Formation and Repair of DNA Lesions: Studies of Oxidized Cytosines and Aflatoxin B_1 Adducts

by

David Wang

B.S. Chemistry
Stanford University, 1992

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGICAL CHEMISTRY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 1998

Signature of Author:_____

Department of Chemistry
January 15, 1998

Certified by:_____

John M. Essigmann
Professor of Chemistry and Toxicology
Thesis Supervisor

Accepted by:_____

Dietmar Seyferth
Chairman, Departmental Committee on Graduate Studies

Thesis Committee

Professor Gerald N. Wogan _____ _____
Chairperson

Professor John M. Essigmann _____ _____
Thesis Supervisor

Professor Peter C. Dedon _____

Formation and Repair of DNA Lesions: Studies of Oxidized Cytosines and Aflatoxin B_1 Adducts

by

David Wang

Submitted to the Department of Chemistry on January 15, 1998 in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Biological Chemistry

ABSTRACT

The persistence of DNA adducts can lead to the induction of mutations and can ultimately contribute to carcinogenesis and aging. Adduct formation and adduct repair are the balancing factors that determine the cellular adduct load, and thus understanding these processes is central to the study of mutagenesis. The adducts derived from two types of DNA damaging agents known to induce mutations, reactive oxygen species and aflatoxin B_1 (AFB_1), were analyzed. Specifically, the *repair* of oxidized cytosine derivatives and the *formation* of AFB_1 guanine adducts were the subjects of investigation aimed at understanding some of the factors contributing to carcinogenesis.

In the first half of this thesis research, the ability of endonuclease III of *Escherichia coli* to excise three mutagenic oxidized cytosine lesions was examined. These lesions are 5,6-dihydroxy-5,6-dihydrouracil (Ug), 5-hydroxycytosine (5-ohC) and 5-hydroxyuracil (5-ohU). Site-specifically modified 40-mer oligonucleotides containing each of the three lesions in the same sequence context were prepared by either chemical synthesis or a combination of chemical and enzymatic steps. Using ^{32}P-labeled substrates and a gel electrophoresis based assay, the order of repair efficiency is Ug > 5-ohC > 5-ohU based on ratios of V_{max}/K_m. Modest effects were observed when the base paired opposite the lesion was changed from G to A. These experiments demonstrate conclusively that endonuclease III can excise these lesions in vitro and thus the enzyme may serve a genoprotective role in vivo.

In the second half of this work, a novel intercalation inhibitor was designed to probe the effects of intercalation by AFB_1 8,9-epoxide on its reaction with DNA. DNA duplexes were prepared consisting of a target strand containing multiple potentially reactive guanines and a non-target strand containing a *cis*-syn thymidine-benzofuran photoproduct. Since the covalently linked benzofuran moiety physically occupies an intercalation site, it was reasoned that such a

site would be rendered inaccessible to AFB_1 epoxide. By strategic positioning of this intercalation inhibitor in the intercalation site 5' to a specific guanine, the adduct yield at that site was greatly diminished, indicating that intercalation by AFB_1 epoxide contributed favorably to adduct formation. Using this approach it was possible to simplify the production of site-specifically modified oligonucleotides containing AFB_1 adducts in a p53 derived sequence, as a step towards understanding the basis of an observed p53 mutational hotspot found in human liver tumors.

Thesis Supervisor: Professor John M. Essigmann

Title: Professor of Chemistry and Toxicology

ACKNOWLEDGMENTS

"Graduate school is nothing more than an endurance test." These were the words of a wizened 5th year graduate student to me when I was a naive 1st year student. At that point in time I had not only no idea what kind of adventure I had just committed to, but no idea how true that statement would prove to be. Thus, I am deeply indebted to many, many people for help, advice, camaraderie, distraction and laughter during the past years.

First and foremost, I must thank my advisor, John, for all of his efforts over the years: for having faith and confidence in me from the very beginning of my studies; for providing me the room to grow as a scientist and the freedom to study, in his words, "anything related to cancer"; for introducing me to the splendors of Lake Mooselookmeguntic; and for having the sense of humor to tolerate yet another Californian in his lab. I must also thank the members of my thesis committee, Dr. Gerald Wogan and Dr. Peter Dedon, for their advice and suggestions, and Dr. Wogan in particular for his pivotal contributions to the aflatoxin project.

Some people who made this work possible include Dr. Richard Cunningham (SUNY Albany) and Dr. Bruce Demple (Harvard), who generously supplied me with purified proteins for my studies. Also, Dr. Bing Wang in Dr. Biemann's laboratory (MIT) and Lisa Marzilli in Dr. Vouros' laboratory (Northeastern) played critical roles by providing mass spectrometric analysis of various modified oligonucleotides. Without the preliminary work done by Marshall and Dipti in synthesizing the oxidized cytosines, I would probably still be doing experiments today.

I cannot thank Bill enough for developing the synthesis of his psoralen adduct and for coming up with the idea of inhibiting intercalation. I can only hope to be lucky enough in the future to have the opportunity to work with an individual who possesses such creativity, enthusiasm and passion for science. Also many thanks in the nonscientific arena: for being an organizer, for embracing new activities like volleyball and skiing, and for being a great friend and housemate. To think he was once "that obnoxious guy from Cal."

I would not have survived to this point in graduate school without the support of many individuals, including the lunch bunch, the volleyball crew, and the spades gang. Many hours of amusement and discourse were spent with Ben, Martha, Dennis, Linda, and Jeff "show me gold" Eckert. Thanks to Matt and Becky for trips to the Cape and New Hampshire and of course, Ubi; to Tanya, for reintroducing "pansy" to my vocabulary; and to Kathy for being a mentor, friend and surrogate sister. Special thanks to Evan, for his unique sense of humor, intellectualism, and for being the most reliable of friends. I am also grateful to Leo, Jeremy, Naomi and Jane for providing outlets from the MIT culture and exploring restaurants in Cambridge and Boston.

In the Essigmann lab, I must acknowledge many individuals both past and present. The old generation: Dipti, Dan, Kevin, Chen, Lisa, XQ and ET, who instilled me with the lab culture

and provided guidance those first clueless years. My contemporaries in the Fantastic Four: Bill, Jim and Debbie, who have witnessed and shared many highs and low through the years. And the Next Generation of the lab: Maria, Maryann and Zoran. Special thanks to Marjie and Debbie for always lending a sympathetic ear and to Jill, for many heartfelt and thoughtful discussions and also for trying to rescue me from my "gloom and doom" phase. Thanks to Paula, who has the dubious distinction of being my baymate longer than anyone else. I cannot imagine having worked with a better group of people over these many years.

I must thank my parents, who taught me the value of education and, most importantly, always supported me in every endeavor I have undertaken. I owe them a debt that can never truly be repaid. I also want to thank my sister Lisa for her encouragement and constant positive attitudes towards science.

Finally, this thesis is dedicated to Mrs. Boyer, Mrs. Sitzman, and Mrs. Ikoma, my first science teachers, who taught me so much about chemistry, biology and life itself when I was at a very impressionable age, and by doing so, propelled me into this scientific odyssey; fortunately I fared better than Odysseus, who needed a full ten years to complete his journey.

"If we couldn't laugh, we would all go insane."
Jimmy Buffet

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5-ohC	5-hydroxycytosine
5-ohU	5-hydroxyuracil
8-oxoA	8-oxoadenine
8-oxoG	8-oxoguanine
8-oxodGTP	8-oxo-2'-deoxyguanosine triphosphate
8-oxoGTP	8-oxo-guanosine triphosphate
A. flavus	*Aspergillus flavus*
AFB_1	Aflatoxin B_1
AFB_1-diol	AFB_1-8,9-diol
AFB_1-N7-Gua	8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1
AFB_1-FAPY	8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB_1
AP	Abasic site
APE	Human AP endonuclease
BER	Base excision repair
Cg	5,6-dihydroxy-5,6-dihydrocytosine
CS	Cockayne's Syndrome
dRpase	deoxyribophosphodiesterase
E. coli	*Escherichia coli*
FAB	Fast atom bombardment
FapyA	4,6-diamino-5-formamidopyrimidine
FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
GC	Gas chromatography
HCC	Hepatocellular carcinoma
HPLC	High pressure liquid chromatography
HPRT	Hypoxanthine guanine phosphoribosyl transferase
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MS	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
NER	Nucleotide excision repair
PNK	Polynucleotide kinase
O^6MeG	O6-methyl guanine
PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Tg	Thymine glycol
Ug	5,6-dihydroxy-5,6-dihydrouracil
XP	Xeroderma Pigmentosum
XRCC	X-ray sensitivity cross complementing

Chapter 1: INTRODUCTION

The defining experiments of Avery, McLeod and McCarty in 1944 demonstrating that DNA rather than protein is the genetic material revolutionized the prevailing dogma of that era (Avery et al., 1944). A corollary concurrently adopted with this concept was the notion that DNA must be a chemically stable structure in order to preserve and faithfully transmit genetic information (Friedberg et al., 1995). Over the past several decades the perception of DNA as static has been supplanted by a dynamic model wherein the DNA continually undergoes many potentially harmful chemical reactions to form what are referred to as DNA adducts or lesions (Lindahl, 1993). Fortunately, the formation of DNA damage is balanced by the existence of specific proteins designed to repair alterations to DNA and maintain the structural and informational integrity of the genome. Since the first observations in the 1950s and 1960s that DNA could be repaired (Rupert et al., 1958; Setlow and Carrier, 1964; Pettijohn and Hanawalt, 1964), a broad range of functionally diverse and versatile repair systems has been discovered that counteract insults from the vast array of endogenous and exogenous DNA damaging agents that threaten the genome (Friedberg et al., 1995). Thus the formation and repair of DNA adducts are phenomenon that are closely intertwined, both historically and biologically.

The balance between adduct formation and adduct repair is a precarious one. It has been estimated that the genome of an average human cell suffers on the order of 10^4 assorted covalent alterations each day (Lindahl, 1993). These include reactions due to the inherent chemical instability of DNA, such as spontaneous depurination and cytosine deamination, as well as reactions with endogenously generated oxidants and alkylators (Lindahl, 1993). Left unrepaired,

these adducts can have numerous deleterious effects on cellular physiology, including inhibition of replication or transcription and mutagenesis. In a normal cell, the action of cellular DNA repair enzymes is sufficient to remove the majority of these lesions and preserve genomic integrity. Perturbing the natural balance, however, either by augmenting adduct formation or decreasing repair capacity, can have severe consequences for the cell. In many instances the loss of a single repair protein results in sensitization towards killing by a particular damaging agent or the generation of a mutator phenotype (Friedberg et al., 1995). Alternatively constant environmental exposure to DNA damaging agents can overwhelm repair systems, leading to increased mutagenesis. In both cases, increased mutations can ultimately lead to carcinogenesis. In fact, specific environmental factors such as UV light and aflatoxin B_1 are associated with characteristic mutations observed in some human cancers (Greenblatt et al., 1994). Similarly, over time, the gradual accumulation of adducts arising from oxidative damage that escape repair has been hypothesized to contribute to aging and degenerative disorders (Halliwell and Gutteridge, 1989; Ames et al., 1993).

Regardless of the type of DNA damaging agent involved, the appearance of a mutation in vivo represents a composite of several determining factors: 1) the rate of reaction between the damaging agent and DNA; 2) the rate of removal of the adduct; 3) the miscoding potential of the adduct; and 4) possible selection for the mutation. The first two parameters, adduct formation and adduct repair, dictate the population of potentially mutagenic adducts present during replication and are critical factors in mutagenesis. The goal of this thesis was to study adduct formation and repair. Two types of adducts were investigated: oxidative lesions formed by reactive oxygen species and DNA adducts formed by the environmental carcinogen aflatoxin

B_1. In particular, the *repair* of a family of oxidized cytosine derivatives and the *formation* of aflatoxin B_1 guanine adducts were the subjects of investigation.

In an aerobic world, oxidative stress is a constant threat to the genome. Both endogenous metabolism and ionizing radiation can generate reactive oxygen species, which can in turn damage DNA and form as many as 100 different DNA adducts (von Sonntag, 1987). In particular, adducts derived from cytosine have been the subject of much recent interest. The three adducts 5-ohC, 5-ohU and Ug have been detected in mammalian tissues (Wagner et al., 1992), and speculation that these lesions may be partially or completely responsible for the C→T transition mutations that dominate the mutational spectra of oxidizing agents (Tkeshelashvili et al., 1991; Moraes et al., 1990; Waters et al., 1991) is supported by data from recent experiments. DNA polymerase extension assays in vitro have determined that both 5-ohC and 5-ohU are capable of base-pairing with A and that the corresponding triphosphates are accepted by polymerases, suggesting that they may be mutagenic in vivo (Purmal et al., 1994b; Purmal et al., 1994a). Indeed, one report indicates that 5-ohC induces a mutation frequency of 2.5% in a reporter construct in *E. coli* (Feig et al., 1994). Finally, recent work in *E. coli* reveals that 5-ohU and Ug, which are oxidative deamination products of cytosine, are both highly mutagenic lesions producing exclusively C→T transitions (Kreutzer and Essigmann, submitted). Given the clear genomic threat presented by these highly mutagenic lesions and their intracellular prevalence, it was of great importance to determine which proteins might be involved in the repair of these lesions. When this investigation began, there was nothing known about the enzymatic repair of these lesions. A likely candidate to repair these oxidized cytosine derivatives based on their structural similarity to previously identified substrates was endonuclease III of *E. coli*.

Endonuclease III is a 24 kDa protein possessing both glycosylase and apurinic/apyrimidinic (AP) endonuclease activity. The enzyme has a broad substrate specificity, acting upon a host of hydrated, oxidized or ring-fragmented pyrimidines (Dizdaroglu et al., 1993; Breimer and Lindahl, 1984). Crystal structure data combined with modeling studies suggest that catalysis involves an intermediate in which the damaged base is rotated out of the helix (Thayer et al., 1995). The *nth* gene of *E. coli*, which encodes endonuclease III, has been cloned and sequenced, and homologs have been identified in yeast and mammals (Asahara et al., 1989; Roldán-Arjona et al., 1996; Augeri et al., 1997; Hilbert et al., 1997; Eide et al., 1996; Aspinwall et al., 1997).

In the first half of this thesis, the repair of the three lesions 5-ohC, 5-ohU and Ug by endonuclease III was investigated. The approach taken herein involved chemical synthesis of defined substrates each containing a single lesion and examination of the ability of specific glycosylases of the base excision repair pathway to excise the lesions. The results of the experiments demonstrate conclusively that 5-ohC, 5-ohU and Ug can all be removed by endonuclease III in vitro, suggesting that this enzyme may play an important role in protecting the genome from oxidative damage.

In the second half of this thesis, the formation of DNA adducts by the potent human carcinogen AFB_1 was examined. Exposure to aflatoxin B_1 (AFB_1), a common contaminant of various foods in certain parts of the world, is a known risk factor for hepatocellular carcinoma (Qian et al., 1994). The molecular mechanism of human hepatocellular carcinogenesis is poorly defined but is at least partially mediated by the DNA adducts formed by AFB_1. Upon metabolic activation to the *exo*-8,9-epoxide, AFB_1 reacts almost exclusively with the N7 of guanine to form guanine DNA adducts (AFB_1-N7-Gua)(Essigmann et al., 1977; Croy et al., 1978). Analysis of

mutational spectra and phage genomes containing a specific AFB_1-N7-Gua adduct reveals G→T substitutions as the predominant mutagenic event (Foster et al., 1983; Levy et al., 1992; Bailey et al., 1996b). In human liver cancer, a striking mutational hotspot has been attributed to AFB_1; approximately 50% of hepatocellular carcinomas in portions of eastern Asia and sub-Saharan Africa, where exposure to AFB_1 through contaminated food is a frequent event, possess G:C→T:A transversions in the third position of codon 249 of the p53 tumor suppressor gene (Hsu et al., 1991; Bressac et al., 1991; Greenblatt et al., 1994; Shen and Ong, 1996).

Work from several groups over the past 10 years suggests that a key step in the formation of AFB_1 DNA adducts is the intercalation of AFB_1 epoxide into the DNA duplex. From kinetic, structural and stoichiometric experiments, a model has been proposed wherein rapid intercalation by the epoxide leads to a conformation highly favorable to attack by the guanine N7 nucleophile and adduct formation (Gopalakrishnan et al., 1989; Gopalakrishnan et al., 1990; Iyer et al., 1994a). Moreover, in the absence of this favorable interaction, rapid hydrolysis of the epoxide to the biologically inactive AFB_1-diol can occur. While this model is highly plausible, it is based predominantly on indirect evidence and has not been directly tested. Thus the goals of this part of the thesis were twofold: to determine whether adduct formation was indeed dependent upon intercalation and, if so, to try and exploit this property in the synthesis of site-specific adducts.

For these purposes, a covalently linked intercalation inhibitor was designed to probe the effects of inhibiting intercalation by AFB_1 epoxide on adduct formation at a specific site. The results of this work confirm the previously hypothesized idea that intercalation does play a significant role in adduct formation. Moreover, modulating the intercalation step can serve as an

effective means of controlling the resulting adduct profile. This approach has been successfully applied to the synthesis of several AFB_1-modified oligonucleotides as a first step towards understanding the basis for the observed p53 mutational hotspot in hepatocellular carcinoma.

Chapter 2: LITERATURE SURVEY

I. DNA REPAIR PROCESSES

The ability to repair DNA is a characteristic of all life forms, from prokaryotes to humans (Friedberg et al., 1995). Even some viruses and bacteriophages encode their own DNA repair enzymes. Clearly, the preservation of genomic integrity is of critical importance, and consequently a diverse array of methods exist to repair damage. The different mechanisms to repair damage can be grouped into four general categories: 1) base excision repair (BER); 2) nucleotide excision repair (NER); 3) direct reversal of damage; and 4) recombinational repair. Some of the details more relevant to this work, in particular base excision repair and to a lesser extent nucleotide excision repair, are described below. Since the clearest understanding of the biochemistry and genetics of repair is in bacteria, *E. coli* repair enzymes will be the focus of this section with some discussion of the analogous, but typically more complex, human repair systems. A comprehensive description of DNA repair processes can be found in (Friedberg et al., 1995).

A. Base Excision Repair (BER).

The BER pathway involves several proteins that act to excise a single damaged nucleobase from DNA and replace it with the correct undamaged nucleotide. Among the activities utilized in BER are: 1) a glycosylase to hydrolyze the glycosidic bond between the modified nucleobase and the deoxyribose moiety; 2) an AP endonuclease to cleave the resulting AP site and form an extendable 3' terminus; 3) a DNA deoxyribophosphodiesterase (dRpase) activity to remove 5' deoxyribose phosphates formed by AP endonucleases; 4) a DNA

polymerase activity to insert an undamaged nucleotide triphosphate into the gap; and 5) a DNA ligase to seal the nick (Figure 1).

The primary role of the glycosylase in BER is to recognize a damaged nucleobase and catalyze hydrolysis of the glycosydic bond to release the adduct. Glycosylases have been identified that remove oxidized, alkylated and deaminated bases (Friedberg et al., 1995). Typically, glycosylases are small, roughly 20-40 kDa proteins that do not require ATP or cofactors for their activity (Friedberg et al., 1995). X-ray crystallography has recently revealed that many glycosylases appear to possess a "base-flipping" motif (Thayer et al., 1995; Vassylyev et al., 1995; Slupphaug et al., 1996; Labahn et al., 1996; Yamagata et al., 1996) that everts the target nucleobase from the helix into an active-site pocket on the enzyme (Roberts, 1995). By "base-flipping," the nucleobase is more accessible to the various side-chain functionalities in the enzyme that contribute to catalysis and substrate discrimination. Of the different glycosylase activities that have been identified, some, such as endonuclease III and alkA display very broad substrate specificities and can excise numerous discrete adducts (Singer and Hang, 1997). In contrast, others such as uracil glycosylase and 3-methyladenine glycosylase I demonstrate exquisite substrate stringency. The X-ray structures of mutant human uracil glycosylases cocrystalized with a uracilated DNA duplex are sufficiently resolved to rationalize the precise substrate specificity of the enzyme (Slupphaug et al., 1996; Slupphaug et al., 1996). The proximity of a specific tyrosine amino acid side chain in the active site binding pocket 3 Å from the 5 position of the bound U precludes the binding of T or other substituted pyrimidines, presumably due to steric constraints (Mol et al., 1995). However, uracil glycosylase is the most exhaustively studied glycosylase to date, and in the other cases, insufficient structural resolution

is available to explain the observed biochemical properties of the glycosylases on the basis of structure.

There are two classes of glycosylases: simple glycosylases and combined glycosylase/AP lyases. The combined glycosylase/AP lyases are able to execute a second reaction, cleavage of the phosphodiester backbone at the nascent AP site. This reaction occurs by β-elimination from a ring-opened isomer of deoxyribose resulting in termini containing a 3' α-β unsaturated aldehyde and a 5' phosphate (Figure 3). Although this cleavage is a lyase activity, enzymes of this type have been classified as class I AP endonucleases. With certain enzymes, β-δ elimination occurs creating end products with 3' and 5' phosphates. A general model has been proposed to explain mechanistically the differences between the glycosylases and the glycosylase/AP lyases (Sun et al., 1995). In this model, the enzymes that act strictly as a glycosylase utilize an activated water molecule or hydroxide as the nucleophile (Figure 2). Upon displacement of the nucleobase, an intact AP site is the end product. By contrast, the combined glycosylase/AP endonucleases are suggested to utilize specific conserved enzymic amino groups as the active nucleophile (Figure 3) (Dodson et al., 1993; Thayer et al., 1995; Zharkov et al., 1997). Formation of a Schiff's base intermediate with the deoxyribose follows release of the base, and the presence of a positive charge on the imine increases the lability of the α-proton, promoting enzyme assisted β-elimination and ultimately strand cleavage (Figure 3). Evidence for the Schiff's base intermediate has been obtained for the enzymes endonuclease III, fapy glycosylase, and T4 endonuclease V by $NaCNBH_3$ or $NaBH_4$ reduction of the imine to form a covalently bound protein-DNA complex (Dodson et al., 1993; Sun et al., 1995; Tchou and Grollman, 1995).

Regardless of the type of glycosylase involved in the excision event, the second step of BER involves processing to create a 3' OH terminus amenable to extension by DNA polymerases. The class II AP endonucleases specifically hydrolyze the phosphodiester bond 5' to intact AP sites formed by either simple glycosylases or spontaneous depurination, resulting in 3' OH and 5' deoxyribose phosphate termini (Figure 2). In addition, they can also hydrolyze various 3' terminal groups, such as phosphate, phosphoglycolate and the α-β unsaturated aldehyde products of class I AP endonucleases (Friedberg et al., 1995). Exonuclease III and endonuclease IV, which are both class II AP endonucleases, are estimated to account for approximately 85-90 % and 10 %, respectively, of the total observed AP endonuclease activity in *E. coli* (Yajko and Weiss, 1975; Ljungquist et al., 1976).

The 5' deoxyribose phosphate residues generated through the action of class II AP endonucleases at intact AP sites must be removed in order for correct repair to occur (Figure 2). These termini are refractory to processing by conventional 5'-3' exonucleases, such as that of polymerase I, and instead require a dedicated enzymatic activity (Franklin and Lindahl, 1988). In *E. coli*, the recJ protein possesses a dRpase activity that catalyzes hydrolysis of the 5' deoxyribose phosphate generating free deoxyribose 5' phosphate and a 5' phosphate (Dianov et al., 1994). In addition, fapy glycosylase (encoded by the *fpg* gene) can excise terminal 5' deoxyribose phosphates by β-elimination yielding a 5' phosphate (Graves et al., 1992). These are the two prokaryotic dRpase activities identified to date; however, *recJ fpg* double mutants are able to repair AP sites, suggesting that other repair activities in *E. coli* can substitute for recJ and fapy glycosylase (Dianov et al., 1994). Not surprisingly, the yeast homolog of fapy glycosylase, Ogg1 also possesses dRpase activity (Sandigursky et al., 1997b).

Once an appropriate 3' OH terminus is available, the action of a DNA polymerase inserts a single nucleotide into the gap. While it is possible that all three DNA polymerases in *E. coli* may play roles in repair synthesis, it has been suggested that polymerase I, due to its low processivity, is eminently suited to the one nucleotide gap-filling necessary in BER (Kornberg and Baker, 1992). In support of this hypothesis, mutants in polymerase I are hypersensitive to DNA damaging agents (Paterson et al., 1971; Zhang et al., 1992). The final step in BER is ligation of the single-stranded nick to produce fully repaired DNA. *E. coli* ligase can join 3'-OH to 5'-phosphate ends using NAD^+ as a cofactor. The complete set of reactions involved in BER has been fully reconstituted in vitro using five purified *E. coli* proteins. Uracil glycosylase, endonuclease IV, recJ, DNA polymerase I, and DNA ligase are sufficient to excise and repair a uracil in an oligonucleotide duplex substrate (Dianov and Lindahl, 1994).

BER in mammals can proceed via two pathways: one that is highly analogous to that in *E. coli* involving a single nucleotide repair patch and an alternative "long-patch" mechanism. The short patch BER has been reconstituted with the four mammalian counterparts of the respective *E. coli* proteins: human uracil DNA glycosylase, human AP endonuclease, polymerase β and DNA ligase I (Nicholl et al., 1997). The major AP endonuclease in mammals, a homolog of exonuclease III, has been cloned by several groups and is referred to as APE, HAP1, or REF1 (Robson and Hickson, 1991; Demple et al., 1991; Xanthoudakis et al., 1992). Polymerase β is the primary polymerase involved in mammalian BER gap filling (Wiebauer and Jiricny, 1990; Dianov et al., 1992; Sobol et al., 1996) and quite interestingly can remove 5' deoxyribose phosphate moieties by a β-elimination mechanism, thus obviating the need for a separate dRpase to complete repair (Matsumoto and Kim, 1995). Homozygous knockout mice

in polymerase β or APE are embryonically lethal, underscoring the importance of these BER proteins to survival (Gu et al., 1994; Xanthoudakis et al., 1996). Following repair synthesis, either DNA ligase I or DNA ligase III can act to complete the repair reactions. Ligation by DNA ligase III is stimulated by the accessory protein XRCC1 that may serve a scaffolding function to facilitate completion of BER. (Kubota et al., 1996).

The "long-patch" BER has been reported to occur in mammalian cell extracts (Frosina et al., 1996) and with purified proteins (Klungland and Lindahl, 1997). In cell extracts, repair synthesis extends to at least the 7th nucleotide 3' of an AP site substrate (Frosina et al., 1996). Synthesis is dependent upon PCNA suggesting that polymerase δ or ϵ is responsible for gap filling in this system. Using purified recombinant proteins, Lindahl and coworkers observed a repair patch of 2-6 nucleotides that required DNase IV (also FEN-1 or "flap endonuclease"), an endonuclease that cleaves 5' overhangs and branches (Klungland and Lindahl, 1997). This repair can be carried out with either purified polymerase δ or β (Klungland and Lindahl, 1997). It is proposed that insertion of multiple nucleotides by polymerase can lead to strand displacement 3' of the original lesion site, forming an overhanging substrate for DNase IV. Interestingly, polymerase β has been shown to physically associate with APE (Bennett et al., 1997) as well as with DNA ligase I and with both XRCC1 and ligase III, raising the possibility that these proteins associate in a multiprotein complex (i.e. a repairosome) that executes BER (Kubota et al., 1996; Prasad et al., 1996).

B. Nucleotide Excision Repair (NER)

The NER pathway has been extensively reviewed (Friedberg et al., 1995; Wood, 1996;

Sancar, 1996). In NER, incision events both 5' and 3' to an adduct result in the removal from DNA of a contiguous tract of nucleotides surrounding the adduct. The resulting gap is subsequently filled in by a DNA polymerase and sealed by DNA ligase (Figure 4). In *E. coli* the repair tract is 12 or 13 nucleotides whereas in mammalian cells it is 24-32 nucleotides. NER is known to repair a number of predominantly bulky or helix distorting lesions, including those of psoralen, pyrimidine dimers, aflatoxin, cisplatin and acetylaminofluorene. In addition small lesions that minimally perturb the structure of the DNA such as AP sites, thymine glycol (Lin and Sancar, 1989; Kow et al., 1990; Reardon et al., 1997) and O^6MeG (Voigt et al., 1989) are in vitro substrates for NER. Given the diversity of structures repaired by Uvr(A)BC, an intriguing issue is the mode of damage recognition utilized by the repair proteins. Since these adducts share essentially no structural similarity, factors other than physical complementarity must be utilized. One current model to explain the broad specificity of NER suggests that the energy of ATP hydrolysis is used to bend and unwind the DNA in damaged regions, which possess a greater propensity to undergo deformation than undamaged DNA, to a conformation that promotes protein binding (Sancar, 1996).

In *E. coli* the incision steps are executed by the Uvr(A)BC excinuclease. In the current model of NER, a $(UvrA)_2(UvrB)_1$ complex forms that specifically recognizes and binds to damaged sites in a process dependent upon ATP hydrolysis. Upon binding to DNA damage, the dimer of UvrA dissociates from the complex leaving a very stable UvrB-DNA complex. Subsequent binding by UvrC to this complex results in UvrB mediated incision at the 4th or 5th phosphodiester bond 3' to the adduct and UvrC mediated incision at the 8th phosphodiester bond 5' to the adduct. After cleavage, the helicase activity of UvrD promotes release of UvrC and the

excised fragment. DNA polymerase I then fills the gap and displaces UvrB. In vitro, all three E coli polymerases are capable of gap filling repair synthesis, although polymerases II and III require additional accessory proteins (Sancar, 1996). In the final step of the repair process, DNA ligase seals the nick.

The NER pathway in humans is significantly more complex, with 16 polypeptides involved in the excision reaction that produces a fragment of 24-32 nucleotides (Wood, 1997a). The autosomal recessive disease Xeroderma Pigmentosum (XP) is caused by defective nucleotide excision repair, and studies of XP have revealed the existence of seven genetic complementation groups (XP-A through XP-G) (Wood, 1996). Proteins in these complementation groups have been characterized and attributed roles in the repair pathway: XPA is a damage recognition protein involved in binding; XPB and XPD are helicases that are also part of the transcription factor TFII-H; XPC may serve an important role in mediating protein-protein interactions in the complex; and XPF and XPG are endonucleases that carry out the 5' and 3' incision events respectively. Since repair synthesis is dependent on PCNA, it is likely that polymerases δ or ϵ carry out the repair synthesis (Wood and Shivji, 1997b).

C. Transcription-Coupled Repair

The NER mediated processing of lesions is not uniform throughout the genome; instead, transcribed genes are repaired more efficiently than untranscribed genes, and the transcribed strand of a gene is preferentially repaired as well (Mellon et al., 1987). The signaling pathway linking transcription to NER is believed to be triggered by the stalling of RNA polymerase II at an adduct. In *E. coli*, a specific transcription-repair coupling factor exists that recognizes the

stalled polymerase and recruits the $(UvrA)_2(UvrB)_1$ complex to the damaged site (Selby and Sancar, 1994). The situation is more complex in humans where several proteins are involved. The human disease Cockayne's Syndrome (CS) is characterized by defects in transcription coupled repair and can arise from defects in either the genes CSA or CSB or in the NER proteins XPD and XPB that are part of transcription factor TFIIH (Sancar, 1996). Until recently, it appeared that NER was the only transcriptionally coupled repair process; however, it was recently reported that the transcription coupled repair of an oxidative lesion is independent of XPA or XPF, suggesting for the first time that BER can also be preferentially directed to transcribed strands of DNA (Cooper et al., 1997).

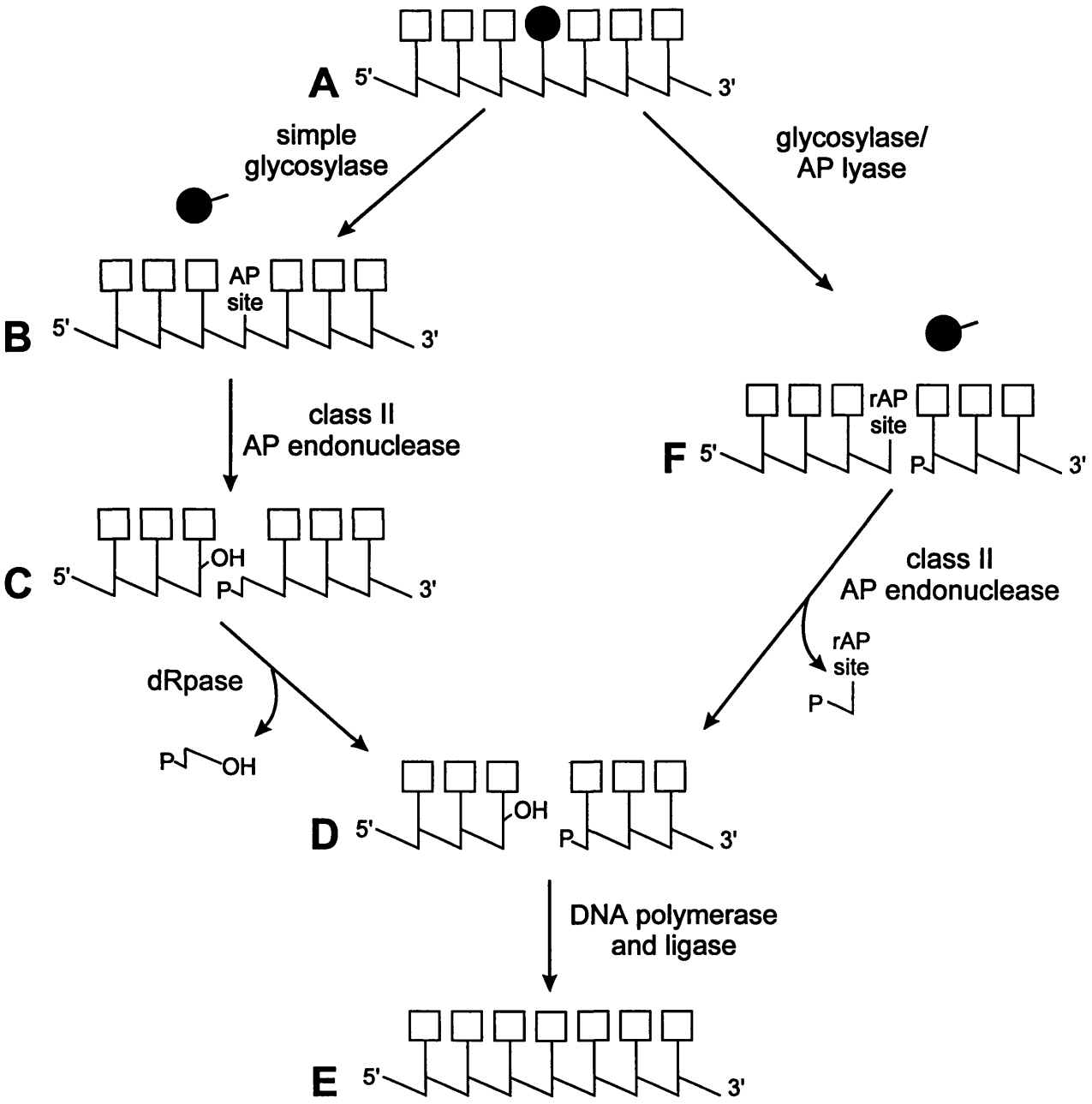

Figure 1. Schematic of BER. The modified base (solid circle) can be removed by either of two pathways. In the 1st pathway (left) a simple glycosylase excises the damaged base forming an AP site (**B**), which is cleaved by an AP endonuclease (**C**). Then a dRpase removes the 5' deoxyribose phosphate from the 3' fragment creating a one nucleotide gap flanked by 3' OH and 5' P (**D**). Alternatively (right), a combined glycosylase/AP lyase excises the damaged base and cleaves the AP site, forming a reduced AP site (rAP) and a 5' P (**F**). Following removal of the 3' sugar fragment, the same product (**D**) is formed. The one nucleotide gap is filled in by DNA polymerase and DNA ligase. Adapted from Friedberg et al.

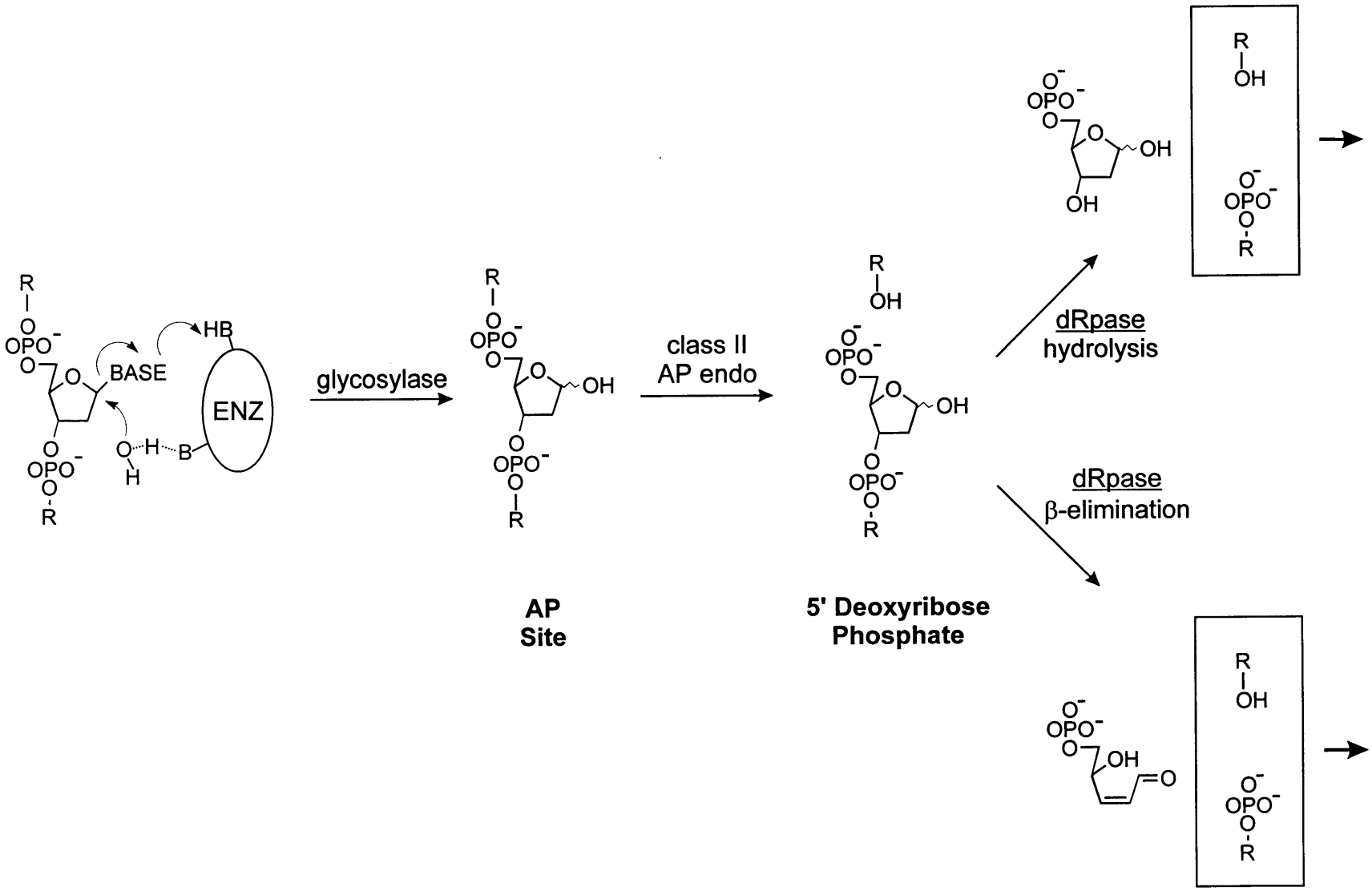

Figure 2. BER executed by a simple glycosylase and class II AP endonuclease (steps A-B-C-D of Figure 1). An activated H_2O molecule is proposed to be the nucleophile. Hydrolysis of the AP site forms 3' OH and 5' deoxyribose phosphate termini. The 5' deoxyribose phosphate can be excised by two different dRpase mechanisms, either hydrolysis or β-elimination.

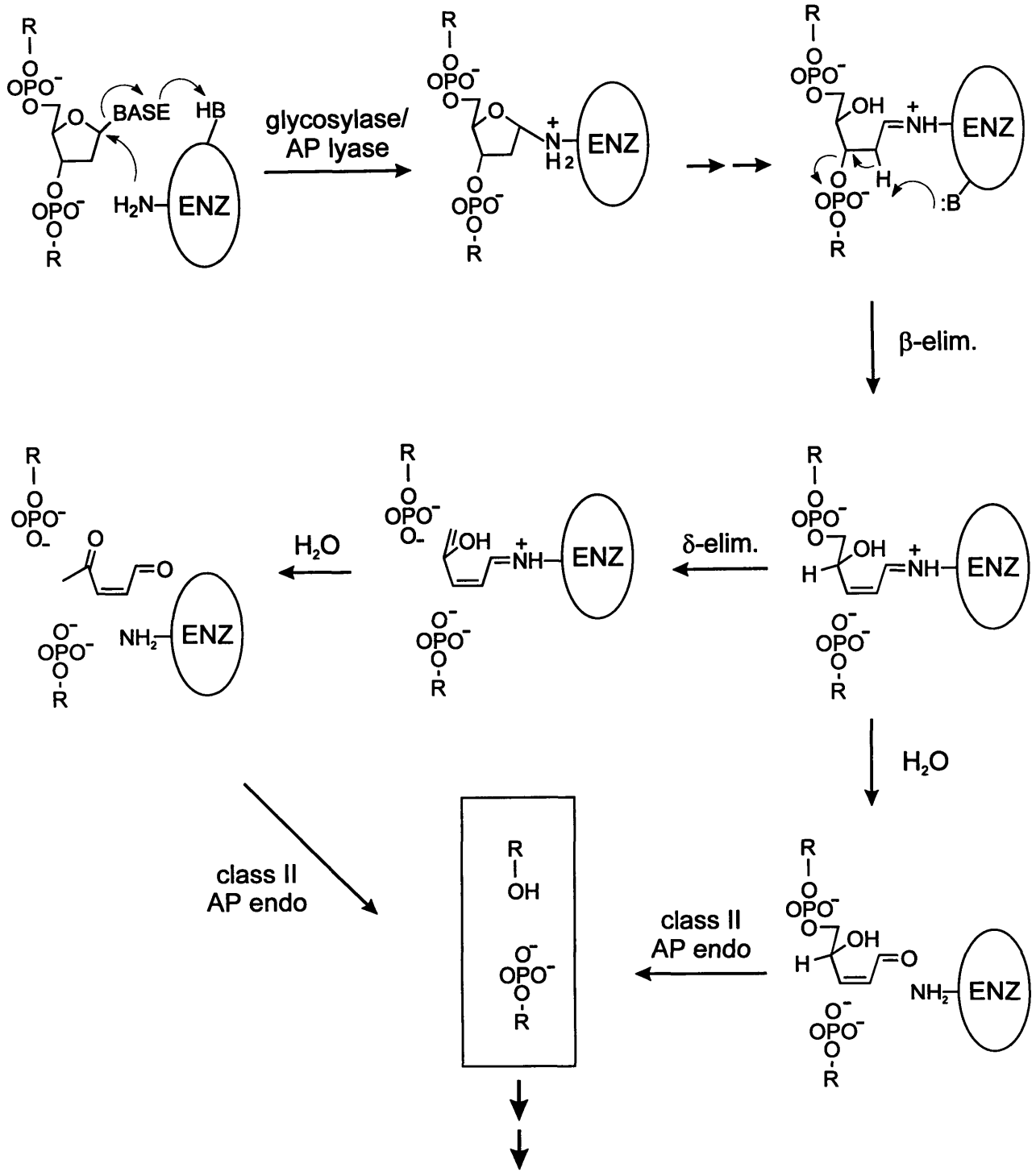

Figure 3. Proposed mechanism of BER involving combined glycosylase/AP lyases (steps A-F -D of Figure 1). Displacement of the modified base is followed by formation of a Schiff's base between the amino nucleophile and C1' that promotes β or β-δ elimination. The resulting 3' P or 3' α-β unsaturated aldehyde must be removed, typically by the 3' phosphatase or 3' exonuclease activities of class II AP endonucleases to produce a 3' OH for repair synthesis.

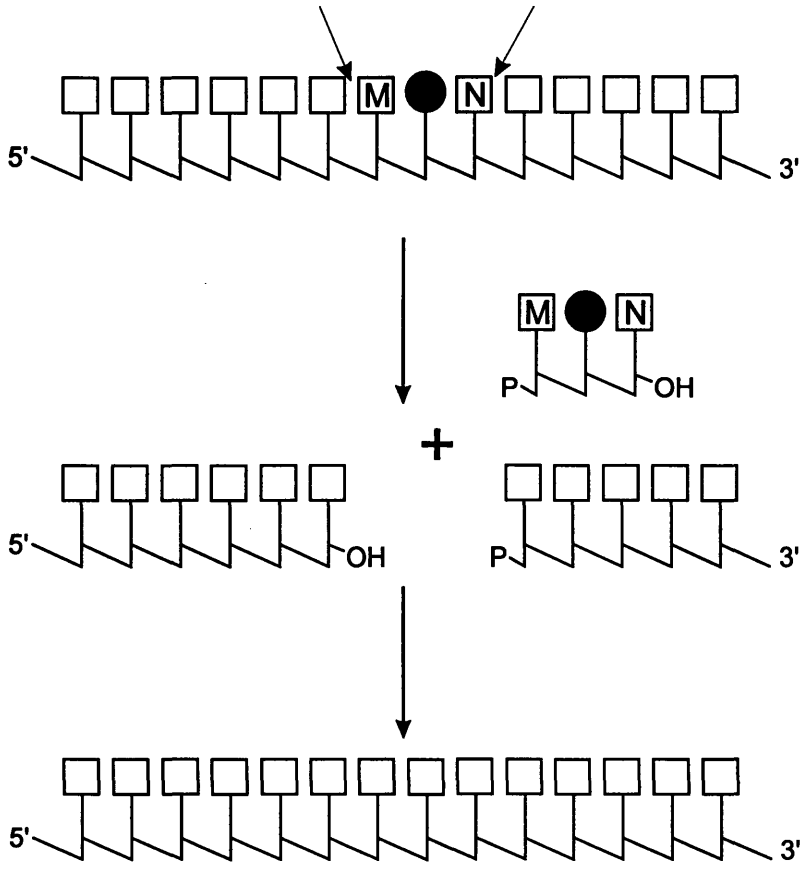

Figure 4. Schematic of NER. Dual incisions occur 5' and 3' to the lesion at variable distances M, N from the lesion. Typically in *E. coli*, M=8 and N=4 or 5 whereas in mammalian systems M≈24, N≈5. An intact fragment containing the lesion is excised and repair synthesis follows. Adapted from Friedberg et al.

II. OXIDANTS AND OXIDATIVE DAMAGE TO DNA

Oxidative stress and damage to DNA is a necessary consequence of aerobic life as many byproducts of endogenous metabolism are highly reactive species. Although all cellular macromolecules are subject to damage by reactive oxygen species (ROS), the primary deleterious consequences of oxidative stress are believed to arise from damage to DNA. The formation of oxidized DNA adducts has been implicated in mutagenesis, carcinogenesis, aging and a number of neurological disorders (Halliwell and Gutteridge, 1989; Ames et al., 1993).

A. Sources of ROS

One important exogenous source of oxidative damage is ionizing radiation. Exposure to ionizing radiation can directly damage DNA, but the predominant pathway arises from radiolysis of H_2O, which results in the formation of reactive species such as •OH that can in turn react with DNA (von Sonntag, 1987). Most of the detailed knowledge regarding the interaction of the DNA with reactive oxygen species has accumulated from extensive studies of ionization radiation (von Sonntag, 1987).

Endogenous metabolic processes, and aerobic respiration in particular, can also contribute significantly to the load of reactive oxygen species. During the four electron reduction of O_2 to H_2O, the reactive intermediates O_2^-, H_2O_2, and •OH, can all leak from the mitochondrial membrane (Figure 5A). In addition, macrophages produce numerous free radicals, including both O_2^- and NO•, which can react to form peroxynitrite. Other endogenous sources of reactive oxygen species include peroxisomes, which produce H_2O_2 during the β-

oxidation of fatty acids, and cytochrome P450s (Hsu et al., 1991).

B. Antioxidant Defenses

Cells possess various small molecule and enzymatic defenses that inactivate reactive oxygen species (Ames et al., 1993). A number of small molecules can act as radical scavengers including ascorbate (vitamin C), tocopherol (vitamin E), β-carotene, bilirubin, and urate. Glutathione will react with O_2^-, •OH or H_2O_2 to form stable glutathione radicals that eventually dimerize to form the disulfide (Farr and Kogoma, 1991). Reduction by glutathione reductase can then regenerate glutathione. Other enzymatic defenses include superoxide dismutase (SOD), which catalyzes the dismutation of O_2^- to H_2O_2 and O_2, and catalase, which converts H_2O_2 to H_2O and O_2 (Figure 5B). In addition, glutathione peroxidase and alkylhydroperoxide reductase can reduce potentially deleterious peroxides (Farr and Kogoma, 1991).

Many types of stress can induce bacterial expression of sets of genes in response to hostile conditions, such as heat shock, starvation, oxidative stress or DNA damage (Gottesman, 1984; Storz et al., 1992). In prokaryotic cells there are two distinct response pathways to oxidative stress (Farr and Kogoma, 1991). While the roles of all of the proteins involved in each pathway are not clear, at least some act directly to counteract the threat of oxidative damage. In the response pathway to high levels of H_2O_2, cellular levels of at least 30 proteins are activated, some of which are under the control of the transcriptional activator oxyR (Christman et al., 1985). OxyR regulates the induction of catalase, alkyl hydroperoxide reductase and glutathione reductase (Christman et al., 1985). Similarly, O_2^- stress can induce an alternative response that is partially regulated by the SoxR and SoxS proteins. Among the proteins induced by SoxRS are

MnSOD, endonuclease IV, heat shock protein GroEL, glucose-6-phosphate dehydrogenase, and alkylhydroperoxide reductase (Walkup and Kogoma, 1989; Greenberg and Demple, 1989). Both OxyR and SoxR are direct sensors of oxidative stress and are transcriptionally active in the oxidized state (Storz et al., 1990; Ding et al., 1996).

C. Damage to DNA

DNA damage resulting from attack of reactive oxygen species include base modifications, sugar damage, strand breaks, AP sites and DNA protein-crosslinks. The strand breaks and AP sites are likely the consequence of initial damage to the sugars and bases, respectively, followed by additional chemical reactions. Studies in vitro have demonstrated that O_2^- and H_2O_2 are themselves fairly inert to reaction with DNA (Aruoma et al., 1989a). In contrast, the •OH is highly reactive, with measured rate constants reaching the diffusion controlled limit (von Sonntag, 1987); this is likely to be the primary reactive species. Much of the observed toxicity of O_2^- and H_2O_2 has been attributed to intracellular reduction of these species to •OH. For example, in the presence of metal ions such as Fe^{2+}, H_2O_2 can be reduced by the Fenton reaction to •OH, or a similar iron bound oxygen species (Henle and Linn, 1997). Similarly, processing of O_2^- by superoxide dismutases produces H_2O_2, which can then be converted to •OH. The primary reactions of •OH with DNA are additions to the π bonds of DNA bases or hydrogen abstractions from deoxyribose (Breen and Murphy, 1995). Analysis of the partitioning of damage in polymeric structures indicates that reaction with the bases is favored by three to four fold over reaction with sugars (von Sonntag, 1987).

1. Sugar Damage

Hydrogen atom abstraction from the deoxyribose moieties of DNA by •OH can lead to base loss and single-strand breaks due to fragmentation of the sugar. A vast array of ring fragmentation products are observed upon gamma irradiation, and the most common resulting termini possess either 3' phosphate or 3' phosphoglycolate groups and a 5' phosphate (Figure 6A) (Henner et al., 1983; von Sonntag, 1987). In principle, any hydrogen from the deoxyribose is susceptible to abstraction, forming a carbon based radical that reacts rapidly in the presence of oxygen to form a peroxyl radical. Subsequent reactions are complex and can produce numerous fragmentation products (Breen and Murphy, 1995). A commonly observed pathway involves initial abstraction of H4', ultimately yielding 3' phosphoglycolate and 5' phosphate termini with release of a base propenal (Breen and Murphy, 1995). Alternatively, the end product of H4' abstraction can be an oxidized AP site (3,5-dihydroxy-4-oxopentanal) (Teebor et al., 1988). Similar types of damage are formed by radiomimetic drugs such as the ene-diynes or bleomycin (Dedon and Goldberg, 1992).

2. Base Damage

Oxygen radical damage to the four nucleobases is highly complex, with many distinct lesions formed from each nucleobase (von Sonntag, 1987). Systematic in vitro studies of the reaction of oxidants or ionizing radiation with individual nucleosides or nucleotides, as well as polynucleotides, have facilitated the identification of many of these oxidized base derivatives (von Sonntag, 1987; Luo et al., 1996; Douki et al., 1996; Henle et al., 1996).

The primary purine oxidation products are 7,8-dihydro-8-oxoguanine (8-oxoG) and 7,8-

dihydro-8-oxoadenine (8-oxoA) and the two corresponding ring opened formamidopyrimidine derivatives (Aruoma et al., 1989a; Aruoma et al., 1989b). The mechanism of formation is likely to involve addition of •OH to C8 of the purine ring system (Figure 6B). This radical putatively exhibits "redox ambivalence" and can act as either an oxidizing or reducing agent (Breen and Murphy, 1995). Oxidation and loss of a proton results in formation of 8-oxoG. In contrast, reduction can promote imidazole ring opening to generate the 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG). The reaction with A is likely to undergo similar chemistry, leading to either 8-oxoA or 4,6-diamino-5-formamidopyrimidine (FapyA). 8-oxoG is the most prevalent oxidized lesion detected in human tissues, at approximately 1 in 10^6 bases (Wagner et al., 1992).

Thymine glycol (Tg) is the major thymine derived adduct detected after oxidation or irradiation of DNA in vitro and in vivo (Teoule et al., 1977; Breimar and Lindahl, 1985). The proposed mechanism of adduct formation involves initial attack of •OH at either the C5 or C6 of the pyrimidine ring, resulting in formation of a carbon based radical (Figure 7A). In an aerobic environment, addition of O_2 leads to formation of a peroxyl species, which can abstract a proton to generate the hydroperoxide. Reduction of the hydroperoxide, perhaps by glutathione peroxidase, results in formation of Tg. Another oxidation product found in vivo is hydroxymethyluracil, which probably arises from H-abstraction from the methyl group and subsequent reduction of the peroxyl radical formed upon addition of O_2 (Teebor et al., 1984). Additional products can be formed from rearrangement or fragmentation of the pyrimidine ring, such as as urea and 5-hydroxy-5-methyl hydantoin.

By analogy to thymine oxidation, cytosine glycol is the initial cytosine oxidation product

formed (Figure 7A). Unlike Tg, however, Cg is believed to be highly unstable and decomposes rapidly (von Sonntag, 1987; Douki et al., 1996). Since saturation of the 5-6 position of C generally enhances the rate of deamination (Hayatsu, 1976), Ug can be readily formed. Ug or Cg can also dehydrate to form 5-ohU or 5-ohC, respectively (Figure 7B). These adducts have been identified in vitro (Dizdaroglu et al., 1986), and moreover Ames and coworkers reported in 1992 that these three adducts are present at steady state levels in human tissues at levels comparable to 8-oxoG (Wagner et al., 1992).

D. Biological Consequences of Oxidative Adducts

Advances in analytical chemistry have facilitated detection and determination of low levels of adducts in vitro and in vivo. The primary methods involve use of either HPLC with electrochemical detection (Shigenaga et al., 1994) or GC-MS (Dizdaroglu, 1994) to detect specific adducts. Application of these methods to tissues samples or urine has identified biologically relevant adducts and provided estimates of the in vivo levels of oxidative damage. From such studies, Ames and coworkers estimate that the DNA in a human cell suffers 10,000 oxidative hits per day (Cathcart et al., 1984; Fraga et al., 1990). Since aerobic respiration is the primary source of endogenous ROS, it is not surprising that the amount of oxidative damage formed in an organism has also been correlated with metabolism (Adelman et al., 1988) and that levels of damage to mitochondrial DNA have been observed to be approximately 10 fold higher than damage to the corresponding nuclear DNA from the same tissues (Richter et al., 1988). In addition, both oxidative lesions and mutations accumulate in cells with increasing age (Mullaart et al., 1990; Fraga et al., 1990; Agarwal and Sohal, 1994; Wang et al., 1995). Consistent with

these observations, life span has been inversely correlated with metabolic rate (and cumulative adduct levels) (Agarwal and Sohal, 1994; Sohal and Weindruch, 1996), and overexpression of SOD and catalase in *Drosophila* is reported to lead to an increase in life span (Orr and Sohal, 1994).

Assessment of the mutational properties of DNA adducts has relied on two complementary approaches: global mutational spectra analysis and site-specific mutagenesis studies (Singer and Essigmann, 1991). A mutational spectrum is the composite of the number, types, and sites of all mutations observed in a given sequence of interest and can be determined in the either the absence or presence of any exogenous DNA damaging agents. A particular genomic or vector sequence is monitored following replication, typically by some form of phenotypic selection, and ultimately mutations are identified by sequencing. Given the significant levels of oxidized lesions produced by endogenous metabolism, the "spontaneous" as well as the oxidant induced mutational spectra should provide insight into the mutational properties of oxidized lesions. In "spontaneous" mutational spectra, the most commonly observed base substitutions are GC→AT transitions followed by GC→TA transversions, reflecting the mutations derived from inherent replication errors, stochastic depurination events, and DNA adducts arising from endogenous sources (Shaaper and Dunn, 1991; Halliday and Glickman, 1992; de Jong et al., 1992). Numerous mutational spectra have also been obtained using different sources of reactive oxygen species, such as ionizing radiation, H_2O_2, and metal ions (Moraes et al., 1990; Tkeshelashvili et al., 1991; McBride et al., 1991; Waters et al., 1991). The results of these studies reveal that the majority of oxidant induced mutations are base substitutions although some deletions, insertions and frameshifts are also induced. Of these base

substitutions, the most prominent are GC→AT transitions followed by GC→TA transversions (Ayaki et al., 1986; Tindall et al., 1988; Moraes et al., 1990; Tkeshelashvili et al., 1991; Waters et al., 1991) suggesting that oxidative damage to G and C can create mutagenic lesions.

Mutational spectra provide important information regarding the frequency and types of mutations that arise from a particular DNA damaging agent but cannot attribute the mutations to particular DNA adducts due to the multiplicity of adducts formed by typical damaging agents. The advent of the site-specific approach towards studying mutations has facilitated deconvolution of the contributions of individual adducts to the overall mutational spectrum (Singer and Essigmann, 1991). This method entails chemical synthesis of a single defined lesion in an oligonucleotide that is subsequently incorporated into a vector by genetic engineering and evaluated for type and frequency of mutation (Yarema and Essigmann, 1995). This approach has been used both in bacteria and mammalian systems to characterize the mutational properties of many adducts (Marnett and Burcham, 1993).

A number of oxidative lesions have been examined using the site-specific approach. Studies of Tg found it to be only very weakly mutagenic, giving rise to T→C substitutions at a frequency of 0.3% (Basu et al., 1989). 8-oxoG has been found to cause primarily G→T transversions with a mutation frequency of 0.5-1.8 % in *E. coli* and a slightly higher 2.3-4.8% in COS cells (Wood et al., 1990; Cheng et al., 1992; Moriya, 1993a). Moreover, the mutation frequency is much higher in certain repair deficient cells (Moriya and Grollman, 1993b). The mutagenicity of 8-oxoG has been attributed to its ability to undergo a conformation switch from anti to syn relative to the deoxyribose sugar. In the syn conformation, the 8-oxoG can direct DNA polymerases to incorporate A opposite it, leading to a G→T mutation after the next cycle of

replication (Shibutani et al., 1991). Although the analogous adduct 8-oxoA can also adopt a syn conformation in vitro and form a promutagenic basepair with G (Leonard et al., 1992), it was not detectably mutagenic in a site specific mutational assay (Wood et al., 1992). At the other extreme, studies of oxidized cytosines indicate that both 5-ohU and Ug give rise to C→T mutations with 80% mutation frequency (Kreutzer and Essigmann, submitted). 5-ohC also induces C→T transitions but is much more weakly mutagenic, only one order of magnitude above background (Kreutzer and Essigmann, submitted).

In addition to possessing miscoding properties, DNA adducts can have more immediate effects on cellular physiology by affecting replication and transcription. Both RNA and DNA polymerases can be inhibited by various lesions. Primer extension assays in vitro have shown that Tg and AP sites in a template strand can stall some polymerases leading to termination of strand synthesis (Sagher and Strauss, 1983; Rouet and Essigmann, 1985; Clark and Beardsley, 1989). Attempts to determine the inhibitory properties of these lesions in vivo have relied predominantly upon a host-cell reactivation assay. This assay measures the ability of bacteria (host-cell) to support replication of exogenously introduced, damaged bacteriophage DNA; the presence of lesions that inhibit replication will inactivate the phage. By this criterion, it has been observed that both AP sites and Tg are lethal (Evans et al., 1993). An indirect effect of oxidative damage can be to increase the load of AP sites in the genome as some oxidized adducts are labile and prone to N-glycosyl bond cleavage.

E. Repair of Oxidative Damage

DNA glycosylases and BER have been implicated as the primary enzymes involved in

protecting the genome from oxidative damage. The repair of oxidative DNA damage has been recently reviewed (Demple and Harrison, 1994; Wallace, 1997; Croteau and Bohr, 1997).

1. Repair of Oxidized Purines

The set of repair enzymes aimed at mitigating the effects of 8-oxoG in *E. coli* have been extensively studied both biochemically and genetically. The interplay of the three genes *mutM* (also *fpg*), *mutY* and *mutT*, and their gene products, MutM (also known as fapy glycosylase), MutY, and MutT, has been elucidated and presents an elegant paradigm of how different proteins can contribute to minimize the consequences of a single adduct (Michaels and Miller, 1992b). MutM is a glycosylase that excises 8-oxoG when it is paired with C, resulting in formation of an AP site and subsequent productive repair synthesis. One recent report suggests that MutM can be induced by the O_2^- generators paraquat and menadione (Kim et al., 1996). MutM is much less active on the 8-oxoG in an 8-oxoG:A pairing since excision of 8-oxoG from such an intermediate would lead to fixation of a G→T mutation. Fortuitously, MutY can act upon the 8-oxoG:A mispair to remove the A (Michaels and Miller, 1992b). Gap-filling by a repair polymerase offers another opportunity for correct insertion of C opposite the 8-oxoG and generation of a suitable substrate for repair by MutM. Cells deficient in either *mutM* or *mutY* are mutators specific for G→T transversions (Cabrera et al., 1988; Nghiem et al., 1988), and the mutator phenotype is greatly exacerbated in *mutM mutY* double mutants (Michaels et al., 1992a). The third protein in this system MutT hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate, thus sanitizing the nucleotide pool and preventing misincorporation of 8-oxo-dGTP into DNA (Maki and Sekiguchi, 1992). Cells deficient in MutT show 1000-fold elevated

levels of A→C mutations (Yanofsky et al., 1966). In addition, since MutT is also capable of hydrolyzing 8-oxoGTP, it has been suggested to play a role in preventing transcriptional misincorporation of 8-oxoGTP into RNAs (Taddei et al., 1997).

The components of this genoprotective system are conserved and identification of homologs in eukaryotes has been the subject of intense recent effort. A number of laboratories has reported the cloning of yeast (Nash et al., 1996; van der Kemp et al., 1996) and mammalian (Radicella et al., 1997; Roldan-Arjona et al., 1997; Rosenquist et al., 1997; Lu et al., 1997) functional homologs of fapy glycosylase designated Ogg1 (for oxo-guanine glycosylase) that excise 8-oxoG from DNA. It appears that there may also be a second discrete Ogg activity in yeast (Ogg2) that differs in its recognition of the base opposite the 8-oxoG lesion (Nash et al., 1996; van der Kemp et al., 1996). Similarly, mammalian homologs of MutY (McGoldrick et al., 1995; Slupska et al., 1996) and MutT (Mo et al., 1992) have been discovered.

Several other repair proteins have been reported to have activity in vitro against 8-oxoG. The mammalian N-methylpurine DNA glycosylase, which has a very broad substrate specificity, can repair 8-oxoG (Bessho et al., 1993), as can the human NER system (Reardon et al., 1997). Interestingly, the ribosomal S3 protein of Drosophila has been observed to possess numerous enzymatic properties, including glycosylase activity for 8-oxoG, class I AP endonuclease activity, and dRpase activities (Sandigursky et al., 1997a). The biological relevance of these activities remains to be determined.

2. Repair of Oxidized Pyrimidines

The primary defense against oxidized pyrimidines has been attributed to the glycosylase

activity of endonuclease III, primarily based on its broad substrate specificity. Endonuclease III is a combined glycosylase/AP lyase believed to operate via "base flipping." Experiments in vitro indicate that thymine glycol, urea, β-ureidoisobutyric acid, dihydrothymine, cytosine hydrate, uracil glycol, 5-ohC, 5-ohU and several other lesions are all excised by endonuclease III (Breimer and Lindahl, 1984; Dizdaroglu et al., 1993; Hatahet et al., 1994). However, *nth* mutants display no sensitivity to ionizing radiation and are only weak mutators, suggesting that additional repair systems may exist that provide functional redundancy (Cunningham and Weiss, 1985). In support of this possibility, another glycosylase designated endonuclease VIII has been identified, and biochemical experiments indicate that endonuclease VIII and endonuclease III share at least some substrate specificity (Melamede et al., 1994). In addition to its glycosylase activity, endo VIII also carries out β-δ elimination, much like fapy glycosylase (Wallace, 1997). Interestingly, analysis of the amino acid sequence reveals that endonuclease VIII shares homology with fapy glycosylase rather than with endonuclease III (Wallace, 1997). Conflicting genetic data have been reported as to the phenotype of cells deficient in both endonucleases III and VIII (*nth nei*). In one study, *nth nei* double mutants are sensitized to killing by oxidative stress but are not mutators (Saito et al., 1997). A second study indicates that *nth nei* cells have nearly wild-type survival characteristics, but have an approximately 20 fold increased mutation frequency (Jiang et al., 1997). Further experimentation is required to reconcile the observed differences in these cell strains. Homologs of endonuclease III have been cloned from yeast (Roldán-Arjona et al., 1996; Eide et al., 1996; Augeri et al., 1997) and humans (Aspinwall et al., 1997; Hilbert et al., 1997). Unlike endonuclease III deficient *E. coli*, yeast deficient in this activity are sensitive to H_2O_2 and the oxygen radical generator menadione, suggesting that this

repair enzyme is physiologically significant as a genoprotectant (Eide et al., 1996).

In addition to endonuclease III and endonuclease VIII, Wallace and coworkers have reported that both uracil glycosylase and fapy glycosylase from *E. coli* are capable of excising certain oxidized pyrimidines in vitro (Hatahet et al., 1994). Furthermore, it is possible that uracil glycosylase from *E. coli* and humans may repair 5-ohU (Hatahet et al., 1994; Dizdaroglu et al., 1996) although others have found otherwise (Zastawny et al., 1995; Fujimoto et al., 1997). There is also biochemical evidence that the Uvr(A)BC as well as the human NER systems can act to repair Tg lesions (Lin and Sancar, 1989; Kow et al., 1990; Reardon et al., 1997). Moreover, in human cells Tg is apparently repaired in a transcriptionally coupled fashion, and this mode of repair may extend to other oxidized lesions (Cooper et al., 1997). Additional repair activities against oxidized pyrimidines include the excision of hydroxymethyluracil by hydroxymethyluracil glycosylase in mammals (Hollstein et al., 1984) and the repair of 5-formyl uracil by bacterial alkA (Bjelland et al., 1994).

3. Repair of Oxidized Sugars

Sugar fragmentation products are typically inhibitory to repair synthesis and must be excised to form extendable 3' termini. In *E. coli* the 3' phosphatase and 3' phosphodiesterase activities of the class II AP endonucleases exonuclease III and endonuclease IV are critical for repair of oxidative damage. The observation that *xth* cells are hypersensitive to H_2O_2 has been attributed to the presence of direct sugar damage or α-β unsaturated aldehydes from class I AP endonucleases activity that require processing by exonuclease III (Demple et al., 1983; Demple et al., 1986). Similarly cells deficient in endonuclease IV (*nfo*) are sensitized to killing by

oxidants; in fact, for certain oxidants, the *nfo* mutants are more sensitive than *xth* mutants, suggesting that endonuclease IV processes some oxidative damage that exonuclease III cannot (Cunningham et al., 1986). In the presence of O_2^-, endonuclease IV can be induced by 10-20 fold (Chan and Weiss, 1987). AP endonucleases from mammalian cells also possess 3' phosphatase and 3' phosphodiesterase activities.

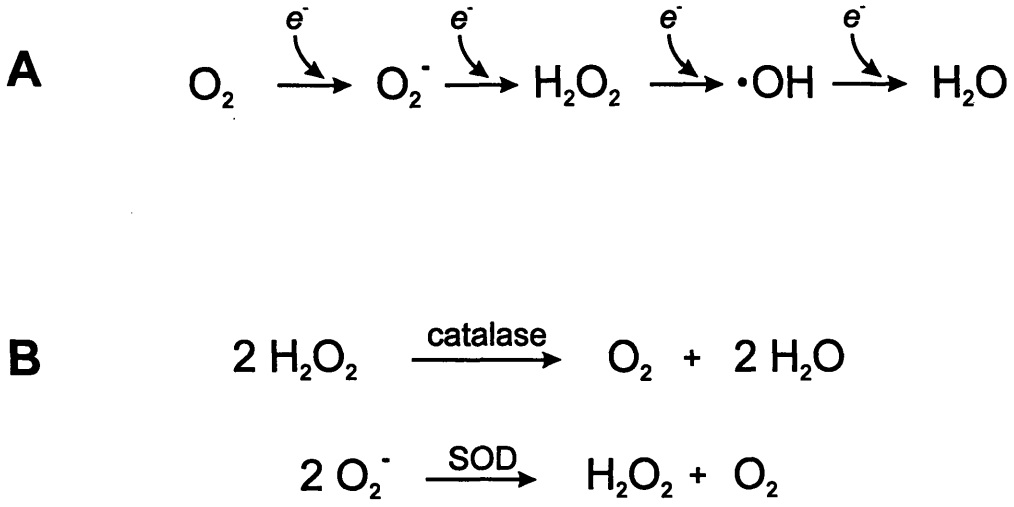

Figure 5. A. Formation of ROS by the sequential one electron reduction of O_2 to H_2O. **B.** Protection against ROS by enzymatic defenses. The reactions catalyzed by the catalase and superoxide dismutase (SOD) detoxify H_2O_2 and O_2^-.

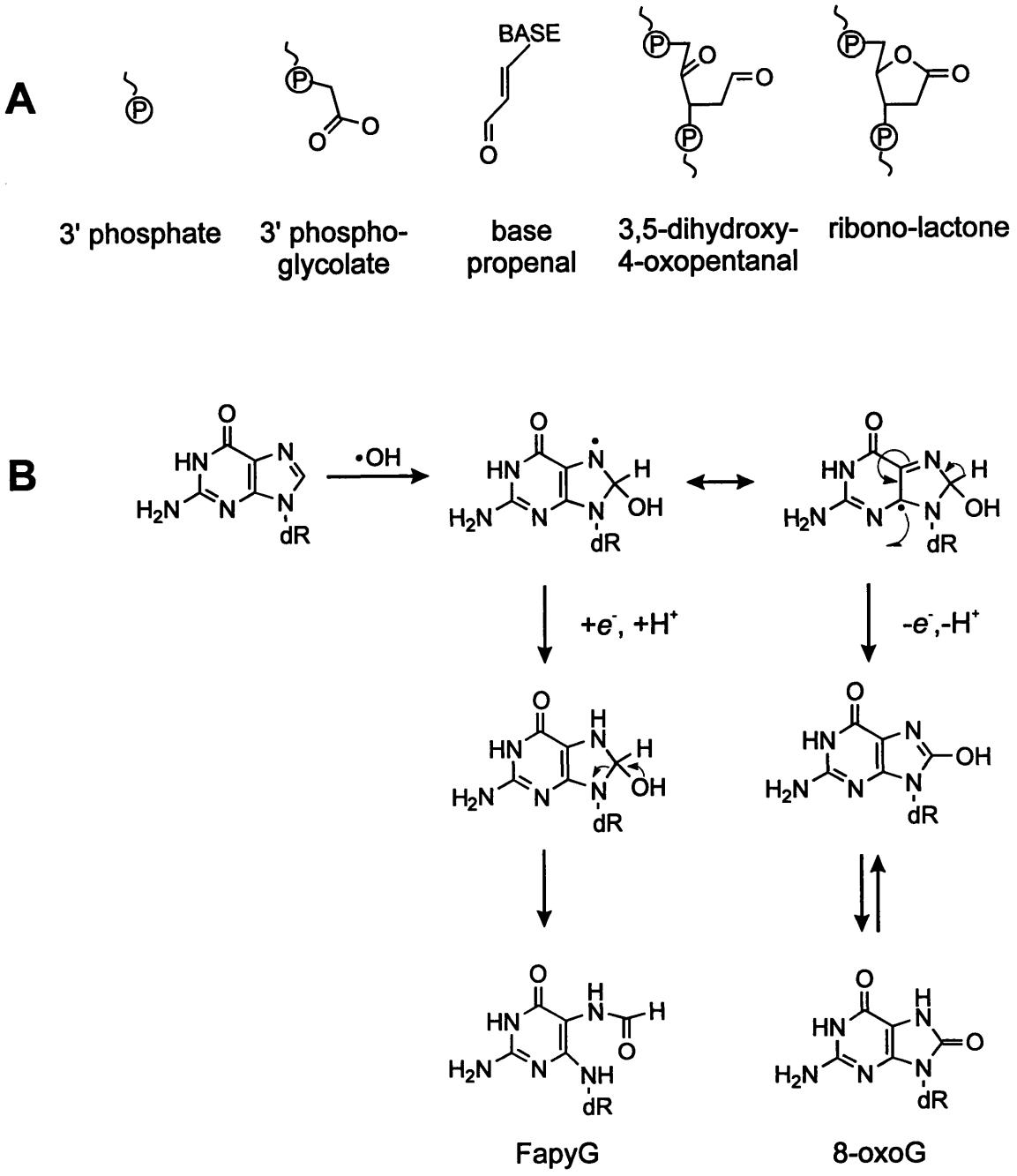

Figure 6. A. Structures of some commonly observed sugar fragmentation products. **B.** The reaction of guanine with OH. Attack primarily at C8 generates a radical species that displays "redox ambivalence." Oxidation and loss of a proton leads to formation of 8-oxoG. Reduction and gain of a proton results in ring opening to give the formamidopyrimidine (FapyG). The reaction with adenine is likely to undergo analogous chemistry.

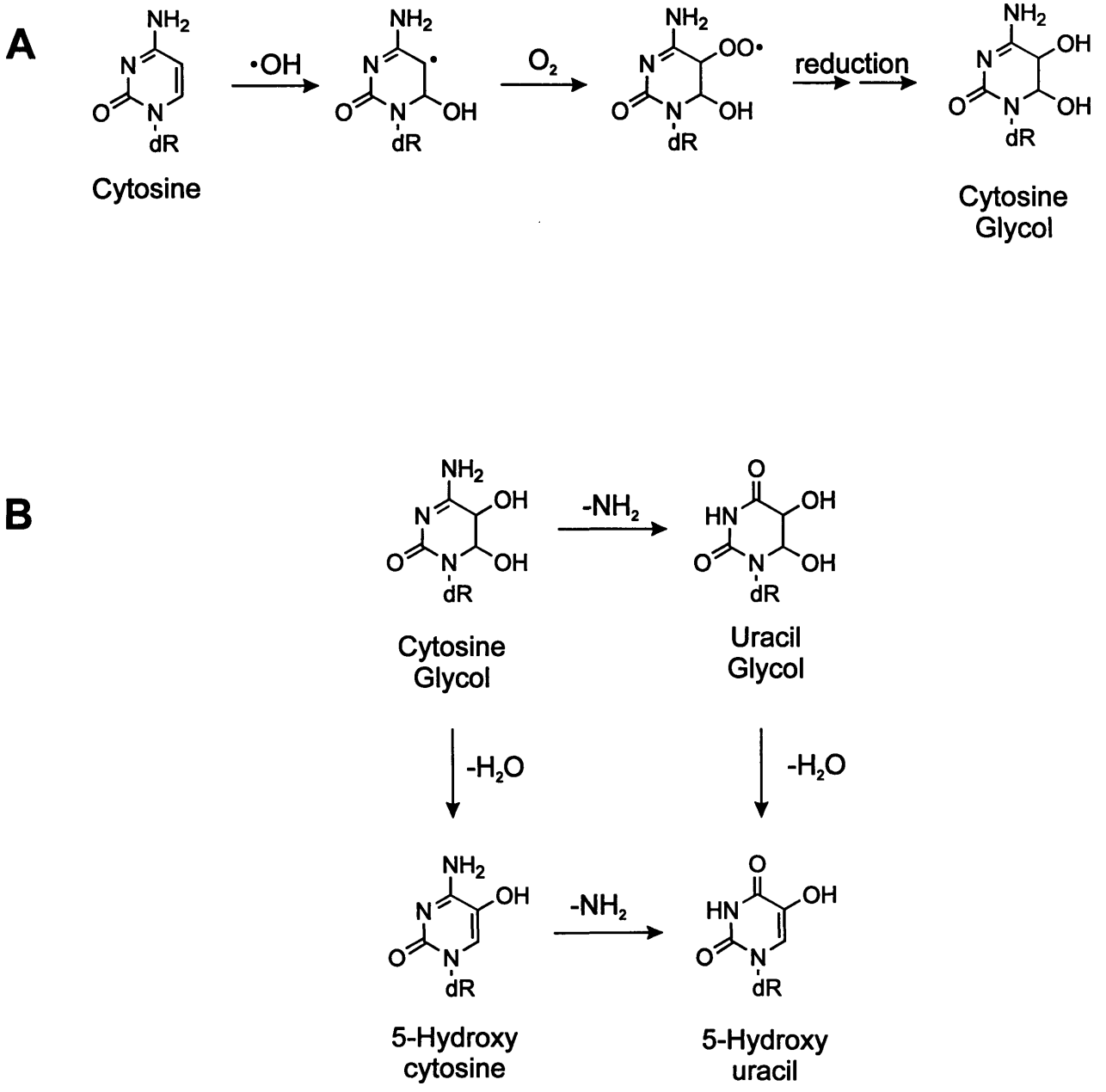

Figure 7. A. Pyrimidine oxidation. Proposed mechanism of formation of cytosine glycol (and by analogy thymine glycol). Radical attack at the 5,6 double bond forms a carbon radical that reacts with O_2 to form the hydroperoxide. Following subsequent reduction, cytosine glycol is produced. **B.** Decomposition of cytosine glycol can occur either by deamination to form Ug or dehydration to form 5-ohC. These two species can subsequently dehydrate or deaminate, repsectively, to form 5-ohU.

III. AFLATOXIN B_1

The potent human carcinogen aflatoxin B_1 (AFB_1) is a known risk factor for hepatocellular carcinoma (HCC) (Qian et al., 1994). A second significant risk factor is infection by hepatitis B virus, and in concert the two act in a synergistic fashion. AFB_1 is a toxic metabolite produced by the fungus *Aspergillus flavus*. The primary route of human exposure to aflatoxin is through dietary intake of foods contaminated by *A. flavus*. Peanuts, corn and other grains and legumes stored under conditions of high temperature and humidity are at high risk for fungal growth (Busby, Jr. and Wogan, 1984). AFB_1 contamination is particularly high in areas such as Mozambique, Southeast Asia and China (Busby, Jr. and Wogan, 1984), and correspondingly, the incidence of HCC is elevated in those regions of the world. Although the molecular mechanism of human hepatocarcinogenesis is poorly defined, the mutagenic DNA adducts formed by AFB_1 are likely to play an important role.

A. AFB_1 DNA Adducts

AFB_1 itself is incapable of covalently reacting with DNA and requires metabolic activation by cytochrome P450s to the *exo*-8,9-epoxide. This requirement, coupled with the restricted expression of cytochrome P450s to specific tissues, provides an explanation for the hepatotropism of AFB_1. Experiments in cultured cells established that five out of 12 human cytochrome P450s can convert AFB_1 to a mutagenic species (Aoyama et al., 1990) and that the two most efficient isoforms are IIIA4 and IA2 (Shimada and Guengerich, 1989; Mace et al., 1997). Although enzymatic oxidation can produce both the *endo* and *exo* epoxides (Raney et al.,

1992), only the *exo* epoxide is capable of reacting with DNA (Iyer et al., 1994a). Once formed, the *exo*-8,9 epoxide is highly reactive and readily hydrolyzes to form AFB_1-diol with a reported rate constant of 0.6/s (Johnson et al., 1996). In addition, the *exo*-8,9 epoxide can be inactivated by a number of detoxifying enzymes, such as glutathione-S-transferases and epoxide hydrolases. Thus the balance between cellular levels of cytochrome P450s and these protective enzymes is important in determining the amount of adducts formed and has been correlated with susceptibility to HCC (McGlynn et al., 1995).

The chemical synthesis and isolation of AFB_1 epoxide in 1988 heralded a new era in the study of AFB_1 (Baertschi et al., 1988). It was previously believed that the AFB_1 epoxide was too reactive to be isolated, and thus early studies of AFB_1 relied on the AFB_1-dichloride as a model compound to mimic AFB_1 epoxide (Swenson et al., 1975). In order to utilize authentic AFB_1 epoxide, it was necessary to use one of several complex and limited approaches such as liver microsomes, biphasic oxidizing systems, or photooxidation to generate AFB_1 epoxide in situ (Baertschi et al., 1988). In contrast the facile synthesis of AFB_1 epoxide from AFB_1 and dimethyldioxirane proceeds in high yield to give primarily (90%) the biologically active *exo* isomer (Raney et al., 1992), which can be isolated and even crystallized (Baertschi et al., 1988).

AFB_1 epoxide reacts almost exclusively with the N7 position of guanine both in vitro and in vivo to form 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua) (Figure 8) (Essigmann et al., 1977; Croy et al., 1978). A minor (< 2%) adenine derived adduct has been identified after in vitro treatment of DNA with AFB_1 epoxide (Iyer et al., 1994b) although this adduct has not been detected in vivo. The principal AFB_1-N7-Gua adduct is highly unstable due to the positive charge on the imidazole ring of guanine and can undergo several fates. At neutral

or acidic pH, spontaneous depurination is the predominant reaction (Groopman et al., 1981; Bailey et al., 1996a). Under mildly alkaline conditions, AFB_1-N7-Gua can undergo imidazole ring opening to form one or more isomers of a product likely to be 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB_1 (AFB_1-formamidopyrimidine or AFB_1-FAPY) (Lin et al., 1977; Hertzog et al., 1982). All available data are consistent with a FAPY adduct, but definitive structural proof has not been reported. The dominant product is believed to arise from ring opening between C8 and C9 although it has been suggested that ring opening between C7 and C8 can also occur to some extent (Hertzog et al., 1982). Moreover, the AFB_1-FAPY adducts are likely to exist as a mixture of rotamers due to restricted rotation around the amide bond. Finally, an alternative reaction of the parental AFB_1-N7-Gua that has been observed in DNA heavily modified by AFB_1 is release of AFB_1-diol from the DNA to regenerate guanine (Groopman et al., 1981).

The reaction of AFB_1 epoxide with double stranded B-form DNA is highly favored over reaction with single stranded DNA or alternative duplex structures (Misra et al., 1983; Raney et al., 1993). Moreover, treatment of DNA with AFB_1 epoxide does not result in the random formation of AFB_1-N7-Gua adducts at guanines. Instead, sequence specificity has been observed that favors runs of guanines by as much as 25 fold compared to other sequences (Muench et al., 1983; Benasutti et al., 1988). Although the basis for this specificity is not understood, empirical rules governing the level of reactivity have been derived based on the 5' and 3' bases nearest a target guanine (Benasutti et al., 1988). It has been hypothesized that formation of the AFB_1-N7-Gua adduct is highly dependent on intercalation by AFB_1 epoxide (discussed below), and thus the favorability of the site for intercalation, as well as the relative

nucleophilicity of the guanine, are likely to be contributing factors to the observed sequence specificity of adduct formation.

B. Intercalation and the Neighbor Exclusion Principle.

The phenomenon of intercalation was first coined by Lerman in 1961 to describe a noncovalent mode of binding between DNA and flat aromatic molecules wherein the planar molecule inserts between base pairs in a double helix (Lerman, 1961). Classically intercalation extends the helix by about 3.4 Å and leads to unwinding of the helix that is dependent on the specific intercalator (Wilson, 1990) Intercalation affects a variety of physical properties of the duplex such as its viscosity and sedimentation coefficient (Wilson, 1990). Measurement of these properties, along with X-ray crystallography have been the primary tools used to distinguish between intercalative and groove-binding interactions of small molecules (Wilson, 1990). The driving forces for intercalation are likely to be desolvation of the aromatic molecule and stacking interactions.

Following the discovery of intercalation, Crothers hypothesized that a neighbor exclusion principle may govern the ability of successive molecules to intercalate into adjacent sites in DNA and that only every other base-pair site can be occupied (Crothers, 1968). Empirical experimentation with simple intercalators has substantiated the neighbor exclusion principle. For example, the binding of ethidium to DNA becomes saturated at a ratio of one intercalator per two base pairs (Nelson and Tinoco, 1984; Wilson, 1990). Unfortunately the basis of this observed neighbor exclusion is not well understood. Moreover, it does not always hold true as some bis-intercalators have been observed to violate the neighbor exclusion principle (Wakelin

et al., 1978; Robledo-Luiggi et al., 1991).

C. Dependence of Adduct Formation on Intercalation

Current evidence suggests that formation of AFB_1-N7-Gua DNA adducts proceeds through a transition state in which the AFB_1 epoxide is intercalated on the 5' side of the target guanine. From NMR structural studies on the adduct, it is speculated that 5' intercalation facilitates adduct formation by positioning the epoxide for in-line nucleophilic attack by the guanine N7 (Gopalakrishnan et al., 1990; Iyer et al., 1994a). Additional evidence for a 5' intercalation event comes from stoichiometric studies with the two self-complementary hexamers ATCGAT and ATGCAT. Reaction with excess AFB_1 epoxide yields only a 1:1 ratio of AFB_1 to the duplex oligonucleotide $(ATCGAT)_2$, in which the two guanines share the same 5' intercalation site; in contrast, in the case where distinct 5' intercalation sites exist in each strand, a 2:1 AFB_1: $(ATGCAT)_2$ ratio is observed (Gopalakrishnan et al., 1989). Furthermore, modifications of the ring systems in AFB_1 that decrease planarity and therefore intercalation ability, a situation that exists with aflatoxin G_1, decrease affinity for DNA and result in lower reactivity (Raney et al., 1990). Finally, addition of the intercalating agent ethidium bromide to target DNA prior to treatment with AFB_1 epoxide greatly reduces guanine reactivity (Misra et al., 1983). These experiments, coupled with results from stopped-flow kinetic analysis, support a model in which the intercalated intermediate provides a kinetic and entropic advantage for productive reaction with DNA over hydrolysis (Johnson and Guengerich, 1997). Moreover, in the absence of this favorable interaction, AFB_1 epoxide can be readily hydrolyzed to the inactive AFB_1-diol.

D. Repair and Mutagenicity of AFB_1 Adducts

The biological responses to the presence of AFB_1 DNA adducts have been examined in prokaryotes and eukaryotes. Cells deficient in UvrA are sensitized to AFB_1 (Sarasin et al., 1977), and in one study, the observation of AFB_1 induced mutations in *E. coli* required functional inactivation of the NER pathway (Foster et al., 1983), suggesting that NER is important for the repair of AFB_1 adducts. In vitro, AFB_1-N7-Gua and AFB_1-FAPY are comparable substrates for purified Uvr(A)BC (Oleykowski et al., 1993). In addition to NER, fapy glycosylase has been demonstrated to act upon AFB_1-FAPY in vitro (Chetsanga and Frenette, 1983; Oleykowski et al., 1993), although *fpg* mutants have not yet been examined for sensitivity to AFB_1. The finding that Uvr(A)BC can repair AFB_1-N7-Gua and AFB_1-FAPY with equal efficiency is not paralleled in mammalian systems. Although removal of AFB_1-N7-Gua is reduced in XP-A cells compared to normal cells, the AFB_1-FAPY adduct is more persistent than AFB_1-N7-Gua, suggesting that AFB_1-FAPY is repaired less efficiently by NER than AFB_1-N7-Gua (Leadon et al., 1981). Consistent with this view, the half-life of AFB_1-N7-Gua in rat liver is approximately 7.5 hr (Croy and Wogan, 1981) whereas levels of AFB_1-FAPY remain relatively unchanged, even after 72 hr (Croy and Wogan, 1981). It is not yet known if any of the mammalian homologs of fapy glycosylase are active against AFB_1-FAPY.

The mutagenic properties of AFB_1 have been investigated both in *E. coli* and in mammalian cells. From the mutational spectrum determined in the *lacI* gene in *E. coli*, the predominant mutations are GC→TA transversions with a small percentage of GC→AT transitions (Foster et al., 1983). Similar mutations are produced by a site-specifically positioned AFB_1-N7-Gua in a M13 bacteriophage vector replicated in *E. coli* (Bailey et al., 1996b). This approach

also revealed the presence of some semi-targeted mutations at the base immediately 5' of the adduct (Bailey et al., 1996b). An in vitro mutational analysis of the human HPRT gene revealed primarily GC→TA transversions (Cariello et al., 1994). The most common mutations observed in mammalian systems are GC→TA transversions followed by a lesser frequency of GC→AT transitions (Trottier et al., 1992; Levy et al., 1992; Aguilar et al., 1993). Interestingly, more than 50% of hepatocellular carcinomas in areas of the world with high levels of AFB_1 exposure possess a characteristic GC→TA transversion in the third position of codon 249 of the p53 tumor suppressor gene (Hsu et al., 1991; Bressac et al., 1991; Greenblatt et al., 1994). This mutation has been attributed to AFB_1 since HCCs from regions of the world with low or no exposure to AFB_1 rarely display this particular mutation (Shen and Ong, 1996).

E. Hepatocellular Carcinoma, Hepatitis B, and AFB_1

In addition to AFB_1 exposure, hepatitis B infection is also an important risk factor for HCC. Most of the regions of the world with high levels of exposure to dietary AFB_1 are also characterized by high levels of hepatitis infection, (Busby, Jr. and Wogan, 1984) and exposure to both of these risk factors confers a synergistic increase in susceptibility to HCC (Qian et al., 1994). Unfortunately, the molecular mechanism of this observed synergy remains elusive. One hypothesis that has been experimentally examined suggests that chronic viral infection can result in the induction of cytochrome P450s, increasing the efficiency of AFB_1 epoxide formation and ultimately the yield of mutagenic adducts (Kirby et al., 1994). Despite these observations, there exists a dearth of mechanistic models to explain this phenomenon.

Even the carcinogenic effects of hepatitis B infection alone are poorly understood. A

generally accepted rationale is that viral infection can cause prolonged liver injury and chronic inflammation, eventually leading to malignant transformation. However this process may involve many diverse mechanisms. For example, viral integration into genomic DNA may activate cellular oncogenes or inactivate tumor suppressors (Ganem and Varmus, 1987). Alternatively, the expression of viral proteins may play more direct roles in carcinogenesis. The hepatitis B viral genome contains four major open reading frames encoding surface antigen, core antigen, polymerase, and the X protein (Ganem and Varmus, 1987). The multifunctional X protein can act as a transcriptional activator and transforms rodent cells in vitro (Twu and Schloemer, 1987; Shirakata et al., 1989). Moreover, it has been found to bind to p53 in vitro and abolish its transactivating ability (Wang et al., 1994) as well as inhibit p53 dependent apoptosis in cell culture (Wang et al., 1995). Thus viral infection with hepatitis B may functionally inactivate p53 providing a selective growth advantage to infected cells. Transgenic mouse models have been developed to study the role of viral proteins in carcinogenesis. Mice that express either the hepatitis surface antigen (Chisari et al., 1989) or X protein (Ueda et al., 1995) are subject to a cascade of neoplastic changes that leads to the development of hepatocellular carcinoma. Such models have also been used to confirm the synergistic effect of AFB_1 exposure on susceptibility to HCC. Treatment of transgenic mice expressing the surface antigen with AFB_1 results in rapid development of tumors in comparison to untreated transgenic mice (Sell et al., 1991). The development of model systems that mimic to some degree conditions during human carcinogenesis offers hope for the future and suggests that elucidating the interplay between AFB_1 and hepatitis in the molecular mechanism of carcinogenesis may become a tractable problem.

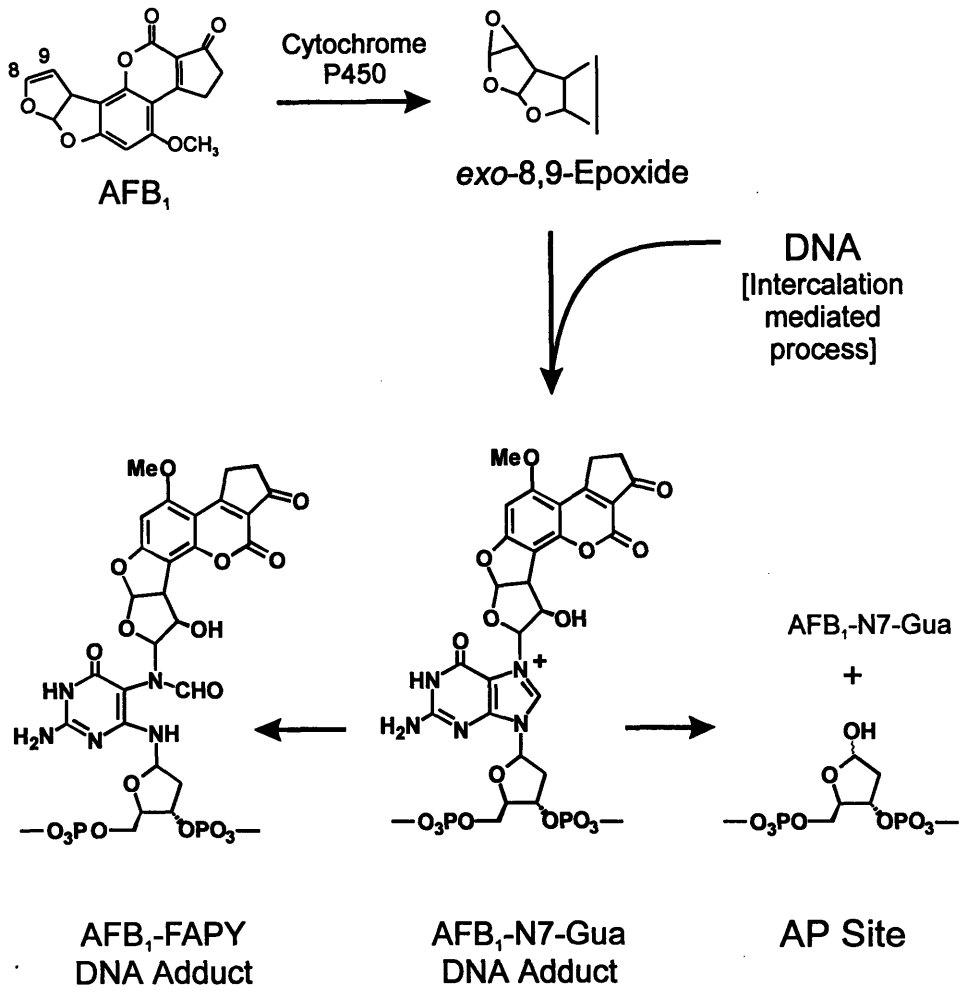

Figure 8. DNA adducts formed by AFB_1. Upon metabolic activation to the *exo*-8,9-epoxide, AFB_1 forms adducts with the N7 position of guanine in a reaction believed to involve an intercalation step. The parental AFB_1-N7-Gua adduct is highly labile and can either depurinate to form an AP site or ring open to form AFB_1-FAPY.

Chapter 3: REPAIR OF OXIDIZED CYTOSINES BY ENDONUCLEASE III OF *E. COLI*

I. INTRODUCTION

Among the many oxidized lesions formed by ROS are three potentially mutagenic cytosine derivatives: 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol or Ug), 5-hydroxycytosine (5-ohC) and 5-hydroxyuracil (5-ohU). Interest in these lesions stems from their relative abundance in mammalian tissues and their observed mutagenicity both in vitro and in vivo (Feig et al., 1994; Purmal et al., 1994a; Purmal et al., 1994b; Kreutzer and Essigmann, submitted). The mutational specificity of each of the lesions is C→T, coincident with the primary mutation observed in the mutational spectra of oxidizing agents (Moraes et al., 1990; Tkeshelashvili et al., 1991; Waters et al., 1991).

Which enzymes repair these mutagenic lesions? When this project was initiated, there was nothing known about the repair of these lesions. The goal of this investigation was to examine the in vitro ability of endonuclease III from *E. coli* to excise these lesions using site-specifically modified oligonucleotides. Endonuclease III is a combined glycosylase/AP lyase known to have a broad substrate specificity for a variety of hydrated, oxidized or ring-fragmented pyrimidines (Breimer and Lindahl, 1984; Dizdaroglu et al., 1993). The substrates used in these experiments were prepared by either chemical synthesis or a combination of chemical and enzymatic steps. The novel preparation of duplexes containing Ug is described in this chapter. Oligonucleotides containing the lesions 5-ohC and 5-ohU were synthesized using protected phosphoramidites of 5-ohC and 5-ohU (Morningstar et al., 1997).

Since the inception of this investigation, several other groups have reported on the repair of these lesions using both site-specifically modified oligonucleotides as well as globally

oxidized DNA. Very recently, a chemical synthesis of 5-ohU phosphoramidite very similar to the one used in this study was reported (Fujimoto et al., 1997). The synthesis of the triphosphates of 5-ohC and 5-ohU and their subsequent enzymatic incorporation into oligonucleotides has also been accomplished (Roy-Burman et al., 1970; Mascarenas et al., 1993). Site specific substrates have been generated enzymatically by appending a single modified nucleotide triphosphate to the 3' end of an oligonucleotide using terminal deoxynucleotidyl transferase and then using DNA polymerases to extend beyond the adduct (Hatahet et al., 1993). In vitro repair assays with these substrates demonstrate that endonuclease III can excise both 5-ohC and 5-ohU from DNA (Hatahet et al., 1994). A complementary approach to the study of adduct repair has been the use of GC-MS to determine the quantity and types of adducts released from DNA. Incubation of a repair enzyme with globally damaged DNA results in release of various modified bases, which are then derivatized, detected and quantitated. This method has been used to determine that Ug, 5-ohC and 5-ohU are removed by endonuclease III (Dizdaroglu et al., 1993; Wagner et al., 1996). There has been much interest in the repair of these oxidized cytosine lesions as evidenced by this rapidly growing body of literature. The contributions of this work to the field were to compare the kinetics of endonuclease III mediated base excision for the lesions Ug, 5-ohC and 5-ohU in two different base-pairing schemes.

II. MATERIALS AND METHODS

Repair enzymes.

Purified endonuclease III from *E. coli* was the gift of Dr. Richard Cunningham, State University of New York, Albany. Purified endonuclease IV from *E. coli* was the gift of Dr. Bruce Demple, Harvard School of Public Health. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard (Bradford, 1976).

5' End-labeling of oligonucleotides.

T4 polynucleotide kinase (PNK) (New England Biolabs) and [γ-^{32}P] ATP (6000Ci/mmol) (New England Nuclear) were incubated with oligonucleotides in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT at 37 °C for 15 min. Exhaustive phosphorylation was achieved by addition of 1 mM ATP to the reaction and incubating for an additional 30 min at 37 °C. PNK was heat inactivated by treating at 65 °C for 15 min.

Oligonucleotides containing 5-ohC and 5-ohU.

All oligonucleotides were synthesized on an Applied Biosystems 391 automated DNA synthesizer. For oligonucleotides containing 5-ohC and 5-ohU, phosphoramidites were prepared (Morningstar et al., 1997) and the 40-mers,

5'- AATTGCGATCTAGCTCGCCAG**X**AGCGACCTTATCTGATGA-3',

where X= 5-ohC or 5-ohU, were synthesized. All oligonucleotides were purified by 20% polyacrylamide gel electrophoresis. Concentrations were determined by measuring the A_{260} and

calculating the extinction coefficient (ϵ) of the single-stranded oligonucleotide as described (Borer, 1975). Duplexes containing 5-ohC or 5-ohU were prepared by mixing ^{32}P-labeled oligonucleotides with 1.1 equivalents of the complementary strand, which contained either G or A at the site opposite the lesion, in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, and 0.1 mg/mL BSA and heating for 2 min at 75 °C. The samples were allowed to cool for 1 hr at room temperature and then 1 hr at 4 °C.

Synthesis and characterization of oligonucleotides containing Ug.

Typically, 375 μg of the 5-mer oligonucleotide GUAGC in NH_4Cl-NH_4OH (pH 9.0) was treated with 2.4% OsO_4 for 30 min at 55 °C to generate the Ug containing pentamer GUgAGC. The modified oligonucleotide was purified by reversed-phase HPLC with UV detection on a Beckman ODS column using a gradient 0-30% B over 60 min (A= 0.1 M NH_4OAc in H_2O; B= 0.1 NH_4OAc in 50% H_2O/acetonitrile) followed by 23% denaturing polyacrylamide gel electrophoresis. DNA was recovered by the method of crush and soak (Maniatis et al., 1989). The oligonucleotide was characterized by MALDI-TOF MS as well as enzymatic digestion to the nucleoside and comparison to standards. The DNA was digested with 0.05 units of nuclease P1 (ICN) in 66 mM NaOAc (pH 5) and 0.13 mM $ZnCl_2$ for 1 hr at 37 °C followed by incubation with 25 units calf intestinal phosphatase (Sigma) in 100 mM Tris HCl (pH 8.8). HPLC analysis of the nucleosides utilized a linear gradient 0-20% B over 40 min (A= H_2O: B= 50% H_2O/acetonitrile). Authentic Ug was synthesized by treating U with OsO_4; the product was purified by HPLC and identified by FAB-MS. Ug was detected by monitoring UV absorbance at 214 nm. To determine the stability of Ug, the oligonucleotide GUgAGC was subjected to the

conditions of duplex construction and examined for formation of degradation products by reversed-phase HPLC.

Construction of duplexes containing Ug.

Duplex oligonucleotide substrates were prepared as follows. Oligonucleotide 1 (Figure 9D) was exhaustively phosphorylated with ATP and GUgAGC was ^{32}P-end labeled as described above. Typically 1800 pmol of each of the three oligonucleotides 1, 2, and 3 (Figure 9D) were annealed by heating to 75 °C in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, followed by cooling to room temperature over 45 min. An equimolar amount of phosphorylated GUgAGC was added along with 2000 units T4 DNA ligase (New England Biolabs) and incubated at 16 °C for 3-4 hours. Five units of endonuclease IV were added and the sample was incubated at 37 °C for 30 min to cleave any abasic sites formed during the construction process. The ligation mixture was concentrated using Microcon 10 filters and then subjected to 20% polyacrylamide gel electrophoresis containing 8 M urea for 3 hr at 15 V/cm. Bands were visualized by autoradiography and the band corresponding to full length duplex was excised and recovered by the method of crush and soak. The samples were desalted and transferred to 1X TE buffer using Centricon 10 membranes. To confirm that the recovered DNA was in duplex form, a sample was electrophoresed on a 12% native acrylamide gel. The purified duplexes were quantitated by measuring the A_{260} in a one cm path length cell and using an ϵ value of 661 L/mmol estimated by summing the individual ϵ values of the single-strands and subtracting 2.0 L/mmol per G:C bp and 3.3 L/mmol per A:T bp to account for hypochromicity (Puglisi and Tinoco, Jr., 1989). Finally, the samples were ethanol precipitated and resuspended

in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT and 0.1 mg/mL BSA.

Enzymatic Reactions and Determination of Kinetic Parameters.

Reactions using endonuclease III were performed in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT and 0.1 mg/mL BSA. Reactions were terminated by addition of an equal volume of formamide gel-loading dye. For initial determinations of enzyme activity, samples were incubated for 30 min at 37 °C at endonuclease III concentration between 6.25-625 nM. For time course experiments 6.25 nM endonuclease III was incubated with 1000 nM DNA in 20 μL, and 2 or 3 μL aliqots were removed and quenched at appropriate time points.

Enzymatic reactions (10 or 20 μL) containing varying amounts of ^{32}P-labeled substrate (100-3000 nM) were incubated with 6.25 nM endonuclease III for 10 min at 37 °C. The samples were electrophoresed on 20% denaturing polyacrylamide gels and the bands corresponding to substrate and product were quantitated using a Molecular Dynamics PhosphorImager. Reaction velocity (V) was calculated as pmol of product formed per minute per nanogram of protein. Substrate concentration [S] was calculated as nM. The apparent K_m and V_{max} values were determined by performing non-linear least squares fitting of the averaged data of at least three determinations to the Michaelis-Menton equation using Microcal Origin.

III. RESULTS

The objective of this work was to study the relative efficiency of removal of three oxidized cytosine derivatives by endonuclease III. A series of site-specifically modified oligonucleotide duplexes was used to determine the kinetics of base excision using phosphodiester bond cleavage as an end-point. The substrates were generated by either total chemical synthesis or by a combination of chemical and enzymatic methods.

Synthesis and Construction of duplexes containing Ug.

The lability of Ug made it necessary to invoke a combination of chemical and enzymatic methods to prepare a duplex containing a single Ug lesion. A small pentamer oligonucleotide was chemically oxidized with OsO_4 to generate a singly modified oligonucleotide. The oxidation proceeded in low yield (5-10%) but gave rise to a single major product peak as determined by HPLC (Figure 9A). Isolation of this material yielded a product possessing physical properties consistent with those expected of an oligonucleotide containing an Ug residue; the purified oligonucleotide eluted earlier on reversed-phase HPLC suggesting that it was more polar than the starting material, and it had retarded mobility relative to the unmodified oligonucleotide during PAGE. MALDI-TOF MS confirmed that the isolated peak had a mass 34 amu greater than the unmodified ($M\text{-}H_{calc}$ 1522.0, $M\text{-}H_{obs}$ 1522.5), which is the expected mass increase upon dihydroxylation of uridine. Enzymatic digestion of the product peak to the nucleoside level revealed the absence uridine in the sample, indicating that the chemical modification was specific for uridine (Figure 9B). Moreover, the uridine derivative, like

authentic Ug, lacked UV absorbance at 260 nM (Douki et al., 1996). Monitoring of the chromatogram at 214 nM revealed two peaks eluting at 4 and 5 min (Figure 9C); the later peak corresponds to authentic Ug nucleoside; it is believed that the earlier peak is residual Ug 5'-monophosphate resulting from incomplete reaction of the calf intestinal phosphatase.

The stability of the Ug containing pentamer was examined under the conditions necessary for preparation of the 40-mer duplex. Treatment at 37 °C for 30 min followed by 65 °C for 15 min (end-labeling), and 16°C for 3 hr (ligation) revealed between 2 and 4.5% degradation to the AP site containing oligonucleotide, which was subsequently removed after the ligation step by endonuclease IV treatment.

Upon phosphorylation, the modified oligonucleotide was ligated into a scaffold construct containing a single-stranded gap complementary in sequence to the modified oligonucleotide (Figure 9D). Using equimolar quantities of each of the four oligonucleotide components, ligation efficiencies ranging between 30-60% were regularly achieved. There was no significant difference in the ligation efficiency of incorporating Ug opposite A or G. The fully ligated material was purified from an 8 M urea polyacrylamide gel. In order to preserve the integrity of the Ug lesion, the gel was electrophoresed at 15 V/cm to minimize heat generation; additionally, the sample was not heated prior to loading on the gel. A consequence of these steps was that a significant fraction of the DNA remained duplex under these conditions. This duplex DNA band could be recovered and subsequent electrophoresis under standard denaturing conditions resulted in the appearance of a band corresponding to a 40-mer. Electrophoresis of the duplex under native conditions revealed > 90% double-stranded material.

Endonuclease III removes Ug, 5-ohC, and 5-ohU from DNA.

As seen in Figure 10, Ug, 5-ohC, and 5-ohU were excised from DNA and the subsequent AP site cleaved regardless of whether the lesions were paired with G or A. The band that appears in lanes treated with endonuclease III migrated as expected as approximately a 21-mer. Endonuclease III had no effect on the control duplexes containing either C:G or C:A. Time course experiments reveal that enzymatic activity was linear for the first 20-30 min (Figure 11), and that the extent of cleavage was greatest for Ug, followed by 5-ohC and then 5-ohU.

Comparative Kinetics of 5-ohC, 5-ohU, and Ug removal.

Assays in which the concentration of substrate was varied were carried out and values of K_m and V_{max} were determined for each lesion in two different base-pairing contexts: paired with G or paired with A (Figure 12). As shown in Table 1, based on values of V_{max}/K_m, Ug was repaired more efficiently than 5-ohC, which was in turn repaired more efficiently than 5-ohU. For each lesion, positioning of an A residue in the opposite strand resulted in both higher K_m values as well as higher V_{max} values. V_{max}/K_m, however, was always greater for the pairing with G. The values of V_{max} for the three lesions varied rather modestly over a 2.6-fold range, whereas the K_m values spanned a slightly greater 6-fold range.

IV. DISCUSSION

The relative kinetics of removal of the oxidized lesions Ug, 5-ohU, and 5-ohC from site-specifically modified DNA substrates were determined using site-specifically modified substrates. In this experimental system, endonuclease III processed the lesions in the order Ug > 5-ohC > 5-ohU. In order to examine the repairability of these lesions, the first synthesis of the thermally labile Ug lesion in an oligonucleotide was developed for this work. Solid-phase methods previously developed in this laboratory were used to synthesize the oligonucleotides containing 5-ohC and 5-ohU (Morningstar et al., 1997). The use of these site-specifically modified substrates also made it possible to examine the effects of changing the base positioned opposite each lesion to reflect potential biologically important pairings.

Although the solid-phase incorporation of a labile saturated pyrimidine, (5R)-5,6-dihydro-5-hydroxythymidine into an oligonucleotide has been reported (Matray and Greenberg, 1994), no analogous synthesis for the structurally similar Ug has been advanced to date. In the absence of such technology, an intact U containing oligonucleotide was selectively oxidized at the U. OsO_4 is widely used as a probe of DNA structure owing to its specific reactivity with the 5,6 double bond of T and has been used to synthesize site-specifically modified oligonucleotides containing thymine glycol (Tg) (Basu et al., 1989). The reaction of OsO_4 with U proceeds analogously, albeit less vigorously, to the reaction with T (Beer et al., 1966). Moreover, OsO_4 has been used to synthesize deoxyuridine glycol nucleoside (Wagner et al., 1996)(Douki et al., 1996). A logical extension of this approach was to treat an oligonucleotide containing a single U with OsO_4. The short oligonucleotide GUAGC was oxidized and then purified and

characterized using HPLC, gel electrophoresis, MALDI-TOF MS, and enzymatic digestion. Subsequent enzymatic manipulations introduced the pentamer into 40 bp duplexes. Similar strategies have been used by others to prepare large duplexes for in vitro DNA repair studies (Chenna et al., 1995).

Oxidation with OsO_4 is known to give exclusively *cis*-glycols (Schröder, 1980). It is likely that oxidation of the oligonucleotide GUAGC affords a mixture of the two diastereomers of *cis*-Ug (5S,6R and 5R,6S) as has been reported for the $KMnO_4$ oxidation of T to Tg (Teebor et al., 1987). Although Douki et al. report that the two diastereomeric nucleosides are separable by reversed-phase HPLC (Douki et al., 1996), under the experimental conditions of this study Ug nucleoside synthesized by treatment of deoxyuridine with OsO_4 eluted as a single peak during reversed-phase HPLC (Figure 9C). Similarly, Luo and coworkers observe only a single peak corresponding to Ug of unknown stereochemistry upon subjecting deoxycytidine to the Fenton reaction (Luo et al., 1996). Thus the kinetic parameters determined herein may reflect enzymatic activity directed towards a mixture of the two diastereomers. Of relevance are studies by Wagner et al., who observe comparable endonuclease III activity against the *cis*- and *trans*-Ug isomers as measured by gas chromatography of the TMS derivatives, suggesting that the relative configuration of the hydroxyl groups may not dramatically perturb the reaction kinetics (Wagner et al., 1996).

The results of the kinetic experiments are summarized in Table 1 and indicate that the relative ability of endonuclease III to process these three lesions varies over a modest 6-fold range (Wang and Essigmann, 1997). Phosphodiester bond cleavage was used as an end-point to measure the reaction kinetics. Other studies have demonstrated that the glycosylase activity of

endonuclease III varies widely depending upon the lesion, suggesting that phosphodiester bond cleavage is not rate limiting (Kow and Wallace, 1987; Boorstein et al., 1989; Ganguly et al., 1990). Comparison of the values of V_{max}/K_m, or the specificity constant, revealed that at low substrate concentrations, the order of preference for repair was Ug:G ≈ Ug:A > 5-ohC:G > 5-ohC:A ≈ 5-ohU:G > 5-ohU:A. Direct comparison of V_{max} values indicates that under saturating conditions, Ug and 5-ohC are repaired at roughly equal rates while 5-ohU is repaired somewhat more slowly. Examination of K_m values reveals that for each lesion, base pairing with A led to slightly higher values of the K_m than when the lesion was paired with G. The V_{max} values observed, 0.12-0.32 pmol/min/ng, fall well within the range of specific activities previously reported for endonuclease III towards various pyrimidine hydrates in homopolymeric DNA. These values vary from 2.8×10^{-5} pmol/min/ng protein for thymine hydrate to 0.044 for cytosine hydrate and 0.35 for uracil hydrate (Boorstein et al., 1988; Ganguly et al., 1990). Although other laboratories have reported that endonuclease III acts upon 5-ohC, 5-ohU and Ug, no absolute kinetic parameters have been previously determined. In addition to placing the repairability of these lesions on an absolute scale, these findings are in good agreement with the observation that 5-ohC is repaired about twice as well as 5-ohU (Hatahet et al., 1994; Wagner et al., 1996) as well as with the observation that *cis*-Ug is a slightly better substrate than 5-ohC (Wagner et al., 1996). Moreover, it has been reported that 5-ohU:G is repaired slightly better than 5-ohU:A, which is also consistent with our data (Hatahet et al., 1994). The only other kinetic parameter reported for any of these lesions is a recent GC-MS study of 5-ohU removal by human uracil glycosylase, which acts with a similar apparent K_m of 450 nM. (Dizdaroglu et al., 1996).

The identity of the base opposite a lesion when it is excised from DNA can have critical

consequences for the welfare of a cell since removal of a lesion when it is mispaired will result in fixation of the mutation rather than productive repair. Enzymes have been ascribed physiological roles in reducing mutation frequency based on their observed ability to discriminate between appropriate and inappropriate base-pairings. For example, hypoxanthine glycosylase removes inosine, the deamination product of adenine, 20X more rapidly from I:T as compared to I:C (Dianov and Lindahl, 1991). Similarly, the MutM protein has an approximately 13-fold lower K_m value for removing 8-oxoG from 8-oxoG:C as compared to 8-oxoG:A (Tchou et al., 1994). In both of these cases, nonproductive repair is disfavored. If one of the physiological roles of endonuclease III is to protect the cell from oxidative damage to C, it might be expected that repair of lesions paired with G would be favored. Based on values of V_{max}/K_m, excision of 5-ohC and 5-ohU when paired opposite G is favored over removal when paired with A, albeit only by 2-3 fold. Comparable levels of discrimination are observed for uracil glycosylase, which demonstrates a slight but consistent preference for removing uridine when paired with G versus A (Slupphaug et al., 1995).

The recent structural characterization of endonuclease III indicates that an extrahelical base can be accommodated in the enzyme crystal structure (Thayer et al., 1995). This motif is becoming increasingly common as more structures of DNA-modifying enzymes are solved (Roberts, 1995). Interestingly, NMR studies have indicated that a site-specifically located Tg residue in duplex DNA displays extrahelicity in the absence of protein (Kao et al., 1993), highlighting an unresolved issue of much current interest; does this class of proteins recognize and bind to an extrahelical base, or does the protein actively participate in the eversion process (Nelson and Bestor, 1996)? In either case, the observed differences in the kinetic parameters

when the opposing base is varied could reflect differential interactions between protein side chains and the orphan base upon binding. Another possibility is that the stability of the Watson-Crick hydrogen bonding between the two bases correlates with the propensity of the base to "flip-out"; a stable interaction is more resistant to the extrusion process. Structural and thermodynamic comparisons of the lesions in different base-pairing schemes might shed further light on the mechanisms of protein-DNA recognition, binding and catalysis.

The physiological relevance of these observations is difficult to assess with certainty. Steady state levels of Ug, 5-ohC, and 5-ohU have been measured, indicating a balance between the rate of oxidative damage and the repair capacity of the cell. The high steady state levels in mammalian tissues, on the order of 1 in 10^6 nucleotides, indicate that between 10^3-10^4 lesions could be present in each genome, posing a serious threat to genomic integrity (Wagner et al., 1992). Unfortunately, direct identification of repair systems responsible for correcting oxidative damage has proven elusive. Although this work clearly demonstrate that endonuclease III is capable of processing all three of these oxidative lesions in vitro, *E. coli* cells with *nth* mutations, which lack endonuclease III, are only weak mutators and are not sensitized towards killing by oxidants (Cunningham and Weiss, 1985), suggesting the existence of alternative repair pathways. The existence of multiple repair systems to repair similar types of damage is well precedented. For example, alkylation damage in the form of O^6MeG can be directly reversed by the repair proteins ada and ogt, and similarly, the base excision repair enzymes alkA and tag act in concert to remove N-alkylated lesions (Friedberg et al., 1995). The finding that the Uvr(A)BC excision repair complex, in addition to endonuclease III, removes Tg lesions supports the notion that there may be functional redundancy in the repair of oxidized pyrimidines (Lin

and Sancar, 1989; Kow et al., 1990), as does the identification of endonuclease VIII, which also appears to repair some modified pyrimidines (Melamede et al., 1994). Interestingly, the recent cloning of an endonuclease III homolog in yeast and the observed sensitivity of the corresponding knockout strain to oxidizing agents indicates that this base excision repair pathway is physiologically important, at least in yeast (Eide et al., 1996). Additionally, it has been reported that repair of Tg in human cells proceeds in a transcriptionally coupled fashion independent of NER proteins (Cooper et al., 1997). This suggests that BER proteins such as the human homolog of endonuclease III may be preferentially directed to the transcribed strands of transcribed genes. The human homolog of endonuclease III has been cloned (Hilbert et al., 1997; Aspinwall et al., 1997), and examination of the repair properties of this enzyme may shed more light as to the true physiological role of this enzyme family.

V. FUTURE STUDIES

Ongoing work in this laboratory is aimed at understanding the role of endonuclease III in the in vivo repair of oxidative damage. Experiments conducted by Deborah Kreutzer are examining the levels of adducts present in *E. coli* of different repair backgrounds to try and assess the importance of different repair enzymes in excising specific adducts.

In addition, the availability of site specific substrates and the recent cloning of eukaryotic homologs of endonuclease III present tremendous opportunities for further experimentation. Comparison of the kinetic parameters in different sequence contexts may provide insight into the mechanism of repair. In particular, if repair does proceed by base flipping, adducts in AT rich regions may be more easily repaired than adducts in GC regions. The substrate specificity and repair kinetics of the recently cloned eukaryotic homologs of endonuclease III should also be examined as should the possibility that there might be an eukaryotic homolog of endonuclease VIII. The phenotypes of knockout mice deficient in the endonuclease III homolog should reveal the relative importance of this protein in mammalian cells as a defense against oxidative damage and whether there are other eukaryotic repair glycosylases with similar substrate specificity.

The observed transcription coupled repair of Tg in human cells suggests that BER proteins can be preferentially directed to the transcribed strand (Cooper et al., 1997). Presumably other oxidized lesions processed by the same BER enzymes will also be repaired in a transcriptionally coupled fashion in those cells. In addition to examining this possibility, it may prove fruitful to use a yeast 2-hydrid system to search for proteins that interact with glycosylases to elucidate the mechanism of transcription coupling. Furthermore, it will be

interesting to determine if the repair of Tg and oxidized pyrimidines is transcriptionally coupled in bacteria and whether the transcription repair coupling factor (Selby and Sancar, 1994) plays a role in the process.

From a diagnostic viewpoint, it may useful to generate antibodies against Ug, 5-ohU, and 5-ohC. Antibodies against Tg and 8-oxoG have been utilized to recover these adducts from human samples and to estimate the levels of adducts. Having additional biomarkers may facilitate the accurate determination of the total cellular damage arising from oxidative stress.

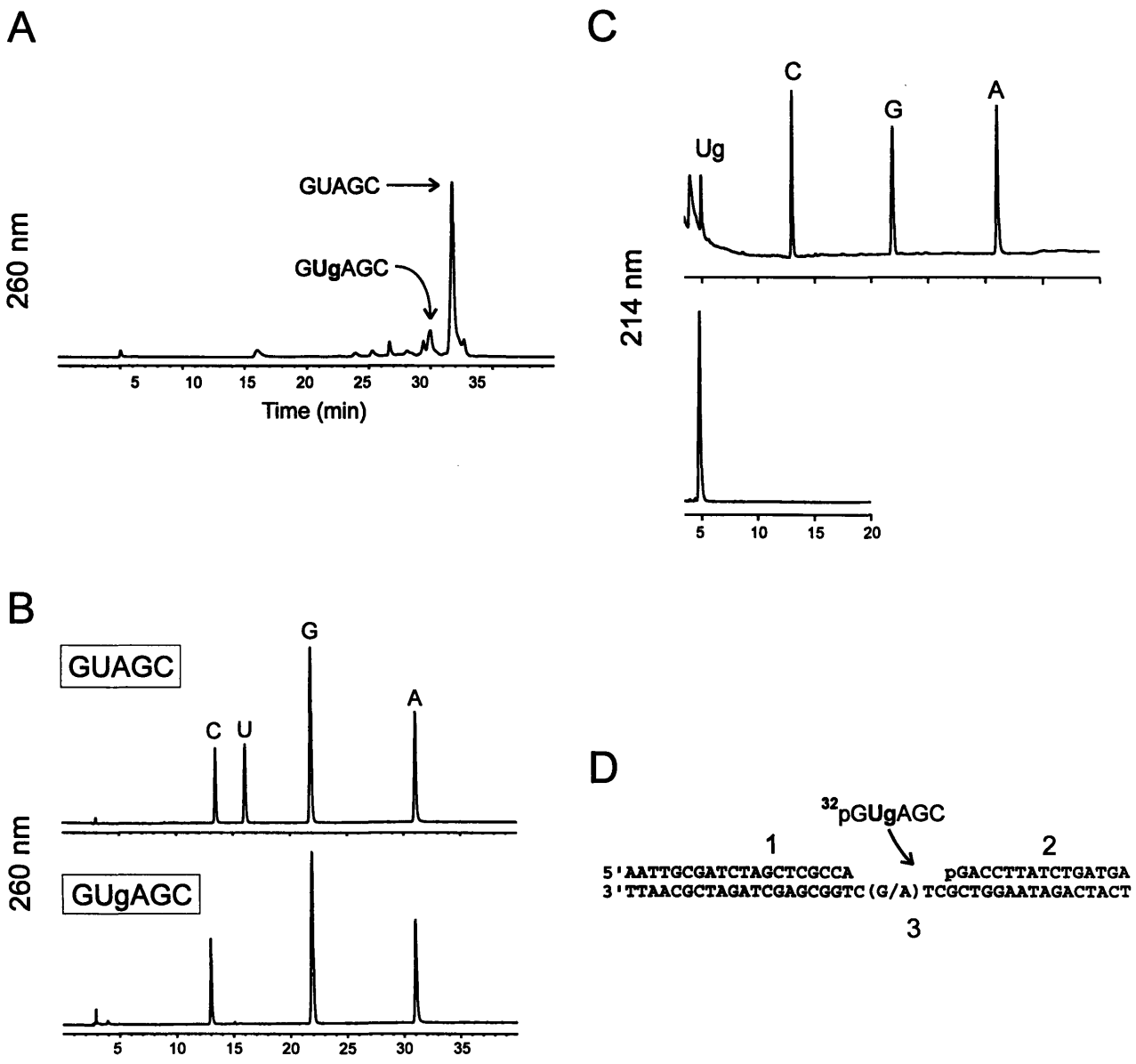

Figure 9. A. HPLC trace of the reaction of OsO_4 with the pentamer GUAGC. **B.** 260 nm HPLC trace after enzymatic digestion of GUAGC (upper) and GUgAGC (lower) to the component nucleosides. The order of elution of the nucleosides is C, U, G, A; **C.** 214 nm HPLC trace after enzymatic digestion of GUgAGC (upper) and authentic Ug standard (lower). **D.** Oligonucleotides used in the ligation of GUgAGC to form 40 base pair duplexes.

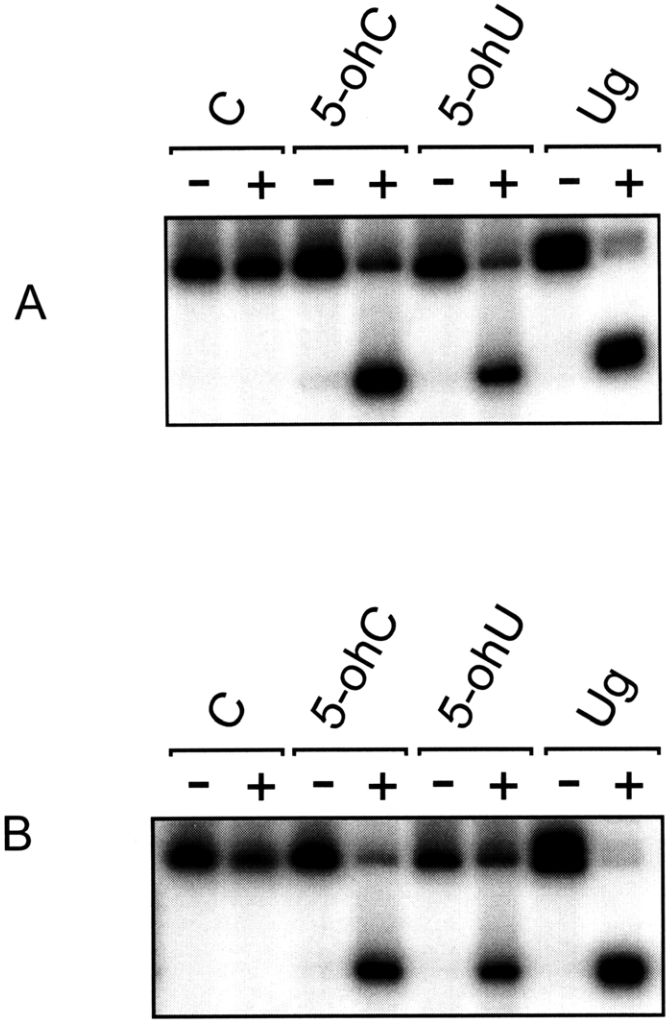

Figure 10. 20% denaturing PAGE analysis of 40 base pair duplexes containing C, 5-ohC, 5-ohU, and Ug incubated with 62.5 nM endonuclease III. **A.** Bases paired opposite G. **B.** Bases paired opposite A.

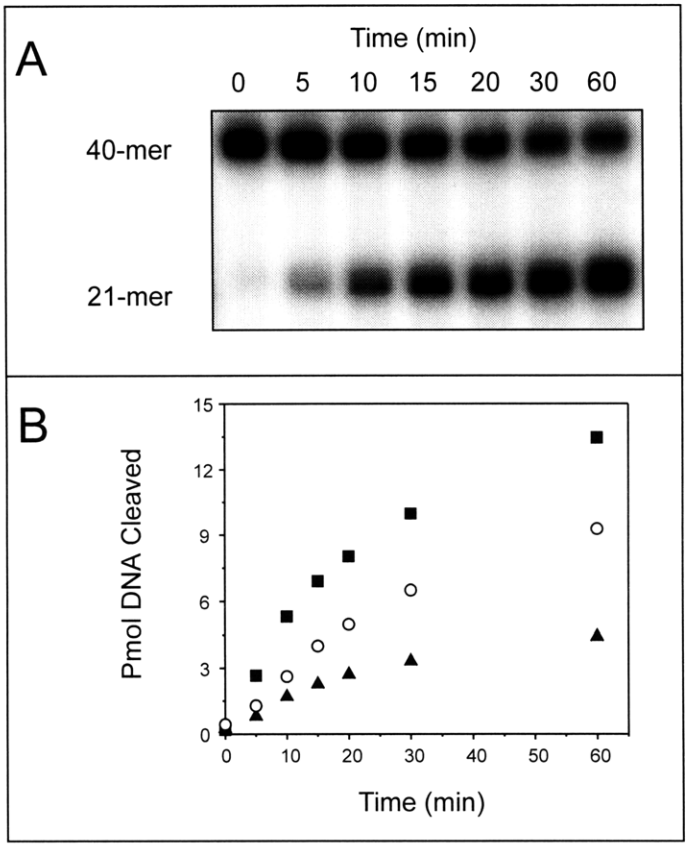

Figure 11. A. 20% denaturing PAGE of a representative time course reaction. Shown is cleavage of 5-ohC:G by endonuclease III. **B.** Extent of cleavage vs. time for Ug (■), 5-ohC (○), and 5-ohU (▲).

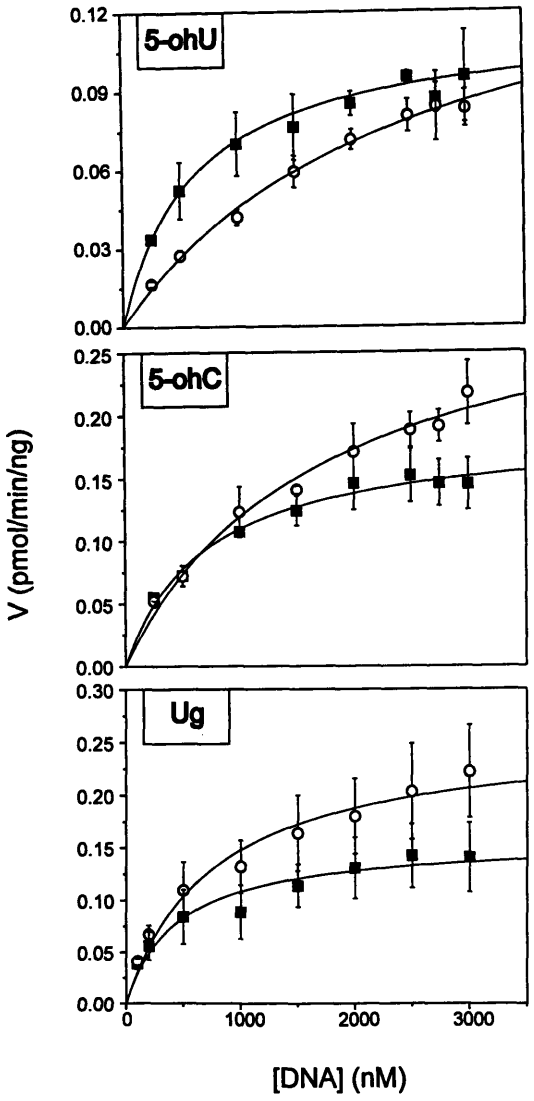

Figure 12. Plots of reaction velocity vs. substrate concentration. Data points are the mean and standard deviation of at least 3 experiments. Curves shown are the best fit of the averaged data to the Michaelis-Menten equation. (■) lesion paired with G; (○) lesion paired with A.

Table 1: Kinetic parameters describing the excision of substrates by endonuclease III.

Substrate	K_m (nM)	V_{max} (pmol/min/ng)	Relative V_{max}/K_m
Ug:G	440	0.16	1.0
Ug:A	740	0.26	0.98
5-ohC:G	710	0.19	0.74
5-ohC:A	1660	0.32	0.54
5-ohU:G	610	0.12	0.53
5-ohU:A	2440	0.16	0.18

Kinetic parameters were determined by fitting the experimental data directly to the Michaelis-Menten equation.

Chapter 4: AN INTERCALATION INHIBITOR ALTERING THE TARGET SELECTIVITY OF AFB_1 EPOXIDE: SYNTHESIS OF SITE SPECIFIC AFB_1 ADDUCTS IN A p53 MUTATIONAL HOTSPOT

I. INTRODUCTION

The carcinogenic effects of AFB_1 have been largely attributed to the mutagenic DNA adducts formed by AFB_1 *exo*-8,9-epoxide. Over the past 10 years, a hypothesis has emerged from kinetic and structural studies suggesting that intercalation by AFB_1 epoxide greatly facilitates AFB_1 adduct formation (Gopalakrishnan et al., 1989; Gopalakrishnan et al., 1990; Iyer et al., 1994a). The goal of this project was to test the validity of the proposed model by directly examining the dependence of AFB_1 adduct formation on intercalation by AFB_1 epoxide. The experimental approach involved comparison of the adduct yields at different guanines that had either vacant or occupied intercalation sites. In order to execute this strategy it was necessary to synthesize a molecule that could be specifically positioned to occupy an intercalation site and prevent further intercalation. The ideal intercalation inhibitor would be easy to synthesize, have a known propensity to occupy intercalation sites, and be readily inserted at any intercalation site. For these purposes, a model compound based on a psoralen thymidine photoproduct was selected. The work described in this chapter was done in collaboration with William R. Kobertz, who synthesized the molecule used as the intercalation inhibitor and played an important role in the design and execution of many of these experiments.

Psoralen is an agent that intercalates prior to its reaction with DNA (Hearst, 1989). Detailed structural analysis of psoralen thymidine adducts reveals that both the *cis*-syn furan-side monoadduct and the crosslink are intercalated in the DNA duplex (Spielmann et al., 1995). The total synthesis of a psoralen thymidine monoadduct and its solid-phase incorporation into oligonucleotides has been reported (Kobertz and Essigmann, 1996; Kobertz and Essigmann,

1997a). A structurally analogous model compound, a *cis*-syn thymidine benzofuran photoproduct, was also synthesized that possessed the same stereo- and regiochemistry as the *cis*-syn psoralen furan-side thymidine monoadduct (Kobertz and Essigmann, 1996); these constraints dictate that the benzofuran moiety occupies an intercalation site when situated in duplex DNA, raising the possibility that such a molecule could be used to inhibit subsequent intercalation by other molecular species. Duplex oligonucleotides with and without the thymidine benzofuran photoproduct were prepared to examine the effect of the presence of the benzofuran moiety in an intercalation site on adduct formation. Results from these experiments support the view that the reaction of DNA with AFB_1 epoxide can be modulated by inhibiting intercalation.

Intercalation inhibitors were subsequently utilized in the preparation of several oligonucleotides containing AFB_1-N7-Gua adducts at specific sites. The availability of these constructs and their use in the generation of site specifically modified vectors will facilitate understanding of the molecular origin of the observed p53 mutational hotspot in hepatocellular carcinoma. Study of the chemical basis of this hotspot has been hampered by the presence of numerous guanines in the nucleotide sequence surrounding codon 249. Due to the formidable obstacle of resolving and purifying highly labile AFB_1 adducts from complex mixtures of multiple reaction products, the synthesis of site-specifically modified AFB_1 adducts has thus far been limited to targets containing a single target guanine (Gopalakrishnan et al., 1989; Bailey et al., 1996b). It was hypothesized that intercalation inhibitors could be used to protect one or more reactive sites selectively in order to achieve a high yield of a given specific adduct. Indeed, results of these experiments revealed that judicious placement of intercalation inhibitors

in a human p53 gene derived sequence resulted in simplification of the local adduct spectrum and improved the yield of individual adducts. In addition, site specific AFB_1-FAPY adducts could be generated from the corresponding parental AFB_1-N7-Gua adducts.

II. MATERIALS AND METHODS

Synthesis of Intercalation Inhibitor.

The synthesis of the suitably protected thymidine-benzofuran phosphoramidite is shown in Figure 14. The synthesis of deoxynucleoside **1** has been previously described (Kobertz and Essigmann, 1996). Treatment of **1** with 4,4'-dimethoxytrityl chloride in the presence of silver nitrate led to rapid protection of the 5' hydroxyl. Removal of the acetate protecting group with 5% 1,8-diazabicyclo[5.4.0]undec-7-ene in freshly distilled methanol afforded the free 3' hydroxyl. Phosphitylation using standard conditions gave phosphoramidite **2**. All reactions were performed under an argon atmosphere and at room temperature. The compounds were characterized by ^{1}H, ^{13}C, and ^{31}P NMR (when applicable) and high resolution MS.

Oligonucleotides.

All oligonucleotides were synthesized on an Applied Biosystems Model 391 DNA synthesizer. Standard conditions were used except for oligonucleotides containing the intercalation inhibitor or 7-deaza-dG. For the thymidine-benzofuran phosphoramidite, the coupling time was extended for the modified base to 15 min. The coupling yield for the modified phosphoramidite **2** was 85-92% as determined by trityl release. Oligonucleotides containing 7-deaza-dG were oxidized by using a 0.5 M solution of (1S)-(+)-(10-camphorsulfonyl)oxaziridine in anhydrous acetonitrile to prevent degradation of the 7-dG residues. The oxidation step was extended to 5 min to ensure complete oxidation. All oligonucleotides were deprotected with NH_4OH for 18 hr at 55°C and subsequently purified by

C18 reversed-phase HPLC prior to use. Oligonucleotides were annealed by heating for 5 min at 80°C in 100 mM sodium phosphate (pH 7.0), 100 mM NaCl. The samples were allowed to cool at room temperature for 1 hr and then at 4°C for 1 hr.

AFB_1 Epoxide Reaction with DNA.

AFB_1 epoxide was generated as reported (Gopalakrishnan et al., 1989). Reactions contained 10 nmol duplex DNA in a total volume of 20 µl (0.5 mM DNA). In a 4°C room, samples were treated with 1-2.5 molar equivalents of AFB_1 epoxide, mixed by vortexing for 5 min, diluted with buffer, and then the AFB_1-diol was removed by extraction with CH_2Cl_2. *Note: Everything contacting AFB_1 or AFB_1 epoxide solutions was treated with bleach to inactivate any residual toxin.*

HPLC Purification.

Samples were loaded onto a C18 reversed-phase analytical column (Beckmann ODS) and eluted with a gradient of 10-30% B over 60 min (A= 0.1 M NH_4OAc in H_2O; B= 0.1 M NH_4OAc in 50% H_2O/acetonitrile) with UV monitoring at both 260 nm and 360 nm. Samples were desalted on a Sep-pak C18 cartridge at 4°C and eluted with 50% acetonitrile.

^{32}P-Labeling and Cleavage of AFB_1 Treated Oligonucleotides.

Purified oligonucleotides were 5'-end labeled with T4 polynucleotide kinase and γ-^{32}P ATP (New England Nuclear, 6000 Ci/mmol) in 70 mM Tris-HCl (pH 7.6) for 5 min at 37°C. Unincorporated label was removed by centrifugation of the sample through a Sephadex G25

column. Assays to identify the position of adducts formed were based upon the known lability of AFB_1 adducts to conditions of alkali and heat (D'Andrea and Haseltine, 1978). One hundred µl of 10% piperidine was added and heated for 30 min at 90 °C. Piperidine was removed by lyophilization, and samples were subsequently electrophoresed through 20% 7 M urea polyacrylamide gels and visualized using a Molecular Dynamics PhosphorImager. The mobilities of bands from samples were compared to those of marker bands generated by the Maxam-Gilbert G-specific reaction (Maniatis et al., 1989).

Conversion to AFB_1-FAPY.

An oligonucleotide containing a site specific AFB_1-FAPY was derived from the corresponding AFB_1-N7-Gua containing oligonucleotide essentially as described (Bailey et al., 1996a). The AFB_1-N7-Gua oligonucleotide was heated for 4 hr at 37 °C in 10 mM sodium phosphate (pH 8.5). The AFB_1-FAPY oligonucleotide was purified using a Nucleopak PA-100 strong ion exchange column (Dionex) eluted at 1.5 mL/min with a gradient of 30%-60% B over 20 min (A = 90:10 water/acetonitrile; B = 1.5 M NH_4OAc in 90:10 water/acetonitrile).

Mass Spectral Characterization of Oligonucleotides.

Both the AFB1-N7-Gua and AFB1-FAPY containing oligonucleotides were characterized by electrospray ionization MS. MS was performed by Lisa A. Marzilli and Dr. Paul Vouros (Northeastern University).

III. RESULTS

Evidence accumulated over the past several years suggests that intercalation of AFB_1 epoxide greatly enhances the reactivity of the potent carcinogen with DNA (Gopalakrishnan et al., 1989; Gopalakrishnan et al., 1990; Raney et al., 1993; Iyer et al., 1994a). Accordingly, if it were possible to reduce the likelihood of intercalation at a given site, one would expect to diminish substantially the adduct yield at that site (Figure 13). A series of duplex oligonucleotides was designed to examine this possibility (Table 2). In each case, one strand contained two or more guanines that were potential adduction sites. In the complementary strand, a *cis*-syn thymidine-benzofuran intercalation inhibitor was situated to occupy the intercalation site immediately 5' to one of the guanines. Chemical synthesis of the phosphoramidite of the intercalation inhibitor (Figure 14) made it possible to control precisely both the number and position of the intercalation inhibitors in the target duplexes. Upon allowing the various DNA duplexes to react with AFB_1 epoxide, HPLC was utilized to separate and isolate the reaction products. The standard elution conditions were sufficient to denature the duplexes, resolving the target strands from their complements. By this method, the complementary strands, which contained the unnatural thymidine-benzofuran moieties, could be removed and the single-stranded site-specifically AFB_1 modified oligonucleotides could be isolated.

The simplest model system consisted of a 10-bp oligonucleotide duplex containing only two G residues in one strand separated by three nucleotides (Table 2, H/QC). In the control sequence, reaction with AFB_1 epoxide afforded two peaks corresponding to AFB_1 monoadducts

and one peak representing the diadduct, as determined by the ratio of their UV absorbances at 360 nm and 260 nm (Figure 15A and 15B). The two monoadduct peaks were isolated, desalted and then 5'-^{32}P end-labeled by using polynucleotide kinase. The known alkaline lability of AFB_1-N7-Gua adducts (D'Andrea and Haseltine, 1978) was utilized to determine the identity of each peak. Electrophoresis of the piperidine treated samples revealed that peak 1 was the G8 AFB_1 adduct while peak 2 was the G4 adduct (Figure 15F). It is known that the reaction of AFB_1 epoxide with guanines in duplex DNA is not random, but rather displays sequence specificity. Although the basis for this discrimination is not well understood, a systematic investigation by Loechler and coworkers has empirically determined reactivity rules based on the immediate 5' and 3' neighboring bases (Benasutti et al., 1988). In the first model system, the two adducts formed in a ratio of 2.4:1, in close agreement to the predicted 2:1 ratio.

Upon altering the complementary strand to position an intercalation inhibitor in the 5' intercalation site of G4 (H/Q7), the ratio of observed products was dramatically shifted; essentially no monoadducts derived from G4 or diadducts were observed (Figure 15D). Conversely, placement of the intercalation inhibitor opposite G8 (H/Q3) greatly reduced the extent of reaction at G8 such that only 11% of the monoadducts arose from G8 (Figure 15E). The asymmetry in the protection afforded is likely due to the inherently higher reactivity of G8, making total abolition of reactivity at that site more difficult.

Since positioning the intercalation inhibitor to occupy the intercalation site 5' to a target guanine necessarily forms a G:T mismatch at that site, the effect of a mismatch alone (H/QT) on adduct distribution was examined. Substitution of a G:T base pair for a G:C afforded an adduct profile that differed only by a slightly increased reactivity of the mismatched G (compare Figure

15B to 15C). Thus, the observed diminished reactivity upon introduction of an intercalation inhibitor can be attributed specifically to the presence of an intercalated moiety and not merely distortions resulting from a mismatched base pair.

In the second model system, reactivity and inhibition in oligonucleotides containing two adjacent guanines (GG) were examined. In the absence of an intercalation inhibitor (GG/R0), two peaks were observed in the HPLC trace, reflecting the two different monoadducts (Figure 16A and 16B). Loechler's rules (Benasutti et al., 1988) predict that the two adducts should form in a 3.25 to 1 ratio, which agrees well with the observed product ratio of 3.3:1 (Figure 16B). Interestingly, there were no diadduct peaks present in the trace. This observation is consistent with the neighbor exclusion principle that has been postulated to govern the ability of molecules to intercalate at adjacent sites (Crothers, 1968; Wilson, 1990). Initial intercalation and reaction of an AFB_1 epoxide molecule at one guanine results in inhibition of intercalation at the other adjacent guanine, thereby preventing the formation of a second adduct. Nevertheless, placement of the intercalation inhibitor at either of the two guanine sites (GG/R5 and GG/R6) resulted in an increase in the yield of adduct at the non-protected guanine (Figure 16C and 16D).

Since the AFB_1 adducts seemed to exhibit neighbor exclusion properties, the benzofuran moiety was tested to see if it possessed similar characteristics. Duplexes were prepared with an intercalation inhibitor occupying the intercalation sites either 3' to or 5' to the actual target intercalation site (Figure 17). Positioning the intercalation inhibitor 3' to the target site (H/Q6) afforded some protection whereas placement of the intercalation inhibitor to the 5' side (H/Q8) had no inhibitory effects.

Finally a sequence representing nucleotides 741 to 749 of the human p53 cDNA, 5'

CCGGAGGCC (P53), was tested (Figure 18). In the complementary strands, guanine bases were replaced with 7-deaza-dG to prevent formation of additional AFB_1 adducts in this strand; such adducts would complicate analysis of the adduct spectrum. Treatment of a control duplex (P53/S0) revealed three HPLC peaks that corresponded to AFB_1 monoadducts (Figure 18B). After ^{32}P labeling and alkaline cleavage, it was determined that peak 1 contained exclusively the G747 adduct, peak 2 contained the G744 adduct, while peak 3 was a mixture of the G743 and G746 (Figure 18A). Under certain conditions, peak 3 could actually be resolved to afford two peaks.

Experiments carried out with two intercalation inhibitors present within several bp of each other yielded reaction profiles consistent with single-stranded DNA, indicating that too much structural distortion was imposed by multiple intercalators or mismatches to maintain duplex structure. Thus, the effects of placing a single intercalation inhibitor at different positions in the DNA were examined. The first experiment examined protection of G743 (P53/S6), the nucleotide predicted to have the greatest inherent reactivity. Protection of that site altered the product profile significantly: peak 3 was greatly reduced; peak 2 increased by approximately 4-fold; and peak 1 was relatively unchanged (Figure 18C). In contrast, protection of G744 (P53/S5) resulted in diminished yield of peak 3 while enhancing peak 1 by 3-fold (Figure 18D). The presence of intact AFB_1 monoadduct in each sample was confirmed by electrospray MS.

The oligonucleotide containing G747 AFB_1-N7-Gua adduct was treated with alkali to generate the corresponding AFB_1-FAPY adduct at that site (Figure 19). Treatment for 4 hr at pH 8.5 lead to the formation of a product with a retention time intermediate to that of the parental

AFB_1-N7-Gua containing oligonucleotide and the unmodified P53 oligonucleotide by ion exchange chromatography. This product was isolated and determined by electrospray ionization MS to have a molecular weight consistent with that expected for the AFB_1-FAPY containing oligonucleotide.

IV. DISCUSSION

Given that the half life of AFB_1 epoxide in water is approximately one second (Johnson et al., 1996), it has been proposed that intercalation of AFB_1 epoxide provides a kinetic and entropic advantage for productive reaction with DNA versus unproductive hydrolysis (Raney et al., 1990; Johnson and Guengerich, 1997). In order to examine directly the effects of intercalation on AFB_1 epoxide reactivity with DNA, a means was devised to occupy intercalation sites with covalently linked benzofuran derivatives (Figure 13). The results of these experiments support the hypothesis that intercalation in DNA by AFB_1 epoxide facilitates adduct formation, as evidenced by the observation that abolishing intercalation at a given site greatly diminished reactivity at that site (Kobertz et al., 1997b). Furthermore, this principle has been applied to the problem of synthesizing specific AFB_1-N7-Gua adducts within a region of the p53 gene and resulted in a several fold increase in the yield of isolatable adducts.

The first set of experiments clearly revealed that intercalation contributes significantly to the ability of AFB_1 epoxide to react with DNA (Figure 15). The relative adduct yields could be shifted from almost exclusively one adduct to almost exclusively the other depending upon the location of the intercalation inhibitor. In addition, diadduct formation was almost completely abolished. There remained, however, some residual adduction at the protected site (Figure 15D and 15E). This observation was not unexpected since both single stranded DNA and mononucleotides are known to react, albeit to a much lesser extent, with AFB_1 epoxide (Raney et al., 1993) indicating that intercalation is not an obligate step for reaction with guanine.

As a control, the effects of introducing a G:T mismatch alone were examined (H/QT).

No inhibition of adduct formation was observed, suggesting that the diminished reactivity observed after introduction of an intercalation inhibitor is due to the presence of the intercalated benzofuran moiety and not merely to distortions induced by a G:T mismatch. An intriguing finding is the slightly increased relative yield of the G4 monoadduct upon substituting T for C in the complementary strand (Figure 15C). One possible explanation is that the guanine is more favorably positioned for nucleophilic attack on the epoxide when it is engaged in a wobble pairing characteristic of G:T base pairs (Patel et al., 1982). Alternatively, the mismatch itself, or the presence of the additional hydrophobic methyl group of T, might facilitate intercalation. As CpG groups in mammalian DNA are often methylated, methylation status could play a significant role in the reaction of AFB_1 epoxide with DNA *in vivo*. Of particular relevance is recent evidence that CpG methylation significantly alters the distribution of DNA adducts of another intercalative carcinogen, benzo(a)pyrene diol epoxide (Denissenko et al., 1997).

The susceptibility of AFB_1 adducts to piperidine cleavage made it possible to assign the HPLC peaks to specific guanine adducts by comparison to Maxam-Gilbert G-reaction markers (Figure 15F). No cross contamination of the two different adducts was observed. However, a minor band (10%) of similar mobility to the unmodified target oligonucleotide was observed in the piperidine treated samples for every monoadduct (Figure 15F, 16E and 18A). This band most likely arises during manipulation of the samples from chemical reversal of the AFB_1-N7-Gua adduct to give free AFB_1-diol and guanine, as has been previously observed in vitro (Hertzog et al., 1980; Groopman et al., 1981). It is unlikely to be a contamination from the HPLC purification process as the band persisted, even after multiple HPLC purifications.

The second model system tested the reactivity of AFB_1 epoxide with a target DNA

containing two adjacent guanines (Figure 16). One interesting observation was that essentially no diadducts were observed with this duplex. The absence of diadducts can be rationalized on the basis of the nearest neighbor exclusion principle, which postulates that occupation of a single intercalation site prevents subsequent intercalation both immediately 5' and 3' to the initial site (Crothers, 1968; Wilson, 1990). In this scenario, initial modification at one guanine results in occupancy of an intercalation site, effectively blocking intercalation at the adjacent guanine target site and thus diminishing reactivity of that guanine. Nevertheless, addition of intercalation inhibitors in the complementary strand had the expected effects. In both cases (GG/R5 and GG/R6), reaction at the protected guanine diminished while the yield of the other adduct increased (Figure 16C and 16D). From these experiments, it is unclear whether the benzofuran moiety possesses neighbor excluding properties and to what extent this principle affects the reaction. If the neighbor exclusion principle is equally applicable to all intercalators, then the expectation upon placing an intercalation inhibitor at one of the two sites would be that all reactivity should be abolished. This was not the case, however, suggesting that the neighbor exclusion principle is not strictly applicable. Indeed, other violations of the neighbor exclusion principle have been reported (Wakelin et al., 1978; Robledo-Luiggi et al., 1991).

Based on the observations with the GG/R0 duplex, it was of interest to test whether the intercalation inhibitor would exhibit neighbor exclusion properties in a simpler system using oligonucleotide H (Figure 17). Duplexes containing an intercalation inhibitor shifted one base to either the 5' or 3' side of the target were treated with AFB_1 epoxide. These experiments revealed an asymmetry in the protection afforded by these constructs. Placing the intercalation inhibitor immediately 3' to the target resulted in some protection (Figure 17B). However, when placed to

the 5' side, the there was no protective effect (Figure 17C). The lack of consistently observable neighbor exclusion by this particular intercalation inhibitor may stem from the size of the intercalation inhibitor or the specific helix-distortion imposed. Expansion of the benzofuran moiety to a bulkier molecule that completely spans the helix may fill the entire intercalation space and serve as a more efficient inhibitor of intercalation. In any case, examination of the basis of the neighbor exclusion principle warrants further investigation.

Having established the feasibility of altering chemical reactivities by intercalation inhibition, the next step was to apply this methodology to help understand the conundrum presented by an observed AFB_1-related p53 mutational hotspot. In greater than 50% of hepatocellular carcinomas in regions of the world with exposure to AFB_1 and hepatitis B, a G→T mutation at G747 (the third position of codon 249) is observed (Hsu et al., 1991; Bressac et al., 1991; Greenblatt et al., 1994). Based on Loechler's predictions, G747 should not be exceptionally prone to adduction; the four potential target guanines in the p53 mutational hotspot sequence (Figure 16E) are expected to have relative reactivities of 3:1.4:1:1.4 (Benasutti et al., 1988). Why, then, are so many mutations observed at G747? Although selective pressures certainly play a role in shaping the mutations observed in end-stage tumors, selection alone is unlikely to explain why G747 mutations are so abundant. One approach towards understanding the factors and processes subsequent to adduct formation that lead to a mutation is the use of oligonucleotides containing adducts at specific sites (Singer and Essigmann, 1991). By synthesizing a series of oligonucleotides each containing an AFB_1 adduct at a different site, one can begin to study the relative ability of each adduct to induce a mutation. In addition, site-specific methods can address the identity of the actual mutagenic species. Since AFB_1-N7-Gua

adducts are highly labile and are capable of undergoing either depurination to afford an abasic site or imidazole ring opening to form the AFB_1-FAPY derivative, there has been uncertainty as to the relative mutagenic properties of the three species (Bailey et al., 1996b). Insertion of defined substrates into an appropriate vector will enable the first site-specific examination of the ability of AFB_1 adducts or their decomposition products to induce mutations in the p53 sequence. However, to date, no study of the synthesis of site-specific AFB_1 adducts in such a sequence or any oligonucleotide containing multiple guanines has been reported. It was our goal to utilize intercalation inhibitors to facilitate the synthesis and purification of specific AFB_1 adducts in such a sequence.

In studies of the GC-rich p53 sequence between nucleotides 741-749 (Figure 18), two alterations had to be incorporated into the experimental design. Due to the high melting temperature of these duplexes, a G:T mismatch, which was shown to have only minor effects on the adduct profile, was incorporated to facilitate denaturation of the two strands during HPLC. In addition, 7-deaza-dG, which lacks a nitrogen at position 7, was used in the place of dG in the complementary strand to prevent adduct formation that might complicate the analysis. In the absence of an intercalation inhibitor (P53/S0), three peaks corresponding to monoadducts were obtained with peak 3 the dominant reaction product (Figure 18B). Electrospray MS confirmed that the isolated products were of the expected molecular weight corresponding to intact AFB_1 monoadducts. As anticipated, placement of two intercalation inhibitors within several bases of each other in a short oligonucleotide appeared to prevent hybridization of the duplex. Thus, the effects of a single intercalation inhibitor on the product profile were examined. Upon protection of the theoretically most reactive site, G743, the yield of the other peaks increased significantly,

with the G744 adduct comprising greater than 50% of the monoadducted species (Figure 18C). Similarly, protection of G744 lead to a large increase in the yield of the G747 adduct (Figure 18D). This is consistent with the observed asymmetry of protection afforded by the intercalation inhibitor, which appears to inhibit intercalation at both the site itself as well as the 5' neighboring intercalation site. Thus, depending upon the location of the intercalation inhibitor, it was possible to alter the adduct distribution such that any of the three monoadduct peaks represented the major reaction product (Table 3). It was also possible to convert the AFB_1-N7-Gua monoadducts into the corresponding AFB_1-FAPY containing oligonucleotides (Figure 19). After alkali treatment, a peak was isolated with properties consistent to that of AFB_1-FAPY: during ion exchange chromatography, the retention time was retarded relative to the parental AFB_1-N7-Gua (consistent with loss of the positive charge on the imidazole ring); the isolated peak was more stable and less prone to depurination than AFB_1-N7-Gua; and the molecular weight of the sample was consistent with that calculated for an AFB_1-FAPY containing oligonucleotide. The availability of oligonucleotides containing both AFB_1-N7-Gua and AFB_1-FAPY will facilitate direct comparison of their repairability and mutagenicity.

This work demonstrates clearly that inhibiting intercalation of AFB_1 epoxide can significantly alter the resulting adduct spectrum and that introduction of intercalation inhibitors into DNA duplexes provides a facile means of manipulating product yields (Table 3). Even in complex sequence environments, such as the p53 mutational hotspot sequence surrounding codon 249, a significant effect on adduct distribution with a single intercalation inhibitor can be demonstrated (Table 3). Moreover, this is the first reported synthesis of site specific AFB_1-N7-Gua and AFB_1-FAPY oligonucleotides in sequence contexts containing multiple guanines.

V. FUTURE STUDIES

Many unanswered questions remain about the role of intercalation in small molecule DNA interactions. It should be possible to use the novel molecules described above to gain a better understanding of the phenomenon of intercalation. Structure-function investigations with intercalation inhibitors may shed more light on factors that contribute to the neighbor exclusion principle, and perhaps provide an understanding of the factors underlying neighbor exclusion. Second generation intercalation inhibitors can be designed with superior inhibitory or neighbor excluding capabilities. Moreover, intercalation inhibitors can be used to probe the intercalation requirements of other carcinogens, such as benzo(a)pyrene and aromatic amines, and facilitate the site-specific synthesis of carcinogen-DNA adducts. Is the intercalation step unique to AFB_1 adduct formation, or will it be a more generalized phenomenon observed in nature with other agents?

The availability of both site-specific AFB_1-N7-Gua and AFB_1-FAPY adducts provides a resource for studies of both DNA repair and mutagenicity. Although biochemical comparison of the processing by Uvr(A)BC of these lesions has been reported, there have not been any biochemical examinations with human or mammalian NER proteins. In light of the observed persistence of AFB_1-FAPY in human cells (Croy and Wogan, 1981; Leadon et al., 1981), in vitro repair studies may provide insight into the basis of this persistence.

Finally, the synthesis of these substrates affords the opportunity to study the relative repairability and mutability of the different adducts in the p53 sequence. Construction of appropriate vectors using these, or similar modified oligonucleotides may clarify the factors that

contribute to the observed mutational hotspot in human liver tumors. Used in conjunction with cell lines expressing hepatitis viral proteins, such vectors may also shed some light on mechanism of the observed synergy between HBV and AFB_1 as risk factors for HCC.

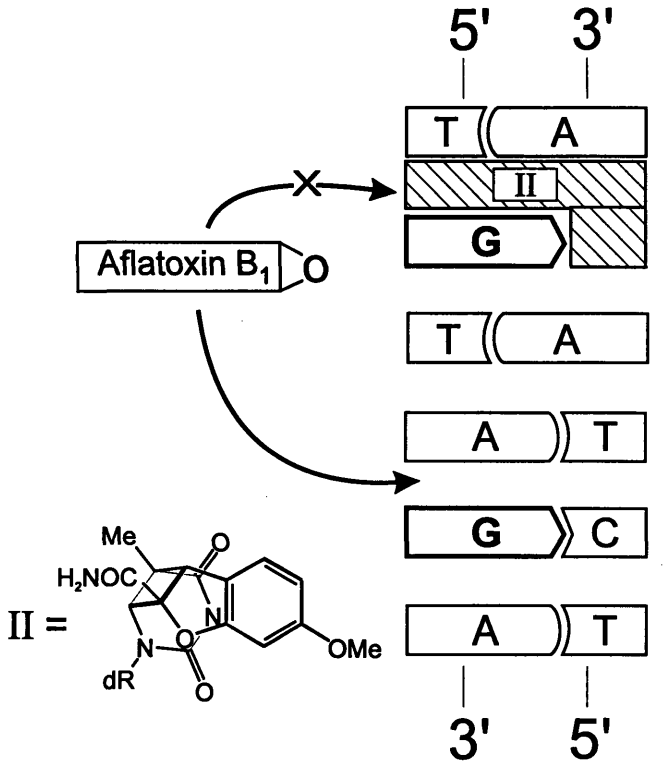

Figure 13. Experimental scheme. Occupation of an intercalation site 5' to a guanine should prevent intercalation by AFB_1 epoxide and reduce reactivity at the protected site. The covalently attached intercalation inhibitor (designated II), a *cis*-syn thymidine-benzofuran photoproduct, is positioned in the complementary strand. The benzofuran moiety is positioned on the 3' side of the T component of the *cis*-syn thymidine-benzofuran adduct.

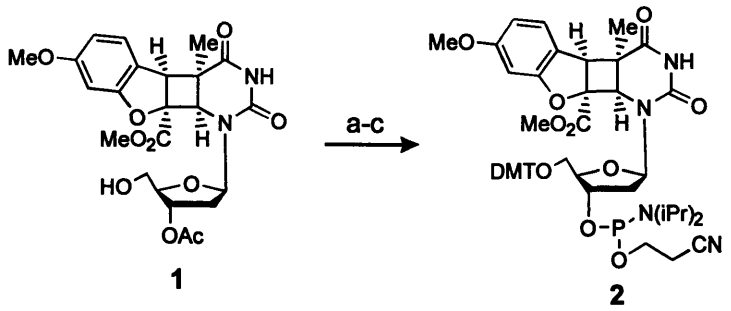

Figure 14. Synthesis of the cis-syn thymidine-benzofuran phosphoramidite (yields in parenthesis). a. 4,4'-dimethoxytrityl chloride, $AgNO_3$, collidine, DMF (84%). b. 5% 1,8-diazabicyclo[5.4.0]undec-7-ene in methanol (98%). c. Bis(isopropylamine)-2-cyanoethyl phosphoramidite, diisopropylammonium tetrazolide, CH_2Cl_2 (74%).

Table 2: Oligonucleotides used in this work.

H	5'-ATAGATTGTA-3'
QC	3'-TATCTAACAT-5'
QT	3'-TATTTAACAT-5'
Q8	3'-TAXCTAACAT-5'
Q7	3'-TATXTAACAT-5'
Q6	3'-TATCXAACAT-5'
Q3	3'-TATCTAAXAT-5'
GG	5'-TATAGGTTAT-3'
R0	3'-ATATCCAATA-5'
R6	3'-ATATXCAATA-5'
R5	3'-ATATCXAATA-5'
P53	5'- CCGGAGGCC-3'
S0	3'-T77TCTCC7-5'
S6	3'-T77XCTCC7-5'
S5	3'-T77CXTCC7-5'

7=7-deaza-dG; X=thymidine-benzofuran photoproduct (intercalation inhibitor)

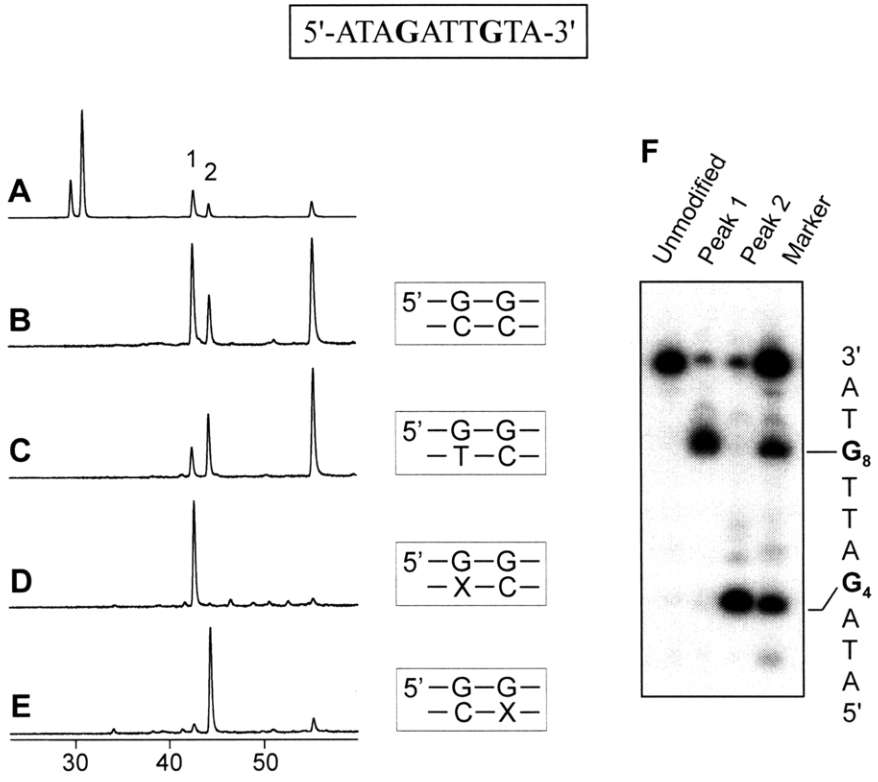

Figure 15. Intercalation inhibition in duplex H/Q series. Only molecules containing AFB_1 have UV absorbance at 360 nm. **A.** 260 nm HPLC trace of the AFB_1 reaction with H/QC. The peaks near 30 min are the unreacted H and QC oligonucleotides, repsectively. Peaks 1 and 2 are AFB_1 monoadducts, and the peak near 55 min is an AFB_1 diadduct. **B.** 360 nm trace of H/Q3. **C.** 360 nm trace of the reaction with H/QT. **D.** 360 nm trace of the reaction with H/Q7. **E.** 360 nm trace of the reaction with H/Q3. **F.** Electrophoretic analysis: piperidine cleavage of purified peaks 1 and 2 and comparison to markers generated by the Maxam and Gilbert G reaction. Piperidine treatment of the unmodified oligonucleotide H is shown in the lane labeled "unmodified."

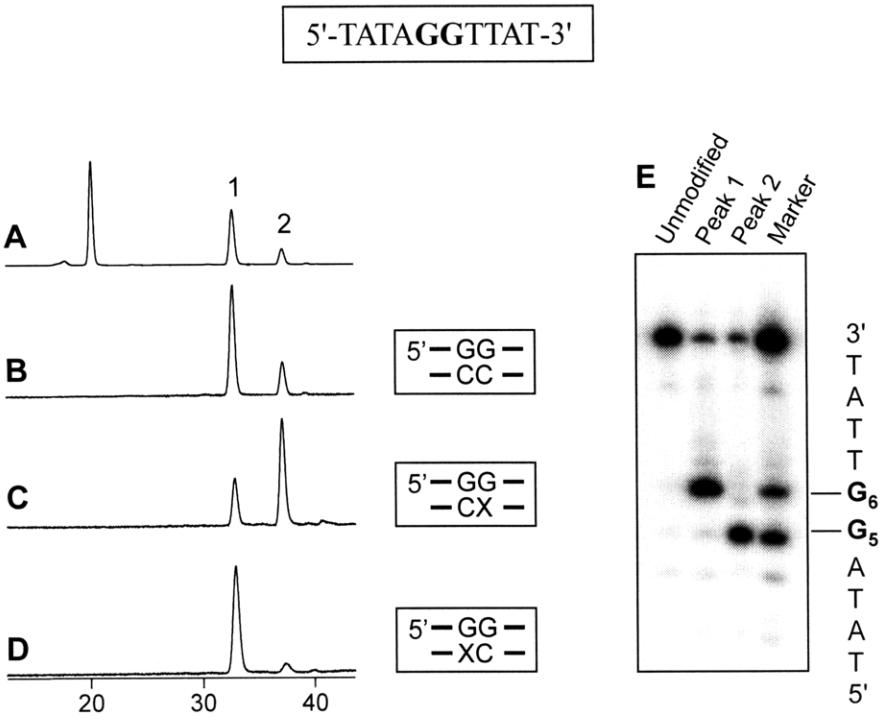

Figure 16. Intercalation inhibition in duplexes GG/R. **A.** 260 nm HPLC trace of the reaction with GG/R0. Large peak near 20 min is R0. Peaks 1 and 2 are AFB_1 monoadducts. **B.** 360 nm trace of reaction with GG/R0. **C.** 360 nm trace of the reaction with GG/R5. **D.** 360 nm trace of the reaction with GG/R6. **E.** Electrophoretic analysis: Piperidine cleavage of purified peaks 1 and 2.

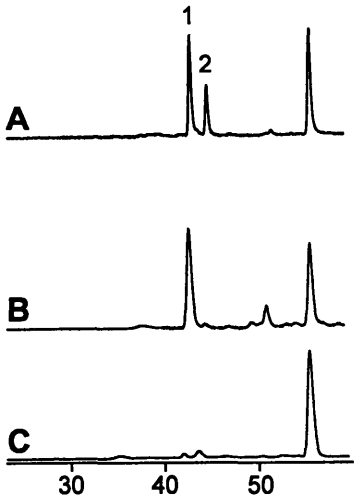

Figure 17. Nearest neighbor inhibition in duplex (H/Q) series. **A.** For reference, 360 nm trace of the reaction with H/QC showing the retention time of the two monoadducts and the diadduct. **B.** 360 nm trace of the reaction with H/Q6. Under these experimental conditions (10 equivalents of AFB_1 epoxide) some protection is afforded by Q6, indicated by the presence of the G8 monoadduct. **C.** 360 nm trace of the H/Q8 reaction showing no inhibition and almost complete conversion to the diadduct species.

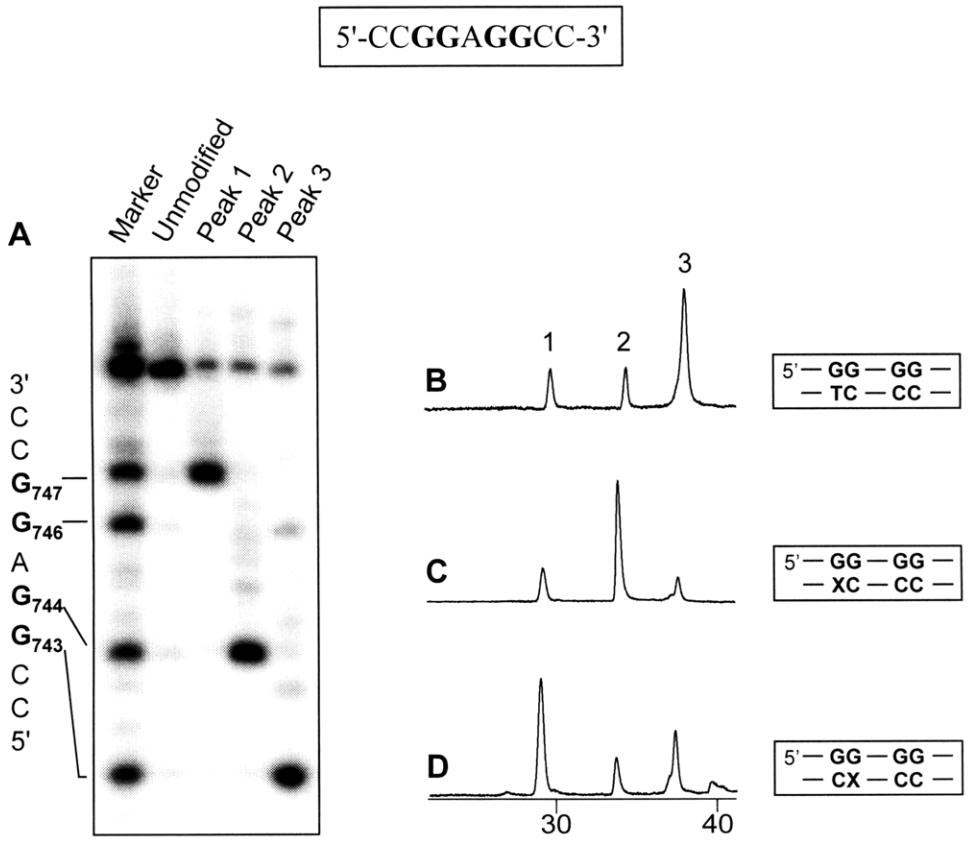

Figure 18. Effects of introducing an intercalation inhibitor into duplexes derived from the human p53 sequence. For clarity, all HPLC traces are at 360 nm and show only the monoadduct region of the trace. **A.** Electrophoretic analysis: piperidine cleavage of peaks 1, 2, and 3 and comparison to markers. The sequence of oligonucleotide P53 is shown (left). Numbers refer to the nucleotide position of each base in the human p53 cDNA. **B.** AFB_1 reaction with duplex P53/S0. Peaks 1, 2 and 3 are all AFB_1 monoadducts. **C.** Reaction with duplex P53/S6. **D.** Reaction with duplex P53/S5.

Table 3: Relative monoadduct yield as a percentage of total monoadducts.

Duplex	% Peak 1	% Peak 2	% Peak 3
H/QC	70	30	N/A
H/QT	34	66	N/A
H/Q7	>95	<5	N/A
H/Q3	11	89	N/A
GG/R0	77	23	N/A
GG/R6	93	7	N/A
GG/R5	33	67	N/A
P53/S0	17	14	69
P53/S6	21	60	18
P53/S5	51	14	35

Peak areas determined by integration of A_{360}.

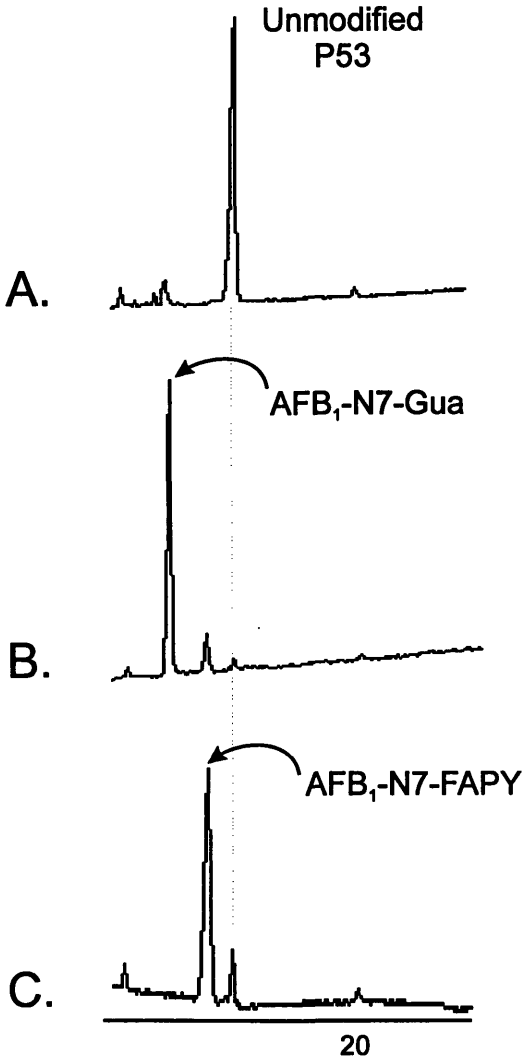

Figure 19. Conversion of AFB_1-N7-Gua to AFB_1-FAPY. 260 nm traces of ion exchange HPLC are shown. **A.** Chromatogram of the umodified P53 oligonucleotide. **B.** Purified P53 oligonucleotide containing AFB_1-N7-Gua modified at G747. A small amount of degradation can be observed. **C.** Following alkali treatment, the AFB_1-N7-Gua is almost entirely converted to a peak of intermediate retention time, consistent with loss of the positive charge. Some reversal of the adduct to afford unmodified P53 is also evident.

REFERENCES

Adelman, R., Saul, R.L., and Ames, B.N. (1988). Oxidative damage to DNA: relation to species metabolic rate and life span. Proc.Natl.Acad.Sci.U.S.A. *85*, 2706-2708.

Agarwal, S. and Sohal, R.S. (1994). DNA oxidative damage and life expectancy in houseflies. Proc.Natl.Acad.Sci.U.S.A. *91*, 12332-12335.

Aguilar, F., Hussain, S.P., and Cerutti, P. (1993). Aflatoxin B_1 induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. Proc.Natl.Acad.Sci.U.S.A. *90*, 8586-8590.

Ames, B.N., Shigenaga, M.K., and Hagen, T.M. (1993). Oxidants, antioxidants and the degenerative diseases of aging. Proc.Natl.Acad.Sci.U.S.A. *90*, 7915-7922.

Aoyama, T., Yamano, S., Guzelian, P.S., Gelboin, H.V., and Gonzalez, F.J. (1990). Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B_1. Proc.Natl.Acad.Sci.U.S.A. *87*, 4790-4793.

Aruoma, O.I., Halliwell, B., and Dizdaroglu, M. (1989a). Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. J.Biol.Chem. *264*, 13024-13028.

Aruoma, O.I., Halliwell, B., Gajewski, E., and Dizdaroglu, M. (1989b). Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. J.Biol.Chem.. *264*, 20509-20512.

Asahara, H., Wistort, P.M., Bank, J.F., Bakerian, R.H., and Cunningham, R.P. (1989). Purification and characterization of *Escherichia coli* endonuclease III from the cloned nth gene. Biochemistry *28*, 4444-4449.

Aspinwall, R., Rothwell, D.G., Roldan-Arjona, T., Anselmino, C., Ward, C.J., Cheadle, J.P., Sampson, J.R., Lindahl, T., Harris, P.C., and Hickson, I.D. (1997). Cloning and characterization of a functional human homolog of *Escherichia coli* endonuclease III. Proc.Natl.Acad.Sci.U.S.A. *94*, 109-114.

Augeri, L., Lee, Y.M., Barton, A.B., and Doetsch, P.W. (1997). Purification, characterization, gene cloning, and expression of *Saccharomyces cerevisiae* redoxyendonuclease, a homolog of *Escherichia coli* endonuclease III. Biochemistry *36*, 721-729.

Avery, O. T., MacLeod, C. M., and McCarty, M. (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J.Exp.Med. *79*, 137-158.

Ayaki, H., Higo, K., and Yamamoto, O. (1986). Specificity of ionizing radiation-induced mutagenesis in the lac region of single-stranded phage M13 mp10 DNA. Nucleic Acids Res. *14*, 5013-5018.

Baertschi, S.W., Raney, K.D., Stone, M.P., and Harris, T.M. (1988). Preparation of the 8,9-epoxide of the mycotoxin aflatoxin B_1: the ultimate carcinogen species. J.Am.Chem.Soc. *110*, 7929-7931.

Bailey, E.A., Iyer, R.S., Harris, T.M., and Essigmann, J.M. (1996a). A viral genome containing an unstable aflatoxin B_1-N7-guanine DNA adduct situated at a unique site. Nucleic Acids Res. *24*, 2821-2828.

Bailey, E.A., Iyer, R.S., Stone, M.P., Harris, T.M., and Essigmann, J.M. (1996b). Mutational properties of the primary aflatoxin B_1-DNA adduct. Proc.Natl.Acad.Sci.U.S.A. *93*, 1535-1539.

Basu, A.K., Loechler, E.L., Leadon, S.A., and Essigmann, J.M. (1989). Genetic effects of thymine glycol: site-specific mutagenesis and molecular modeling studies. Proc.Natl.Acad.Sci.U.S.A. *86*, 7677-7681.

Beer, M., Stern, S., Carmalt, D., and Mohlhenrich, K.H. (1966). Determination of base sequence in nucleic acids with the electron microscope. V. The thymine-specific reactions of osmium tetroxide with deoxyribonucleic acid and its components. Biochemistry *5*, 2283-2288.

Benasutti, M., Ejadi, S., Whitlow, M.D., and Loechler, E.L. (1988). Mapping the binding site of aflatoxin B_1 in DNA: systematic analysis of the reactivity of aflatoxin B_1 with guanines in different DNA sequences. Biochemistry *27*, 472-481.

Bennett, R.O., Wilson, D.M., Wong, D., and Demple, B. (1997). Interaction of human apurinic endonuclease and DNA polymerase beta in the base excision repair pathway. Proc.Natl.Acad.Sci.U.S.A. *94*, 7166-7169.

Bessho, T., Roy, R., Yamamoto, K., Kasai, H., Nishimura, S., Tano, K., and Mitra, S. (1993). Repair of 8-hydroxyguanine in DNA by mammalian N-methylpurine-DNA glycosylase. Proc.Natl.Acad.Sci.U.S.A. *90*, 8901-8904.

Bjelland, S., Birkeland, N.K., Benneche, T., Volden, G., and Seeberg, E. (1994). DNA glycosylase activities for thymine residues oxidized in the methyl group are functions of the AlkA enzyme in *Escherichia coli*. J.Biol.Chem.*269*, 30489-30495.

Boorstein, R.J., Hilbert, T.P., Cadet, J., Cunningham, R.P., and Teebor, G.W. (1989). UV-Induced pyrimidine hydrates in DNA are repaired by bacterial and mammalian DNA glycosylase activities. Biochemistry *28*, 6164-6170.

Borer, P.N. (1975). CRC Handbook of Biochemistry and Molecular Biology (Cleveland: CRC Press).

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. *72*, 248-254.

Breen, A.P. and Murphy, J.A. (1995). Reactions of oxyl radicals with DNA. Free Radic.Biol.Med. *18*, 1033-1077.

Breimar, L.H. and Lindahl, T. (1985). Thymine lesions produced by ionizing radiation in double-stranded DNA. Biochemistry *24*, 4018-4022.

Breimer, L.H. and Lindahl, T. (1984). DNA glycosylase activities for thymine residues damaged by ring saturation, fragmentation, or ring contraction are functions of endonuclease III in *Escherichia coli*. J.Biol.Chem. *259*, 5543-5548.

Bressac, B., Kew, M., Wands, J., and Ozturk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature *350*, 429-431.

Busby, W.F., Jr. and Wogan, G.N. (1984). Aflatoxins. In Chemical Carcinogens. C.E. Searle, ed. (Washington, D. C.: Am. Chem. Soc.), pp. 945-1136.

Cabrera, M., Nghiem, Y., and Miller, J.H. (1988). mutM, a second mutator locus in *Escherichia coli* that generates GC→TA transversions. J.Bacteriol *170*, 5405-5407.

Cariello, N., Cui, L., and Skopek, T.R. (1994). *In vitro* mutational spectrum of aflatoxin B_1 in the human hypoxanthine guanine phosphoribosyltransferase gene. Cancer Res. *54*, 4436-4441.

Cathcart, R., Schwiers, E., Saul, R.L., and Ames, B.N. (1984). Thymine glycol and thymidine glycol in human and rat urine: a possible assay for oxidative DNA damage. Proc.Natl.Acad.Sci.U.S.A. *81* 5633-5637.

Chan, E. and Weiss, B. (1987). Endonuclease IV of *Escherichia coli* is induced by paraquat. Proc.Natl.Acad.Sci.U.S.A. *84*, 3189-3193.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992). 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. J.Biol.Chem. *267*, 166-172.

Chenna, A., Hang, B., Rydberg, B., Kim, E., Pongracz, K., Bodell, W.J., and Singer, B. (1995). The benzene metabolite *p*-benzoquinone forms adducts with DNA bases that are excised by a repair activity from human cells that differs from an enthenoadenine glycosylase. Proc.Natl.Acad.Sci.U.S.A. *92*, 5890-5894.

Chetsanga, C.J. and Frenette, G.P. (1983). Excision of aflatoxin B_1-imidazole ring opened guanine adducts from DNA by formamidopyrimidine-DNA glycosylase. Carcinogenesis *4*, 997-1000.

Chisari, F.V., Klopchin, K., Moriyama, T., Pasquinelli, C., Dunsford, H.A., Sell, S., Pinkert, C.A., Brinster, R.L., and Palmiter, R.D. (1989). Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell *59*, 1145-1156.

Christman, M.F., Morgan, R.W., Jacobson, F.S., and Ames, B.N. (1985). Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. Cell *41*, 753-762.

Clark, J.M. and Beardsley, G.P. (1989). Template length, sequence context, and 3'-5' exonuclease activity modulate replicative bypass of thymine glycol lesions in vitro. Biochemistry *28*, 775-779.

Cooper, P.K., Nouspikel, T., Clarkson, S.G., and Leadon, S.A. (1997). Defective transcription-coupled repair of oxidative base damage in Cockayne syndrome patients from XP group G. Science *275*, 990-993.

Croteau, D.L. and Bohr, V.A. (1997). Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J.Biol.Chem.*272*, 25409-25412.

Crothers, D.M. (1968). Calculation of binding isotherms for heterogeneous polymers. Biopolymers *6*, 575-584.

Croy, R.G., Essigmann, J.M., Reinhold, V.N., and Wogan, G.N. (1978). Identification of the principal aflatoxin B_1-DNA adduct formed in vivo in rat liver. Proc.Natl.Acad.Sci.U.S.A. *75*, 1745-1749.

Croy, R.G. and Wogan, G.N. (1981). Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of aflatoxin B_1. Cancer Res. *41*, 197-203.

Cunningham, R.P., Saporito, S.M., Spitzer, S.G., and Weiss, B. (1986). Endonuclease IV (nfo) mutant of *Escherichia coli.* J.Bacteriol *168*, 1120-1127.

Cunningham, R.P. and Weiss, B. (1985). Endonuclease III (*nth*) mutants of *Escherichia coli.* Biochemistry *82*, 474-478.

D'Andrea, A.D. and Haseltine, W.A. (1978). Modification of DNA by aflatoxin B_1 creates alkali-labile lesions in DNA at positions of guanine and adenine. Proc.Natl.Acad.Sci.U.S.A. *75*, 4120-4124.

de Jong, P.J., Grosovsky, A.J., and Glickman, B.W. (1992). Spectrum of spontaneous mutation at the APRT locus of Chinese hamster ovary cells: an analysis at the DNA sequence

level. Proc.Natl.Acad.Sci.U.S.A. *85*, 3499-3503.

Dedon, P.C. and Goldberg, I.H. (1992). Free-radical mechanisms involved in the formation of sequence-dependent bistranded DNA lesions by the antitumor antibiotics bleomycin, neocarzinostatin, and calicheamicin. Chem.Res.Toxicol. *5*, 311-332.

Demple, B., Halbrook, J., and Linn, S. (1983). *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide. J.Bacteriol *153*, 1079-1082.

Demple, B. and Harrison, L. (1994). Repair of oxidative damage to DNA: enzymology and biology. Annu.Rev.Biochem. *63*, 915-948.

Demple, B., Herman, T., and Chen, D.S. (1991). Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes. Proc.Natl.Acad.Sci.U.S.A. *88*, 11450-11454.

Demple, B., Johnson, A., and Fung, D. (1986). Exonuclease III and endonuclease IV remove 3' blocks from DNA synthesis primers in H_2O_2-damaged *Escherichia coli*. Proc.Natl.Acad.Sci.U.S.A. *83*, 7731-7735.

Denissenko, M.F., Chen, J.X., Tang, M., and Pfeifer, G.P. (1997). Cytosine methylation determines hot spots of DNA damage in the human p53 gene. Proc.Natl.Acad.Sci.U.S.A. *94*, 3893-3898.

Dianov, G. and Lindahl, T. (1991). Preferential recognition of I·T base-pairs in the initiation of excision-repair by hypoxanthine-DNA glycosylase. Nucleic Acids Res. *19*, 3829-3833.

Dianov, G. and Lindahl, T. (1994). Reconstitution of the DNA base excision-repair pathway. Curr.Biol. *4*, 1069-1076.

Dianov, G., Price, A., and Lindahl, T. (1992). Generation of single-nucleotide repair patches following excision of uracil residues from DNA. Mol.Cell Biol. *12*, 1605-1612.

Dianov, G., Sedgwick, B., Daly, G., Olsson, M., Lovett, S., and Lindahl, T. (1994). Release of 5'-terminal deoxyribose-phosphate residues from incised abasic sites in DNA by the *Escherichia coli* RecJ protein. Nucleic Acids Res. *22*, 993-998.

Ding, H., Hidalgo, E., and Demple, B. (1996). The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor. J.Biol.Chem.*271*, 33173-33175.

Dizdaroglu, M. (1994). Chemical determination of oxidative DNA damage by gas chromatography- mass spectrometry. Methods Enzymol. *234:3-16*, 3-16.

Dizdaroglu, M., Holwitt, E., Hagan, M.P., and Blakely, W.F. (1986). Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium

tetroxide. Biochem.J. *235*, 531-536.

Dizdaroglu, M., Karakaya, A., Jaruga, P., Slupphaug, G., and Krokan, H.E. (1996). Novel activities of human uracil DNA *N*-glycosylase for cytosine-derived products of oxidative DNA damage. Nucleic Acids Res. *24*, 418-422.

Dizdaroglu, M., Laval, J., and Boiteux, S. (1993). Substrate specificity of the *Escherichia coli* endonuclease III: excision of thymine- and cytosine-derived lesions in DNA produced by radiation-generated free radicals. Biochemistry *32*, 12105-12111.

Dodson, M.L., Schrock, R.D., and Lloyd, R.S. (1993). Evidence for an imino intermediate in the T4 endonuclease V reaction. Biochemistry *32*, 8284-8290.

Douki, T., Delatour, T., Paganon, F., and Cadet, J. (1996). Measurement of oxidative damage at pyrimidine bases in gamma-irradiated DNA. Chem.Res.Toxicol. *9*, 1145-1151.

Eide, L., Bjorås, M., Pirovano, M., Alseth, I., Berdal, K.G., and Seeberg, E. (1996). Base excision of oxidative purine and pyrimidine DNA damage in *Saccharomyces cerevisiae* by a DNA glycosylase with sequence similarity to endonuclease III from *Escherichia coli*. Proc.Natl.Acad.Sci.U.S.A. *93*, 10735-10740.

Essigmann, J.M., Croy, R.G., Nadzan, A.M., Busby, W.F., Jr., Reinhold, V.N., Buchi, G., and Wogan, G.N. (1977). Structural identification of the major DNA adduct formed by aflatoxin B_1 *in vitro*. Proc.Natl.Acad.Sci.U.S.A. *74*, 1870-1874.

Evans, J., Maccabee, M., Hatahet, Z., Courcelle, J., Bockrath, R., Ide, H., and Wallace, S. (1993). Thymine ring saturation and fragmentation products: lesion bypass, misinsertion and implications for mutagenesis. Mutat.Res. *299*, 147-156.

Farr, S.B. and Kogoma, T. (1991). Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol.Rev. *55*, 561-585.

Feig, D.I., Sowers, L.C., and Loeb, L.A. (1994). Reverse chemical mutagenesis: identification of the mutagenic lesions resulting from reactive oxygen species-mediated damage to DNA. Proc.Natl.Acad.Sci.U.S.A. *91*, 6609-6613.

Foster, P.L., Eisenstadt, E., and Miller, J.H. (1983). Base substitution mutations induced by metabolically activated aflatoxin B_1. Proc.Natl.Acad.Sci.U.S.A. *80*, 2695-2698.

Fraga, C.G., Shigenaga, M.K., Park, J.W., Degan, P., and Ames, B.N. (1990). Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc.Natl.Acad.Sci.U.S.A. *87*, 4533-4537.

Franklin, W.A. and Lindahl, T. (1988). DNA deoxyribophosphodiesterase. EMBO J. *7*, 3617-3622.

Friedberg, E.C., Walker, G.C., and Siede, W. (1995). DNA Repair and Mutagenesis (Washington, DC: ASM Press).

Frosina, G., Fortini, P., Rossi, O., Carrozzino, F., Raspaglio, G., Cox, L.S., Lane, D.P., Abbondandolo, A., and Dogliotti, E. (1996). Two pathways for base excision repair in mammalian cells. J.Biol.Chem. *271*, 9573-9578.

Fujimoto, J., Tran, L., and Sowers, L.C. (1997). Synthesis and cleavage of oligodeoxynucleotides containing a 5-hydroxyuracil residue at a defined site. Chem.Res.Toxicol. *10*, 1254-1258.

Ganem, D. and Varmus, H.E. (1987). The molecular biology of the hepatitis B viruses. Annu.Rev.Biochem. *56*, 651-693.

Ganguly, T., Weems, K.M., and Duker, N.J. (1990). Ultraviolet-induced thymine hydrates in DNA are excised by bacterial and human DNA glycosylase activities. Biochemistry *29*, 7222-7228.

Gopalakrishnan, S., Harris, T.M., and Stone, M.P. (1990). Intercalation of aflatoxin B_1 in two oligodeoxynucleotide adducts: comparative ^{1}H NMR analysis of d(ATC^{AFB}GAT)·d(ATCGAT) and d(AT^{AFB}GCAT)$_2$. Biochemistry *29*, 10438-10448.

Gopalakrishnan, S., Stone, M.P., and Harris, T.M. (1989). Preparation and spectroscopic characterization of aflatoxin B_1 adducts with oligodeoxynucleotide d(ATCGAT)$_2$. J.Am.Chem.Soc. *111*, 7232-7239.

Gottesman, S. (1984). Bacterial regulation: global regulatory networks. Ann.Rev.Genet. *18*, 415-441.

Graves, R.J., Felzenszwalb, I., Laval, J., and O'Connor, T.R. (1992). Excision of 5'-terminal deoxyribose phosphate from damaged DNA is catalyzed by the Fpg protein of *Escherichia coli*. J.Biol.Chem. *267*, 14429-14435.

Greenberg, J.T. and Demple, B. (1989). A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. J.Bacteriol *171*, 3933-3939.

Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. *54*, 4855-4878.

Groopman, J.D., Croy, R.G., and Wogan, G.N. (1981). *In vitro* reactions of aflatoxin B_1-adducted DNA. Proc.Natl.Acad.Sci.U.S.A. *78*, 5445-5449.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., and Rajewsky, K. (1994). Deletion of a DNA

polymerase beta gene segment in T cells using cell type-specific gene targeting. Science *265*, 103-106.

Halliday, J.A. and Glickman, B.W. (1992). Mechanisms of spontaneous mutation in DNA repair-proficient *Escherichia coli*. Mutat.Res. *250*, 55-71.

Halliwell, B. and Gutteridge, J.M.C. (1989). Free radicals in biology and medicine (Oxford: Clarendon Press).

Hatahet, Z., Kow, Y.W., Purmal, A.A., Cunningham, R.P., and Wallace, S.S. (1994). New substrates for old enzymes. 5-hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine are substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA *N*-glycosylase, while 5-hydroxy-2'-deoxyuridine is a substrate for uracil DNA *N*-glycosylase. J.Biol.Chem. *269*, 18814-18820.

Hatahet, Z., Purmal, A.A., and Wallace, S.S. (1993). A novel method for site specific introduction of single model oxidative DNA lesions into oligodeoxyribonucleotides. Nucleic Acids Res. *21*, 1563-1568.

Hayatsu, H. (1976). Bisulfite modification of nucleic acids and their consituents. Prog.Nucleic Acid Res.Mol.Biol. *16*, 75-124.

Hearst, J.E. (1989). Photochemistry of the psoralens. Chem.Res.Toxicol. *2*, 69-75.

Henle, E.S. and Linn, S. (1997). Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J.Biol.Chem. *272*, 19095-19098.

Henle, E.S., Luo, Y., Gassmann, W., and Linn, S. (1996). Oxidative damage to DNA constituents by iron-mediated fenton reactions. The deoxyguanosine family. J.Biol.Chem. *271*, 21177-21186.

Henner, W.D., Rodriguez, L.O., Hecht, S.M., and Haseltine, W.A. (1983). Gamma ray induced deoxyribonucleic acid strand breaks. J.Biol.Chem. *258*, 711-713.

Hertzog, P.J., Smith, J.R.L., and Garner, R.C. (1980). A high pressure liquid chromoatography study on the removal of DNA-bound aflatoxin B_1 in rat liver and in vitro. Carcinogenesis *1*, 787-793.

Hertzog, P.J., Smith, J.R.L., and Garner, R.C. (1982). Characterisation of the imidazole ring-opened forms of *trans*-8,9-dihydro-8-(7-guanyl)-9-hydroxy aflatoxin B_1. Carcinogenesis *3*, 723-725.

Hilbert, T.P., Chaung, W., Boorstein, R.J., Cunningham, R.P., and Teebor, G.W. (1997). Cloning and expression of the cDNA encoding the human homologue of the DNA repair enzyme, *Escherichia coli* endonuclease III. J.Biol.Chem. *272*, 6733-6740.

Hollstein, M.C., Brooks, P., Linn, S., and Ames, B.N. (1984). Hydroxymethyluracil DNA glycosylase in mammalian cells. Proc.Natl.Acad.Sci.U.S.A. *81*, 4003-4007.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., and Harris, C.C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature *350*, 427-428.

Iyer, R.S., Coles, B.F., Raney, K.D., Thier, R., Guengerich, F.P., and Harris, T.M. (1994a). DNA adduction by the potent carcinogen aflatoxin B_1: mechanistic studies. J.Am.Chem.Soc. *116*, 1603-1609.

Iyer, R.S., Voehler, M.W., and Harris, T.M. (1994b). Adenine adduct of aflatoxin B_1 epoxide. J.Am.Chem.Soc. *116*, 8863-8869.

Jiang, D., Hatahet, Z., Blaisdell, J.O., Melamede, R.J., and Wallace, S.S. (1997). *Escherichia coli* endonuclease VIII: cloning, sequencing, and overexpression of the nei structural gene and characterization of nei and nei nth mutants. J.Bacteriol. *179*, 3773-3782.

Johnson, W.W. and Guengerich, F.P. (1997). Reaction of aflatoxin B_1 *exo*-8,9-epoxide with DNA: kinetic analysis of covalent binding and DNA-induced hydrolysis. Proc.Natl.Acad.Sci.U.S.A. *94*, 6121-6125.

Johnson, W.W., Harris, T.M., and Guengerich, F.P. (1996). Kinetics and mechanism of hydrolysis of aflatoxin B_1 *exo*-8,9-epoxide and rearrangement of the dihydrodiol. J.Am.Chem.Soc. *118*, 8213-8220.

Kao, J.Y., Goljer, I., Phan, T.A., and Bolton, P.H. (1993). Characterization of the effects of a thymine glycol residue on the structure, dynamics, and stability of duplex DNA by NMR. J.Biol.Chem. *268*, 17787-17793.

Kim, H.S., Park, Y.W., Kasai, H., Nishimura, S., Park, C.W., Choi, K.H., and Chung, M.H. (1996). Induction of *E. coli* oh8Gua endonuclease by oxidative stress: its significance in aerobic life. Mutat.Res. *363*, 115-123.

Kirby, G.M., Chemin, I., Montesano, R., Chisari, F.V., Lang, M.A., and Wild, C.P. (1994). Induction of specific cytochrome P450s involved in aflatoxin B_1 metabolism in hepatitis B virus transgenic mice. Mol.Carcinog. *11*, 74-80.

Klungland, A. and Lindahl, T. (1997). Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). EMBO J. *16*, 3341-3348.

Kobertz, W.R. and Essigmann, J.M. (1996). Total synthesis of a *cis*-syn 2-carbomethoxypsoralen furan-side thymidine monoadduct. J.Am.Chem.Soc. *118*, 7101-7107.

Kobertz, W.R. and Essigmann, J.M. (1997a). Solid-phase synthesis of oligonucleotides containing a site-specific psoralen derivative. J.Am.Chem.Soc. *119*, 5960-5961.

Kobertz, W.R., Wang, D., Wogan, G.N., and Essigmann, J.M. (1997b). An intercalation inhibitor altering the target selectivity of DNA damaging agents: synthesis of site-specific aflatoxin B_1 adducts in a p53 mutational hotspot. Proc.Natl.Acad.Sci.U.S.A. *94*, 9579-9584.

Kornberg, A. and Baker, T.A. (1992). DNA replication (New York: W.H. Freeman).

Kow, Y.W. and Wallace, S.S. (1987). Mechanism of action of *Escherichia coli* endonuclease III. Biochemistry *26*, 8200-8206.

Kow, Y.W., Wallace, S.S., and Van Houten, B. (1990). UvrABC nuclease complex repairs thymine glycol, an oxidative DNA base damage. Mutation Res. *235*, 147-156.

Kubota, Y., Nash, R.A., Klungland, A., Schar, P., Barnes, D.E., and Lindahl, T. (1996). Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. EMBO J. *15*, 6662-6670.

Labahn, J., Scharer, O.D., Long, A., Ezaz-Nikpay, K., Verdine, G.L., and Ellenberger, T.E. (1996). Structural basis for the excision repair of alkylation-damaged DNA. Cell *86*, 321-329.

Leadon, S.A., Tyrrell, R.M., and Cerutti, P.A. (1981). Excision repair of aflatoxin B_1-DNA adducts in human fibroblasts. Cancer Res. *41*, 5125-5129.

Leonard, G.A., Guy, A., Brown, T., Téoule, R., and Hunter, W.N. (1992). Conformation of guanine·8-oxoadenine base pairs in the crystal structure of d(CGCGAATT(O8A)GCG). Biochemistry *31*, 8415-8420.

Lerman, L.S. (1961). Structural considerations in the interaction of DNA and acridines. J.Mol.Biol. *3*, 18-30.

Levy, D.D., Groopman, J.D., Lim, S.E., Seidman, M.M., and Kraemer, K.H. (1992). Sequence specificity of aflatoxin B_1-induced mutations in a plasmid replicated in xeroderma pigmentosum and DNA repair proficient human cells. Cancer Res. *52*, 5668-5673.

Lin, J.-K., Miller, J.A., and Miller, E.C. (1977). 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B_1, a major acid hydrolysis product of aflatoxin B_1 -DNA or -ribosomal RNA adducts formed in hepatic microsome-mediated reactions in rat liver *in vivo*. Cancer Res. *37*, 4430-4438.

Lin, J.J. and Sancar, A. (1989). A new mechanism for repairing oxidative damage to DNA: (A)BC excinuclease removes AP sites and thymine glycols from DNA. Biochemistry.

28, 7979-7984.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709-715.

Ljungquist, S., Lindahl, T., and Howard-Flanders, P. (1976). Methyl methane sulfonate-sensitive mutant of *Escherichia coli* deficient in an endonuclease specific for apurinic sites in deoxyribonucleic acid. J.Bacteriol *126*, 646-653.

Lu, R., Nash, H.M., and Verdine, G.L. (1997). A mammalian DNA repair enzyme that excises oxidatively damaged guanines maps to a locus frequently lost in lung cancer. Curr.Biol. *7*, 397-407.

Luo, Y., Henle, E.S., and Linn, S. (1996). Oxidative damage to DNA constituents by iron-mediated Fenton reactions. J.Biol.Chem. *271*, 21167-21176.

Mace, K., Aguilar, F., Wang, J.-S., Vautravers, P., Gomez-Lechon, M., Gonzalez, F.J., Groopman, J.D., Harris, C.C., and Pfeifer, A.M.A. (1997). Aflatoxin B_1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. Carcinogenesis *18*, 1291-1297.

Maki, H. and Sekiguchi, M. (1992). MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature *355*, 273-275.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989). Molecular Cloning, A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Marnett, L.J. and Burcham, P.C. (1993). Endogenous DNA adducts: potential and paradox. Chem.Res.Toxicol. *6*, 771-785.

Mascarenas, J.L., Hayashibara, K.C., and Verdine, G.L. (1993). Template-directed interference footprinting of protein-thymine contacts. J.Am.Chem.Soc. *115*, 373-374.

Matray, T.J. and Greenberg, M.M. (1994). Site-specific incorporation of the alkaline labile, oxidative stress product (5R)-5,6-dihydro-5-hyydroxythymidine in an oligonucleotide. J.Am.Chem.Soc. *116*, 6931-6932.

Matsumoto, Y. and Kim, K. (1995). Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair. Science *269*, 699-702.

McBride, T.J., Preston, B.D., and Loeb, L.A. (1991). Mutagenic spectrum resulting from DNA damage by oxygen radicals. Biochemistry *30*, 207-213.

McGlynn, K.A., Rosvold, E.A., Lustbader, E.D., Hu, Y., Clapper, M.L., Zhou, T., Wild, C.P., Xia, X.-L., Baffoe-Bonnie, A., Ofori-Adjei, D., Chen, G.-C., London, W.T., Shen, F.-M., and Buetow, K.H. (1995). Susceptibility to hepatocellular carcinoma is associated with

genetic variation in the enzymatic detoxification of aflatoxin B_1. Proc.Natl.Acad.Sci.U.S.A. *92*, 2384-2387.

McGoldrick, J.P., Yeh, Y.C., Solomon, M., Essigmann, J.M., and Lu, A.L. (1995). Characterization of a mammalian homolog of the *Escherichia coli* MutY mismatch repair protein. Mol.Cell Biol. *15*, 989-996.

Melamede, R.J., Hatahet, Z., Kow, Y.W., Ide, H., and Wallace, S.S. (1994). Isolation and characterization of endonuclease VIII from *Escherichia coli*. Biochemistry *33*, 1255-1264.

Mellon, I., Spivak, G., and Hanawalt, P.C. (1987). Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell *51*, 241-249.

Michaels, M.L., Cruz, C., Grollman, A.P., and Miller, J.H. (1992a). Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc.Natl.Acad.Sci.U.S.A. *89*, 7022-7025.

Michaels, M.L. and Miller, J.H. (1992b). The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J.Bacteriol. *174*, 6321-6325.

Misra, R.P., Muench, K.F., and Humayun, M.Z. (1983). Covalent and noncovalent interactions of aflatoxin with defined deoxyribonucleic acid sequences. Biochemistry *22*, 3351-3359.

Mo, J.Y., Maki, H., and Sekiguchi, M. (1992). Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. Proc.Natl.Acad.Sci.U.S.A. *89*, 11021-11025.

Mol, C.D., Arvai, A.S., Slupphaug, G., Kavli, B., Alseth, I., Krokan, H.E., and Tainer, J.A. (1995). Crystal structure and mutational analysis of human uracil-DNA glycosylase: structural basis for specificity and catalysis. Cell *80*, 869-878.

Moraes, E.C., Keyse, S.M., and Tyrrell, R.M. (1990). Mutagenesis by hydrogen peroxide treatment of mammalian cells: a molecular analysis. Carcinogenesis *11*, 283-293.

Moriya, M. (1993a). Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted GC→TA transversions in simian kidney cells. Proc.Natl.Acad.Sci.U.S.A. *90*, 1122-1126.

Moriya, M. and Grollman, A.P. (1993b). Mutations in the mutY gene of *Escherichia coli* enhance the frequency of targeted GC→TA transversions induced by a single 8-oxoguanine residue in single-stranded DNA. Mol.Gen.Genet. *239*, 72-76.

Morningstar, M.L., Kreutzer, D.A., and Essigmann, J.M. (1997). Synthesis of oligonucleotides containing two putatively mutagenic DNA lesions: 5-hydroxy-2'-deoxyuridine and 5-hydroxy-2'-deoxycytidine. Chem.Res.Toxicol. *10*, 1345-1350.

Muench, K.F., Misra, R.P., and Humayun, M.Z. (1983). Sequence specificity in aflatoxin B_1-DNA interactions. Proc.Natl.Acad.Sci.U.S.A. *80*, 6-10.

Mullaart, E., Lohman, P.H.M., Berends, F., and Vijg, J. (1990). DNA damage metabolism and aging. Mutat.Res. *237*, 189-210.

Nash, H.M., Bruner, S.D., Schaeer, O.D., Kawate, T., Addona, T.A., Spooner, E., Lane, W.S., and Verdine, G.L. (1996). Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. Curr.Biol. *6*, 968-980.

Nelson, H.C.M. and Bestor, T.H. (1996). Base eversion and shuffling by DNA methytransferases. Chemistry and Biology *3*, 419-423.

Nelson, J.W. and Tinoco, I.J. (1984). Intercalation of ethidium ion into DNA and RNA oligonucleotides. Biopolymers *23*, 213-233.

Nghiem, Y., Cabrera, M., Cupples, C.G., and Miller, J.H. (1988). The mutY gene: a mutator locus in *Escherichia coli* that generates GC→TA transversions. Proc.Natl.Acad.Sci.U.S.A. *85*, 2709-2713.

Nicholl, I.D., Nealon, K., and Kenny, M.K. (1997). Reconstitution of human base excision repair with purified proteins. Biochemistry *36*, 7557-7566.

Oleykowski, C.A., Mayernik, J.A., Lim, S.E., Groopman, J.D., Grossman, L., Wogan, G.N., and Yeung, A.T. (1993). Repair of aflatoxin B_1 DNA adducts by the UvrABC endonuclease of *Escherichia coli*. J.Biol.Chem. *268*, 7990-8002.

Orr, W.C. and Sohal, R.S. (1994). Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. Science *263*, 1128-1130.

Patel, D.J., Kozlowski, S.A., Marky, L.A., Rice, J.A., Broka, C., Dallas, J., Itakura, K., and Breslauer, K.J. (1982). Structure, dynamics, and energetics of deoxyguanosine-thymidine wobble base pair formation in the self-complementary d(CGTGAATTCGCG) duplex in solution. Biochemistry *21*, 437-444.

Paterson, M.C., Boyle, J.M., and Setlow, R.B. (1971). Ultraviolet- and X-ray induced responses of a deoxyribonucleic acid polymerase-deficient mutant of *Escherichia coli*. J.Bacteriol *107*, 61-67.

Pettijohn, D.E. and Hanawalt, P.C. (1964). Isolation of partially density-labeled DNA molecules from bacteria. J.Mol.Biol. *8*, 170-174.

Prasad, R., Singhal, R.K., Srivastava, D.K., Molina, J.T., Tomkinson, A.E., and Wilson, S.H. (1996). Specific interaction of DNA polymerase beta and DNA ligase I in a multiprotein base excision repair complex from bovine testis. J.Biol.Chem. *271*, 16000-16007.

Puglisi, J.D. and Tinoco, I., Jr. (1989). Absorbance melting curves of RNA. Methods Enzymol. *180*, 304-325.

Purmal, A.A., Kow, Y.W., and Wallace, S.S. (1994a). 5-Hydroxypyrimidine deoxynucleoside triphosphates are more efficiently incoporated into DNA by exonuclease-free Klenow fragment than 8-oxopurine deoxynucleoside triphosphates. Nucleic Acids Res. *22*, 3930-3935.

Purmal, A.A., Kow, Y.W., and Wallace, S.S. (1994b). Major oxidative products of cytosine, 5-hydroxycytosine and 5-hydroxyuracil, exhibit sequence context-dependent mispairing *in vitro*. Nucleic Acids Res. *22*, 72-78.

Qian, G.S., Ross, R.K., Yu, M.C., Yuan, J.M., Gao, Y.T., Henderson, B.E., Wogan, G.N., and Groopman, J.D. (1994). A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiology, Biomarkers and Prevention *3*, 3-10.

Radicella, J.P., Dherin, C., Desmaze, C., Fox, M.S., and Boiteux, S. (1997). Cloning and characterization of hOGG1, a human homolog of the OGG1 gene of Saccharomyces cerevisiae. Proc.Natl.Acad.Sci U.S.A. *94*, 8010-8015.

Raney, K.D., Coles, B., Guengerich, F.P., and Harris, T.M. (1992). The *endo*-8,9-epoxide of aflatoxin B_1: a new metabolite. Chem.Res.Toxicol. *5*, 333-335.

Raney, K.D., Gopalakrishnan, S., Byrd, S., Stone, M.P., and Harris, T.M. (1990). Alteration of the aflatoxin cyclopentenone ring to a δ-lactone reduces intercalation with DNA and decreases formation of guanine N7 adducts by aflatoxin epoxides. Chem.Res.Toxicol. *3*, 254-261.

Raney, V.M., Harris, T.M., and Stone, M.P. (1993). DNA conformation mediates aflatoxin-B_1-DNA binding and the formation of guanine N7 adducts by aflatoxin B_1 8,9-*exo*-epoxide. Chem.Res.Toxicol. *6*, 64-68.

Reardon, J.T., Bessho, T., Kung, H.C., Bolton, P.H., and Sancar, A. (1997). In vitro repair of oxidative DNA damage by human nucleotide excision repair system: possible explanation for neurodegeneration in xeroderma pigmentosum patients. Proc.Natl.Acad.Sci.U.S.A. *94*, 9463-9468.

Richter, C., Park, J., and Ames, B.N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc.Natl.Acad.Sci.U.S.A. *85*, 6465-6467.

Roberts, R.J. (1995). On base flipping. Cell *82*, 9-12.

Robledo-Luiggi, C., Wilson, W.D., Pares, E., Vera, M., Martinez, C.S., and Santiago, D. (1991). Partial intercalation with DNA of peptides containing two aromatic amino acids. Biopolymers *31*, 907-917.

Robson, C.N. and Hickson, I.D. (1991). Isolation of cDNA clones encoding a human apurinic/apyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in *E. coli* xth (exonuclease III) mutants. Nucleic Acids Res. *19*, 5519-5523.

Roldan-Arjona, T., Wei, Y.F., Carter, K.C., Klungland, A., Anselmino, C., Wang, R.P., Augustus, M., and Lindahl, T. (1997). Molecular cloning and functional expression of a human cDNA encoding the antimutator enzyme 8-hydroxyguanine-DNA glycosylase. Proc.Natl.Acad.Sci.U.S.A. *94*, 8016-8020.

Roldán-Arjona, T., Anselmino, C., and Lindahl, T. (1996). Molecular cloning and functional analysis of a *Schizosaccharomyces pombe* homologue of *Escherichia coli* endonuclease III. Nucleic Acids Res. *24*, 3307-3312.

Rosenquist, T.A., Zharkov, D.O., and Grollman, A.P. (1997). Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. Proc.Natl.Acad.Sci.U.S.A. *94*, 7429-7434.

Rouet, P. and Essigmann, J.M. (1985). Possible role for thymine glycol in the selective inhibition of DNA synthesis on oxidized DNA templates. Cancer Res. *45*, 6113-6118.

Roy-Burman, S., Roy-Burman, P., and Visser, D.W. (1970). Studies on the effect of triphosphates of 5-aminouridine and 5- hydroxydeoxyuridine on ribonucleic acid and deoxyribonucleic acid polymerases. Biochem.Pharmacol. *19*, 2745-2756.

Rupert, C.S., Goodgal, S.H., and Herriott, R.M. (1958). Photoreactivation in vitro of ultraviolet inactivated *Hemophilus infuenzae* transforming factor. J.Gen.Physiol. *41*, 451-471.

Sagher, D. and Strauss, B. (1983). Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during in vitro synthesis: uniqueness of adenine nucleotides. Biochemistry *22*, 4518-4526.

Saito, Y., Uraki, F., Nakajima, S., Asaeda, A., Ono, K., Kubo, K., and Yamamoto, K. (1997). Characterization of endonuclease III (nth) and endonuclease VIII (nei) mutants of *Escherichia coli* K-12. J.Bacteriol. *179*, 3783-3785.

Sancar, A. (1996). DNA excision repair. Annu.Rev.Biochem. *65*, 43-81.

Sandigursky, M., Yacoub, A., Kelley, M.R., Deutsch, W.A., and Franklin, W.A. (1997a). The *Drosophila* ribosomal protein S3 contains a DNA deoxyribophosphodiesterase (dRpase) activity. J.Biol.Chem. *272*, 17480-17484.

Sandigursky, M., Yacoub, A., Kelley, M.R., Xu, Y., Franklin, W.A., and Deutsch, W.A. (1997b). The yeast 8-oxoguanine DNA glycosylase (Ogg1) contains a DNA deoxyribophosphodiesterase (dRpase) activity. Nucleic Acids Res. *25*, 4557-4561.

Sarasin, A., Goze, A., Devoret, R., and Moule, Y. (1977). Induced reactivation of UV-damaged phage lambda in *E. coli* K12 host cells treated with aflatoxin B_1 metabolites. Mutat.Res. *42*, 205-214.

Schröder, M. (1980). Osmium tetraoxide cis hydroxylation of unsaturated substrates. Chem.Rev. *80*, 187-213.

Selby, C.P. and Sancar, A. (1994). Mechanisms of transcription-repair coupling and mjutation frequency decline. Microbiol.Rev. *58*, 317-329.

Sell, S., Hunt, J.M., Dunsford, H.A., and Chisari, F.V. (1991). Synergy between hepatitis B virus expression and chemical hepatocarcinogens in transgenic mice. Cancer Res. *51*, 1278-1285.

Setlow, R.B. and Carrier, W.L. (1964). The disappearance of thymine dimers from DNA: an error-correcting mechanism. Proc.Natl.Acad.Sci U.S.A. *51*, 226-231.

Shaaper, R.M. and Dunn, R.L. (1991). Spontaneous mutation in the *Escherichia coli lacI* gene. Genetics *129*, 317-326.

Shen, H.-M. and Ong, C.-N. (1996). Mutations of the p53 tumor suppressor gene and *ras* oncogenes in aflatoxin hepatocarcinogenesis. Mutat.Res. *366*, 23-44.

Shibutani, S., Takeshita, M., and Grollman, A.P. (1991). Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature *349*, 431-434.

Shigenaga, M.K., Aboujaoude, E.N., Chen, Q., and Ames, B.N. (1994). Assays of oxidative DNA damage biomarkers 8-oxo-2'-deoxyguanosine and 8-oxoguanine in nuclear DNA and biological fluids by high-performance liquid chromatography with electrochemical detection. Methods Enzymol. *234:16-33*, 16-33.

Shimada, T. and Guengerich, F.P. (1989). Evidence for cytochrome $P\text{-}450_{NF}$, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. Proc.Natl.Acad.Sci.U.S.A. *86*, 462-465.

Shirakata, Y., Kawada, M., Fujiki, Y., Sano, H., Oda, M., Yaginuma, K., Kobayashi, M., and Koike, K. (1989). The X gene of hepatitis B virus induced growth stimulation and tumorigenic transformation of mouse NIH3T3 cells. Jpn.J.Cancer Res. *80*, 617-621.

Singer, B. and Essigmann, J.M. (1991). Site-specific mutagenesis: retrospective and prospective. Carcinogenesis *12*, 949-955.

Singer, B. and Hang, B. (1997). What structural features determine repair enzyme specificity and mechanism in chemically modified DNA? Chem.Res.Toxicol. *10*, 713-732.

Slupphaug, G., Eftedal, I., Kavli, B., Bharati, S., Helle, N.M., Haug, T., Levine, D.W., and Krokan, H.E. (1995). Properties of a recombinant human uracil-DNA glycosylase from the *UNG* gene and evidence that *UNG* encodes the major uracil-DNA glycosylase. Biochemistry *34*, 128-138.

Slupphaug, G., Mol, C.D., Kavli, B., Arvai, A.S., Krokan, H.E., and Tainer, J.A. (1996). A nucleotide-flipping mechanism from the structure of human uracil-DNA glycosylase bound to DNA. Nature *384*, 87-92.

Slupska, M.M., Baikalov, C., Luther, W.M., Chiang, J.H., Wei, Y.F., and Miller, J.H. (1996). Cloning and sequencing a human homolog (hMYH) of the *Escherichia coli* mutY gene whose function is required for the repair of oxidative DNA damage. J.Bacteriol. *178*, 3885-3892.

Sobol, R.W., Horton, J.K., Kuhn, R., Gu, H., Singhal, R.K., Prasad, R., Rajewsky, K., and Wilson, S.H. (1996). Requirement of mammalian DNA polymerase-beta in base-excision repair. Nature *379*, 183-186.

Sohal, R.S. and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. Science *273*, 59-63.

Spielmann, H.P., Dwyer, T.J., Sastry, S.S., Hearst, J.E., and Wemmer, D.E. (1995). DNA structural reorganization upon conversion of a psoralen furan-side monoadduct to an interstrand cross-link: implications for DNA repair. Proc.Natl.Acad.Sci.U.S.A. *92*, 2345-2349.

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

Storz, G., Tartaglia, L.A., Farr, S.B., and Ames, B.N. (1992). Bacterial defenses against oxidative stress. Trends.Genet.1990.Nov. *6*, 363-368.

Sun, B., Latham, K.A., Dodson, M.L., and Lloyd, R.S. (1995). Studies on the catalytic mechanism of five DNA glycosylases. Probing for enzyme-DNA imino intermediates. J.Biol.Chem. *270*, 19501-19508.

Swenson, D.H., Miller, J.A., and Miller, E.C. (1975). The reactivity and carcinogenicity of aflatoxin B_1-2,3-dichloride, a model for the putative 2,3-oxide metabolite of aflatoxin B_1. Cancer Res. *35*, 3811-3823.

Taddei, F., Hayakawa, H., Bouton, M., Cirinesi, A., Matic, I., Sekiguchi, M., and Radman, M. (1997). Counteraction by MutT protein of transcriptional errors caused by oxidative

damage. Science *278*, 128-130.

Tchou, J., Bodepudi, V., Shibutani, S., Antoshechkin, I., Miller, J., Grollman, A.P., and Johnson, F. (1994). Substrate specificity of fpg protein. J.Biol.Chem. *269*, 15318-15324.

Tchou, J. and Grollman, A.P. (1995). The catalytic mechanism of Fpg protein. Evidence for a Schiff base intermediate and amino terminus localization of the catalytic site. J.Biol.Chem. *270*, 11671-11677.

Teebor, G., Cummings, A., Frenkel, K., Shaw, A., Voituriez, L., and Cadet, J. (1987). Quantitative measurement of the diastereoisomers of cis thymidine glycol in gamma-irradiated DNA. Free Radic.Res.Commun. *2*, 303-309.

Teebor, G. W., Boorstein, R. J., and Cadet, J. (1988). The repairability of oxidative free radical mediated damage to DNA: a review. Int.J.Radiat.Biol. *54*, 131-150.

Teebor, G.W., Frenkel, K., and Goldstein, M.S. (1984). Ionizing radiation and tritium transmutation both cause formation of 5-hydroxymethyl-2'-deoxyuridine in cellular DNA. Proc.Natl.Acad.Sci.U.S.A. *81*, 318-321.

Teoule, R., Bert, C., and Bonicel, A. (1977). Thymine fragment damage retained in the DNA polynucleotide chain after gamma irradiation in aerated solutions. II. Radiat.Res. 72, 190-200.

Thayer, M.M., Ahern, H., Xing, D., Cunningham, R.P., and Tainer, J.A. (1995). Novel DNA binding motifs in the DNA repair enzyme endonuclease III crystal structure. EMBO J. *14*, 4108-4120.

Tindall, K.R., Stein, J., and Hutchinson, F. (1988). Changes in DNA base sequence induced by gamma-ray mutagenesis of lambda phage and prophage. Genetics *118*, 551-560.

Tkeshelashvili, L.K., McBride, T., Spence, K., and Loeb, L.A. (1991). Mutation spectrum of copper-induced DNA damage. J.Biol.Chem. *266*, 6401-6406.

Trottier, Y., Waithe, W.I., and Anderson, A. (1992). Kinds of mutations induced by aflatoxin B_1 in a shuttle vector replicating in human cells transiently expressing cytochrome P450IA2 cDNA. Mol.Carcinog. *6*, 140-147.

Twu, J.S. and Schloemer, R.H. (1987). Transcriptional trans-activating function of hepatitis B virus. J.Virol. *61*, 3448-3453.

Ueda, H., Ullrich, S.J., Gangemi, J.D., Kappel, C.A., Ngo, L., Feitelson, M.A., and Jay, G. (1995). Functional inactivation but not structural mutation of p53 causes liver cancer. Nat.Genet. *9*, 41-47.

van der Kemp, P.A., Thomas, D., Barbey, R., de Oliveira, R., and Boiteux, S. (1996). Cloning and expression in *Escherichia coli* of the OGG1 gene of Saccharomyces cerevisiae, which codes for a DNA glycosylase that excises 7,8-dihydro-8-oxoguanine and 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine. Proc.Natl.Acad.Sci.U.S.A. *93*, 5197-5202.

Vassylyev, D.G., Kashiwagi, T., Mikami, Y., Ariyoshi, M., Iwai, S., Ohtsuka, E., and Morikawa, K. (1995). Atomic model of a pyrimidine dimer excision repair enzyme complexed with a DNA substrate: structural basis for damaged DNA recognition. Cell *83*, 773-782.

Voigt, J.M., Van, H.B., Sancar, A., and Topal, M.D. (1989). Repair of O6-methylguanine by ABC excinuclease of *Escherichia coli* in vitro. J.Biol.Chem. *264*, 5172-5176.

von Sonntag, C. (1987). The Chemical Basis of Radiation Biology (London: Taylor & Francis).

Wagner, J.R., Blount, B.C., and Weinfeld, M. (1996). Excision of oxidative cytosine modifications from gamma-irradiated DNA by *Escherichia coli* endonuclease III and human whole-cell extracts. Anal.Biochem. *233*, 76-86.

Wagner, J.R., Hu, C.C., and Ames, B.N. (1992). Endogenous oxidative damage of deoxycytidine in DNA. Proc.Natl.Acad.Sci.U.S.A. *89*, 3380-3384.

Wakelin, L.P.G., Romanos, M., Chen, T.K., Glaubiger, D., Canellakis, E.S., and Waring, M.J. (1978). Structural limitations on the bifunctional intercalation of diacridines into DNA. Biochemistry *17*, 5057-5063.

Walkup, L.K.B. and Kogoma, T. (1989). *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. J.Bacteriol *171*, 1476-1484.

Wallace, S.S. (1997). Oxidative damage to DNA and its repair. In Oxidative Stress and the Molecular Biology of Antioxidant Defenses. J.G. Scandalios, ed. (Cold Spring Harbor Laboratory Press), pp. 49-90.

Wang, D. and Essigmann, J.M. (1997). Kinetics of oxidized cytosine repair by endonuclease III of *Escherichia coli*. Biochemistry *36*, 8628-8633.

Wang, X.W., Forrester, K., Yeh, H., Feitelson, M.A., Gu, J.-R., and Harris, C.C. (1994). Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc.Natl.Acad.Sci.U.S.A. *91*, 2230-2234.

Wang, X.W., Gibson, M.K., Vermeulen, W., Yeh, H., Forrester, K., Sturzbecher, H.W., Hoeijmakers, J.H., and Harris, C.C. (1995). Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. Cancer Res. *55*, 6012-6016.

Wang, Y.J., Ho, Y.S., Lo, M.J., and Lin, J.K. (1995). Oxidative modification of DNA bases in rat liver and lung during chemical carcinogenesis and aging. Chem.Biol.Interact. *94*, 135-145.

Waters, L.C., Sikpi, M.O., Preston, R.J., Mitra, S., and Jaberaboansari, A. (1991). Mutations induced by ionizing radiation in a plasmid replicated in human cells. Radiat.Res. *127*, 190-201.

Wiebauer, K. and Jiricny, J. (1990). Mismatch-specific thymine DNA glycosylase and DNA polymerase beta mediate the correction of G.T mispairs in nuclear extracts from human cells. Proc.Natl.Acad.Sci.U.S.A. *87*, 5842-5845.

Wilson, W.D. (1990). Reversible interactions of nucleic acids with small molecules. In Nucleic Acids in Chemistry and Biology. G.M. Blackburn and M.J. Gait, eds. (New York: Oxford University Press), pp. 295-336.

Wood, M.L., Dizdaroglu, M., Gajewski, E., and Essigmann, J.M. (1990). Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. Biochemistry *29*, 7024-7032.

Wood, M.L., Esteve, A., Morningstar, M.L., Kuziemko, G.M., and Essigmann, J.M. (1992). Genetic effects of oxidative DNA damage: comparative mutagenesis of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine in *Escherichia coli*. Nucleic Acids Res. *20*, 6023-6032.

Wood, R.D. (1996). DNA repair in eukaryotes. Annu.Rev.Biochem. *65*, 135-167.

Wood, R.D. (1997a). Nucleotide excision repair in mammalian cells. J.Biol.Chem. *272*, 23465-23468.

Wood, R.D. and Shivji, M.K. (1997b). Which DNA polymerases are used for DNA-repair in eukaryotes? Carcinogenesis *18*, 605-610.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.C., and Curran, T. (1992). Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. EMBO J. *11*, 3323-3335.

Xanthoudakis, S., Smeyne, R.J., Wallace, J.D., and Curran, T. (1996). The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc.Natl.Acad.Sci.U.S.A. *93*, 8919-8923.

Yajko, D.M. and Weiss, B. (1975). Mutations simultaneously affecting endonuclease II and exonuclease III in *Escherichia coli* . Proc.Natl.Acad.Sci.U.S.A. *72*, 688-692.

Yamagata, Y., Kato, M., Odawara, K., Tokuno, Y., Nakashima, Y., Matsushima, N., Yasumura, K., Tomita, K., Ihara, K., Fujii, Y., Nakabeppu, Y., Sekiguchi, M., and Fujii, S. (1996). Three-dimensional structure of a DNA repair enzyme, 3-methyladenine DNA glycosylase II, from *Escherichia coli.* Cell *86*, 311-319.

Yanofsky, C., Cox, E.C., and Horn, V. (1966). The unusual mutagenic specificity of an *E. coli* mutator gene. Proc.Natl.Acad.Sci.U.S.A. *55*, 274-281.

Yarema, K.J. and Essigmann, J.M. (1995). Evaluation of the genetic effects of defined DNA lesions formed by DNA-damaging agents. Methods *7*, 133-146.

Zastawny, T.H., Doetsch, P.W., and Dizdaroglu, M. (1995). A novel activity of E. coli uracil DNA N-glycosylase excision of isodialuric acid (5,6-dihydroxyuracil), a major product of oxidative DNA damage, from DNA. FEBS Lett. *364*, 255-258.

Zhang, Q.M., Yonei, S., and Kato, M. (1992). Multiple pathways for repair of oxidative DNA damages caused by X rays and hydrogen peroxide in *Escherichia coli.* Radiat.Res. *132*, 334-338.

Zharkov, D.O., Rieger, R.A., Iden, C.R., and Grollman, A.P. (1997). NH_2-terminal proline acts as a nucleophile in the glycosylase/AP-lyase reaction catalyzed by *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg) protein. J.Biol.Chem. *272*, 5335-5341.

BIOGRAPHY

David Wang was born on July 26, 1970 in Panorama City, California. He was raised in Northridge, California and attended Chatsworth High School, where he graduated as valedictorian in 1988. Thereafter, he enrolled at Stanford University and majored in Chemistry. While at Stanford, he spent time studying overseas in West Berlin, Germany shortly after the fall of the Berlin Wall. In 1991 he was elected to Phi Beta Kappa, and in 1992 he earned a B.S. in Chemistry with distinction and departmental honors. As an undergraduate, David worked in the laboratory of Professor Robert Waymouth, studying organometallic catalysts. He subsequently began his doctoral studies as a National Science Foundation predoctoral fellow in the Chemistry department at the Massachusetts Institute of Technology. During his five and a half years at MIT, he worked with Professor John M. Essigmann examining the formation and repair of DNA damage. After receiving his Ph.D., he will continue his career in science as a postdoctoral associate in the laboratory of Professor Don Ganem at the University of California, San Francisco.