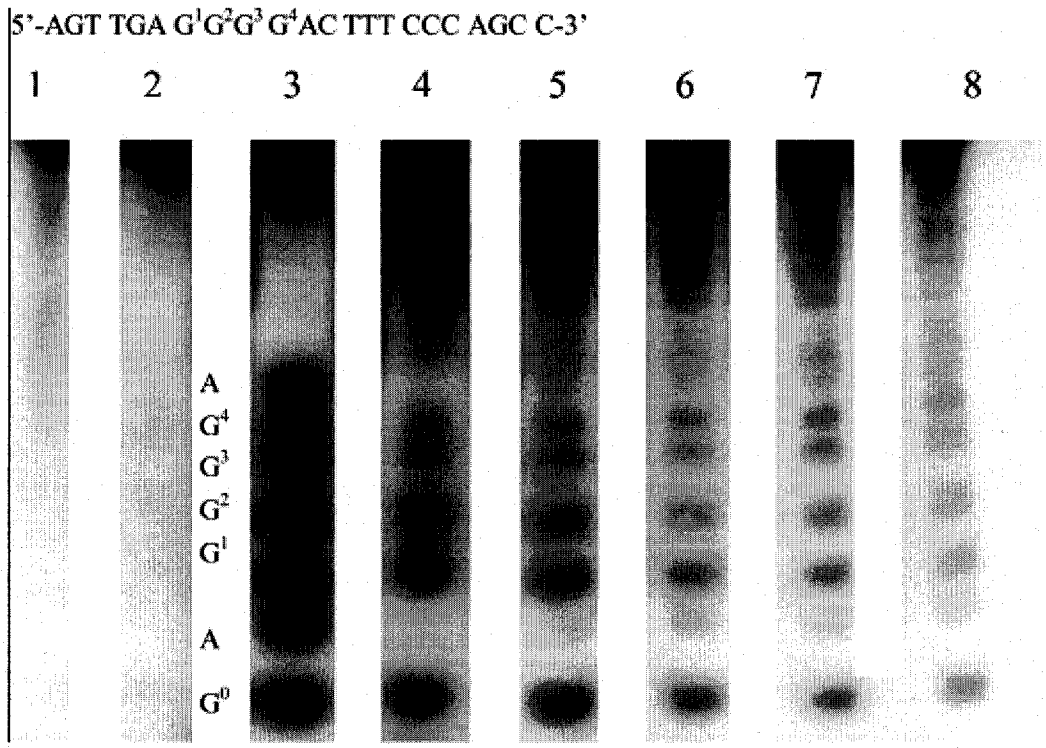

Figure 4.1:. Sequencing gel showing the concentration dependence and site-specificity of DNA oxidation by Cr(VI) and ascorbate. Lanes 1 and 2 are untreated DNA control with and without piperdine treatment, respectively. Lane 3 shows the Maxam-Gilbert G/A lane. Lanes 4-8 show the Cr(VI)/Asc treated DNA following piperdine cleavage in order of decreasing concentrations of Cr(VI)/Asc of (50/500, 25/250, 12.5/125, 6.2/62, 3.1/31 μM).

4.3 Lesion Identification by Reductive Trapping of BER Glycosylases.

Oxidized base lesions are recognized by specific BER enzymes that initiate the repair process through base removal and formation of an abasic site. The BER enzyme forms a Schiff base intermediate at the C1' of the damaged nucleotide sugar that can be covalently trapped as the stable amine using a suitable reductant such as sodium cyanoborohydride.[16] BER glycosylases have affinities for a specific lesion or a subset of lesions, and reductive trapping can be used to identify the class of DNA lesions formed in the reaction. The human BER glycosylase hOGG1 has shown specificity toward the recognition of a family of oxidized guanine residues including 8-oxoG in DNA.[17] We have shown that the recently characterized BER enzymes, NEIL1 and NEIL2, recognize the further-oxidized lesion of guanine, spiroiminodihydantoin (Sp), but have little affinity for 8-oxoG (Results Ch.3).[13] Duplex DNA containing a single 8-oxoG or Sp lesion was synthesized as described in Materials and Methods. Unmodified DNA, DNA containing a single 8-oxoG lesion, or DNA with a single Sp lesion was incubated with hOGG1, or NEIL2 and trapped with $NaCNBH_3$. Figure 4.2 shows a typical gel shift obtained upon trapping of the glycosylases. The results show that NEIL2 was almost exclusively trapped to DNA containing the Sp lesion, and hOGG1 was exclusively trapped to DNA containing the 8-oxoG lesion.

This same trapping system was used to analyze for specific glycosylases being produced from reactions of Cr(VI)/Asc treated duplex DNA. This analysis of lesion formation was carried out using 15, 25, and 50 μM Cr(VI) and a 10-fold excess of ascorbate. Each sample was incubated with $NaCNBH_3$ and hOGG1, NEIL1, or NEIL2 in a manner identical to that in Figure 4.2. Trapping with NEIL1 and NEIL2 under these

conditions exhibited a distinct gel shift, whereas hOGG1 showed essentially no trapping (Figure 4.3). Since hOGG1 is relatively specific for the 8-oxoG lesion and NEIL1 and NEIL2 have little or no affinity for 8-oxoG, this result suggested that the majority of the lesions formed from the oxidation of duplex DNA by Cr(VI)/Asc was not 8-oxoG but a further oxidized guanine lesion such as Sp.

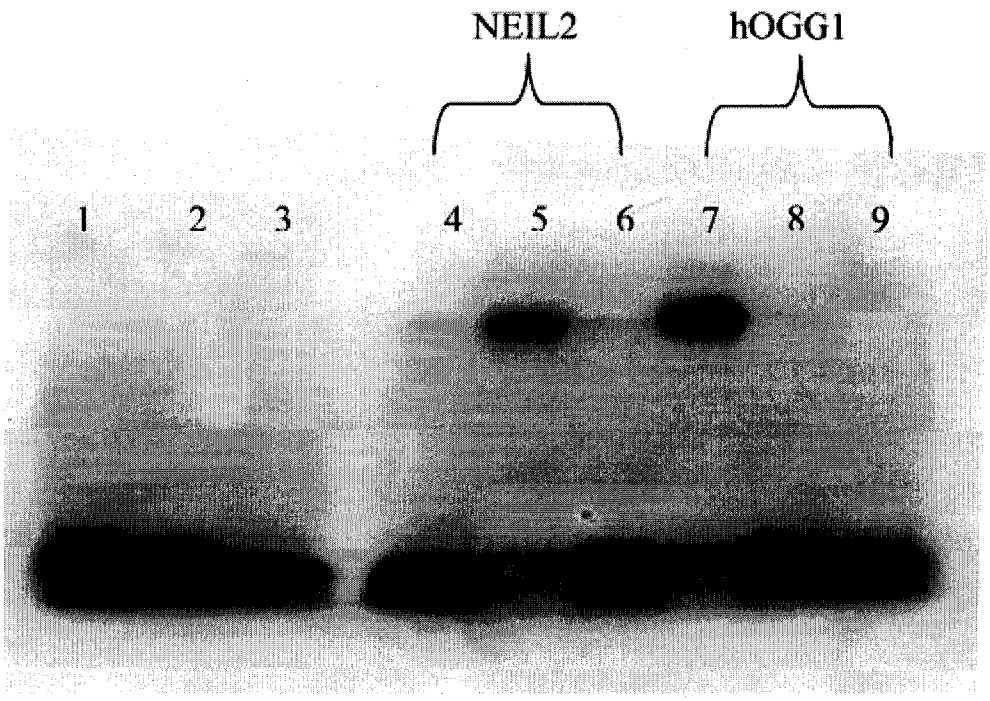

Figure 4.2: SDS-PAGE gel showing lesion-specific reductive trapping of 8-oxoG and Sp in duplex DNA by the BER glycosylases NEIL2 and hOGG1. Lane 1, 8-oxoG + $NaCNBH_3$ control; lane 2, Sp + $NaCNBH_3$ control; lane 3, unmodified DNA + $NaCNBH_3$ control; lane 4, 8-oxoG-contining DNA + NEIL2 + $NaCNBH_3$; lane 5, Sp-containing DNA + NEIL2 + $NaCNBH_3$; lane 6, unmodified DNA + NEIL2 + $NaCNBH_3$; lane 7, 8-oxoG-containing DNA + hOGG1 + $NaCNBH_3$; lane 8, Sp-containing DNA + hOGG1 + $NaCNBH_3$; lane 9, unmodified DNA + hOGG1 + $NaCNBH_3$.

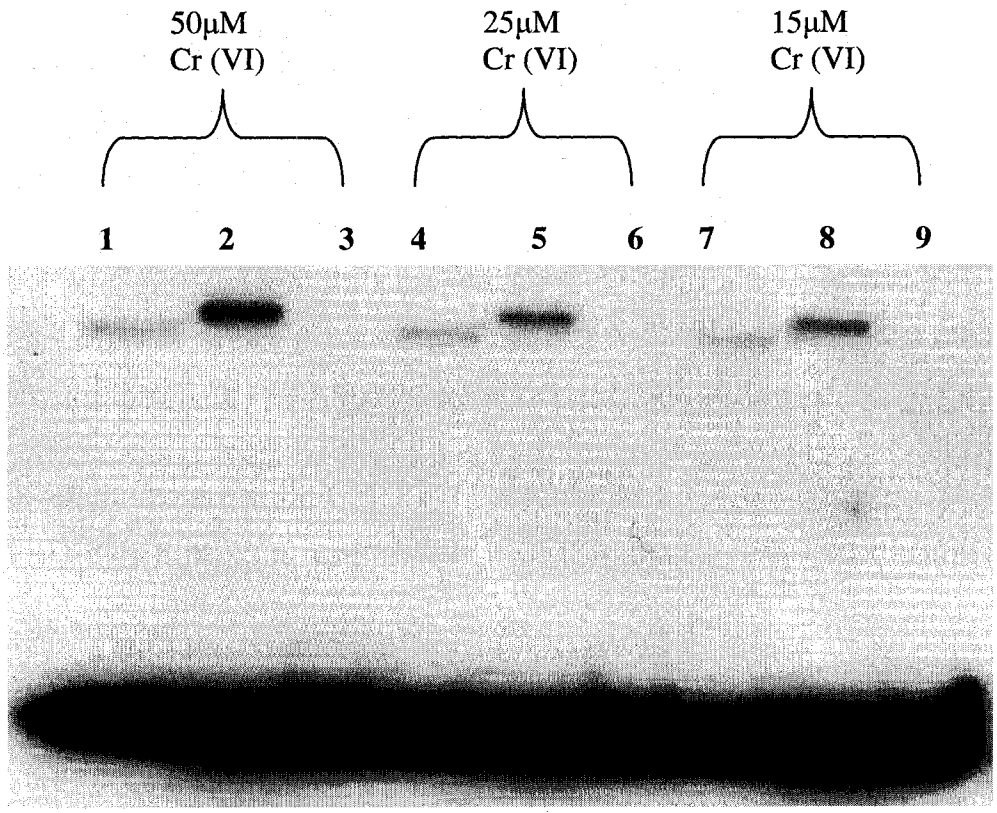

Figure 4.3: Trapped BER glycosylases in Cr(VI)/Asc-oxidized duplex DNA. Lanes 1-3, 50/500 μM Cr(VI)/Asc with NEIL1, NEIL2, and hOGG1 respectively; lanes 4-6, 25/250 μM Cr(VI)/Asc with NEIL1, NEIL2, and hOGG1 respectively; lanes 7-9, 15/150 μM Cr(VI)/Asc with NEIL1, NEIL2, and hOGG1 respectively.

4.4 Detecting Sp Formation in a Cellular System

Growth inhibition of BER deficient *E. coli* strains as a function of chromate treatment. This study focused on a set of known BER deficient bacterial cell lines that included the MutM$^-$, MutY$^-$, the MutM$^-$/MutY$^-$ double mutant, and a Nei deficient *E. coli* cell line. The MutM$^-$ (CM1319), MutY$^-$ (CM1307), and the MutM$^-$/MutY$^-$ double mutant (CM1322) showed no change in growth with respect to the wild-type strain (WP2) as Cr(VI) concentration was increased. A representative growth curve for the single and double mutants vs its wild-type cell line are shown in Figures 4.4 and 4.5.

In contrast, the Nei deficient strain (TK3D11) showed a significant change in growth inhibition over that of its wild-type control (CSR06) following chromate treatment (Figure 4.6). Growth of the Nei deficient strain at the 8 hr time point for the 100 μM and 250 μM Cr(VI) treatments was observed to be inhibited by 27 and 67% with respect to its wild-type control strain. To our knowledge, this the first example of a BER deletion mutant to show differential growth sensitivity toward Cr(VI).

HPLC-ESI-MS analysis for the Sp lesion in genomic DNA of BER deficient *E. coli*. The ability of the Nei deficient *E. coli* (TK3D11) and the MutM$^-$/MutY$^-$ double mutant (CM1322) to accumulate the Sp lesion in genomic DNA in response to chromate treatment was studied by HPLC-ESI-MS. An isotopically labeled ^{18}O-Sp internal standard allowed the identification and quantification of ^{16}O-Sp in digested DNA samples as our group has described previously.[18] Accumulation of Sp in the genomic DNA was assessed in high density cell cultures (OD_{600} = 0.5) of the Nei deficient (TK3D11), the MutM$^-$/MutY$^-$ double mutant (CM1322), and their matched wild-type controls treated with 250 and 500 μM chromate. HPLC-ESI-MS analysis of the digested genomic DNA

for Sp formation, using the ^{18}O-Sp internal standard for quantification showed a dose-dependent accumulation of Sp with increasing chromate concentration only in the Nei deficient (chromate sensitive) *E. coli* (Figure 4.7). Accumulation of the Sp lesion in the Nei deficient *E. coli* was observed to be approximately 20-fold greater than that observed for its wild-type counterpart (CSR06). The MutM$^-$/MutY$^-$ 8-oxoG double mutant (CM1322), which is Nei proficient, and its wild-type counterpart (WP2) showed no significant accumulation of Sp with chromate treatment over that of the control. This result correlated well with our previous studies on in vitro Sp formation in Cr(VI)-oxidized DNA and suggests that the Nei BER enzyme is the primary glycosylase that recognizes Sp in bacterial DNA.

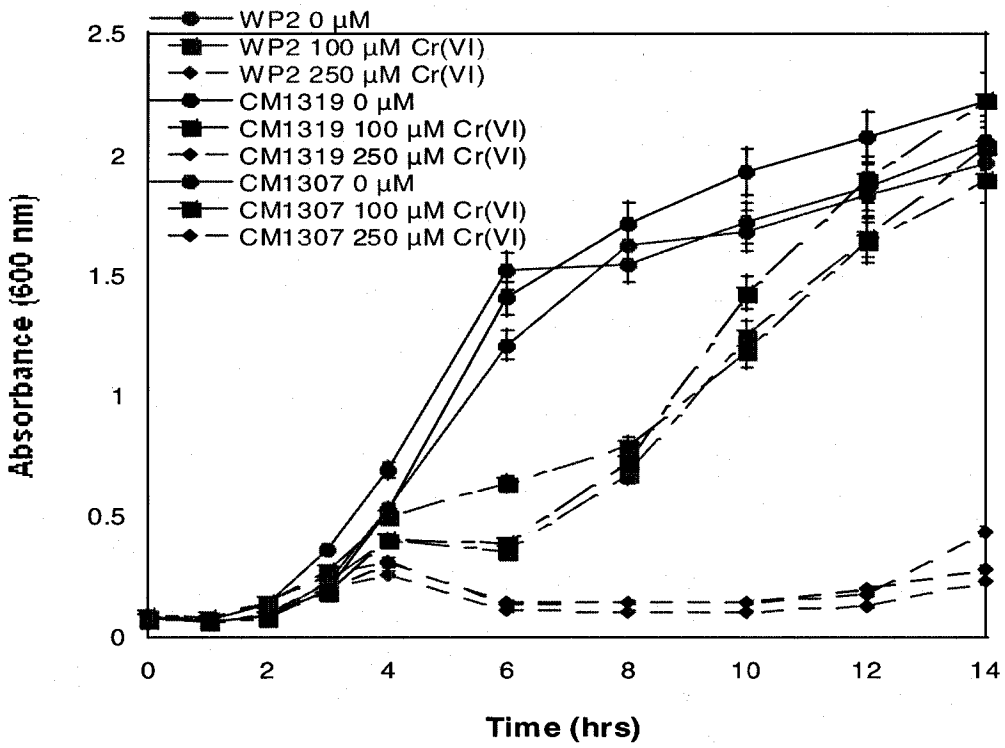

Figure 4.4: Growth inhibition of the MutM$^-$ (CM1319) and MutY$^-$ (CM1307) single mutants with their corresponding wild type (WP2) with increasing chromate treatment. Growth inhibition for the wild type is shown in red symbols, and the MutM$^-$ and MutY$^-$ mutants are shown with identical symbols as the wild type but in blue and green colors respectively.

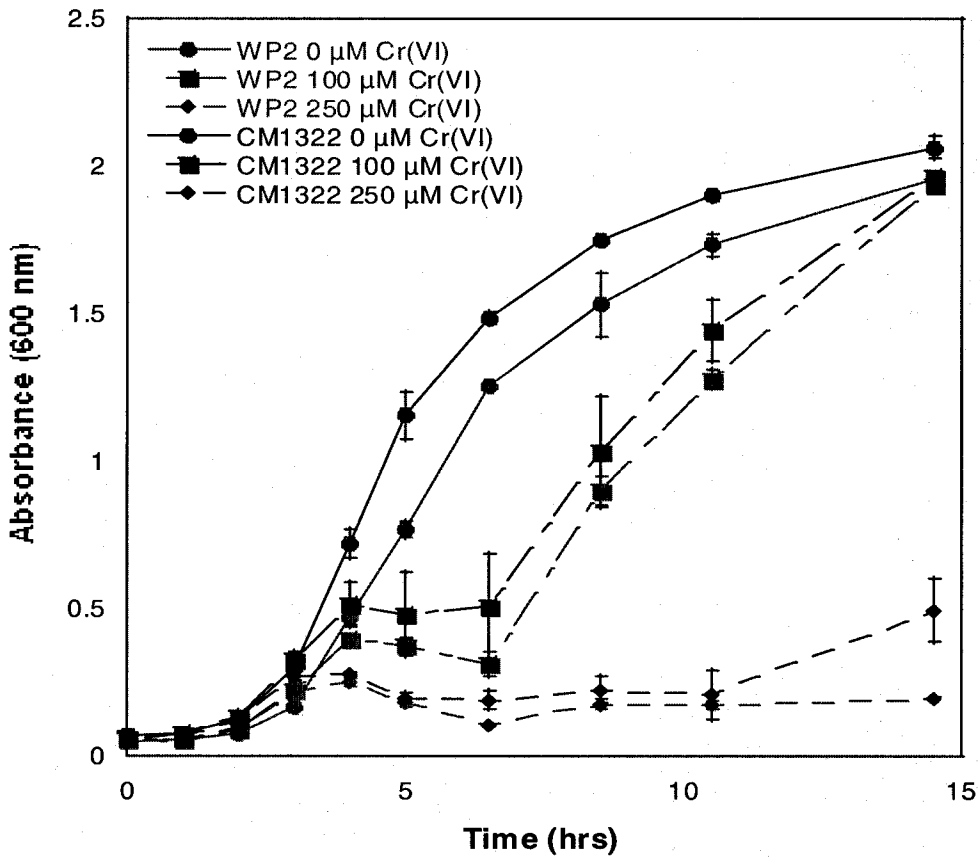

Figure 4.5: Growth inhibition of the MutM⁻/MutY⁻ double mutant (CM1322) and its corresponding wild type (WP2) with increasing chromate treatment. Growth inhibition data for the wild type are represented by the red symbols and the mutant with the identical symbols but in blue for each chromium concentration. The error bars represent the standard deviation of three replicates.

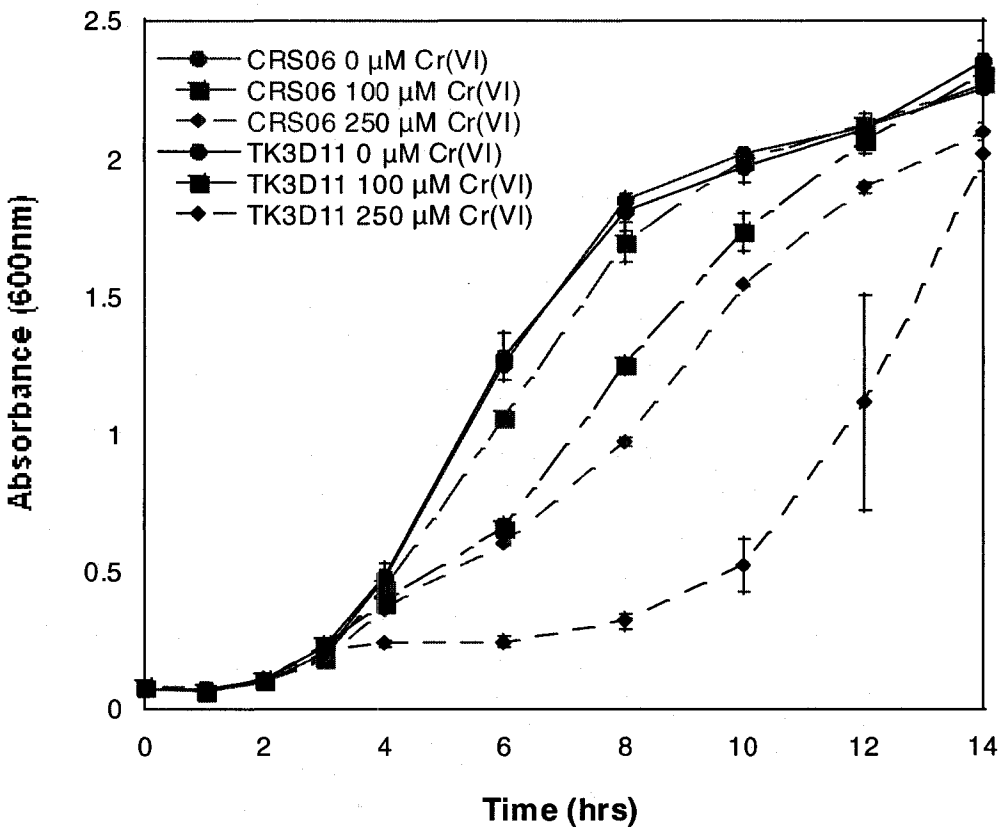

Figure 4.6: Growth inhibition of the Nei$^-$ mutant (TK3D11) and its corresponding wild type (CSR06) with increasing chromate treatment. Growth inhibition data for the wild type are represented by the red symbols and the mutant with the identical symbols but in blue for each chromium concentration. The error bars represent the standard deviation of three replicates.

The toxicity of chromate in these high-density cell cultures (OD_{600} = 0.5) was assessed by a plating assay as growth curves cannot be conducted on high-density cultures. At the 250 μM concentration of chromate, no overt toxicity was observed for any of the cell lines in the plating assay (Figure 4.8). At the 500 μM chromate treatment the Nei deficient (TK3D11) cell line showed an 18% survival rate vs 40% for its matched wild type (CSR06) in the plating assay. The $MutM^-/MutY^-$ double mutant (CM1322) displayed a 35% survival in the plating assay with its matched wild type (WP2) showing 43% survival at the 500 μM chromate treatment.

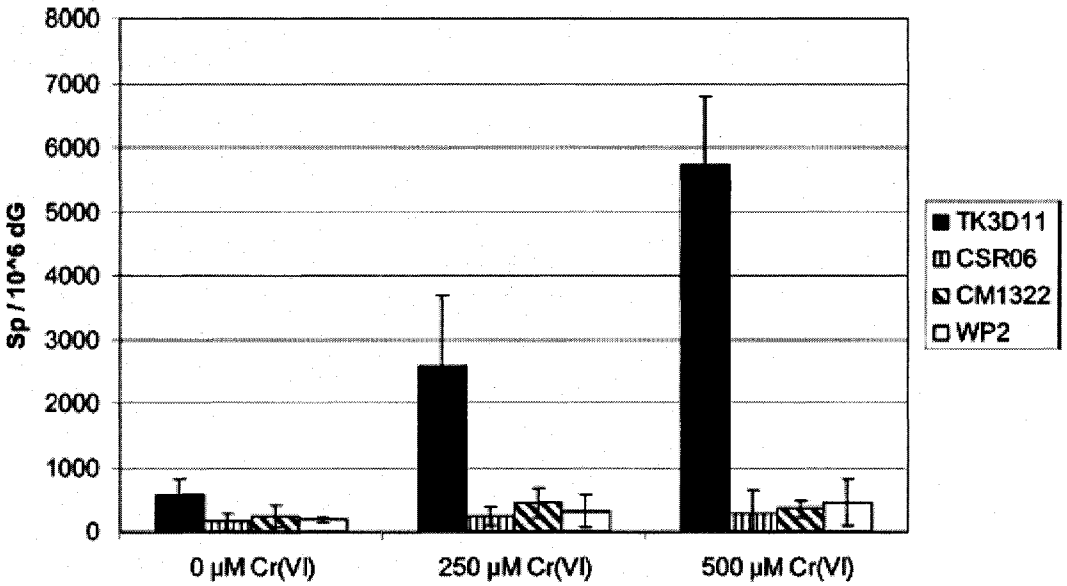

Figure 4.7: Formation of Sp in chromate-treated *E. coli*. The Nei deficient (TK3D11) *E. coli* show a dose dependence for Sp formation with an increasing chromate concentration. The data are normalized on a per million dG basis, and each data point is a minimum of n = 5 replicates. Figure from: Hailer, M.K., Slade, P.G., Martin, B.D. and Sugden, K.D. (2005) *Chem. Res. Toxicol.* **18**, 1378-1383.

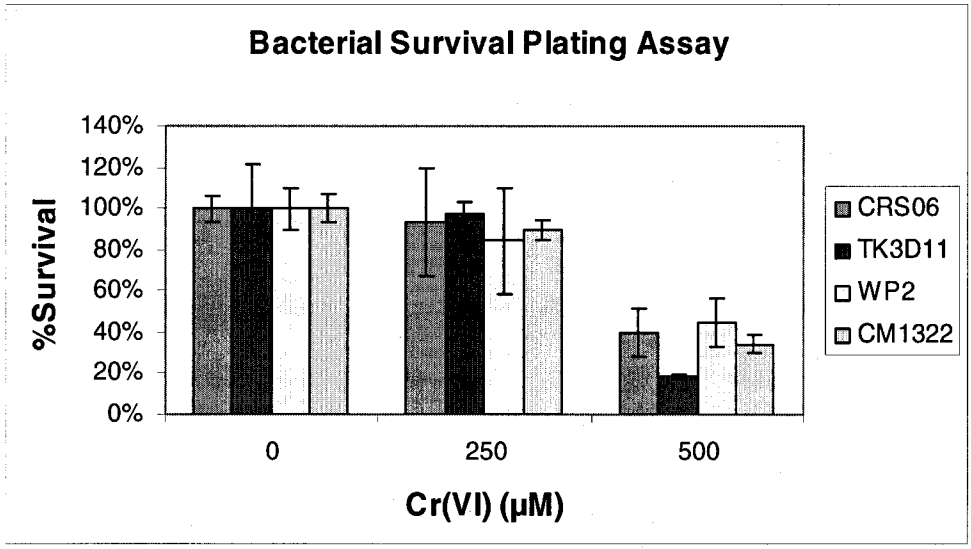

Figure 4.8: Plating assay to determine the toxicity of chromate in high-density cell cultures (OD_{600} = 0.5). Percent survival when exposed to increasing levels of chromate was determined in two repair deficient strains (TK3D11 and CM1322) and compared to their respective wild-type strains (CRS06 and WP2). The samples were normalized and the error bars represent the standard deviation of three replicates.

HPLC-ECD Analysis for the 8-oxoG Lesion in the Genomic DNA of BER Deficient *E. coli.* Enzymatically digested genomic DNA from the different *E. coli* strains were also analyzed for 8-oxoG [19] using HPLC coupled with electrochemical detection (HPLC-ECD). 8-oxoG is the putative intermediate of Sp formation and is primarily removed from genomic DNA by the MutM (Fpg) and MutY BER enzymes.[20] Figure 4.9 shows that the MutM/MutY proficient bacterial cell lines, TK3D11, CSR06, and WP2 all gave typical levels of 8-oxoG (typical background levels for 8-oxoG have been established by the ESCODD to be 0.3-4.2 8-oxoG/10^6 dG; [21]) even at the highest concentration of chromate used (500 μM). Not surprisingly, the $MutM^-/MutY^-$ double

mutant (CM1322) was the only cell line studied that showed accumulation of 8-oxoG in its genomic DNA following chromate treatment. The amount of 8-oxoG accumulation, however, was modest in the MutM$^-$/MutY$^-$ double mutant with respect to the level of Sp accumulated in the Nei deficient cell line and was not differentially toxic to the cell by either a plating assay or growth curves.

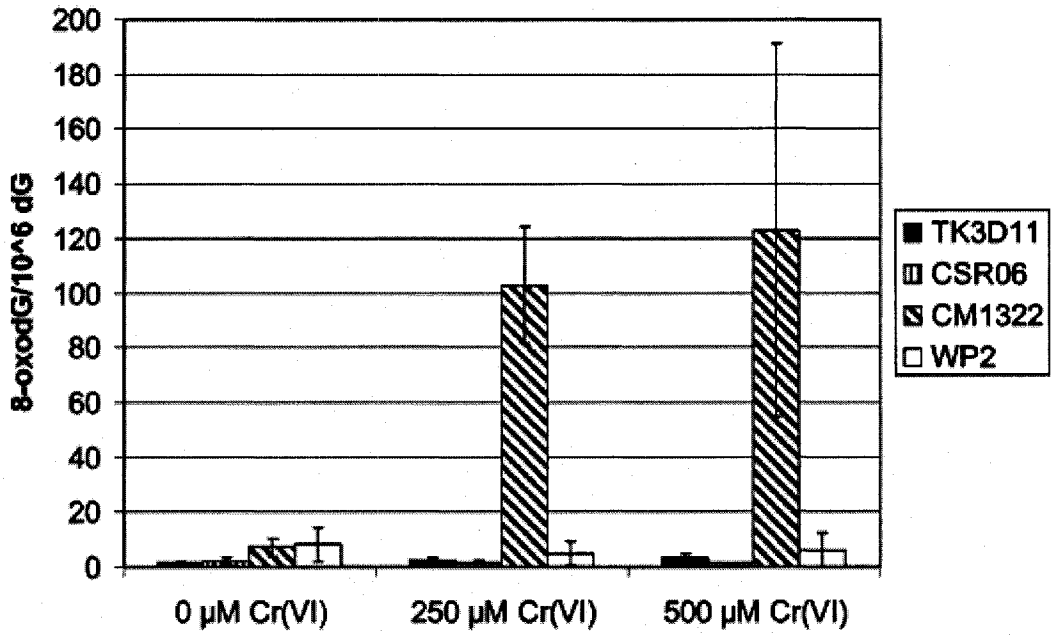

Figure 4.9: Formation of 8-oxoG in chromate-treated wild-type and BER deficient *E. coli* genomic DNA. Each data point is normalized on a per million dG basis and has a minimum of n = 3 replicates. Figure from: Hailer, M.K., Slade, P.G., Martin, B.D. and Sugden, K.D. (2005) *Chem. Res. Toxicol.* **18**, 1378-1383.

4.5 Discussion and Conclusions

The reaction of Cr(VI) with a 10-fold excess of ascorbate resulted in guanine-specific oxidation in a duplex DNA oligonucleotide. The products of this oxidation reaction were determined using BER-specific lesion trapping. These results were further verified by other members of the lab by HPLC-ECD and HPLC-ESI-MS. BER trapping with hOGG1 showed little or no formation of 8-oxoG, which correlated with the low levels of 8-oxoG subsequently identified using the sensitive HPLC-ECD method. BER trapping was observed with the NEIL2 glycoylase, which showed a dose-dependence of trapping with Cr(VI)/Asc treatment. NEIL2 recognizes the Sp lesion in duplex DNA, and the formation of Sp was further verified and quantified using HPLC-ESI-MS by other lab members. The levels of Sp generated in the Cr(VI)/Asc system was approximately 20 times greater than that of 8-oxoG. These findings suggest that the Sp lesion may be a common lesion in DNA oxidation and could play a significant role in the initiation of cancer by carcinogenic Cr(VI) compounds. The formation of Sp directly from guanine in DNA has previously only been observed for carbonate radical anions.[3] To our knowledge, this study is the first example of Sp formation arising discretely from guanine in duplex DNA from reaction with a known carcinogenic metal.

The mechanism of Sp formation directly from guanine is a four-electron process with Sp being the terminal product that predominates at pH 7 or greater.[22] 8-oxoG is the two electron intermediate in this oxidation process, which is further oxidized to the 5-hydroxy-8-oxoguanine species, 5-OH-8-oxoG, and ultimately Sp. Figure 4.10 shows the putative reaction scheme for the formation of Sp from guanine with sequential one-electron oxidation by Cr(IV). The reduction of Cr(VI) with a 10-fold molar excess of

ascorbate has been observed to initially form a transient Cr(IV) species and the oxidized form of ascorbate, dehydroascorbate (DHA) [23], followed by a second reduction reaction of Cr(IV) with Asc to give rise to the ascorbyl radical, $Asc^{\bullet -}$, and a kinetically inert Cr(III) species as shown in equations 1 and 2 below:

$$Cr(VI) + Asc \rightarrow Cr(IV) + DHA \qquad (1)$$

$$Cr(IV) + Asc \rightarrow Cr(III) + Asc^{\bullet -} \qquad (2)$$

A second terminal product of this guanine oxidation pathway, guanidinohydantoin (Gh), may also be formed during this oxidation reaction. However, this product is generally formed under more acidic conditions, while this study was carried out at neutral pH.[22] It is important to note that the oxidation process used in this study shows, for the first time, guanine-specific products caused by the direct oxidation of unmodified dsDNA by Cr(VI) without the introduction of exogenous H_2O_2.

A number of studies on the reaction of Cr(VI) with DNA have shown a bias toward reaction with guanine.[24] These results have often been interpreted as the formation of chromium adducts with guanine through a phosphodiester backbone-ligand intermediate.[25] However, no adduct of chromium with the guanine base has ever been discretely identified. We suggest that guanine-specific reactions in DNA give rise primarily to further –oxidized lesions such as Sp, although formation of these ternary adducts cannot be ruled out in our system.

Sp is known to be considerably more piperidine labile than 8-oxoG and has shown the ability to induce high levels of arrest in polymerase processing.[26] The enhancement in cleavage that we have observed in guanines that are 5' in a run of purines also supports this oxidative mechanism. We postulate that many of the effects of Cr(VI)

treatment observed in cellular systems may be adequately explained by the formation of these further oxidized lesions.

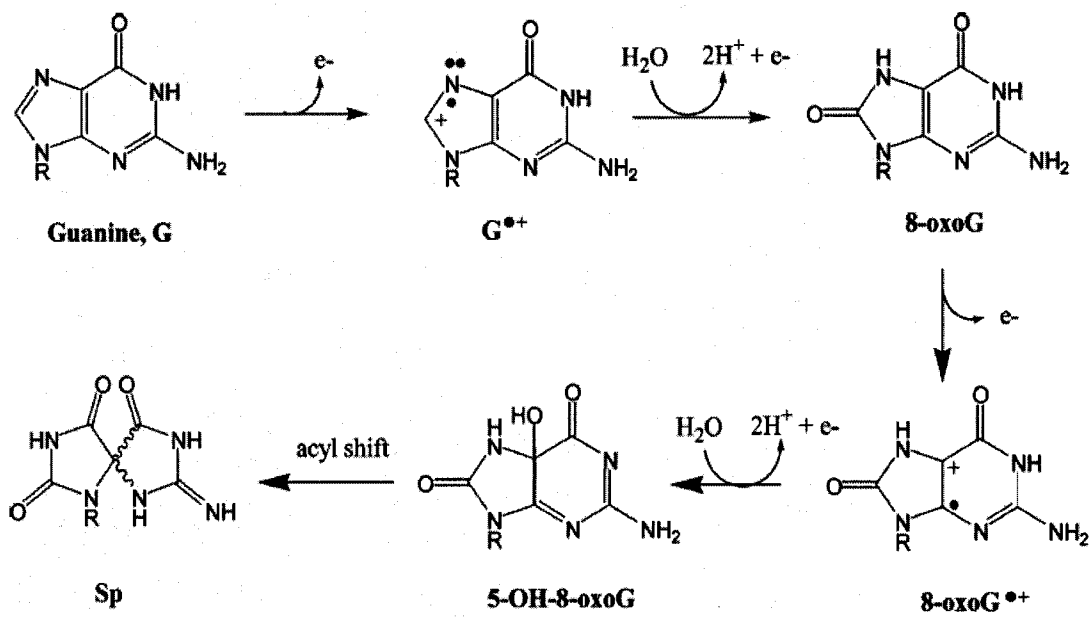

Figure 4.10: General reaction mechanism proposed for the formation of Sp from guanine.

The primary focus of this study was to identify and quantify the formation of the further-oxidized guanine lesions, spiroiminodihydantoin (Sp), arising from oxidation of guanine in unmodified duplex DNA. Clearly, the formation of these further oxidized lesions from oxidative attack on guanine in DNA instead of in an 8-oxoG modified DNA strand was necessary to establish their potential relevance in biological systems. To identify these lesions as arising in unmodified DNA from Cr(VI) oxidation, we exploited the specific affinity of trapping by the base excision repair enzymes NEIL1 and NEIL2 and have further unambiguously identified their formation using HPLC-ESI-MS. The

NEIL family of genes (Nei-like) have only recently been identified, and their affinities for oxidatively damaged bases are still being defined. As described in chapter 3 of this thesis, NEIL1 and NEIL2 were shown to have high affinities for the further oxidized guanine lesions Sp and Gh but little or no affinity for 8-oxoG in duplex DNA. When Sp formation was compared with 8-oxoG in this study, the prevalence of the glycosylase recognition was specific for further-oxidized lesions and not 8-oxoG, suggesting that Sp was formed at significantly greater levels than 8-oxoG.

Growth inhibition in repair deficient strains of bacteria by a toxicant that exceeds that observed for the wild-type bacterial strain implies both reaction with DNA and a role for the repair gene in ameliorating its genotoxic effects. The reaction of chromate with DNA in cellular systems is thought to produce a number of different adducts including those derived from an oxidative pathway (strand breaks, abasic sites, 8-oxoG; [27-29]) and those derived from a metal-binding pathway (DNA inter-strand cross-links and DNA-protein cross-links; [30,31]). Little is known about the exact nature of many of these adducts or their mechanism of repair. One recent study has shown that a human XP-A fibroblast cell line, deficient in nucleotide excision repair (NER), was sensitive to chromate.[32] This chromate sensitivity in the XP-A fibroblasts was attributed to the formation of DNA cross-links arising from the metal-binding pathway.

Even though Cr(VI) is a known intracellular oxidant, no systematic analysis of the impact of Cr(VI) exposure on BER deficient bacterial strains had previously been undertaken. We studied the ability of BER deficient *E. coli* to grow in the presence of Cr(VI) since the formation of 8-oxoG has long been considered to be a relevant lesion induced by Cr(VI) in a variety of in vitro and cellular systems. If Cr(VI) exposure in *E.*

coli generated 8-oxoG as a key genotoxic lesion, it would be expected that strains that are deficient in MutM (Fpg) and/or MutY would exhibit significant growth inhibition. MutM recognizes and excises 8-oxoG opposite cytosine while MutY recognizes and excises adenine opposite 8-oxoG (adenine is the base that is most often misincorporated for cystosine opposite an 8-oxoG upon DNA replication).[20] MutM recognizes Sp in duplex DNA when paired opposite cytosine, C, as a complementary base but shows little or no recognition of this lesion when paired opposite guanine, G, or adenine, A [12] (Figure 4.11). In fact, it has been recently shown that a functional MutY repair enzyme effectively eliminates MutM recognition of Sp opposite G.[12] The BER enzyme, Nei, has shown the ability to recognize Sp in duplex DNA opposite C, G, and A with the greatest affinity for Sp opposite G [12] (Figure 4.11). Nei is also the only BER enzyme known to recognize Sp opposite A. This finding is of considerable significance since recent cellular mutation studies have suggested that G→ C transversion mutations, and to a lesser degree G → T transversion mutations, predominate when Sp is formed in duplex DNA.[6-9] Interestingly, these transversion mutations are the primary mutations observed in the lung tumors of chromate-exposed workers and in shuttle vector replication assays in Cr(VI)-treated mammalian cells.[10,11] On the basis of these lesion specific mutation profiles, we propose that the formation of the Sp lesion is more consistent with known Cr(VI) mutation patterns than is the 8-oxoG lesion.

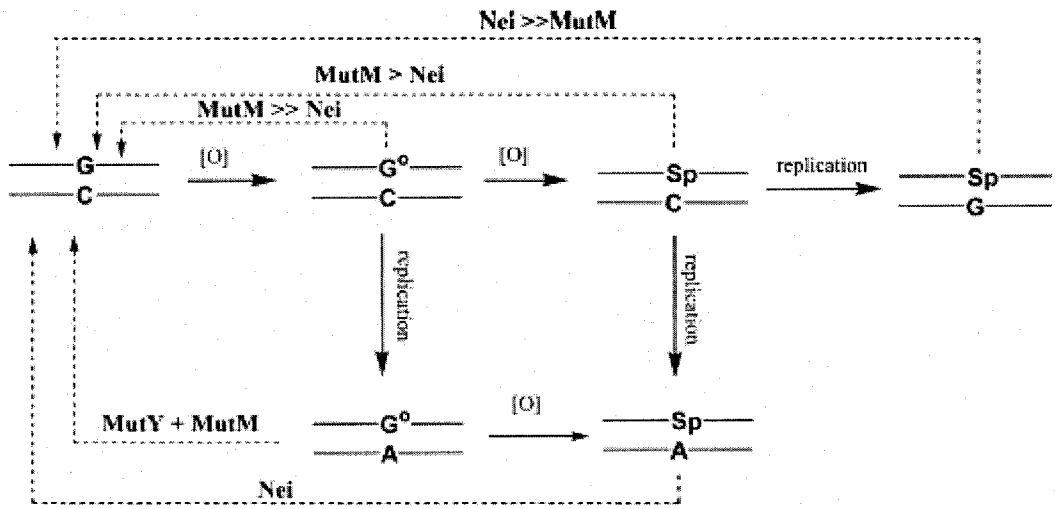

Figure 4.11: Schematic representation of 8-oxoG (G^{o}) and Sp formation and lesion repair by E. coli BER enzymes in genomic DNA. The recognition specific enzyme initiating each repair sequence is indicated above each pathway.

DNA repair studies in *E. coli* are generally considered significant to humans due to the homology and substrate overlap between the *E. coli* and mammalian BER enzymes. Recently, NEIL BER genes were found in both the human and the mouse genome. These genes have been designated as *NEIL1*, *NEIL2*, and *NEIL3*. The mammalian NEIL genes are homologous to *E. coli* Nei and Fpg, and we have hypothesized that they play a fundamental role in repair of chromium-damaged DNA.

The formation of Sp in DNA in vitro has been observed in a wide variety of oxidizing systems including reactions with chromium. The identification and quantification of the Sp lesion in cellular systems have been complicated by it refractory nature toward enzymatic digestion and the polarity of the resulting nucleoside that makes it difficult to separate by HPLC. At the highest dose of chromate used in this study, 500 μM, an approximate 20-fold increase in Sp formation was observed for the Nei deficient

E. coli over its matched wild-type control. In this study, the chromate was added to *E. coli* in the logarithmic growth phase. We hypothesize that these quickly replicating cells accumulated Sp opposite the misincorporated bases guanine and adenine, conditions under which Nei has been shown to play the major role in repair. The failure of the Nei deficient cell line to accumulate even modest amounts of 8-oxoG, while accumulating large quantities of Sp, indicates that chromate oxidation of 8-oxoG effectively and efficiently out-competes the proficient MutM and MutY repair systems in this cell line. This is not unexpected since 8-oxoG is kinetically and thermodynamically prone to oxidation to Sp. In fact, our group has previously shown that chromium preferentially oxidized 8-oxoG in DNA over any other nucleic acid base, including guanine. These results suggest, however, the additional possibility that chromate forms Sp within genomic DNA directly without going through an 8-oxoG intermediate. While there has not been an established mechanistic route for such an oxidative event with chromate, our current results cannot rule out this possibility.

Lesion formation in the MutM$^-$/MutY$^-$ double mutant, which has a functional Nei BER gene, can remove the Sp lesion from the genomic DNA pool, resulting in no accumulation of Sp and thus no sensitivity toward chromate. However, the lack of the MutM and MutY BER glycosylases in this strain explains the observed accumulation of 8-oxoG within the genomic DNA.

In conclusion, the deletion of the Nei BER enzyme in *E. coli* results in a differential sensitivity of this cell line toward chromate. The high level of Sp measured in the genomic DNA of this strain almost certainly contributes to the chromate sensitivity of these cells. On the basis of human mutation data and BER homologies between

humans and bacteria, these data suggest that the Sp lesion may play a major role in chromate-induced lung tumors.

4.6 Experimental Procedures

4.6.1 In vitro Formation and Detection of Oxidized Guanine Lesions

Deoxyribonucleotides. Oligonucleotides used in this study were purchased from Integrated DNA Technologies or Trilink Biotechnologies. The oligonucleotide sequences were d(5'-AGTTGAGXGGACTTTCCCAGCC-3'), where X denotes G or 8-oxoG, and its complement d(5'-GGCTGGGAAAGTCCCCTCAACT-3'). Purification of the oligonucleotides prior to use was accomplished by HPLC using a Dionex Nucleopac PA-100, 4 mm X 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 6.0, in 10% acetonitrile) to 100% mobile phase B over 31 min. Eluting oligonucleotides were monitored at 268 nm. The fraction containing the oligomer was eluted as a single peak. The collected samples were lyophilized and purified into deionized water using a Bio-Rad Micro Bio Spin 6 Column. Pure oligonucleotides were stored at -20 °C until needed.

DNA oxidation using Cr(VI) and ascorbate. Ascorbate (>99%) and sodium dichromate dihydrate were purchased from Sigma-Aldrich. Purified duplex DNA oligonucleotides (2.5 μM) were oxidized by the addition of a 10:1 molar ratio of ascorbate to Cr(VI) in PBS buffer (pH 7.0). Concentrations of Cr(VI) varied from 3.12 to 50 μM with 31-500 μM ascorbate. Reactions were incubated for a minimum of 1 hr at which time UV-vis monitoring at 372 nm had shown complete reduction of Cr(VI) to

Cr(III). The oxidized DNA was then purified into DI water using a Bio-Rad Micro Bio-Spin 6 column.

Analysis of site- and sequence-specific oxidation of DNA by Cr(VI) and ascorbate. Piperidine-labile cleavage sites on the DNA were analyzed by treating lyophilized samples of the Cr(VI)/Asc-oxidized DNA (2.5 μM) with 100 μL of a 1.0 M solution of freshly distilled piperidine followed by heating at 90 °C for 30 min. Samples were loaded on a 20%, 0.4 mm thickness, 21 cm X 50 cm denaturing (7M urea) polyacrylamide gel with 4 μL of 80% formamide loading buffer containing 0.05% xylene cyanol and bromophenol blue. Electrophoresis was carried out at 2200V and 24 mA with 1X TBE as the running buffer. Visualization of the DNA cleavage products was carried out by autoradiography using Kodak X-Omat Ar-5 film.

Analysis of trapped Schiff base complexes of hOGG1 and mNEIL2. The Cr(VI)/Asc-treated, ^{32}P-labeled dsDNA (23 μM) was incubated with mNEIL2 or hOGG1 (15 nM) in 60 μL of reaction buffer (20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA) in the presence of 50 mM $NaCNBH_3$ at 37 °C for 90 min. An amount of 25 μL of the trapped enzyme/DNA complex was removed, and the reaction was stopped by the addition of 5 μL of 6 X SDS loading buffer. Samples were heated to 95 °C for 10 min and flash-cooled on ice. Samples were separated on an 8-12% stacking SDS-PAGE gel and visualized by autoradiography.

4.6.2 Treatment of *E. coli* Strains

Bacterial Strains. The strains WP2 and CM1322 were generous gifts from Dr. Bryn Bridges and Dr. Andy Timms of MRC (University of Sussex, United Kingdom). WP2 is *trpE65-(oc)*, *lon-11*, *sulA1* [33], CMI322 is as WP2 but *mutY68::kan*^{R}, *mutM::Tn10*

(source P1(TT101 X CM1307) (personal communication), CM1319 is as WP2 but *mutM::Tn10*, CM1307 is as WP2 but *mutY68::kan*^{R}.[34] TK3D11 (Δ*kdpFABC-gltA*) 219, Δ(*galK-bioD*) 76, *trkA405*, *trkD1*, *rha-4*, *thi-1* and its control strain CSR06 (*thr-1*, *araC14*, *leuB6*(*Am*), Δ(*gpt-proA*) 62, *lacY1*, *tsx-33*, *glnV44*(*AS*), *phr-1*, *galK2*(*Oc*), *LAM-*, *Rac-O*, *hisG4*(*Oc*), *rfbC1*, *mgl-51*, *rpsL31* (*strR*), *kdgK51*, *xylA5*, *mtl-1*, *arg E3(Oc)*, *thi-1*, *uvrA6*) were obtained from the *E. coli* Genetic Stock Center at Yale University (New Haven, CT). The Δ*kdpFABC-gltA* encompasses the five genes of the Nei operon [35], and TK3D11 is designated herein as Nei^{-} in order to reflect the deletion of this operon.

Differential Growth of BER Deficient *E. coli* Following Chromate Treatment. Fourteen hour growth curves were conducted on the BER deficient strains of *E. coli* and their wild-type controls (WP2 for MutM^{-}/MutY^{-} CM1322 and CSR06 for the Nei^{-} TK3D11). The *E. coli* strains were grown with either 0 (control), 100 or 250 μM Cr(VI) over a time course of 14 hr in LB broth at 37 °C. Chromate, Cr(VI), as sodium dichromate (J.T. Baker Chem. Co., Phillipsburg, NJ) was added at an OD_{600} of 0.05, and measurements of cell density were taken every hour for 8 hr and then every 2 hr for the next 6 hours.

4.6.3 Detecting Sp Formation in an *E. coli* System

Extraction of Genomic DNA for Mass Spectral and Electrochemical Analysis of DNA Lesions. Cultures of CSR06, TK3D11, WP2, and CM1322 were grown in 50 mL of LB broth at 37 °C until they reached an OD_{600} of 0.5. Cell densities were normalized to ensure identical numbers of cells in each treatment. When an OD_{600} of 0.5 was reached, Cr(VI) was added to the cell/LB mixture to give concentrations of 0, 250, and 500 μM. Cells were allowed to grow for 3 hr after the addition of Cr(VI) and prior to

harvesting. Cells were harvested and lysed using the Qiagen bacterial lysis buffer (Qiagen Inc., Valencia, CA), and genomic DNA was extracted using phenol/chloroform as previously described.[36]

HPLC-ESI-MS Analysis of Oxidized Duplex DNA. Hydrolyzed DNA samples were analyzed using a Waters 2790 HPLC coupled to a Micromass LCT mass spectrometer with electrospray ionization. Separations were performed using a C18 reverse phase Microsorb MV 2.1 mm X 250 mm column, with a 0.5% aqueous acetonitrile/min gradient mobile phase and a flow rate of 0.4 mL/min. Mass spectral analysis was performed using selected ion monitoring (SIM) with a cone voltage of 30 V, a capillary voltage of 3000 V, a desolvation temperature of 250 °C, a source temperature of 150 °C, and an aperture of 15. Sp was identified and quantified through the introduction of the stable isotopic ^{18}O internal standard of ^{18}O described previously.[19] A linear calibration curve was obtained using 1.0 nmol of ^{18}O-Sp as the internal standard and variable amounts of ^{16}O-Sp ranging from 0.01 to 1.0 nmol (slope = 1.0, $r^2 = 0.997$). For the measurements of Sp in hydrolyzed DNA samples, the amount of ^{18}O-Sp internal standard was chosen according to the limits of this standard curve. Levels of Sp in hydrolzyed DNA samples were expressed as the number of Sp per 10^6 dG.

HPLC-ECD Analysis of Genomic DNA from *E. coli*. HPLC-ECD identification of 8-oxoG was performed using a previously established method.[36] Briefly, 8-oxoG and dG in enzymatically digested DNA samples were separated by HPLC with a 4.6 mm X 150 mm reverse phase YMC basic column and quantified using a CoulArray electrochemical detection system (ESA Inc., Chelmsford, MA). Nucleosides were eluted from the column using an isocratic mobile phase of 100 mM sodium acetate, pH 5.2, in

4% methanol. Potentials of the four coulometric analytical cells of the CoulArray system place in series were as follows: 50, 125, 175, 200, 250, 380, 500, 700, 785, 850, 890, and 900 mV. Calibration curves were generated from a dG standard (0.1-2 μg) and an 8-oxoG standard (50-1000 pg). The amount of 8-oxoG was calculated by comparing the peak area from a 50 μL injection of enzymatic hydrolysate of the oxidized DNA sample with the calibration curve. Levels of 8-oxoG in the genomic DNA were expressed as the number of 8-oxoG per million dG.

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Appendix 1: Identifying Common Science Misconceptions in 5th Grade Science Classes at Lewis and Clark Elementary School

A1.1 The ECOS Program

This appendix topic is presented in fulfillment of the funding requirements for the ECOS fellowship for the 2005-2006 school year. The ECOS program at the University of Montana is funded by the National Science Foundation. ECOS promotes hands-on science education in schoolyards and adjacent open areas in western Montana, and brings graduate researchers into K-12 county schools in Missoula, Montana. The purposes of this fellowship are two-fold. First, the fellowship gives graduate students the opportunity to develop teaching skills and curriculum writing expertise. Opportunities for true teaching and curriculum development are a rare occurrence in the science fields in graduate institutions, making this fellowship a golden opportunity for students who may want to pursue post-graduate teaching careers. The second purpose of the ECOS fellowship is to bring research from the University of Montana to the local school system. It is a hope that this program will inspire school age children to pursue degrees in science, and will also expose teachers to new ideas and techniques for classroom teaching. In this sense, both graduate students and K-12 teachers learn from each other. Graduate students may learn tried and true teaching techniques, and the teachers can learn ways to integrate cutting-edge research into their existing curriculum.

One of the requirements of the ECOS fellowship is to write a paper focusing on some aspect of these new skills learned as a "resident ecologist" in K-12 classrooms. As per the fellowship guidelines, this paper will be added to the thesis of the fellow. To

fulfill this requirement, I chose to focus on science misconceptions in the three 5th grade classes at Lewis and Clark Elementary School, because as a potential educator it is important to understand the learning process and try to identify areas where students may be struggling to understand specific concepts or ideas. Once misconceptions are identified they can be addressed and hopefully changed to fit true scientific concepts. If misconceptions are not identified and addressed, they can easily persist until college or even well into adulthood when there would be little chance of ever changing the misconception into something more valid.

A1.2 Identifying Science Misconceptions

A misconception is defined as a mistaken thought, idea or notion; a misunderstanding.[1] This idea of misconceptions is also known as an alternative concept or a naïve concept.[2] Students form misconceptions based on their experiences and what they see and hear. Misconceptions can come from a variety of factors and unfortunately, these interpretations have been shown to impede learning of fundamental scientific concepts. Students develop misconceptions as mechanisms for understanding phenomena in the world around them, and the sources for acquiring this information are often not science-based. In addition to this, misconceptions can even arise from incorrect teaching in school, especially when the concepts are never challenged again and persist into adulthood. A students' prior knowledge can be used as a building block for acquiring new knowledge, when this information is correct. When the information is incorrect however, prior knowledge interferes with a student's ability to process new science concepts.[2]

In order to correct science misconceptions, these concepts need to first be identified so that the students can replace them with new information. Several studies have shown that it can be difficult to convince a student to give up a long held misconception[3] unless the new concepts "are more valid, more powerful, more useful, or in some other way preferable to their existing concepts."[4] One way to identify and correct misconceptions is to assess the students' prior knowledge, keeping in mind that giving the student the "correct" information will not necessarily cause them to abandon their misconceptions and adopt this new information.

The most predominant and accepted model for instruction in the U.S. school system is lecture based, which is founded on the assumption that knowledge can be transferred intact from the mind of the teacher to the mind of the learner. This lecture method of teaching is another way that misconceptions can form. As educators, teachers must be aware that teaching and learning are not synonymous; we can "teach" without having the students "learn". This realization is based on the Constructivist Learning Theory [5] which states that knowledge is constructed in the mind of the learner and misconceptions play a role in this process of acquiring knowledge. Many misconceptions encountered in science are no more than a reflection of poorly differentiated concepts that have developed naturally. Students often hold the correct concept but have not applied it properly to problems in the classroom and the real world. This is where identification and hands on application can be used to change the misconception into a valid and working concept.

A1.3 ECOS Experiences from a 5th Grade Classroom

The ECOS program is designed to bring a hands-on approach to understanding science. One of the goals of the ECOS program was to develop curricula pertaining to ecological science topics in a very hands-on, inquiry-based format. These curricula were developed to provide background information to the students on a particular topic; students then determined the "answers" to questions presented in the activity. In this sense, students were allowed to make initial hypotheses or guesses and then work their way through the activity to come to some sort of a conclusion. Generally, there was a concluding session to help students solidify their observations into answers. The goal of the ECOS program was never to identify and alleviate science misconceptions, but working with these students during the year did provide the opportunity to identify some misconceptions that were prevalent in the 5th grade science classrooms.

To identify some misconceptions that exist amongst the 5th graders at Lewis and Clark Elementary, students were given a worksheet (Figure A.1) with several questions on topics that have been identified previously as areas where misconceptions are known to exist.[6,7] The questions were set up as either true/false or multiple choice. The students were told that they would not be graded on the worksheet in hopes that this would alleviate any stress they might feel about getting the "correct" answer. This worksheet was given to not only the two ECOS classes that we had worked with all year, but it was also given to a non-ECOS class of 5th graders with whom we had not interacted with at all during the year.

Science Trivia Questions

Check yes or no, or circle the correct answer

1. If you drop a 1 pound weight and a 10 pound at the same time from the same height, the 10 pound weight will hit the ground first.

□ Yes □ No

2. It is warmer during the summer in North America because the Earth is closer to the Sun.

□ Yes □ No

3. The moon increases and decreases in size throughout the month.

□ Yes □ No

4. Plants get food from:

a.) fertilizer
b.) the ground water
c.) making their own food internally
d.) absorbing it from the soil via the roots

5. Plants, fungi, eggs and seeds are not living.

□ Yes □ No

6. What are the bubbles in boiling water composed of?

a.) air
b.) water vapor
c.) oxygen
d.) nothing

7. We can see objects because:

a.) light reflects off the object and our eyes focus the reflecting light.
b.) they are bathed in light.
c.) light travels from our eyes to the object.
d.) light travels faster at night.

8. Whales, jellyfish, and starfish are all fish.

□ Yes □ No

9. A species high on the food web is a predator to everything below it.

□ Yes □ No

10. Air and oxygen are the same gas.

□ Yes □ No

Figure A.1: Worksheet given to 5th grade students at Lewis and Clark to determine common misconceptions.

The goal of this worksheet was two-fold. First, the worksheet was given out to hopefully determine any misconceptions that are commonly held among the 5th grade classrooms at Lewis and Clark Elementary School. Identifying misconceptions is the first step in changing a misconception into a valid concept. The hope would be to give this information to the teachers so they could potentially work to identify and alleviate some of these misconceptions in upcoming 5th grade science classes. The second goal was a very qualitative goal. We had intentionally added misconception questions based on topics that we had specifically worked on with the two ECOS classes (questions 4, 6,and 7; Fig. A.1) while others concerned areas that we had not discussed. Again, when these topics were presented early in the year, the goal was not to change an existing misconception, and misconceptions had not previously been identified in these classrooms. Although the science trivia quiz is structured such that a positive response indicates that the student had the misconception and a negative response indicates the correct answer, we felt that the high prevalence of misconceptions would negate any effect of consistency in the answers.

A1.4 Results of the Misconception Worksheet

The first question on our misconception worksheet concerned gravity, and the false idea that a heavier object will fall faster than a lighter object of the same size. This misconception was just as common among both study groups (approximately 35% of all students). Little difference existed among ECOS and non-ECOS student responses to the misconception regarding the idea that the seasons occur because of earth's changing distance from the sun, rather than its tilt. This idea again was relatively common, as 27% of the students identified this as a true statement. The third misconception, that the moon

changes size over the course of the month, also did not produce a different proportion of student responses between the two study groups. Nearly 50% of both ECOS and non-ECOS students believed this to be true. However, upon further examination, we realized that the wording of the question is poor and most likely caused confusion amongst the students, as they may have thought that we were referring to the appearance of the moon rather than its actual shape.

The fourth common misconception, a multiple choice question about plant food sources, did show different responses between our study groups. The non-ECOS students responded equally to the three incorrect statements, which identified fertilizer, ground water, and soil as the primary food source of a plant. However, only one non-ECOS student correctly identified the true source (internal), while the vast majority of ECOS students selected the correct answer (Figure A.2). The next misconception included on our worksheet was the idea that plants, eggs, and seeds are non-living. Very few of the students surveyed in either group believed this to be true. ECOS students again had a greater proportion of correct responses to the next multiple choice question, which asked for the composition of the bubbles in boiling water. Most of the non-ECOS students incorrectly believed that the bubbles are made of air, while the majority of ECOS students knew that they are made of water vapor (Figure A.3).

Both study groups performed equally well on the question testing for the misconception that we see objects because they are bathed in light or because light travels from our eyes to the object. A substantial proportion of students, approximately 30% of both groups, did not understand that light reflects off an object and our eyes focus the reflecting light. In addition, 19% of ECOS and non-ECOS students believed that whales,

jellyfish, and starfish are all fish. Again, there was little to no difference in responses between the two groups. One question for which the non-ECOS students had a greater proportion of correct answers than ECOS students regarded the idea that a species high on the food web is a predator to everything below it. 45% of non-ECOS students correctly identified this idea as false, while only 33% of the ECOS students answered false. Lastly, the students were asked whether air and oxygen were the same gas. As with several of the other questions, ECOS students and non-ECOS students responded similarly: approximately 50% of the students in both groups believed this to be true.

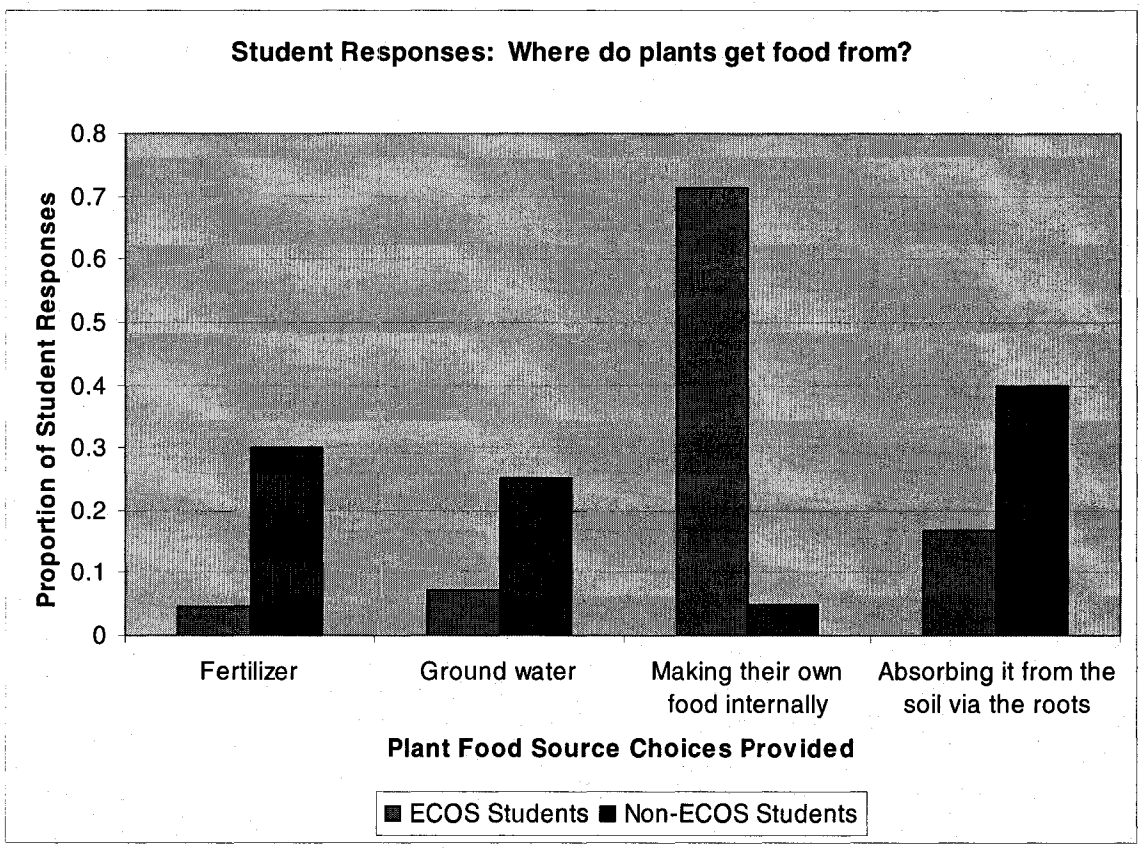

Figure A1.2: Graphical representation of the responses to multiple choice question number 4 of the misconception worksheet. This question asked students to identify where plants produce their food from and the available answers are shown below each bar set. Responses were normalized and ECOS student responses are shown in green and non-ECOS student responses are shown in blue.

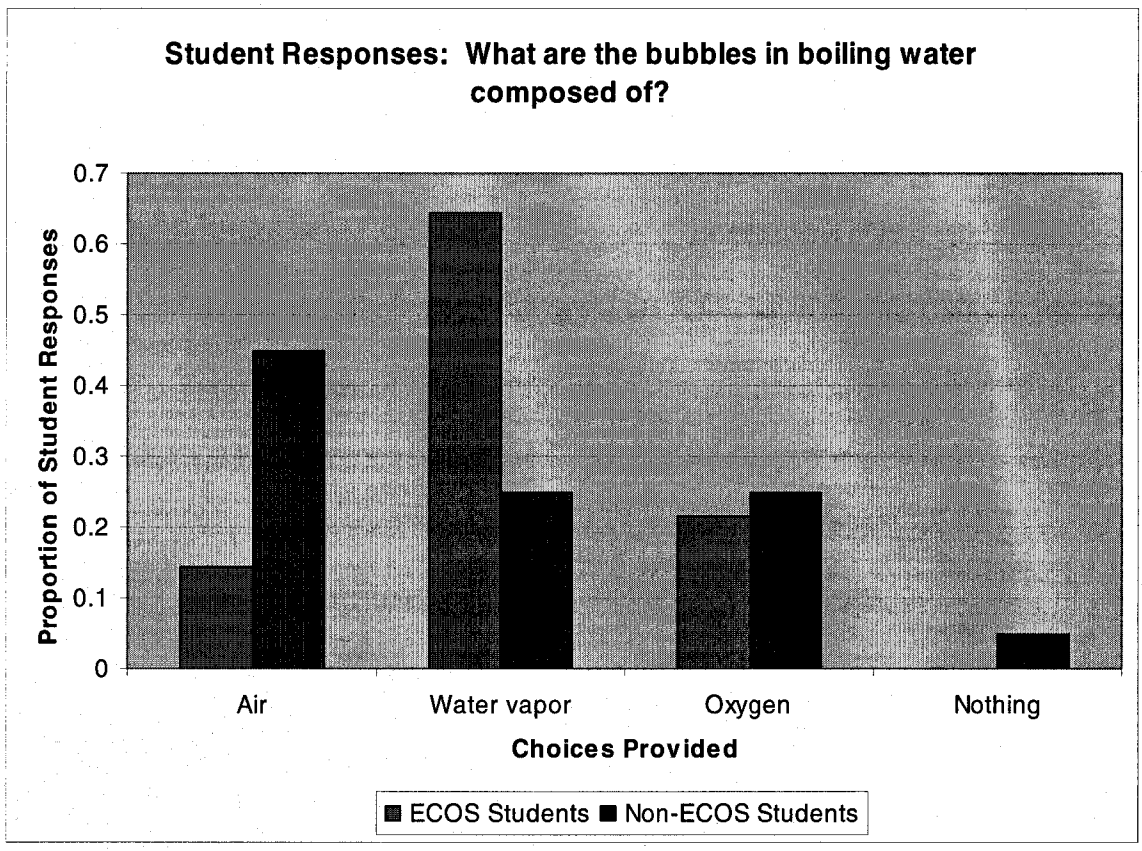

Figure A1.3: Graphical representation of the responses to multiple choice question number 6 of the misconception worksheet. This question asked students to identify what the bubbles in boiling water are composed of and the available answers are shown below each bar set. Responses were normalized and ECOS student responses are shown in green and non-ECOS student responses are shown in blue.

A1.5 Discussion

The results from our misconception worksheet indicate that the majority of students at Lewis and Clark Elementary School hold a variety of scientific misconceptions. These misconceptions could exist for a variety of reasons. For instance, some of the topics on the worksheet may be areas of study that they have never been exposed to, or may be topics they have learned about from non-traditional teaching sources (e.g. museum, TV program, parent or friend, etc.). The worksheet did not

identify how the students came to the answer that they indicated. However, while this misconception worksheet does not identify reasons behind the misconception, it does prove that misconceptions exist among the 5th graders tested. This worksheet also indicates areas of study that could be further evaluated to help change the misconceptions that these students hold.

The misconceptions identified are similar in all three classes signifying that all the students are roughly at the same educational level and have been exposed to similar topics. However, from our worksheet, we are able to see that in two of the three areas for which we specifically developed lessons, there are significant differences between ECOS and non-ECOS classes. During the school year, we introduced a variety of curriculum pieces oriented towards understanding plants. The awareness that plants need a variety of factors from the environment to live but actually make their food internally was identified to a much higher extent by ECOS students than by the non-ECOS students (Figure A.2). In addition, curriculum pieces were also designed to specifically illustrate the phases of matter, and water was used as a specific example several times. When asked what the bubbles are composed of in boiling water, the majority of ECOS students answered correctly. This was not the case in the responses from the non-ECOS students (Figure A.3).

In contrast to the first two questions, the third curriculum piece that was developed did not yield similar results. This curriculum piece pertained to light. The students in the ECOS classes were able to work through an experiment on light and were given some information on how our eyes perceive light and color, yet these students did not answer the light question correctly (question #7) any more so than the non-ECOS

students. This could be interpreted as an instance in which introducing students to a particular concept caused more misconceptions than actually creating knowledge and valid concepts in the minds of students. This is something that both the ECOS fellows and teachers should be aware. Students can regularly be assessed to determine whether knowledge is being organized into valid concepts or misconceptions, and if confusion still exists with a particular topic more time can be spent to alleviate this confusion.

If students are taught a concept correctly, why are misconceptions so resistant to change through instruction? One explanation is that each of us constructs knowledge that "fits" our experiences. Once we have constructed this knowledge, simply being told that we are wrong is not enough to make us change our (mis)concepts.[8] The only way to get rid of an old theory is to construct a new theory that does a better job of explaining the experimental evidence or finds a more appropriate set of experimental facts to explain.[8] We as educators must design lessons that clarify and direct students into different and more valid thought patterns.

Following some of the ideas put forth in the constructivist model of knowledge there are important implications for the way students are taught. Social knowledge such as the days of the week can be taught by direct instruction. It can even be argued that this is the only way that children can learn social conventions.[9] However, physical and logical knowledge cannot be transferred from the mind of the teacher to the mind of the learner. Following this idea would cause a shift in the classroom from someone who "teaches" to someone who tries to facilitate learning; a shift from teaching by imposition to teaching by negotiation.[10]

The goal of the ECOS program has been to loosely follow this way of teaching by negotiation. The curriculum that was developed for Lewis and Clark Elementary was designed to allow students to discover ideas and concepts and come to original conclusions. We worked to develop these concepts with the students so that their conclusions would be based upon valid observations. However, we did not specifically work to change any preconceived notions or misconceptions. Our goal was to design the lessons in such a way that the correct or valid conclusion would be the most obvious and make the most real world sense. Again, we have no true assessments that might indicate that our goals were reached other than conversations with our students at the end of each lesson and discussions on conclusions presented to us from the class.

A1.6 Future Directions

While the idea of identifying science misconceptions is incredibly important, it is equally important to test for those misconceptions in the proper way. In order to correctly determine the naïve concepts held by the students, they should be first pre-tested with a very sound worksheet or test. Our worksheet falls short of this goal, but with some revision would work well. For instance, question number 3 is very poorly written and most likely caused a great amount of confusion amongst the students. In addition to this, the worksheet should have been thoroughly reviewed by the 5^{th} grade teachers at Lewis and Clark. With their advice, only questions with which students have had significant experience would have been included on the misconceptions worksheet.

Once a number of misconceptions have been identified, new lessons will need to be developed to convince the students that there is a more valid way of understanding and using a particular concept. This is where the next group of ECOS fellows could make a

significant impact. Once the misconceptions are identified, the fellows could work to change the misconceptions into more valid concepts through different hands-on experimental activities. Providing a hands-on activity allows the student to experiment with different "real-world" variables that would hopefully allow the student to re-evaluate his or her current (mis)concept. In addition to providing hands-on lessons, fellows should have the students describe their thought process and how they have determined a new way of thinking about a concept (or why they believe their current concept is still valid). At the end of the year, students could again be tested to see if their misconceptions have changed into working concepts.

Misconceptions are prevalent in all age groups of students, and if unidentified, these misconceptions can persist well into adulthood. The ECOS program provides the prefect platform to identify and work to change naïve concepts into valid working concepts. This study should certainly be continued during the 2006/2007 school year. Not only could valuable thinking skills be taught today's youth, but new ways of accurately teaching concepts could be developed and implemented in the school curricula.

A1.7 References

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