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Oxidative DNA Damage: Mutagenic Properties of 5-Hydroxycytosine, 5-Hydroxyuracil, and Uracil Glycol in *Eschericia coli*

by

Deborah Anne Kreutzer

B. A. Chemistry
Wheaton College, 1992

SUBMITTED TO THE DIVISION OF TOXICOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN TOXICOLOGY

AT THE

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 1998

Signature of Author:_____
Division of Toxicology
May 11, 1998

Certified by:_____
John M. Essigmann
Professor of Chemistry and Toxicology
Thesis Supervisor

Accepted by:_____
Peter C. Dedon
Chairperson, Committee on Graduate Students

Thesis Committee

Professor Gerald N. Wogan_____
Chairperson

Professor John M. Essigmann___
Thesis Supervisor

Professor Peter C. Dedon__

Professor Steven R. Tannenbaum_____

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ABSTRACT

Oxidative DNA damage has been implicated in mutagenesis, carcinogenesis and aging. Endogenous cellular processes such as aerobic metabolism generate reactive oxygen species (ROS) that interact with DNA to form dozens of different types of DNA damage products. If unrepaired, these lesions can exert a number of deleterious effects including the induction of mutations. In an effort to understand the genetic consequences of cellular oxidative damage, many laboratories have determined the patterns of mutations generated by the interaction of ROS with DNA. Compilation of these mutational spectra has revealed that GC→AT is the most commonly observed mutation resulting from oxidative damage to DNA. Until now, however, the chemical nature of the altered DNA base giving rise to this mutation has remained elusive. Since a single oxidizing agent causes multiple lesions, and since mutational spectra convey only the end result of a complex cascade of events (which includes formation of lesions, repair processing, and polymerase errors) it is impossible to assess the mutational specificity of individual DNA adducts directly from mutational spectra. The task of assigning specific features of mutational spectra to individual DNA lesions has been made possible with the advent of a technology to analyze the mutational properties of single defined adducts *in vitro* and *in vivo*. Using this technology, the oxidative adducts 5-hydroxy-2'-deoxycytidine (5-OH-dC), 5-hydroxy-2'-deoxyuridine (5-OH-dU) and 5,6-dihydro-5,6-dihydroxy-2'-deoxyuridine (dUg) are herein examined. 5-OH-dU and dUg are found to be highly mutagenic in *Eschericia coli* (*E. coli*), producing exclusively C→T transition mutations, whereas 5-OH-dC is found to be weakly mutagenic, inducing C→T as well as C→G mutations. The implications of these finding are discussed in terms of the literature and in terms of a future program of *in vivo* repair studies.

Thesis Supervisor: John M. Essigmann
Title: Professor of Chemistry and Toxicology

Acknowledgments

I must first thank Ed Wintner because, as he said to me in his thesis, "This thesis simply would not have been completed without your help." From playing volleyball, to collaborating in the lab, to editing each other's theses, your friendship has been a solid support through some very trying, as well as very exciting, times. Your ability to gently challenge me both inside and outside the lab has helped me to become a fuller person, both scientifically and emotionally. I must also thank you and your family for introducing me to the wonders of Tamworth and for sharing this magical place with me; weekend trips to New Hampshire were a necessary escape from the hectic pace at MIT.

Scientific research is not done single-handedly, and therefore I am grateful to the many colleagues that have influenced this thesis: Dipti Mathur for spearheading the oxidized cytosine project at the very beginning, David Wang for many interesting discussions regarding oxidized cytosine repair and for working out the details of the uracil glycol synthesis, Marshall Morningstar for synthesizing the 5-OH-C and 5-OH-U phosphoramidites, Lisa Bailey for developing the alternative genome construction method for labile adducts, Annie Lee for being a very hardworking and flexible UROP, Pete Wishnok for mass spectrometry data on the modified oligonucleotides, and Kevin Yarema, Marjie Solomon, Bill Kobertz and Paula Collins for many helpful technical discussions.

I am very fortunate that the people with whom I worked in graduate school were not only scientific collaborators, but also good friends. In this regard, I must also thank the many members of the Essigmann and Wogan laboratories for making graduate school fun on a daily basis; from summer and winter trips to Maine, to skiing in Vermont, to random beach days, to volleyball, to lunchtime conversations.

My scientific education began many years ago, and a number of people have influenced me along this path. I thank my mother, Karen Kreutzer, for "expanding my horizons" in math and science when I was young and for encouraging my interests in these fields. Thanks also to the Chemistry Department at Wheaton College including Margaret Farrar, Herb Ellison and Myrna Pearson. Most especially, I thank Elita Pastra-Landis, my college professor, mentor, confidant and friend, who was *the* person that sparked my interests in chemistry and encouraged me into graduate studies. At MIT, I had the great pleasure to learn from, and work with, a number of people including those professors that constituted my thesis committee, Professors Gerald Wogan, Steve Tannenbaum and Pete Dedon.

I am fortunate that my years in graduate school have been balanced by a wonderful group of people that provided me with a life outside the laboratory. I am gratefully indebted to Claude and Martha Wintner for their endless support and encouragement through my graduate education, for their timely scholarly advice, and for welcoming me into their

family, particularly during the holidays. Thanks also to Tom Wintner with whom I enjoyed sharing perspectives on graduate school as well as good German beer.

Thanks to Wen Wissler who, among other things, provided me with a second home on Commonwealth Avenue during the early years of my research, and also to Heather Corbett for cultivating my interest in baseball by indoctrinating me in the history of the Red Sox through our many trips to Fenway Park. Thanks also to Steve Schenkel, Larry Goldberg and Dan Hall for being three of the best college roommates that I never had! To my college roommate, Jill Molinaro Silvestri, I am greatly indebted for her friendship and support, and Suzanne Stone Holden's innate ability to make me laugh, even though we're separated by 3000 miles, has been invaluable.

A hearty thank you to my extended family including my great aunt and uncle, Dick and Ann Thomason, my great grandmother, Edna Thomason, and my cousins, Jim, Sharon, Cara and Chris Santucci, for providing me the sunshine of Southern California during many grey New England Februaries. It was during a memorable hike in the dessert canyons of Palm Springs in which I put the process of graduate school in perspective.

Finally, I thank my thesis advisor, John Essigmann, for not only providing the resources that made this thesis research possible, but also for enabling me to work in a laboratory that encouraged independent thinking. I've learned not only how to ask the right questions, but also how to present the answers in a clear, colorful and meaningful way. During the final weeks of this work, your edits to my dissertation and helpful and *artistic* suggestions to my thesis presentation were much appreciated. Thanks also to you, Ellen, Amy and Nolan for sharing your cabin in Maine with us, as well as for hosting numerous lab parties in your home.

Some of the best advice that I received during my graduate years, certainly the advice that helped me during the final weeks of this dissertation, came from Paula Collins as she moved on from MIT. It has served me well, and may it continue to do so:

"Stay focused, stay happy."

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INTRODUCTION

Throughout history, humans have always feared certain diseases more than others, from leprosy in biblical times to the bubonic plague in the Middle Ages to tuberculosis in the nineteenth century (Pitot, 1993). Thanks to modern advances in the medical sciences, people in industrialized countries today do not hold the same level of fear toward infectious diseases that they once did. Today, as people are living longer lives, it is cancer that most affects us. A first step towards controlling this disease should be to acquire a basic understanding of its genesis. Although we do not yet fully comprehend the molecular mechanisms that lead to cancer development, studies of the past two decades have provided insights into the constellation of genes that play roles as enhancers or suppressors of cancer.

Civilizations as far back as the ancient Egyptians recognized the presence of tumors and, in fact, autopsies of mummies reveal the existence of bone tumors (Pitot, 1993). The term *cancer* has its roots in Latin, and is based on the Greek work *karkinoma*, or crab. It was initially coined by Hippocrates who, around 400 BC, described the long, distended veins radiating from some breast tumors as the limbs of a crab (Varmus and Weinberg, 1993). Traditionally *descriptive* in nature, biology has been transformed in the twentieth century into a science capable of *explaining* complex phenomena such as cancer. This new power stems largely from the merging of two distinct fields, genetics and biochemistry, into the science of molecular biology.

The field of genetics was born in the late nineteenth century with the publishing of Gregor Mendel's pea breeding experiments, bringing to the forefront of biology the

concept of the gene as the hereditary unit (Mendel, 1866). Seminal experiments by O. T. Avery in 1944 (Avery et al., 1944) and by A. D. Hershey and M. Chase in 1952 (Hershey and Chase, 1952) showed definitively that deoxyribonucleic acid (DNA) is the molecule that comprises the genetic material of a cell. Together with these discoveries, the elucidation of the double-helical structure of DNA by J. T. Watson and F. H. Crick in 1953 (Watson and Crick, 1953a; Watson and Crick, 1953b) catalyzed the development of the field of molecular biology. The advancement of this field has been further propelled by the development of biochemical methods for purifying molecules and characterizing enzymes, by the advent of procedures for growing cells and viruses under controlled conditions, and by the improvement in physical techniques for describing molecules at the atomic level (Varmus and Weinberg, 1993). All of these approaches have played major roles in furthering our knowledge of the molecular origins of cancer.

In the field of cancer research, it is widely believed that alterations in the nucleotide sequence of DNA are the underlying cause of cancer development. This is one of nature's great double-edged swords: while DNA mutations are a necessary part of adaption and natural selection, they may also lead to the development of serious disease.

The question thus becomes, what is the underlying cause of DNA mutation? First and foremost, there is the simple fact that the copying of the nucleotide sequence of DNA is not perfect (for a review, see Vogelstein and Kinzler, 1993), and hence a low level of mutation is introduced into the genome by DNA polymerases. In addition, the experiments of H. J. Muller in 1927 demonstrating that X-rays cause DNA mutations (Muller, 1927) led to the investigation of other exogenous chemical agents, such as UV

radiation from sunlight, as potential sources of mutagenesis. More recently, however, attention has been directed toward the role that chemicals produced within the body may play in mutagenesis (Marnett and Burcham, 1993). Much effort is currently aimed at understanding the cellular fate of the reactive metabolites of molecular oxygen that are formed during the process of aerobic respiration. Thus, the focus of this work rests on the DNA damaging effects of reactive oxygen species.

This dissertation will review what is known about oxidative damage to DNA and what questions remain to be answered. It proposes, tests, and suggests an answer to one such question: What is the molecular cause of the most frequently observed DNA mutation, GC→AT transitions?

The final answer to this question, beyond its immediate scientific value, will help to complete an emerging picture of oxidative DNA damage that lays the foundation for the understanding of cancer and the eventual eradication of one of today's most prevalent diseases.

CHAPTER I:

Mutagenicity and Repair of Oxidative DNA Damage: Insights from Studies Using Defined Lesions

A. INTRODUCTION

Life in an aerobic environment provides organisms with enormous benefits, but also presents significant dangers. Through the process of oxidative phosphorylation, the energy currency of the cell, ATP, is generated, but ironically, the highly reactive oxygen species (ROS) necessarily produced to fuel this process can also cause deleterious chemical changes within the cell. When the levels of cellular ROS increase, a state of oxidative stress can occur resulting in covalent modifications to cellular macromolecules. Although all macromolecules are subject to damage by ROS, the primary deleterious consequences of oxidative stress probably arise from damage to DNA because DNA is the source of information for all other macromolecules. The formation of oxidized DNA adducts has been implicated in mutagenesis, carcinogenesis, aging and a number of neurological disorders (Ames et al., 1993; Halliwell and Gutteridge, 1989). (Note: the terms adduct and lesion will be used here interchangeably to refer to covalently modified DNA bases.)

There are various endogenous and exogenous sources of oxygen radicals, and oxygen has been appropriately called the "sink" for electrons generated in various redox reactions of aerobic metabolism (Riley, 1994). Aerobic respiration involves the step-wise four electron reduction of O_2 to H_2O. The reduced intermediates generated through this process, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and OH radical, are highly reactive species, and it has been suggested that a small percentage of these species generated during aerobic respiration may leak from the mitochondrial membrane and thus contribute significantly to the intracellular load of ROS (Riley, 1994). An additional

source of ROS is macrophages; these cells produce numerous free radicals including 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 (Ames et al., 1993).

The most 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 radical that can in turn react with DNA (von Sonntag, 1987). It is noteworthy that most of our detailed knowledge regarding the interaction of DNA with ROS has accumulated from extensive studies of ionizing radiation (von Sonntag, 1987).

DNA damage resulting from attack by ROS includes base modifications, sugar damage, strand breaks, abasic sites and DNA protein-crosslinks. Studies *in vitro* have demonstrated that O_2^- and H_2O_2 are themselves fairly inert to reaction with DNA (Aruoma et al., 1989). In contrast, the OH radical is highly reactive, with measured rate constants reaching the diffusion controlled limit (von Sonntag, 1987). Much of the observed toxicity of O_2^- and H_2O_2 has been attributed to intracellular reduction of these species to OH radicals. For example, in the presence of metal ions such as Fe^{2+}, H_2O_2 can be reduced by the Fenton reaction to OH radical, 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 radical.

The primary reactions of OH radical with DNA are hydrogen abstractions from deoxyribose or additions to the π bonds of DNA bases (Breen and Murphy, 1995).

Hydrogen atom abstraction from the deoxyribose moieties of DNA by OH radical can lead to base loss and single-strand breaks due to fragmentation of the sugar (Henner et al., 1983; von Sonntag, 1987). An 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). Thus, it is oxygen radical damage *to the four nucleobases* that appears to be the primary cause of DNA mutations, with many distinct lesions formed from each nucleobase (von Sonntag, 1987). Systematic *in vitro* studies of the reaction of oxidants (Douki et al., 1996b; Henle et al., 1996; Luo et al., 1996) or ionizing radiation (von Sonntag, 1987) with individual nucleosides or nucleotides, as well as polynucleotides, have facilitated the identification of many of these oxidized base derivatives (Figure 1).

Advances in analytical chemistry have been applied to the quantitative measurement of adduct levels. The primary methods involve use of either high pressure liquid chromatography (HPLC) with electrochemical detection (Shigenaga et al., 1994) or gas chromatography coupled with mass spectrometry (GC-MS) (Dizdaroglu, 1994) to detect specific adducts. Initial estimates suggest that DNA in a single human cell suffers approximately 10,000 oxidative hits per day (Fraga et al., 1990) and that the level of damage to mitochondrial DNA is approximately 10-fold higher than damage to the corresponding nuclear DNA from the same tissues (Richter et al., 1988). More recently, these estimates have been revised downward by approximately one order of magnitude as artifactual oxidation during the analytical processes has been uncovered (Cadet et al., 1997; Helbock et al., 1998).

Cells possess defense systems that prevent formation of DNA damage by intercepting ROS as well as repair enzymes that excise damaged nucleobases or sugars (Ames et al., 1993). Small molecules that act as radical scavengers include ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene and glutathione (Farr and Kogoma, 1991) (Figure 2). Enzymatic defenses include superoxide dismutase, 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. In addition, glutathione peroxidase can reduce potentially deleterious peroxides (Farr and Kogoma, 1991).

Cellular DNA repair systems act to remove DNA damage and ultimately preserve the informational integrity of the genome. Since the cellular adduct load is dictated by the balance between adduct formation and adduct repair, repair processes play a critical role in determining the biological consequences of oxidative stress. Although several discrete repair pathways exist, the base excision repair (BER) pathway is believed to be the primary defense against oxidative damage. BER has been the subject of several recent reviews (Friedberg et al., 1995; Krokan et al., 1997; Seeberg et al., 1995), as has BER of oxidative damage (Croteau and Bohr, 1997; Demple and Harrison, 1994; Wallace, 1997). The BER pathway involves several proteins that act to excise a single damaged nucleobase from DNA and replace it with the correct undamaged nucleotide (Figure 3). The first protein in this process is a glycosylase, whose role is to recognize a damaged nucleobase and catalyze hydrolysis of the glycosydic bond to release the adduct. Typically, glycosylases are small, roughly 20-40 kDa proteins that do not require ATP or cofactors for their activity (Friedberg et al., 1995). In the past three years, X-ray

crystallography has revealed that many glycosylases appear to possess a "base-flipping" motif that turns out the target nucleobase from the helix into an active-site pocket on the enzyme (Krokan et al., 1997). By "base-flipping," the damaged nucleobase is presumably more accessible to the various side-chain functionalities in the enzyme that contribute to catalysis and substrate discrimination. Subsequent enzymatic activities necessary to complete the repair process include cleavage of the resulting abasic site, creation of an extendable 3' terminus, repair synthesis, and ligation (Friedberg et al., 1995).

Although efficient, DNA repair enzymes are by no means perfect, and in spite of their existence, low levels of adducts persist in DNA. These unrepaired DNA adducts can inhibit replication and transcription, and cause mutations. Assessment of the mutational properties of DNA adducts has relied on two complementary approaches: global mutational spectra analysis and site-specific mutagenesis studies (Loechler, 1996; 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 either the absence or presence of an exogenous DNA damaging agent (providing spontaneous or induced spectra, respectively). A particular genomic or vector sequence is monitored following replication, typically by some form of phenotypic selection, and ultimately the frequency, type and position of mutations are determined by sequencing. Mutational spectra can be determined with either double-stranded (ds) or single-stranded (ss) DNA. One advantage to examining ss mutational spectra is that observed mutations can be ascribed to modifications at a specific base whereas with ds DNA the modification can

only be localized to either of the two bases in the mutated base pair.

Both spontaneous and oxidant-induced mutational spectra provide insight into the mutational properties of oxidized lesions (Table 1). Most oxidant-induced mutations are base substitutions, although some deletions, insertions and frameshifts are also induced. 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 (Glickman et al., 1980; Moraes et al., 1990; Sargentini and Smith, 1994; Shaaper and Dunn, 1991).

Numerous induced mutational spectra have been obtained using different sources of oxidizing equivalents, such as ionizing radiation, $O_2^{\cdot-}$, H_2O_2, and metal ions (Ayaki et al., 1986; Glickman et al., 1980; Grosovsky et al., 1988; McBride et al., 1991; Moraes et al., 1990; Ono et al., 1995; Sargentini and Smith, 1994; Tkeshelashvili et al., 1991). The results of these studies reveal that just as in the spontaneous mutational spectra, the most prominent base substitutions are GC→AT transitions followed by GC→TA transversions, hinting that the primary pathways to oxidative damage of DNA are independent of the source of oxidation.

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 specific DNA adducts due to the multiplicity of lesions formed by typical damaging agents. Deconvolution of complex mutational spectra has been facilitated by the advent of technology to prepare synthetic oligonucleotides containing a single DNA

lesion. These single lesion substrates can be used directly to study replication *in vitro* by DNA polymerases, or they can be incorporated into vectors, introduced into cells, and evaluated for mutational frequency and specificity (Singer and Essigmann, 1991). From these experiments, the mutagenicity of a number of discrete oxidative adducts has been analyzed. Compilation of the available data to date reveals an aggregate profile that begins to resemble at least one aspect of the overall mutational spectrum, namely the types of mutations observed. Another important use of the singly modified substrates has been in the determination of repair enzyme substrate specificity. Collectively, these studies have provided much insight into the properties of individual oxidative DNA adducts.

In the quest to identify lesions responsible for the prominent mutations observed in mutational spectra, this lab and others initially focused on the primary guanine oxidation product, 8-OXO-dG. Since then, the emphasis has evolved to other purine and pyrimidine derived lesions. The strategies used to synthesize singly modified oligonucleotides will be discussed later in this dissertation; therefore this review will focus on the results from many experiments employing these substrates. For each of the following nine oxidatively derived adducts (Figure 1), this discussion will relate the known pertinent structural features, behavior in *in vitro* polymerase replication assays, *in vivo* mutagenicity, and repairability.

B. OXIDATIVE PURINE ADDUCTS

1. 7,8-Dihydro-8-oxo-2'-deoxyguanosine (8-OXO-dG)

Oxidation of guanine at the C-8 position has been demonstrated following treatment of DNA with various oxidizing agents, metals, and γ-radiation (Dizdaroglu, 1985; Floyd, 1990; Kasai et al., 1986; Kasai and Nishimura, 1984). 8-OXO-dG is highly abundant in DNA. In fact, it is the most commonly observed oxidized adduct in γ-irradiated mammalian chromatin (Gajewski et al., 1990), as well as in human tissues (Wagner et al., 1992).

Of all the oxidized lesions found in DNA, 8-OXO-dG has been the most extensively analyzed from the structural standpoint. NMR spectroscopy has determined that free 8-OXO-dG in solution adopts a syn conformation about the glycosidic bond and that the modified base exists predominantly as the 6,8-diketo tautomer (Figure 4A) (Aida and Nishimura, 1987; Culp et al., 1989; Uesugi and Ikehara, 1977).

The diketo form of 8-OXO-dG also predominates in duplex DNA (Cho et al., 1990), but the conformation about the glycosidic bond depends upon the identity of its base pair partner. When paired opposite dC, 8-OXO-dG resides in an anti orientation and is capable of forming a stable Watson-Crick pair (Lipscomb et al., 1995; Oda et al., 1991). However, when 8-OXO-dG assumes a syn conformation, it can form a stable Hoogsteen mispair with dA in DNA (Figure 5) (Kouchakdjian et al., 1991; McAuley-Hecht et al., 1994). The observed mutagenicity of 8-OXO-dG has been attributed to its ability to undergo this conformational switch from anti to syn relative to the deoxyribose sugar.

The mutagenic potential of 8-OXO-dG was initially assessed by primer extension assays *in vitro* which suggested that 8-OXO-dG could mispair with any nucleotide and also cause misinsertions at adjacent pyrimidines (Kuchino et al., 1987). Subsequent studies, however, demonstrated that the primary mutagenic activity of 8-OXO-dG arose from its mispairing with dA. Additional studies of both bacterial and mammalian DNA polymerases *in vitro* revealed that the replicative and repair DNA polymerases exhibit differential degrees of dATP incorporation opposite 8-OXO-dG. DNA pol α, pol δ and pol III have greater tendencies to misincorporate dATP, whereas DNA pol I and pol β preferentially insert dCTP (Shibutani et al., 1991).

Upon examining the mutagenicity of 8-OXO-dG by replication of a single adduct in *Eschericia coli* (*E. coli*), 8-OXO-dG was determined to be 0.5-1% mutagenic and exclusively gave G→T transversions at the site of the adduct, presumably through 8-OXO-dG:dA mispairing as shown in Figure 5 (Wood et al., 1990). The biological importance of the 8-OXO-dG:dA base pairing intermediate was confirmed by subsequent *in vivo* experiments (Cheng et al., 1992; Moriya et al., 1991).

Studies of 8-OXO-dG mutagenicity in mammalian systems are consistent with those in bacteria. Replication in HeLa cells of a single 8-OXO-dG adduct in a ds shuttle vector induces targeted G→T transversions at a frequency of 1-2% (Klein et al., 1990; Klein et al., 1992). Interestingly, when the vector is introduced into excision repair deficient XP-A cells, the mutation frequency of the adduct increases by three to five fold. A similar mutation frequency for 8-OXO-dG (2.5-4.8%) is observed when the lesion is replicated in a ss shuttle vector in COS cells (Moriya, 1993a). The effect of sequence

context on the mutagenicity of 8-OXO-dG in mammalian cells has also been investigated (Kamiya et al., 1992; Kamiya et al., 1995a); 8-OXO-dG was incorporated into multiple sites in the c-Ha-ras gene and then transfected into NIH3T3 cells. Depending upon the sequence context examined, 8-OXO-dG induces G→T transversions and G→A transitions, as well as random substitution mutations at the 5' flanking base.

In addition to direct oxidation of bases within the DNA duplex, oxidized nucleotide triphosphates may also be erroneously incorporated from the nucleotide pool by DNA polymerases. Viral, bacterial and mammalian polymerases efficiently incorporate 8-OXO-dGTP into DNA opposite dC and, to a lesser extent, opposite dA (Cheng et al., 1992; Pavlov et al., 1994). Thus depending upon the origin of the 8-OXO-dG residue in DNA, two distinct mutations can arise; misincorporation of 8-OXO-dGTP opposite dA leads to A→C transversions, and misinsertion of dATP opposite template 8-OXO-dG results in G→T transversions.

Fortunately, cellular repair systems exist to counteract oxidative damage in both template DNA and the nucleotide pool. The set of repair enzymes aimed at mitigating the effects of 8-OXO-dG in *E. coli* has 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, but for clarity we shall refer hereafter to the protein as MutM), 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 (reviewed in, Michaels and Miller, 1992b). The *E. coli* MutM protein is a glycosylase that excises 8-OXO-dG most efficiently when it is paired with dC,

resulting in formation of an abasic site and subsequent productive repair synthesis. In contrast, MutM is much less active on 8-OXO-dG when it is present in an 8-OXO-dG:dA pairing. This discriminatory power is biologically advantageous since excision of 8-OXO-dG from such an intermediate would actually lead to fixation of a G→T mutation. Fortunately, the second enzyme in this repair system, MutY, can act efficiently upon the 8-OXO-dG:dA mispair to remove the A. Gap-filling by a repair polymerase offers another opportunity for insertion of dC opposite the 8-OXO-dG and generation of a suitable substrate for repair by MutM. Not surprisingly, 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). Consistent with these properties, a strikingly increased mutation frequency is observed when a single 8-OXO-dG is replicated in a *mutM mutY E. coli* strain (Moriya and Grollman, 1993b).

The third protein in this system, MutT, hydrolyzes 8-OXO-dGTP to 8-OXO-dGMP and pyrophosphate, thus purifying the nucleotide pool and preventing misincorporation of 8-OXO-dGTP into DNA (Maki and Sekiguchi, 1992). Cells deficient in MutT show significantly elevated levels of A→C mutations (Yanofsky et al., 1966). In addition, since MutT is also capable of hydrolyzing the ribonucleoside triphosphate 8-OXO-GTP, it has been suggested to play a role in preventing transcriptional misincorporation of 8-OXO-GTP into RNA (Taddei et al., 1997).

The components of the genoprotective system described above 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 (Aburatani et al., 1997; Arai et al., 1997; Lu et al., 1997; Radicella et al., 1997; Roldan-Arjona et al., 1997; Rosenquist et al., 1997) functional homologs of MutM designated Ogg1 (for oxo-guanine glycosylase) that excise 8-OXO-dG from DNA. Disruption of the yeast Ogg1 gene results in a mutator phenotype with enhanced levels of GC→TA transversions (Thomas et al., 1997). It appears that there may also be a second discrete Ogg activity in yeast (Ogg2) that differs in its recognition of the base opposite 8-OXO-dG (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 (Kakuma et al., 1995; Sakumi et al., 1993) exist.

Several other repair proteins have activity *in vitro* against 8-OXO-dG. The mammalian alkylpurine DNA glycosylase has a very broad *in vitro* substrate specificity, including 8-OXO-dG (Bessho et al., 1993); however, in recent experiments, extracts derived from mice deficient in this enzyme still retain activity against 8-OXO-dG, suggesting that it is not a major glycosylase for 8-OXO-dG (Engelward et al., 1997; Hang et al., 1997). In addition, the human nucleotide excision repair (NER) system has been reported to process 8-OXO-dG (Reardon et al., 1997), and the ribosomal S3 protein of Drosophila has been observed to possess glycosylase activity for 8-OXO-dG (Sandigursky et al., 1997). The biological relevance of these activities remains to be determined.

2. 7,8-Dihydro-8-oxo-2'-deoxyadenosine (8-OXO-dA)

The formation of 8-OXO-dA, the adenine analog of 8-OXO-dG, has also been observed in DNA (Malins and Haimanot, 1990; Stillwell et al., 1989). Similar to 8-OXO-dG, free 8-OXO-dA in solution resides in the syn conformation (Giessner-Prettre and Pullman, 1977; Uesugi and Ikehara, 1977). In DNA, however, 8-OXO-dA resides in the anti orientation and causes only minor conformational changes of the duplex (Guschlbauer et al., 1991). Although 8-OXO-dA can adopt several tautomeric forms, under physiological conditions the 6-amino-8-keto conformation predominates (Figure 4B) (Cho and Evans, 1991).

Replication of DNA templates containing 8-OXO-dA by the bacterial polymerases DNA pol I (KF^{exo-}) and Taq DNA pol, reveals that the lesion is not a block to DNA synthesis and that exclusive incorporation of thymine occurs opposite the damage (Guschlbauer et al., 1991). The lack of 8-OXO-dA mutagenicity in bacteria is confirmed by replication of a bacteriophage genome containing a single 8-OXO-dA lesion in *E. coli* cells (Wood et al., 1992). Although not mutagenic in bacterial systems, some studies suggest that 8-OXO-dA may cause mispairing in mammalian cells. *In vitro* replication of oligonucleotides containing 8-OXO-dA shows that in addition to dTTP, mouse DNA pol α can misinsert dGTP while recombinant rat DNA pol β can misinsert both dATP and dGTP (Kamiya et al., 1995; Shibutani et al., 1993). When a single 8-OXO-dA is incorporated into the c-Ha-ras gene and transfected into mammalian cells, A→G and A→C mutations are induced at a mutation frequency of approximately 1% (Kamiya et al., 1995).

Due to the relatively benign nature of 8-OXO-dA, analysis of the repair of 8-OXO-dA has been limited for the most part to examinations of the substrate specificity of MutM. One initial study found 8-OXO-dA to be refractory to cleavage by MutM (Tchou et al., 1991) but a more recent study indicates that small amounts of 8-OXO-dA are released by this enzyme (Boiteux et al., 1992). It has also been reported that MutY can remove A from a dA:8-OXO-dA pairing (Michaels et al., 1992c).

3. 2-Hydroxy-2'-deoxyadenosine (2-OH-dA)

Another oxidation product of adenine, 2-OH-dA, has also been observed in oxidatively damaged DNA (Kamiya and Kasai, 1995; Mori et al., 1993; Nackerdien et al., 1991; Olinski et al., 1992). Because the yield of 2-OH-dA is 70-80 times higher following oxidation of dA and dATP as compared to oxidation of duplex DNA, it has been suggested that the presence of 2-OH-dA in cellular DNA most likely arises through its incorporation from the nucleotide pool (Kamiya and Kasai, 1995). Modification of the C2 position of adenine may affect the base pairing properties of the base. Moreover, 2-OH-dA exists in two tautomeric forms in aqueous solution (Figure 4C) with the ratio of the keto tautomer (N1-H, C2-keto) to the enol tautomer (C2-OH) approximating 9:1 (Sepiol et al., 1976).

The *in vitro* and *in vivo* mispairing properties of 2-OH-dA in template DNA have been investigated (Kamiya and Kasai, 1996; Kamiya et al., 1995b; Switzer et al., 1993). Collectively, the results of these studies suggest that formation of 2-OH-dA in DNA can lead to all of the base substitution mutations involving A (A→G transition, and A→T and

A→C transversions). Efficient insertion of 2-OH-dATP by DNA pol I (KF^{exo-}) and by calf thymus DNA pol α has also been demonstrated *in vitro* (Kamiya and Kasai, 1995; Switzer et al., 1989) and, in addition to incorporation opposite dT, 2-OH-dATP is misinserted opposite dC. Subsequent replication of this mispair would result in the formation of a GC→AT transition.

The mutation frequency and specificity of 2-OH-dA was evaluated in *E. coli* by using site-specifically modified ds and ss vectors (Kamiya and Kasai, 1997). 2-OH-dA showed little to no genotoxicity, and the mutation frequency of the adduct varied with its placement in a particular DNA sequence or strand. When placed in the (+) strand, 2-OH-dA exhibited a mutation frequency between 0.07 and 0.8%, and the primary mutation induced was a -1 deletion; no increase in mutation frequency was observed for ss versus ds DNA. The mutation frequency of 2-OH-dA when situated in the (-) strand was comparable to that of the (+) strand (between 0.1 and 0.3%), but the mutation specificity differed. The predominant mutations observed from 2-OH-dA in the (-) strand were A→T and A→G substitutions. Unfortunately very little is known about the repair of 2-OH-dA; the only suggestion of repair activity thus far is a report that a ds oligonucleotide containing 2-OH-dA is weakly nicked by an *E. coli* extract (Kamiya and Kasai, 1997).

C. OXIDATIVE PYRIMIDINE ADDUCTS

1. 5,6-Dihydro-5,6-dihydroxy-2'-deoxythymidine (Thymidine Glycol or dTg)

dTg is the major thymine derived adduct detected after oxidation or irradiation of DNA *in vitro* and *in vivo* (Breimar and Lindahl, 1985; Teoule et al., 1977). Although both *cis*- and *trans*-isomers of dTg exist in DNA (Gajewski et al., 1990), the most common laboratory syntheses of dTg involve oxidation of thymidine by OsO_4 or $KMnO_4$, which predominantly provide a mixture of the *cis*-isomers that cannot be readily resolved by standard chromatographic techniques (Frenkel et al., 1981; Iida and Hayatsu, 1971; Teebor et al., 1987). Thus, studies that have introduced dTg into DNA via chemical oxidation have measured the combined effects of both *cis*-lesions. Structurally, NMR studies reveal that dTg induces significant, localized change to duplex DNA, and that the lesion as well as the opposing base reside in a largely extrahelical conformation (Kao et al., 1993; Kung and Bolton, 1997).

In vitro studies show that dTg inhibits primer elongation by bacterial and viral polymerases, and that polymerase arrest occurs at the site of the oxidized lesion (Hayes and LeClerc, 1986; Ide et al., 1985; Rouet and Essigmann, 1985). Interestingly, certain sequence contexts such as 5'-C(Tg)A-3' and, to a lesser extent, 5'-C(Tg)C-3', permit bypass by polymerases *in vitro* (Clark and Beardsley, 1989; Hayes and LeClerc, 1986). When positioned in such a by-passable sequence, a single dTg induces T→C transitions at a frequency of 0.3-0.4% upon replication in *E. coli* (Basu et al., 1989). One additional mutagenic outcome can arise that is dependent on the base precursor of dTg. The primary product of 5-methylcytosine oxidation is dTg (Zuo et al., 1995) and, given the propensity

of dTg to pair with dA, the presence of such an adduct would cause a CG→TA transition.

In addition to base excision repair by endonuclease III and endonuclease VIII, there is biochemical evidence that Uvr(A)BC as well as the human NER system also can act to repair dTg lesions (Kow et al., 1990; Lin and Sancar, 1989; Reardon et al., 1997). Moreover, in human cells dTg is apparently repaired in a transcriptionally coupled fashion, raising the intriguing possibility that this mode of repair may extend to other oxidized lesions (Cooper et al., 1997).

2. 5-Hydroxy-2'-deoxycytidine (5-OH-dC), 5-hydroxy-2'-deoxyuridine (5-OH-dU) and 5,6-dihydro-5,6-dihydroxy-2'-deoxyuridine (Uridine Glycol or dUg)

Oxidation of cytidine initially leads to formation of cytidine glycol (dCg), a highly unstable species that is believed to decompose rapidly (Douki et al., 1996b; von Sonntag, 1987). Deamination of dCg is enhanced by saturation of the 5-6 position of cytosine resulting in formation of dUg. dUg and dCg can also dehydrate to form 5-OH-dU and 5-OH-dC, respectively (Figure 6). These adducts have been identified in vitro (Dizdaroglu et al., 1986) and in mammalian tissues (Wagner et al., 1992).

In vitro replication studies have shown that DNA pol I (KF^{exo-}) incorporates dA opposite 5-OH-dU; in the same experimental system, 5-OH-dC pairs mainly with dG, but also to a lesser extent with dA and dC. The same mispairing specificities are observed when 5-OH-dCTP and 5-OH-dUTP are incorporated into newly synthesized DNA (Purmal et al., 1994a; Purmal et al., 1994b). In addition, the efficient insertion of dUgTP opposite dA has been demonstrated (Purmal et al., 1998).

3. 5-Hydroxymethyl-2'-deoxyuridine (5-HM-dU)

5-HM-dU is a less-studied oxidative adduct that is detected in DNA *in vivo* (Frenkel et al., 1985; Teebor et al., 1984) as well as in human and rat urine at levels comparable to those of dTg (Cathcart et al., 1984). Since the thymine-like base pairing properties of this lesion are unaltered by the addition of a C5-OH group, the mutagenicity of 5-HM-dU may be weak. However, X-ray crystallographic and NMR solution studies have demonstrated that the nucleoside resides in an unusual conformation in which the pucker at the C1' position of the 2'-deoxyribose moiety of 5-HM-dU is changed to *exo* instead of the normal *endo* pucker (Birnbaum et al., 1980).

Studies on the mutagenic potential of 5-HM-dU show conflicting results. Although one study demonstrates the lack of mutagenicity of a single 5-HM-dU in *E. coli* (Levy and Teebor, 1991), others show the SOS dependent mutagenicity of 5-HM-dU, as well as the mutagenicity of the lesion in the Ames assay (Bilimoria and Gupta, 1986; Shirname-More et al., 1987). The base pairing properties of 5-HM-dU in DNA have been examined by NMR (Mellac et al., 1993) and suggest that 5-HM-dU can pair with both dA and dG. When paired with dA, the 5-HM-dU:dA base pair is in Watson-Crick geometry stabilized by an interresidue hydrogen bond between the hydroxymethyl group and the neighboring 5' base. Alternatively, when 5-HM-dU is paired opposite dG, a wobble base pair can be formed, which is stabilized by an intramolecular hydrogen bond between the hydroxymethyl group and the O4-carbonyl. Mispairing *in vivo* of 5-HM-dU:dG could subsequently result in the formation of T→C transition mutations. Since mammalian cells possess a specific glycosylase responsible for the removal of 5-HM-dU from DNA

(Hollstein et al., 1984), more careful investigation into the mutational properties of this lesion is warranted.

4. 5-Formyl-2'-deoxyuridine (5-FO-dU)

Another minor oxidation product of thymidine that may possess mutagenic potential is 5-FO-dU. This lesion can form directly as a product of oxidative damage to DNA (Kasai et al., 1990) and may also result from the decomposition of thymidine hydroperoxides (Tofigh and Frenkel, 1989). In addition, 5-FO-dU may also result via the oxidation of 5-HM-dU. Whereas thymidine contains an electron donating methyl group at the 5-position, 5-FO-dU contains an electron withdrawing group, predicting increased ionization of the N3 imino proton under physiological conditions (Privat and Sowers, 1996).

In vitro studies have shown that 5-FO-dUTP can be incorporated by viral and bacterial DNA polymerases opposite template dA and, to a lesser extent, opposite dG, which would subsequently result in the formation of a G→A mutation (Yoshida et al., 1997). Interestingly, the efficiency of incorporation opposite dG increases with increasing pH, strongly suggesting that the base-ionized form of 5-FO-dU is involved in mispairing (Privat and Sowers, 1996; Yoshida et al., 1997). Furthermore, primer extension assays reveal that DNA polymerases also can direct the misincorporation of dC opposite the lesion, resulting in the formation of a T→G transversion (Zhang et al., 1997). The biological significance of 5-FO-dU is underscored by the presence of repair enzymes able to remove the lesion from DNA. Bacterial AlkA, originally discovered as a 3-

methyladenine glycosylase, has been shown to release 5-FO-dU from DNA (Bjelland et al., 1994) and DNA glycosylase activities for the removal of 5-FO-dU have recently been identified in cell extracts from mouse and rat liver (Zhang et al., 1995), as well as from human cells (Bjelland et al., 1995).

D. CONCLUSION AND PROSPECTIVE

Over the past decade or so, a wealth of structural and biological data has been amassed on oxidative DNA adducts, much of it garnered through the use of singly modified substrates (Table 2). With this knowledge, the task of deconvoluting observed mutational spectra has begun. The two dominant base substitutions observed in spontaneous and oxidant-induced mutational spectra are G→T transversions and C→T transitions (Table 1). Which lesions are responsible for these mutations? *In vitro* experiments followed by *in vivo* studies have now solved a large part of the problem: the G→T transversions observed in mutational spectra could be the result of oxidative damage to dG in the form of 8-OXO-dG. 8-OXO-dG levels are normally controlled by the repair proteins MutM, MutY and MutT, and loss of these protein functions results in high levels of genomic mutation.

At the start of this work, evidence for the lesion(s) that gives rise to the C→T half of the mutational spectrum was just beginning to accumulate. In 1992, Wagner et al. reported the detection of 5-OH-dC, 5-OH-dU and dUg in tissue from rats and in human cells, immediately hypothesizing that these lesions could give rise to C→T transitions

(Wagner et al., 1992). Subsequent *in vitro* polymerization studies supported the mutagenic potential of 5-OH-dC, 5-OH-dU and, more recently, dUg (Purmal et al., 1998; Purmal et al., 1994a; Purmal et al., 1994b).

The balance of this thesis work details the design and execution of experiments which seek to investigate the mutagenicity of 5-OH-dC, 5-OH-dU and dUg *in vivo*, and which uncover molecular origins of the C→T transitions so prevalent in the mutational spectra of living systems.

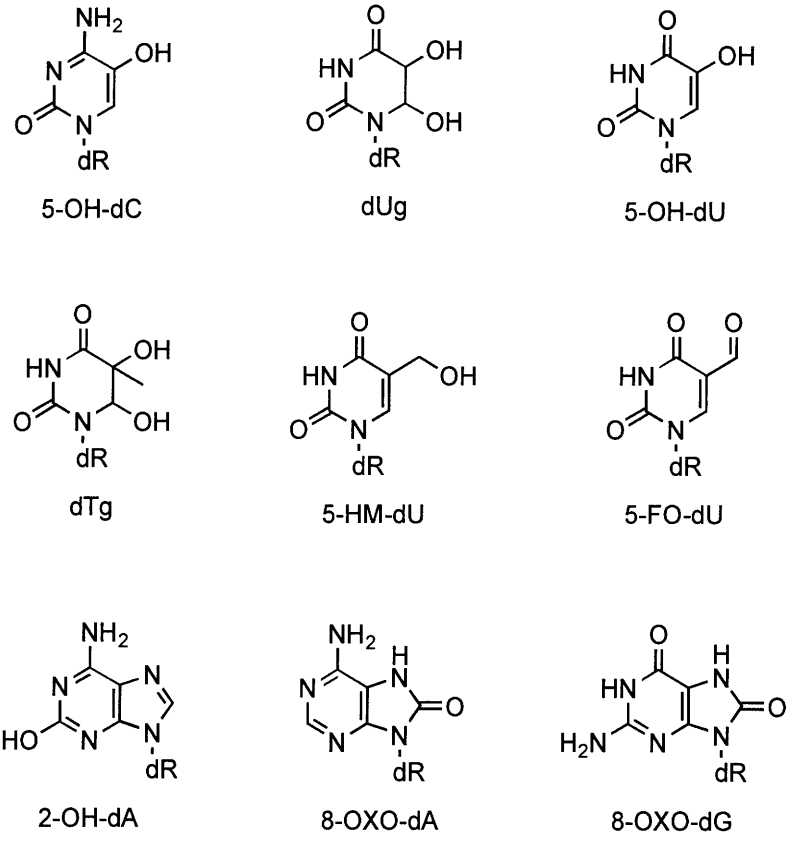
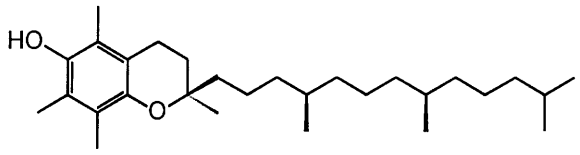
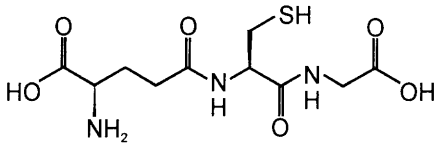


Figure 1: Structures of some oxidative DNA adducts.

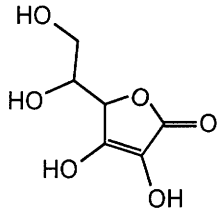
Small Molecules



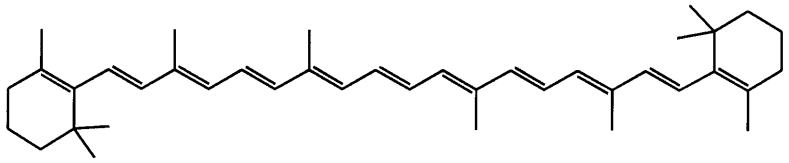
α-tocopherol
(Vitamin E)



glutathione (GSH)



ascorbic acid
(Vitamin C)



β-carotene

Enzymatic Reactions

Superoxide dismutase: $O_2^- + O_2^- \longrightarrow H_2O_2 + O_2$

Catalase: $2H_2O_2 \longrightarrow 2H_2O + O_2$

Glutathione peroxidase: $H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$

Figure 2: Some cellular antioxidant defenses including small molecule radical scavengers and enzymes.

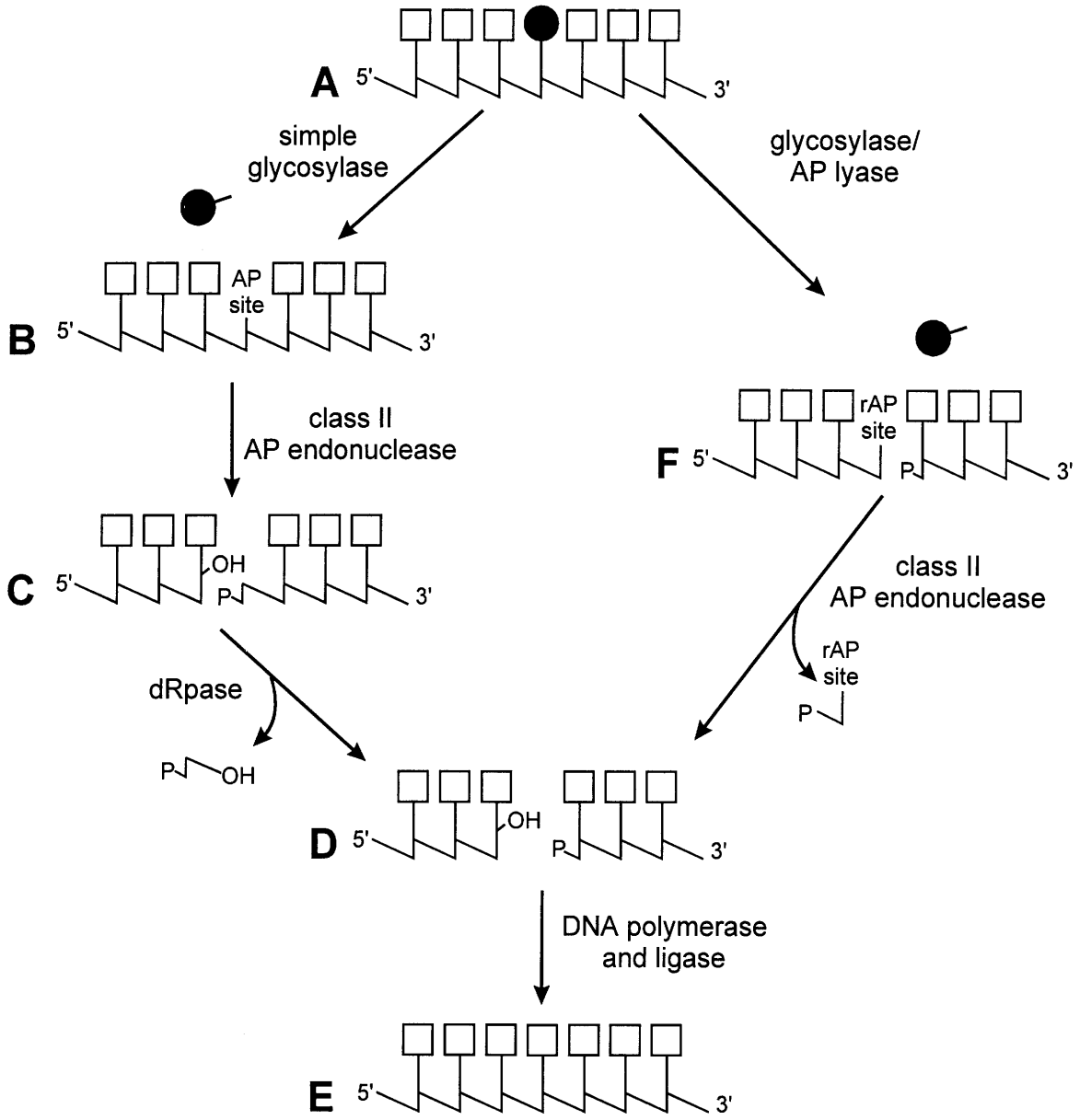


Figure 3: Schematic of BER. The modified base (solid circle) can be removed by either of two pathways. In the 1st pathway (left) a simple glycosylase excises the damaged base forming an AP site (**B**), which is cleaved by an AP endonuclease (**C**). Then a dRpase removes the 5' deoxyribose phosphate 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.

Table 1: Base substitutions observed in spontaneous and oxidant-induced mutational spectra. Mutations are listed from top to bottom in decreasing order of occurrence.

Spontaneous				γ-radiation				O_2^-	H_2O_2	Fe^{2+}	Cu^{2+}
a	b	c	d	e	f	g	h	i	j	k	l
GC→AT	GC→AT	GC→AT	GC→AT	GC→AT	C→T	GC→AT	GC→AT	GC→AT	GC→AT	G→C	C→T
GC→TA	GC→TA	AT→CG	GC→TA	GC→TA	G→T	TA→GC	AT→GC	GC→TA	GC→CG	C→T	G→T
AT→CG	GC→CG	AT→GC	AT→TA	AT→CG	C→A	AT→TA	GC→TA	AT→CG	GC→TA	G→T	CC→TT
AT→TA	AT→GC	AT→TA	AT→CG	AT→TA	G→C	AT→GC	GC→CG	GC→CG	AT→CG	T→C	T→C
GC→CG	AT→TA	GC→TA	AT→GC	GC→CG	A→C	CG→GC	AT→TA	AT→GC	AT→TA	A→G	A→C
		GC→CG	GC→CG		T→C	GC→CG	AT→CG	AT→TA		A→C	A→T
					C→G					A→T	
										C→A	
										C→G	

(a) (Glickman et al., 1980); (b) (Moraes et al., 1990); (c) (Shaaper and Dunn, 1991); (d) (Sargentini and Smith, 1994); (e) (Glickman et al., 1980); (f) (Ayaki et al., 1986); (g) (Grosovsky et al., 1988); (h) (Sargentini and Smith, 1994); (i) (Ono et al., 1995); (j) (Moraes et al., 1990); (k) (McBride et al., 1991); (l) (Tkeshelashvili et al., 1991).

[1] The studies listed are a representative fraction of the total mutational spectra published in the literature. For compilations of mutational spectra see http://info.med.yale.edu/mutbase/html and http://sunsite.unc.edu/dnam/mainpage.

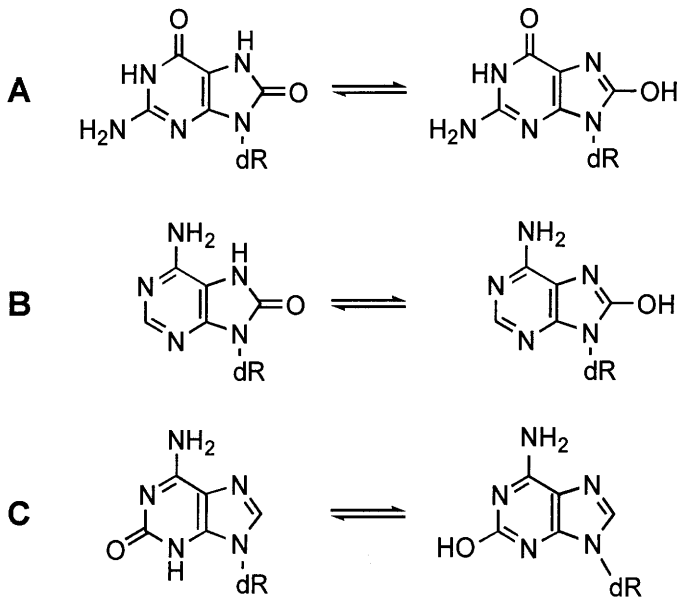


Figure 4: Keto-enol tautomerization of purine adducts. (**A**) 8-OXO-dG; (**B**) 8-OXO-dA; (**C**) 2-OH-dA.

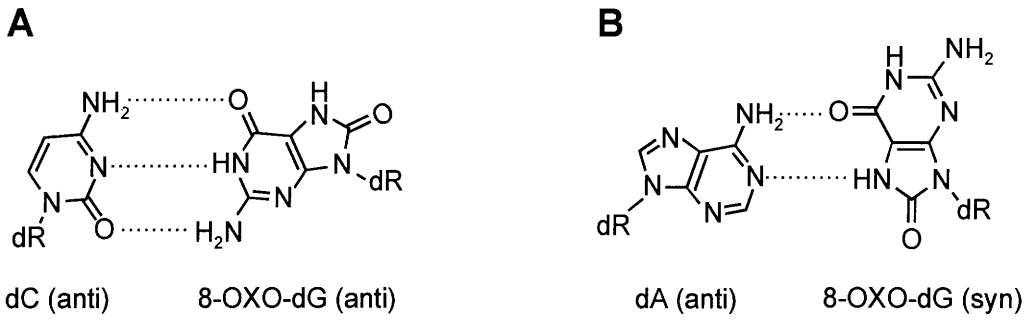


Figure 5: Normal (**A**) and potentially mutagenic (**B**) base-pairing properties of 8-OXO-dG.

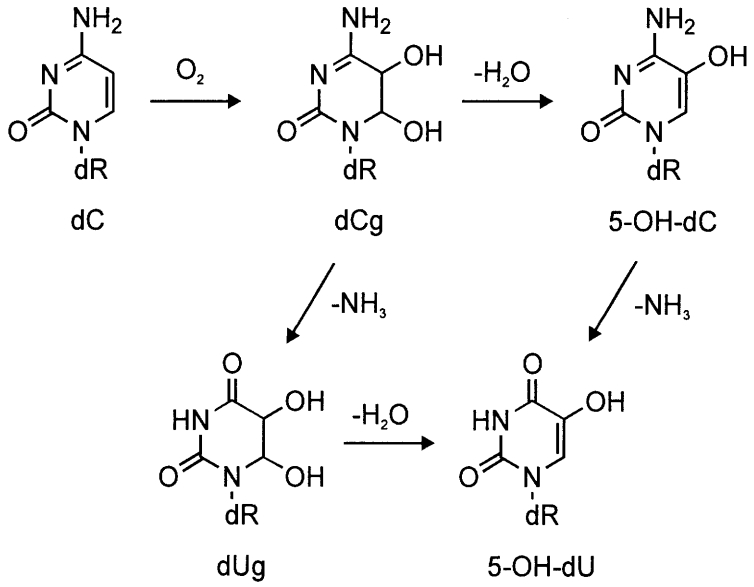


Figure 6: Pathways of oxidized pyrimidine formation. Initial oxidation of dC can form the relatively unstable species, dCg, that can either dehydrate, deaminate, or undergo both reactions to form 5-OH-dC, dUg, or 5-OH-dU, respectively.

Table 2: Mutagenicity and repair of oxidative DNA adducts as determined through site-specific analyses.

	Mutagenic Specificity			Repair Processes	
DNA Adduct	Bacterial Systems	Mammalian Systems	*in vitro*	*in vivo*	*in vitro*
8-OXO-dG	G→T	G→T	G→T, A→C[a]	MutM, MutY, NER	MutM, MutY, MutT, NER
8-OXO-dA	no mutation	A→G, A→C	A→C, A→T[b]	nd	MutM, MutY
2-OH-dA	A→T, A→G	nd[c]	A→G, A→T A→C, C→T[a]	nd	nd
dTg	replication block, T→C	nd	replication block, G→A[a], C→T[d]	nd	endo III, NER, endo VIII
5-OH-dC	nd	nd	C→T, C→G[e], A→G[a]	nd	endo III, endo VIII
5-OH-dU	nd	nd	C→T	nd	endo III, MutM, endo VIII, UDG
dUg	nd	nd	C→T	nd	endo III, MutM, endo VIII
5-HM-dU	no mutation	nd	T→C[f]	nd	HMU glycosylase
5-FO-dU	nd	nd	T→G, G→A[a]	nd	AlkA

[a] Mutation resulting from incorporation of the modified nucleoside from the nucleotide pool.
[b] No mispairing was observed when bacterial polymerases were used.
[c] Not yet determined.
[d] dTg formed as a result of 5-Me-dC oxidation would lead to a C→T mutation.
[e] Mutation resulting from translesional synthesis as well as from incorporation from the nucleotide pool.
[f] Mutations suggested by NMR stability studies of 5-HM-dU mispairs.

CHAPTER II:

Preparation of 5-OH-dC, 5-OH-dU and dUg Singly-Modified Oligonucleotides and Bacteriophage Genomes

A. INTRODUCTION

A program of adduct site-specific mutagenesis was undertaken in order to probe the mutagenic properties of 5-OH-dC, 5-OH-dU and dUg. This technique, previously employed to effect in the study of many DNA lesions, allows a chemically modified DNA adduct to be incorporated at a defined site into DNA and then introduced into a cell. Thus, a single base change to the genome may be monitored in the context of the entire organism.

Especially in the case of oxidative lesions, adduct site-specific mutagenesis is a powerful method by which to study the mutagenic properties of a particular DNA lesion. While several methods exist to oxidize DNA for *in vivo* studies (e.g., treatment with H_2O_2 and Fe^{2+}), such procedures suffer from the unavoidable formation of multiple copies of multiple oxidative products (e.g., dC is converted to 5-OH-dC, 5-OH-dU, dUg, among other lesions) thereby diminishing the precision with which resulting mutagenic data may be interpreted. By contrast, adduct site-specific mutagenesis allows the study of a single copy of a specific type of synthesized adduct.

Adduct site-specific mutagenesis involves the synthesis of an oligonucleotide that contains a lesion of known structure at a defined position, and the use of recombinant DNA techniques to incorporate this oligonucleotide into a plasmid or viral genome. This vector can be studied *in vitro* or introduced into cells, where replication of the genome can be monitored by analyzing the progeny generated. The techniques of constructing site-specifically modified oligonucleotides and genomes has been recently reviewed (Yarema and Essigmann, 1995a), and is summarized below.

An important step in designing an adduct site-specific mutagenesis study is the construction of a singly modified oligonucleotide. Three factors are important to consider: (1) oligonucleotide size and base composition, (2) synthesis strategy, and (3) purification and characterization techniques.

First, the choice of oligonucleotide size must strike a balance between the advantages of a short oligonucleotide for purification and characterization purposes, and the advantages of a longer oligonucleotide for more efficient annealing and ligation during the construction of the genome. In practice, many site-specific mutagenesis studies have made use of modified oligonucleotides of approximately 4-15 bases in length. In addition to oligonucleotide length, the DNA sequence of the modified oligonucleotide must be considered. Often, the selection of oligonucleotide sequence is influenced by the mutability of a lesion in a particular context and by the technique used to select or screen for mutants. While it may be desirable to position an adduct in a sequence that is known to be a preferred site of adduct formation, slow DNA repair, and a mutational hot spot, this information is not always readily discernable. Thus, the choice of sequence context is often determined by selecting a sequence that will facilitate the eventual separation of mutant and wild-type populations. Often, DNA adducts are situated within a restriction endonuclease site so that any mutation arising from the adduct will prevent cleavage of that site by a restriction enzyme.

A second factor to be considered in the construction of a singly modified oligonucleotide is the synthesis strategy to be employed. The two most common techniques used are postsynthetic chemical modification and total chemical synthesis.

The former technique is useful in constructing modified oligonucleotides that contain either labile DNA adducts such as those formed by aflatoxin B_1 that would not survive solid phase DNA synthesis (Gopalakrishnan et al., 1989), or DNA adducts that form intra- and inter-strand crosslinks such as those formed by cisplatin (Toney et al., 1989). Following reaction of the oligonucleotide with a DNA damaging agent, extensive purification to isolate the oligonucleotide containing the particular adduct of interest is required. A main drawback to this approach is the limitation in sequence flexibility since it is favorable to have an oligonucleotide sequence containing only one site of reactivity toward a particular DNA damaging agent. A preferable method of modified oligonucleotide construction is by total chemical synthesis, which involves creating a modified, protected nucleotide that can be directly used for solid phase, automated DNA synthesis (McBride and Caruthers, 1983). The advantages of this approach are threefold: (1) a well characterized adduct is produced, (2) large amounts of modified oligonucleotide are easily obtained, and (3) placement of the lesion in any sequence context is possible.

Once synthesized, it is important to purify and characterize modified oligonucleotides thoroughly prior to their use in genetic experiments. Typically, modified oligonucleotides can be resolved from their unmodified counterparts by using standard high pressure liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE) techniques. Characterization of the modified substrates generally involves digestion of the oligonucleotide to its corresponding nucleosides followed by reverse phase (RP) HPLC analysis with chemically synthesized standards. Mass spectrometry

(MS) is often used to confirm the molecular weight of a modified oligonucleotide and has recently been used to determine the exact position of an adduct within an oligonucleotide (for a review, see Miketova and Schram, 1997). Other characterization approaches include NMR, X-ray crystallography, and a variety of enzymatic and chemical methods that depend upon the nature of the particular DNA adduct.

To perform an adduct site-specific mutagenesis study *in vivo*, a biologically viable singly modified DNA substrate must be constructed. To accomplish this, multiple methods have been developed based upon the use of recombinant DNA technology to incorporate a modified oligonucleotide into a plasmid or viral genome (for a review, see Yarema and Essigmann, 1995a). The basic genome construction method employed in this thesis research was developed by C. W. Lawrence (Banerjee et al., 1988) and has been used to study the mutagenesis of many DNA adducts such as an abasic site (Lawrence et al., 1990), the AFB_1 N7 guanine adduct (Bailey et al., 1996b), the intra-strand DNA crosslink adducts of cisplatin (Yarema et al., 1995b) and the *cis-syn* T-T cyclobutane dimer (Banerjee et al., 1988). Regardless of the particular construction method employed, in the end each results in the creation of a biologically viable singly modified DNA substrate. More important than the particular genome construction method, however, is whether a single-stranded (ss) or a double-stranded (ds) DNA vector is used to study adduct mutagenesis.

Although a duplex genome more closely represents the true environment of a DNA adduct in a cell, ds DNA substrates can severely hinder the study of adduct mutagenesis *in vivo* for two reasons. First, the repair efficiency of a DNA adduct is

increased when situated in ds versus ss DNA. Since many repair systems, including the NER pathway (Sancar, 1996) and many glycosylases such as endonuclease III (Breimer and Lindahl, 1984), have been shown to remove preferentially adducts from DNA duplexes, a lesion may be repaired prior to mutation fixation. Second, some singly modified genomes, particularly those containing bulky, helix-distorting lesions exhibit strand bias during DNA replication that results in favored recovery of progeny from the non-adduct containing strand (Koffel-Schwartz et al., 1987). Since progeny DNA would primarily be generated from the unmodified strand, the measured mutation frequency of an adduct would be greatly diminished. To eliminate the complications of strand bias and DNA repair, ss genomes are often used for mutagenesis studies.

The combined efforts of organic synthesis and recombinant DNA techniques have enabled the construction of both short oligonucleotides and entire genomes containing a single modified DNA base, and these DNA substrates have been used to elucidate the biological properties of many DNA adducts. In this chapter, the synthesis and characterization of short oligonucleotides containing a single 5-OH-dC, 5-OH-dU or dUg lesion shall be described, followed by the construction and characterization of singly modified bacteriophage genomes.

B. METHODS

1. Materials

Restriction enzymes and bacteriophage T4 polynucleotide kinase were from New England Biolabs. Sephadex G-50 Quick Spin Columns were from Boehringer Mannheim. Bacteriophage T4 DNA ligase and exonuclease III were from Pharmacia. Uracil DNA glycosylase (UDG) was from GIBCO/BRL. [γ^{32}P]dATP (6000 Ci/mmol) was from New England Nuclear. Plasmid preparation kits were from Qiagen. Bacteriophage M13mp7L2 was from C. W. Lawrence (Banerjee et al., 1990). OsO_4 (4% solution in H_2O) was from Sigma.

2. Preparation and Purification of Modified Oligonucleotides.

Novel phosphoramidite analogs of 5-OH-dC and 5-OH-dU were synthesized by Marshall L. Morningstar (Morningstar et al., 1997; see also, Fujimoto et al., 1997 and Romieu et al., 1997). The 5-OH-dC and 5-OH-dU phosphoramidites were used to prepare oligonucleotides of the sequence 5'-CAXGCAG-3', where X is 5-OH-dC or 5-OH-dU, on an Applied Biosystems 391 DNA Synthesizer (Figure 7). To protect the modified nucleosides during solid-phase DNA synthesis, the exocyclic hydroxyl group of the 5-OH-dU nucleoside was protected with an acetyl group, and the exocyclic amino and hydroxyl groups of 5-OH-dC were protected with benzyl groups. Following synthesis, the resin bound oligonucleotides were deprotected by treatment with NH_4OH for 5 hr at 60°C, filtered, and then concentrated to dryness. 5-OH-dC and 5-OH-dU oligonucleotides were purified by C18 RP-HPLC. The collected fractions were further

purified by 23% denaturing gel electrophoresis and the oligonucleotides were extracted from the gel by the Crush and Soak method (Maniatis et al., 1989).

The dUg containing oligonucleotide was prepared by oxidation of the corresponding uridine containing oligonucleotide with OsO_4 (Wang and Essigmann, 1997). Three hundred μg of 5'-CAUGCAG-3' (0.5 mM final concentration) were mixed with 6 mg OsO_4 (from a 4% solution in H_2O; 93 mM final concentration) in a buffer of 20 mM NH_4Cl adjusted to pH 9.0 with NH_4OH. The total volume of the reaction was 253 μl. The sample was incubated at 55°C for 30 min, on ice for 60 min, and then extracted 3 times with 250 μl ether, and centrifugally evaporated for 2 min to remove excess ether. The reaction mixture was purified by RP-HPLC on a C18 Beckman Ultrasphere column with a gradient of 0-10% B for 10 min and then 10-40% B for 60 min (A: 0.1 M NH_4OAc and B: 0.1 M NH_4OAc, 1:1 CH_3CN). Homogeneity of the oligonucleotide was confirmed by 23% denaturing gel electrophoresis.

3. Characterization of Modified Oligonucleotides.

Oligonucleotides of the sequence 5'-CAXGCAG-3' where X is dC, 5-OH-dC, 5-OH-dU or dUg were anlayzed by negative ion electrospray mass spectrometry (ES-MS) by Dr. J. Wishnok of the Division of Toxicology Mass Spectrometry Facility. dC, 5-OH-dC and 5-OH-dU containing oligonucleotides were further characterized by enzymatic digestion to the corresponding nucleosides. 10 μg of oligonucleotide, 1μl of 1 M NaOAc (pH 5.0), 1 μl of 20 mM $ZnCl_2$, and 1 μl of nuclease P1 (1.7 U/μl, Sigma) were incubated at 37°C for 1 hr. The reaction was cooled on ice and then 1.3 μl of 1 M Tris-HCl (pH

9.0), 1.0 μl snake venom phosphodiesterase (0.05 U/μl, ICN) and 1 μl of alkaline phosphatase (25 U/μl, Sigma) were added. After a 90 min incubation at 37°C, the sample was analyzed by C18 RP- HPLC using a photodiode array detector.

4. Construction ss M13mp7L2 Genomes

Before genome construction was attempted, it was essential to devise conditions that would maintain the integrity of the modified nucleosides. The integrity of 5-OH-dC, 5-OH-dU and dUg was assessed by subjecting the 7-mer oligonucleotides to the following conditions: 500 ng oligonucleotide were dissolved in a buffer of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ and 50 μg/ml BSA. After incubation at 16°C for 105 min, 65°C for 10 min and 100°C for 2.5 min, the sample was analyzed by C18 RP-HPLC with a gradient of 0-30% B over 60 min (A: 0.1 M NH_4OAc and B: 0.1 M NH_4OAc, 1:1 CH_3CN). While integrity of 5-OH-dC and 5-OH-dU was maintained, degradation of the dUg containing oligonucleotide was observed under these conditions, and thus an alternative method for genome construction was required to construct the dUg containing genome. Success was achieved with 500 ng of dUg containing oligonucleotide using the same buffer of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ and 50 μg/ml BSA, but incubating only at 16°C for 2 hr 15 min. The dUg sample was analyzed by C18 RP-HPLC as described above.

M13mp7L2 ss DNA was obtained by infection of *E. coli* cells with the phage DNA. To 500 ml 2xYT were added 5 ml of an overnight GW5100 cell culture (one colony grown in 5 ml media) and one phage plaque. Following a 9 hr incubation at 37°C

with shaking, the cell suspension was incubated on ice for 10-15 min, and then the cells were pelleted by spinning at 10,000 x g for 30 min. To precipitate the phage, the supernatant (500 ml) was combined with 20 g PEG and 14.6 g NaCl and stirred overnight at 4 °C. The phage were pelleted by spinning at 10,000 x g for 30 min, and then resuspended in TE buffer (10 mM Tris•HCl, 1 mM EDTA pH 8.0). Following phenol extraction and ethanol precipitation, the ss DNA was resuspended in 0.7 M K_2HPO_4/KH_2PO_4 buffer (8 ml) and loaded onto a hydroxylapatite column. The ss DNA was eluted from the column with 0.14 M K_2HPO_4/KH_2PO_4 buffer. 1 ml aliquots were collected in tubes containing 10 µl of 0.5 M EDTA, and 3 µl of each fraction were spotted on an agarose plate containing ethidium bromide (22.5 ml H_2O, 0.2 g agarose, 2.5 ml 10x TBE [TBE buffer: 89 mM Tris base, 89 mM H_3BO_3, 2 mM EDTA pH 8.0] and 1.25 µl ethidium bromide [10 mg/ml]). The fractions containing ss DNA (#3-9) were combined and dialyzed into TE buffer as follows using freshly made buffer each time: 30 min in 1 l TE, 30 min in 1 l TE, overnight in 2 l TE and 1 hr in 1 l TE. The DNA was then butanol extracted twice and then ethanol precipitated and resuspended in TE buffer.

M13mp7L2 ss DNA (130 ng/µl) was linearized by restriction enzyme digestion with *EcoRI* (1.7 U/µl) in a buffer containing 50 mM NaCl, 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 100 µg/µl BSA for 2 hr at 23 °C (Figure 8). The linear genome was diluted 1.5-fold with H_2O (90 ng/µl), and the 47-mer oligonucleotide scaffold was annealed to the linear ss DNA by heating at 80 °C for 5 min and allowing the reaction to cool to room temperature over overnight. The resulting circular M13 molecule contained a 7-base gap that was complementary to the sequence 5'-CACGCAG-3'. Gapped duplex

formation was confirmed by the conversion of ss linear DNA to ss circular DNA as analyzed by electrophoresis on a 1% agarose gel at 120 V for 5 hr in TBE buffer. The gel was stained in ethidium bromide (1 μg/μl) for 1 hr and photographed. The circular gapped-duplex yield was estimated by visual inspection.

Approximately 1 pmol of unmodified (dC), 5-OH-dC, 5-OH-dU and dUg 7-mer oligonucleotide was phosphorylated in a total volume of 10 ml by using T4 polynucleotide kinase (10U) and 1 mM ATP in a buffer of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ and 50 μg/ml BSA for 45 min at 16°C. Oligonucleotides were ligated into an equimolar amount of the freshly prepared 7-base gapped molecules (8 pmol/ml) by using T4 DNA ligase (0.05 Weiss units/μl) for 1 hr at 16°C. The 47-mer oligonucleotide scaffold was removed by heating the sample at 100°C for 2 min in the presence of a 2-fold molar excess of the 47-mer scaffold's complement, resulting in the construction of a uniquely modified, ss circular genome.

Due to the lability of the dUg lesion, a modified genome construction scheme was employed (Bailey et al., 1996a) (Figure 8). M13mp7L2 ss DNA was linearized as described above. The circular M13 molecule containing a 7-base gap was constructed as described except that a uracilated 47-mer oligonucleotide scaffold was used. The dUg containing oligonucleotide was removed by incubation with UDG (0.04 U/μl) and exonuclease III (0.4 U/μl) for 90 min at 16°C.

Finally, singly modified genomes were spun at 2,000 x g for 4 min through a G-50 Quick Spin column that was pre-equilibrated with H_2O; the genomes eluted in the void volume. A 30 μl portion of each genome preparation mixture was examined by gel

electrophoresis, and the presence of completely ligated genome was confirmed by comigration with ss circular DNA.

5. Characterization of ss M13mp7L2 Genomes

Singly modified genomes were constructed and characterized by restriction enzyme digestion in order to assess the amount of oligonucleotide fully ligated in the genome (Bailey et al., 1996a). The genomes were constructed as previously described except that the singly modified heptamers were phosphorylated by using [γ^{32}P]dATP. Following ligation, the genomes were digested with *HinfI* (100 U) and *HaeIII* (75 U) in a buffer of 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM DTT (pH 7.5) at 37°C for 1.5 hr. The digestion products were then electrophoresed through a denaturing 23% polyacrylamide gel. In parallel, a known amount of the labeled oligonucleotides was also electrophoresed. Ligation efficiencies were approximated by comparing the amount of radioactivity in the bands from the digested sample to that of the known amount of oligonucleotide.

C. RESULTS AND DISCUSSION

1. Preparation and Purification of Modified Oligonucleotides

Two factors influenced the selection of an oligonucleotide sequence for this study. First, the synthetic method used to construct the dUg containing oligonucleotide precluded the presence of dT in the sequence of the oligonucleotide, since OsO_4 reacts with both dT and dU (Beer et al., 1966) and the presence of dT in the oligonucleotide would result in the formation of oxidized thymidines as well as oxidized uridines. Second, the lesion had to exist within a unique restriction site such that mutations induced by the lesion would render the site refractory to digestion, thus providing a means to separate mutant sequences (DNA unaltered by digestion) from wild-type (DNA linearized by digestion) sequences. It was impossible to design an oligonucleotide composed of only dC, dG and dA that would create a unique restriction site in M13mp7L2. Therefore, it was necessary to combine nucleotides from the M13mp7L2 genome with those from the oligonucleotide to establish a new restriction site. Three bases on the 3' end of the *EcoRI* digested M13mp7L2 were combined with three bases on the 5' end of the oligonucleotide to create an unique *ApaLI* restriction site, 5'-GTGCAC-3', where the underscored C represents the site of modification (Figure 9). It is important to note that by using *ApaLI* restriction digestion for mutant selection in this system, only mutations induced at, and up to five bases 5' of, the site of modification were selected.

Oligonucleotides of the sequence 5'-CAXGCAG-3' where X is either 5-OH-dC or 5-OH-dU were prepared on a 1-μmole scale by automated solid phase DNA synthesis. All couplings, including those with the modified phosphoramidites, were >97% as

indicated by release of trityl cation (Morningstar et al., 1997). Following DNA synthesis, the modified oligonucleotides and a control oligonucleotide, where X is dC, were deprotected in concentrated aqueous ammonia at 60°C for 5 hr. The deprotected oligonucleotides were subsequently purified by using C18 RP-HPLC.

The lability of dUg precluded the synthesis of a singly modified oligonucleotide through standard solid phase synthesis techniques. To generate a dUg containing oligonucleotide, a uracilated 7-mer of the sequence 5'-CAUGCAG-3' was synthesized and chemically oxidized with OsO_4 to create a dUg modified oligonucleotide (Wang and Essigmann, 1997). Reaction of OsO_4 with DNA is known to give exclusively *cis*-glycols (Schröder, 1980), and it is likely that oxidation of the oligonucleotide 5'-CAUGCAG-3' produces a mixture of the two diastereomers (5*S*, 6*R* and 5*R*, 6*S*) of *cis*-dUg as has been reported for the $KMnO_4$ oxidation of dT to dTg (Teebor et al., 1987). The oxidation reaction of dU to dUg did not proceed in high yield (average yield was 14%) and a number of peaks corresponding to various oxidation products were observed; however, the major oxidation peak was easily separated and corresponded to the dUg modified oligonucleotide. Facile separation is an advantage of short modified oligonucleotides.

2. Characterization of Modified Oligonucleotides

The molecular weight of 5-OH-dC, 5-OH-dU and dUg containing heptamer oligonucleotides was confirmed by negative ion ES-MS. This procedure involves gentle ionization of an oligonucleotide to prevent fragmentation, thus producing molecular ions with one or more charge. For example, a 7-mer oligonucleotide can exist in six different

charge states corresponding to the six phosphodiester groups on the DNA backbone. The instrument measures the signal of [(m-z)/z], where m is the molecular weight of the oligonucleotide and z is the number of charges on the phosphodiester backbone, and one can thereby back-calculate the molecular weight of the oligonucleotide at zero charge. The ES-MS results for the oligonucleotides are shown in Figure 10 and Table 3.

Quantitative assessment of the base composition of the 5-OH-dC and 5-OH-dU modified oligonucleotides was performed by HPLC integration of the nucleoside peaks following digestion of the oligonucleotides to their corresponding nucleosides (Figure 11). Standard curves of the amount of nucleoside versus peak area were constructed and used to quantitate the amount of each nucleoside present in the digested sample. The nucleoside peaks for the oligonucleotides were observed in the correct ratios based upon integration of the peak areas at both 254 and 280 nm. HPLC analysis additionally showed that the modified nucleosides co-eluted and had identical UV spectra to those of standards.

The use of ammoniacal deprotection of the 5-OH-dC and 5-OH-dU oligonucleotides could have presented a complication in that it recently has been observed that 5-acetoxy and 5-benzoyloxy nucleosides can undergo, albeit as a minor reaction, an addition/elimination reaction to yield 5-amino-substituted nucleosides (Yu et al., 1993). In the present study, oligonucleotides containing 5-OH-dC and 5-OH-dU would be partially converted to 5-amino-2'-deoxycytidine (5-NH_2-dC) and 5-amino-2'-deoxyuridine (5-NH_2-dU), respectively. To determine if these side reactions were significant events, 5-NH_2-dC and 5-NH_2-dU were synthesized by M. Morningstar from

the corresponding 5-bromo derivatives (Ferrer et al., 1996; Morningstar et al., 1997). C18 RP-HPLC analysis revealed that the 5-amino derivatives of dC and dU had retention times that would easily allow their detection if they were contaminants in enzymatic hydrolysates of 5'-CA(5-OH-C)GCAG-3' and 5'-CA(5-OH-U)GCAG-3' (Figure 12). The absence of the 5-amino nucleosides (detection limit, approximately 1%) suggests that the amination reaction is rare, if indeed it occurs at all, under the conditions used here.

Characterization of the dUg oligonucleotide confirmed that it possessed physical properties consistent with that of an oxidized product. The dUg heptamer eluted earlier by C18 RP-HPLC and had retarded mobility during electrophoresis through a denaturing polyacrylamide gel compared to the dU containing oligonucleotide.

3. Stability of Modified Oligonucleotides

To assess the stability of the lesions, the modified oligonucleotides were subjected to the conditions used for genome construction. The heptamers were incubated under the reaction temperatures and in the buffers used for genome construction, and then the samples were analyzed by C18 RP-HPLC (Figure 13). Under these conditions, no detectable degradation of the 5-OH-dC and 5-OH-dU modified oligonucleotides was observed. However, the dUg containing oligonucleotide was not stable under these conditions and was found to suffer approximately 43% degradation. Based on earlier degradation studies of a dUg containing pentamer, degradation of the oligonucleotide most likely involves depyrimidination of the lesion and fragmentation at the AP site. Because of the lability of the dUg containing oligonucleotide, it was necessary to employ

milder conditions for the construction of a dUg containing genome. As discussed previously, the milder genome construction method required that the lesion only be incubated at 16°C for 2 hr 15 min (Bailey et al., 1996a). Under these conditions, no detectable degradation of the dUg containing oligonucleotide was observed.

4. Construction of ss M13mp7L2 Genomes

Single-stranded M13mp7L2 DNA was obtained by isolating phage from GW5100 *E. coli* cells. Following phenol extraction and ethanol precipitation, purification of the ss DNA through a hydroxylapatite column was found to be a critical step in the genome construction process; DNA not purified by using such a column was found to degrade, specifically during digestion by *EcoRI* and heat annealing to form a gapped molecule. While hydroxylapatite columns are primarily used to separate ds from ss DNA, it is believed that during the passage of the sample through the column, proteins that degrade DNA may also be removed.

Purified M13mp7L2 ss DNA was digested with *EcoRI*, and the linearized DNA was annealed to a 47-mer scaffold oligonucleotide that brought the ends of the linearized DNA to within 7 nucleotides of one another. The gapped genomes were analyzed by agarose gel electrophoresis, and a typical yield for gapped genome formation was 50%, with 50% linear M13mp7L2 remaining. Subsequently, the 5'-phosphorylated heptamer containing either dC, 5-OH-dC, 5-OH-dU or dUg was ligated into the gapped molecule. Removal of the 47-mer scaffold by heat denaturation, or of the uracilated 47-mer scaffold by UDG and exonuclease III, yielded the ss modified genomes.

It was important to completely remove the scaffold from the ss genomes to ensure replication of the lesion following transfection of the genomes into *E. coli*. Although untested, the possibility that the DNA replication machinery of the cell could use the scaffold as a primer for DNA synthesis did exist. In the case of the unmodified, 5-OH-dC and 5-OH-dU genomes, removal of the scaffold was accomplished by the addition of a 2-fold molar excess of a 47-mer oligonucleotide complementary to the scaffold. Following heat denaturation of the scaffold from the circular genome, it was believed that if reannealing were to occur, it would preferentially do so with the excess complementary DNA strand, and not with the circular genome.

Because of the instability of dUg, the scaffold of the dUg containing genome was unable to be denatured by heating without compromising the integrity of the lesion. A milder genome construction scheme was used in which a uracilated scaffold was used to construct the gapped molecule (Bailey et al., 1996a). Following ligation of the modified oligonucleotide, the uracilated scaffold was removed by the concerted efforts of UDG and exonuclease III. Oligonucleotides containing dUg are refractory to the action of UDG.

5. Characterization of ss M13mp7L2 Genomes.

Singly modified genomes were constructed and characterized by restriction enzyme digestion in order to assess the ligation efficiency of the modified oligonucleotides (Figure 14). The restriction enzymes *HinfI* and *HaeIII* were used to excise a section of DNA surrounding the modified oligonucleotide; an *HinfI* site exists 8 nucleotides (nt) away from the 5' end of the oligonucleotide, and an *HaeIII* site is located

10 nt away from the 3' end of the oligonucleotide. If the modified heptamer were ligated on both its 5' and 3' ends, a 25 nt DNA fragment would be excised following restriction digestion. Likewise, ligation at only the 5' or 3' end would produce a 15 nt or 17 nt fragment, respectively. Any oligonucleotide not ligated into the gapped genome would run as a 7 nt fragment.

The efficiencies of genome construction for dC, 5-OH-dC, 5-OH-dU, and dUg were determined to be 85%, 80%, 44%, and 32%, respectively. Oligonucleotides containing dC or 5-OH-dC were ligated more efficiently than those containing oxidized uridines. One explanation for the increased efficiency is that during ligation of the oligonucleotides into the gapped genome, the lesions were placed opposite dG; perhaps ligation of 5-OH-dU and dUg would be more favorable opposite a template dA. In addition, the low ligation efficiency of the dUg containing heptamer could be compounded by the bulkiness of the lesion.

D. CONCLUSION

The work described above details the construction of biologically viable DNA substrates containing a single oxidized lesion. The procedures used enabled complete control over the DNA sequence surrounding the adduct and allowed the chemical purity of the lesion to be monitored at every stage. With the construction of single-stranded genomes containing either 5-OH-dC, 5-OH-dU or Ug within a specific *ApaLI* site, the stage was now set to examine lesion mutagenicity in *E. coli*.

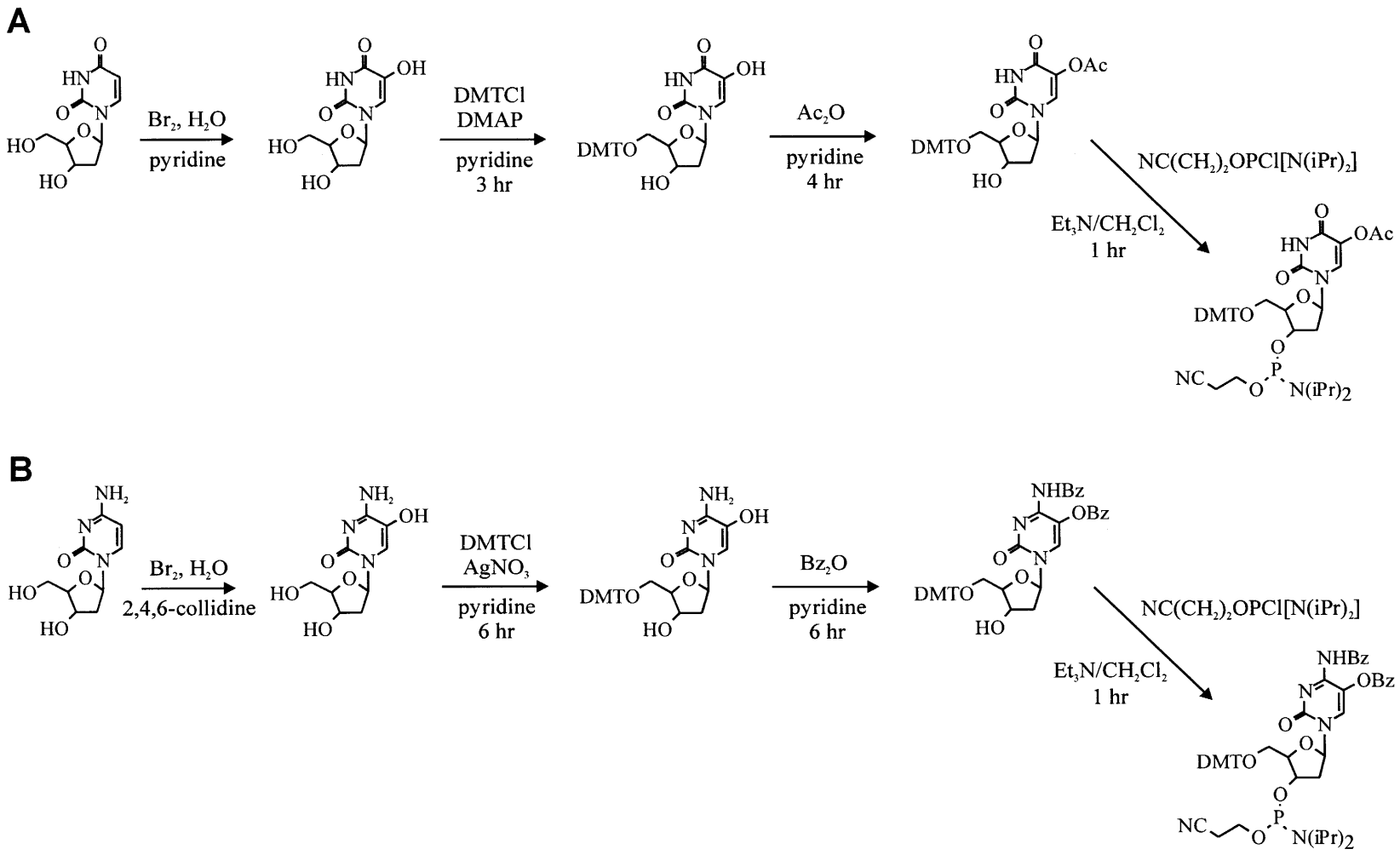


Figure 7: Synthesis of 5-OH-dU (**A**) and 5-OH-dC (**B**) phosphoramidites.

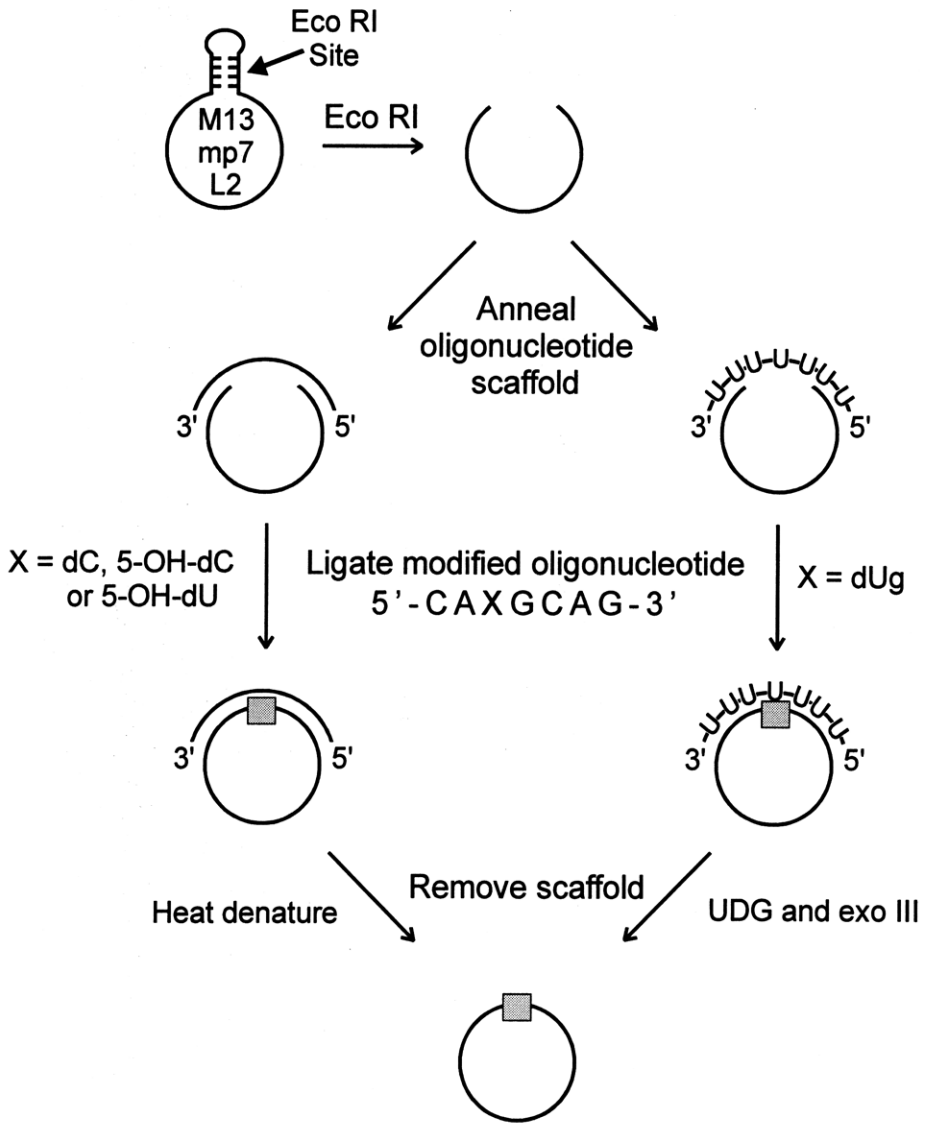


Figure 8: Construction of singly modified M13mp7L2 ss genomes. Bacteriophage M13mp7L2 was linearized by digestion of the *EcoRI* site located in a duplex hairpin region of the genome. An oligonucleotide scaffold with ends complementary to the ends of the linearized genome was annealed to the linear M13 molecule to form a circular construct with a gap complementary to the modified 7-mer oligonucleotide. The modified oligonucleotide was annealed into this gap and then ligated. The scaffold oligonucleotide was removed to yield a singly modified ss genome. Because of the instability of Ug, a milder construction method was employed.

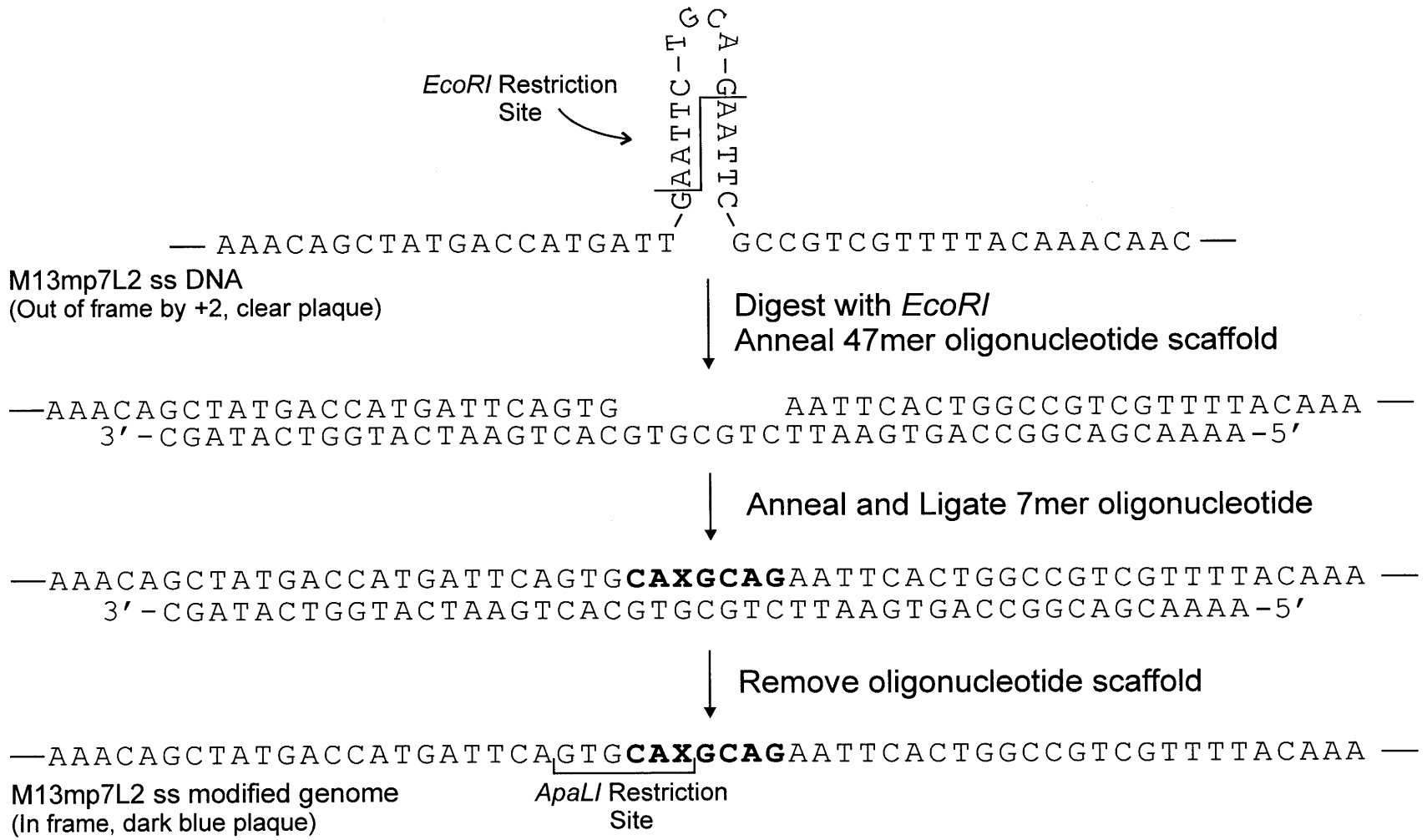


Figure 9: DNA sequence of the *lacZ* α gene containing the modified cytosine lesion.

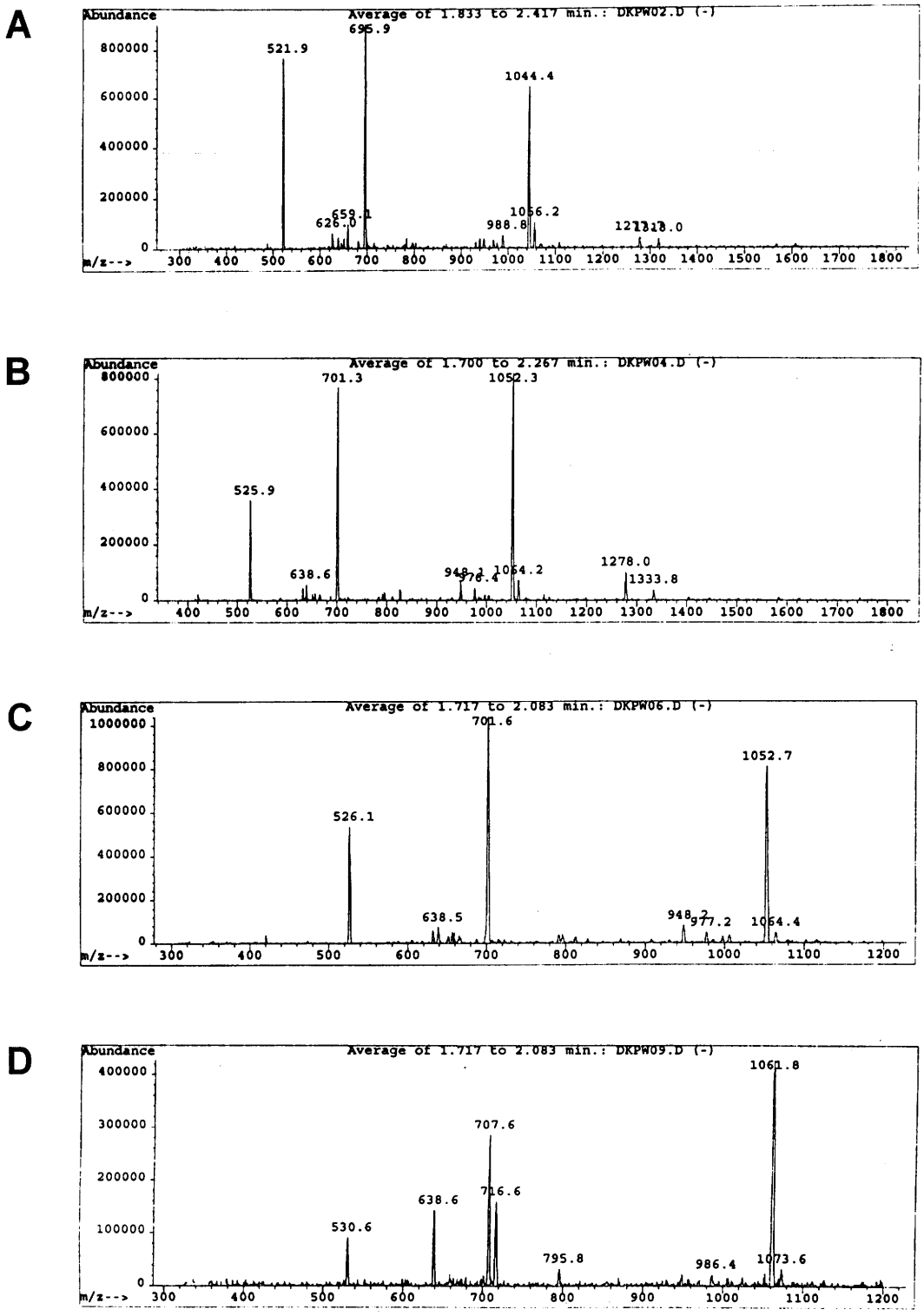


Figure 10: Negative ion ES-MS of the oligonucleotide, 5'-CAXGCAG-3'. (**A**) X = dC; (**B**) X = 5-OH-dC; (**C**) X = 5-OH-dU; (**D**) X = dUg.

Table 3: Results from negative ion ES-MS analyses[a] of the singly modified oligonucleotides, 5'-CAXGCAG-3', where X = dC, 5-OH-dC, 5-OH-dU or dUg.

CAXGCAG		m-z / z		
X	Molecular Weight	z = 2	z = 3	z = 4
dC	2090.9 (2091.4)[b]	1044.4 (1044.7)	695.9 (696.1)	521.9 (521.9)
5-OH-dC	2106.7 (2106.4)	1052.3 (1052.2)	701.3 (701.1)	525.9 (525.6)
5-OH-dU	2107.7 (2107.4)	1052.8 (1052.7)	701.6 (701.5)	526.1 (525.9)
dUg	2125.6 (2125.6)	1061.8 (1061.7)	707.6 (707.5)	530.6 (530.4)

[a] Analyses were performed by Dr. J. Wishnok.
[b] Numbers are presented as "observed (calculated)".

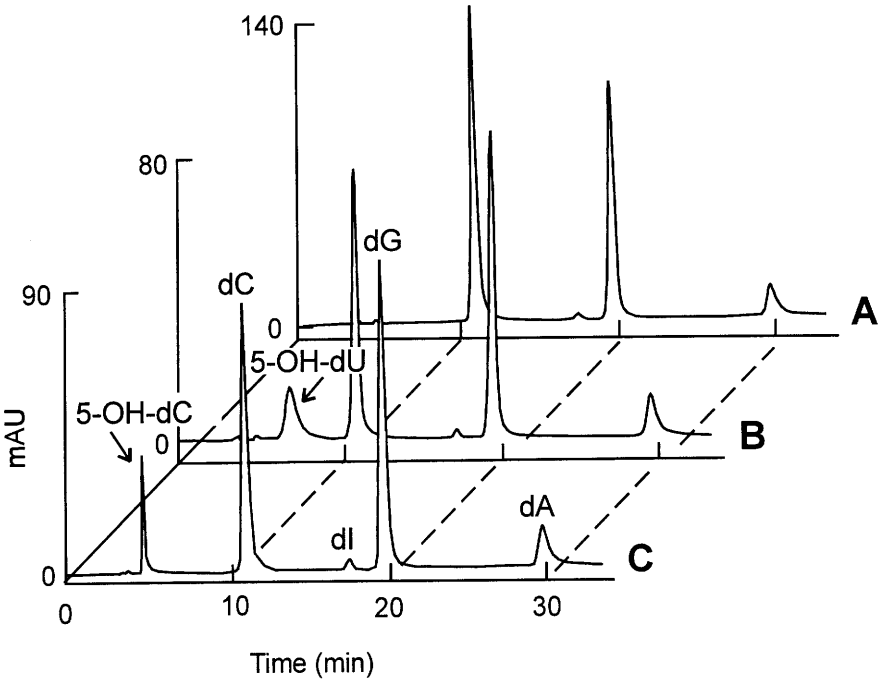


Figure 11: RP-HPLC (280 nm) of deoxynucleosides following digestion of 5'-CAXGCAG-3'. (**A**) X = dC; (**B**) X = 5-OH-dU; (**C**) X = 5-OH-dC. The labels indicate the identity of the peaks as determined by coincident retention times and identical UV spectrum with authentic samples. Retention times: 5-OH-dC, 4.5 min; 5-OH-dU, 6.9 min; dC, 11.1 min; dG, 19.9 min; dA, 30.0 min.

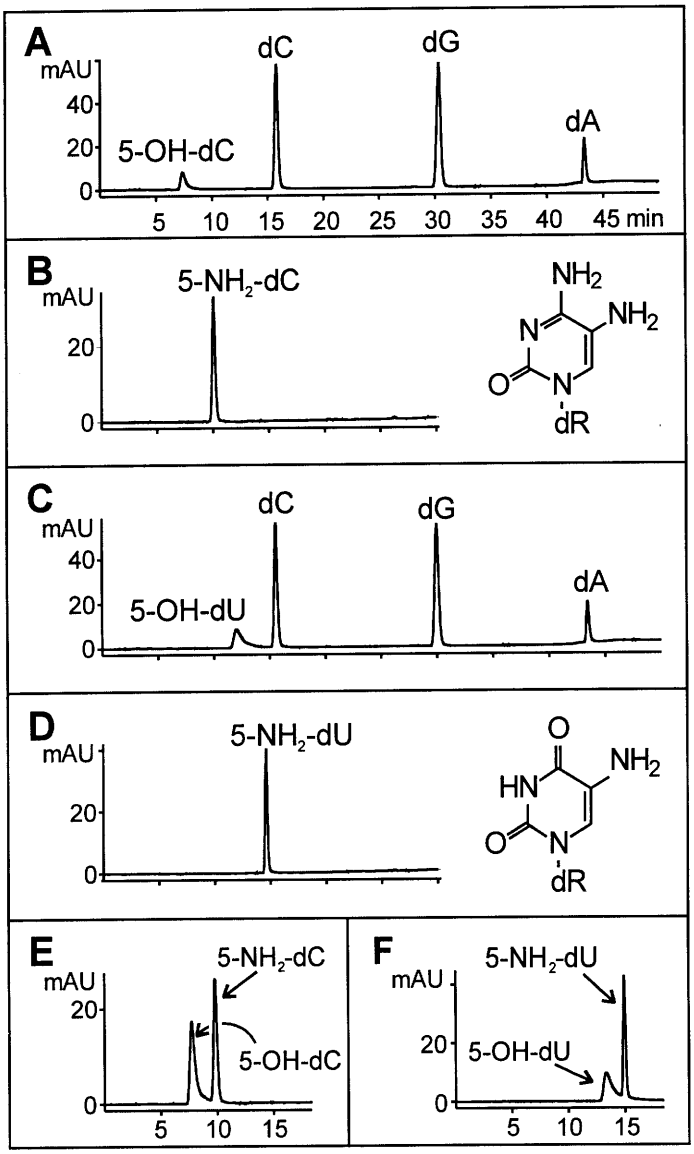


Figure 12: RP-HPLC analyses with detection at 280 nm: (**A**) deoxynucleosides following enzymatic digestion of 5'-CA(5-OH-C)GCAG-3'; (**B**) 5-NH_2-dC standard; (**C**) deoxynucleosides following enzymatic digestion of 5'-CA(5-OH-U)GCAG-3'; (**D**) 5-NH_2-dU standard; (**E**) coinjection of 5-OH-dC; 5-NH_2-dC; (**F**) coinjection of 5-OH-dU and 5-NH_2-dU. Retention times: 5-OH-dC, 7.4 min; 5-NH_2-dC, 10.0 min; 5-OH-dU, 12.0 min; 5-NH_2-dU, 14.6 min; dC 15.8 min; dG, 30.3 min; dA, 43.4 min.

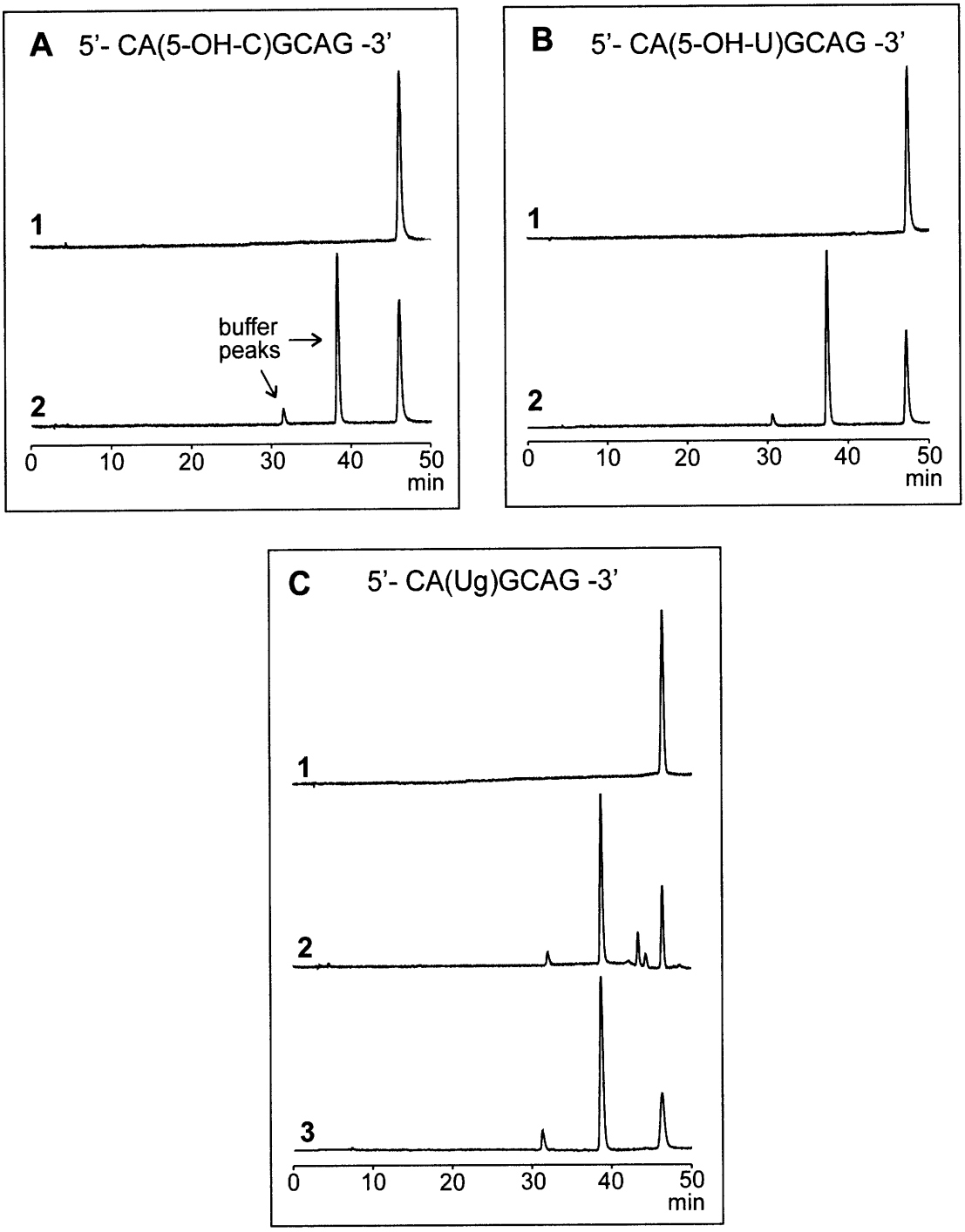


Figure 13: (**1**) RP-HPLC profiles of the 7-mer oligonucleotide, 5'-CAXGCAG-3' where (**A**) X = 5-OH-dC; (**B**) X = 5-OH-dU; (**C**) X = dUg. (**2**) The same oligonucleotide after incubation under standard genome construction conditions (16°C for 105 min, 65°C for 10 min and 100°C for 2.5 min). Under these conditions, 43% of dUg modified oligonucleotide was found to degrade. (**3**) The same oligonculeotide incubated under milder genome construction conditions (16°C for 135 min).

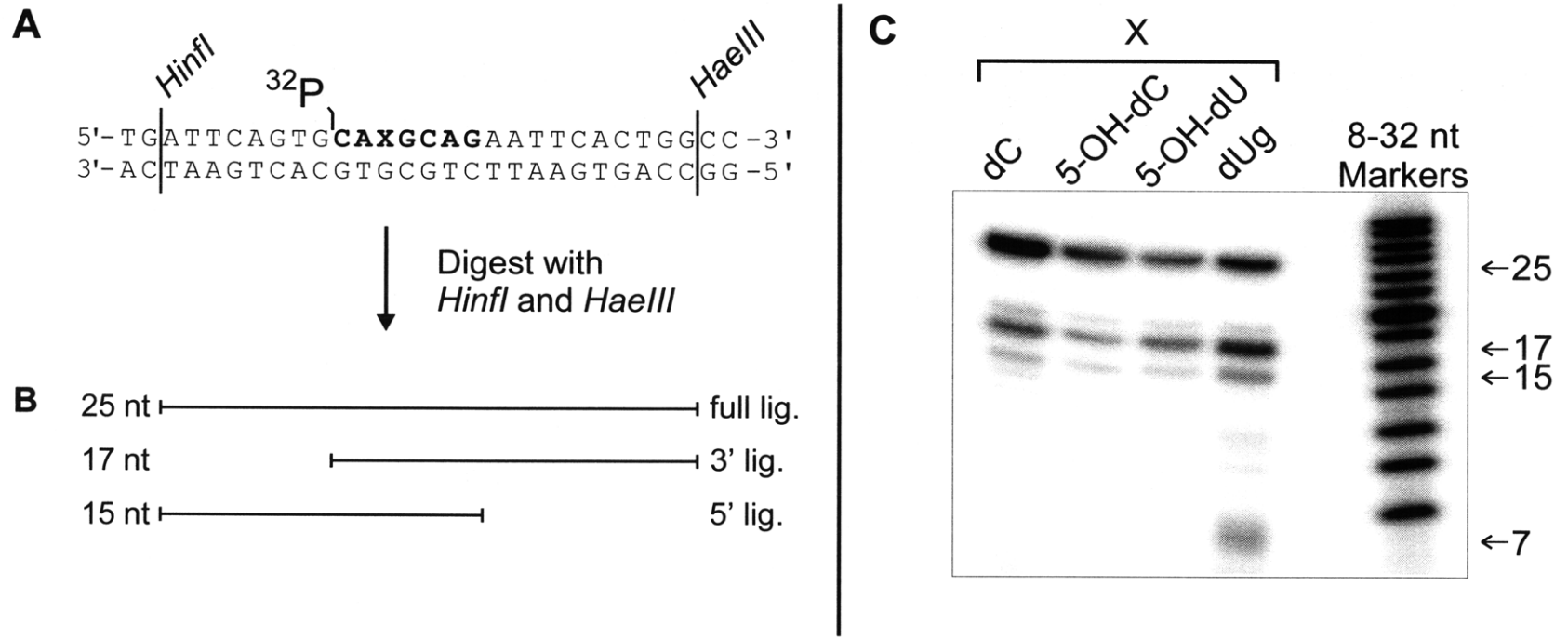


Figure 14: Restriction endonuclease characterization of singly modified genomes. **(A)** Map of the *HinfI* and *HaeIII* restriction sites. **(B)** Following digestion of the genomes with *HinfI* and *HaeIII*, complete 5' and 3' ligation of the 7-mers into the gapped molecule yielded a ^{32}P-labeled 25 nt fragment, whereas ligation on either the 5' or 3' side produced ^{32}P-labeled fragments of 15 and 17nt, respectively. **(C)** Phosporimage of the reaction products electrophoresed on a 23% denaturing polyacrylamide gel.

CHAPTER III:

Mutational Properties of 5-OH-dC, 5-OH-dU and dUg Bacteriophage Genomes in *Eschericia coli*

A. INTRODUCTION

The filamentous bacteriophages of *E. coli* consist of extra-chromosomal ss circular molecules of DNA that naturally occur in bacteria. Filamentous phages are useful molecular cloning vehicles for a number of reasons: (1) phage can tolerate the packaging of foreign DNA up to seven times the unit length of a single viral DNA molecule during replication, (2) bacteriophage do not lyse the host bacterium, and (3) phage DNA can be recovered in ss circular or ds circular forms (Maniatis et al., 1989). In Chapter 2, the construction of filamentous bacteriophage genomes containing a single oxidized cytosine lesion was described. This chapter shall focus on the replication of the singly modified bacteriophage genomes in *E. coli* and the determination of the mutation frequencies and specificities of 5-OH-dC, 5-OH-dU and dUg.

Filamentous phages, such as the M13mp7L2 strain used in this study, are thread-like viruses containing a ss copy of DNA approximately 6400 nucleotides in length (Figure 15). In order to replicate, filamentous phages must infect *E. coli* and, since these phages are male specific, they can only infect *E. coli* cells bearing hair-like F pili on the cell surface (for a review, see Kornberg and Baker, 1992). The first step in viral infection involves attachment of one of the minor phage coat proteins to the receptor at the tip of the F pilus of the host *E. coli*. Upon binding, the pilus is thought to retract, bringing the phage in contact with the bacterial cell surface. The coat proteins are removed from the phage particle and the ss circular DNA, the (+) strand of the phage DNA, is inserted into the cell (Figure 16).

Phage DNA replication occurs in three stages within the infected cell. In stage

one (complementary strand synthesis), a complementary (-) strand of phage DNA is synthesized. This synthesis converts the infecting ss circular DNA, the (+) strand, into ds replicative-form (RF) DNA. Synthesis of the (-) strand begins at the (-) strand origin and is carried out by the host *E. coli* replicative enzymes. In the second stage of replication, the intracellular pool of RF DNA is increased by the sequential action of both replication origins. Rolling-circle type replication from the (+) strand origin is followed by conversion of the progeny ss circles to double strands by replication starting and the (-) strand origin. The resulting RF DNA molecules are intermediates in DNA synthesis and transcription templates for the synthesis of the phage-encoded proteins. The third stage of replication (single-strand production) takes place late in infection. This stage of DNA replication is asymmetric because the (-) strand origin functions at a reduced level late in the infection and therefore mainly (+) strands are produced. These ss circles, (+) strands, are packaged into phage particles for export instead of being converted into RF DNA. The protein coat that protects phage DNA is composed of two proteins: (1) the product of gene VIII which is the major structural protein of bacteriophage particles that forms a filamentous rod, and (2) the product of gene III which is a minor coat protein that is located at one end of the rod. Thus packaged, the (+) strand viral particles are prepared for further infection of bacteria. Ordinarily, infection with filamentous phages is not lethal and the host cells do not lyse, although their growth rate slows approximately 2-fold.

All genes of filamentous bacteriophages are essential, but there is an approximately 500 nucleotide region located between genes II and IV into which

segments of foreign DNA may be inserted (Figure 15). The mp series of bacteriophage M13 vectors were developed by Messing and co-workers to contain a short segment of *E. coli* DNA in this intergenic region (Messing, 1983). Construction of these vectors allowed for the use of a simple color test, known as an α-complementation assay, to distinguish between vectors that carry a segment of foreign DNA and those that do not.

The α-complementation assay was first developed by Ullmann, Jacob and Monod in 1967 (Ullmann et al., 1967), and is based upon the ability of a cell to synthesize the β-galactosidase (β-gal) protein. Normally this protein serves to cleave lactose into glucose and galactose, which the cell can then utilize for energy production. However, expression of β-gal can be induced in the presence of IPTG (isopropylthio β-D-galactoside), and the protein is capable of cleaving other lactose analogs such as X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside); the color component of this assay arises from cleavage of X-gal by β-gal resulting in the formation of a chromogenic compound that is blue in color.

The monomer of the β-gal protein can be dissected into two parts: the N-terminal, called the α-fragment, and the C-terminal, referred to as the ω-fragment. Ullmann et al. showed that a cell bearing any number of deletions of the 5' end of the *lacZ* gene synthesized an inactive ω-fragment of β-galactosidase, and that a cell bearing a deletion of the 3' end of *lacZ* encodes an inactive α-fragment (Ullmann et al., 1967). However, if a cell contained two genes, one directing the synthesis of the α-fragment and the other directing synthesis of the ω-fragment, the two fragments were able to complement one another and β-gal activity was restored.

Many vectors contain the *lac* α gene sequence, and exploitation of these vectors for α-complementation requires the use of a bacterial strain carrying the *lac* ω gene fragment which is usually harbored on the extra-chromosomal F' plasmid (Figure 17). The vectors of the M13mp series contain densely packed restriction sites in the *lac* α sequence, enabling their further usefulness in molecular cloning. The presence of this polycloning region has little effect on the ability of the β-gal peptide to carry out α-complementation. Thus, M13mp vectors plated on bacterial hosts carrying the appropriate F' episome will form blue plaques when the medium contains IPTG and X-gal. Insertions of additional DNA into the polycloning site can shift the peptide sequence out of frame, and thus destroy α-complementation. Plaques generated from these recombinant bacteriophages are colorless when grown in the presence of IPTG and X-gal.

The experiments of this chapter were performed using ss M13mp7L2 bacteriophage genomes into which was inserted a specific oligonucleotide containing a single 5-OH-dC, 5-OH-dU or dUg. The lesion was situated within a unique restriction site and the oligonucleotide was itself situated within the α-fragment of the *lacZ* gene. In this chapter, the following experiments shall be described: (1) Transfection and replication of the singly modified genomes in wild-type *E. coli*, (2) Selection of phage containing the modified oligonucleotide and production of RF DNA, (3) Selection of mutant from wild-type DNA by restriction enzyme digestion, and (4) Determination of the mutation frequencies and specificities of 5-OH-dC, 5-OH-dU and dUg.

B. MATERIALS AND METHODS

1. Materials

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were from Gold Biotechnology. [α^{35}S]dATP and Sequenase version 2.0 sequencing reagents were from Amersham. The cell line used for transfection was DL7 (AB1157; (*lac*) ΔU169) (Lasko et al., 1988) and for plating was GW5100 (JM103, P1-) from Graham Walker (MIT).

2. Transfection of ss M13mp7L2 Genomes into *E. coli*

E. coli DL7 cells were prepared for transfection by electroporation as described (Wood et al., 1990; Bailey et al., 1996b). Briefly, 2 ml of an overnight culture of DL7 cells were diluted in 100 ml LB media and incubated with shaking at 37°C to a density of $8x10^8$ cells/ml. The cells were cooled on ice for 10-15 min and then spun at 4,000 x g for 5 min. (Note: all cell spins were performed at 4°C.) Next, the cells were resuspended in an equal volume of ice cold sterile deionized H_2O (100 ml) and then immediately spun at 8,000 x g for 10 min. Finally, the cells were resuspended in 1/2 volume cold H_2O (50 ml) and then spun at 13,000 x g for 10 min. The cells were resuspended in 1-2 ml cold H_2O and then used directly for transfection.

A 190 µl portion of cell suspension was added to 5-10 µl of the DNA solution containing the prepared genomes. The mixture was transferred to a cold Bio-Rad gene pulser cuvette (0.2 cm) and electroporations were performed with a BTX electro cell

manipulator 600 system set at 50 mF and 129Ω. The electroporation field strength optimal for cell survival was 12.5 kV/cm. Immediately after electroporation, 1 ml of room temperature SOC medium (Hanahan, 1985) was added and a portion of the bacterial suspension was plated in the presence of GW5100 cells, X-Gal and IPTG to determine the number of infective centers. Following plating, the remaining electroporation mixture was incubated for 2-3 h at 37°C, after which the progeny phage were isolated from the supernatant.

3. Mutant Enrichment Protocol

The pooled progeny phage were used to produce RF DNA by using the Qiagen midi-plasmid purification system. RF DNA was purified by 0.6 % agarose gel electrophoresis in TAE buffer (40 mM Tris-CH_3CO_2, 2 mM Na_2 EDTA, pH 8.5) for 1 hr at 100 V. The RF DNA band was visualized by soaking the gel in a 1 μg/ml solution of ethidium bromide. The band was excised from the gel and the DNA was extracted by electroelution with Centricon 10s.

The pool of purified RF DNA was enriched for mutants by digestion with the restriction enzyme, *ApaLI*. Equimolar amounts of RF DNA (250 ng) were treated either in the presence or absence of *ApaLI* (20 U/μl) at 37°C for 4 h in a buffer consisting of 50 mM $K(CH_3CO_2)$, 20 mM Tris-CH_3CO_2, 10 mM $Mg(CH_3CO_2)_2$, 1 mM DTT and 100 μg/ml acetylated BSA. The two fractions, labeled *ApaLI*+ and *ApaLI*-, respectively, were diluted to 100 μl and 10 μl (12 ng of DNA) were used to transfect 190 μl of *E. coli* DL7

cells by electroporation as described above.

4. Determination of Mutation Frequency

The mutation frequency was defined as the ratio of mutant to wild-type plus mutant progeny. The ratio of infective center plaques produced from the *ApaLI+* fraction to the *ApaLI-* fraction gave the percentage not digested by *ApaLI*, the first approximation of mutation frequency. Individual plaques from the *ApaLI+* fraction were sequenced by the Sanger dideoxy method to determine the mutational specificity of each lesion (Sanger et al., 1977). Since the *ApaLI+* fraction included some wild-type DNA that had evaded digestion (as determined by sequence analysis), the true mutation frequency was determined by multiplying the percentage not digested by *ApaLI* by the ratio of mutants sequenced.

C. RESULTS AND DISCUSSION

1. Transfection of ss M13mp7L2 Genomes into *E. coli*

The modified genomes were transfected into DL7 *E. coli* cells (Figure 18). The DL7 strain is what our laboratory refers to as a wild-type cell strain (i.e., it has no known repair or replication defects). As compared to other strains (e.g., AB1157), it contains a chromosomal *lac* gene deletion preventing its interference with the α-complementation assay between the vector and the GW5100 cells. Following transfection, the cell/DNA/phage mixture was plated with GW5100 *E. coli* cells in the presence of IPTG and X-Gal. This cell strain contains a full chromosomal *lac* gene deletion, but carries an extra-chromosomal copy of the ω-fragment of the β-gal gene, and can therefore be used along with the appropriate vector in an α-complementation assay. DNA used in the above transfections gave rise to dark-blue and colorless plaques. Dark-blue plaques resulted from restoration of the M13mp7L2 *lacZ* reading frame (which is normally out of frame by +2) upon ligation of the 7-mer oligonucleotide. The sequence context of the system was such that both wild-type and mutant progeny exhibited a dark-blue phenotype. As determined by sequence analysis, the colorless plaques resulted from undigested parental M13mp7L2 DNA.

Neither 5-OH-dC, 5-OH-dU nor dUg appear to be cytotoxic lesions since the numbers of infective centers generated from transfection of similar amounts of fully ligated genomes are roughly the same. For each lesion, progeny phage representing a total of 50,000 independent transformational events (dark-blue plaques) generated in at

least 9 transfections and at least 3 separate genome construction experiments were pooled for further analysis

2. Enrichment of Mutant DNA by Restriction Enzyme Digestion

The M13mp7L2 genomes were constructed such that the oxidized cytosine was situated within a unique *ApaLI* restriction sequence. If replication past the lesion did not result in mutation, the *ApaLI* restriction sequence would remain intact and, upon digestion with the enzyme and transfection into *E. coli*, the DNA molecule would be linearized and biologically inactivated. If, however, a mutation did occur, the DNA molecule would be refractory to cleavage by *ApaLI* and hence retain its ability to replicate in *E. coli* and form a plaque.

A population of progeny RF DNA generated from unmodified (dC), 5-OH-dC, 5-OH-dU and dUg containing genomes was cleaved by *ApaLI* indicating that none of the lesions were 100% mutagenic in this system. The percentage of DNA not cleaved by *ApaLI* provided an initial estimate of the mutation frequency of each lesion. In the case of 5-OH-dU and dUg, these initial estimates (83% and 80%, respectively) were accurate. However, in the case of the unmodified and 5-OH-dC containing genomes, these estimates (0.08% and 0.09%, respectively) were higher than the true mutation frequencies (0.003% and 0.05%, respectively). This discrepancy arises because the *ApaLI* enzyme is only 99.9% efficient under these conditions, and thus approximately 0.1% of the dC and 5-OH-dC genomes were left uncleaved not because of a mutation but because of enzyme inefficiency. The true mutation frequencies were thus the percentage of uncleaved DNA

multiplied by the percentage of mutant genomes in that DNA population.

3. Determination of Spontaneous Mutation Frequency

The background mutation level of the experiment was defined by the observed mutation frequency of the control genome containing unmodified cytidine (Table 4). Keeping in mind that only the 6 bases comprising the *ApaLI* restriction site could be monitored, the spontaneous mutation frequency in this system was found to be 0.003%, which corresponds to 3 mutational events per 100,000 bases or 3×10^{-5}. The handling of the unmodified oligonucleotide (heating, air exposure) and the genetic engineering techniques used for genome construction may contribute to the spontaneous mutation frequency as observed in this study. Although only 3 spontaneous mutants were observed, it is interesting to note that all were C→T transitions, identical to those observed for the oxidized cytidines. While the number of observed spontaneous events is admittedly small, the data are in accord with the view that these lesions may play a role in spontaneous mutation. The frequency and specificity of spontaneous mutation observed in this site-specific mutagenesis study is consistent with that found for replication of a ss M13 genome in *E. coli* (Yatagai and Glickman, 1990).

The spontaneous mutation frequency of the *lacI* gene of *E. coli* has been previously determined by cloning the gene into ss bacteriophage M13 (Yatagai and Glickman, 1990) and by placing the gene in the F' episome (Schaaper et al., 1986). In the context of M13, the spontaneous mutation frequency of the *lacI* gene was determined to be 1.4×10^{-4} with 80% of the mutations being base substitutions, primarily GC→AT

transistions (Yatagai and Glickman, 1990); in contrast, the mutation frequency of the gene in the F' episome was shown to be 2.0×10^{-6} (Schaaper et al., 1986). Interestingly, the frequency of transition mutations in the M13 system was shown to be 9.4×10^{-5}, which is similar to the value obtained for the spontaneous transition mutations observed in this work. The discrepancy between these results may be a reflection of the enhancement of dC deamination in ss versus ds DNA (Lindahl and Nyberg, 1972; Lindahl and Nyberg, 1974).

4. Mutation Frequency of 5-OH-dC

Although one order of magnitude greater than background, a modest mutation frequency (0.05%) was observed for 5-OH-dC (Table 4). The predominant mutation observed was a C→T transition, and several C→G transversions were also observed. The data presented here for 5-OH-dC are in agreement with *in vitro* polymerase bypass and dNTP incorporation studies demonstrating that this lesion is processed without significant error by the Klenow fragment of DNA polymerase I (Purmal et al., 1994b; Purmal et al., 1994a).

Although one study by Feig et al. suggests that 5-OH-dC is significantly mutagenic in *E. coli* (2.5% mutation frequency), the technique used to generate 5-OH-dC in that study also allowed for the generation of other oxidized products (Feig et al., 1994). Whereas the work presented here employed a direct chemical synthesis to generate 5-OH-dC, in the Feig experiment dCTP was oxidized with Fe^{2+} and H_2O_2, and the "5-OH-dCTP" peak was isolated by HPLC. The putative cytidine oxidation product was

incorporated into a viral genome by the low fidelity HIV reverse transcriptase (Preston et al., 1988). In order to cause a C→T mutation, the oxidized species had to be incorporated opposite template dGs. Given the high mutagenicity of 5-OH-dU and dUg, it is possible that interpretation of the results of the earlier study could be skewed considerably if only a small amount of a highly mutagenic oxidized uridine or readily deaminated oxidized cytosine species were present after HPLC purification. Furthermore, due to the low fidelity of HIV reverse transcriptase, the possibility that an oxidized uridine or readily deaminated cytidine was incorporated opposite template dGs cannot be excluded. The present study was a more direct approach to the problem and it is unlikely to have given rise to ambiguous results.

The molecular mechanisms of 5-OH-dC mutagenesis have yet to be investigated. In its enol conformation, 5-OH-dC is not expected to cause mispairing since it retains cytidine-like base pairing properties (Figure 19). However, it is plausible that a minor conformation of 5-OH-dC such as the imino tautomer exists in DNA and that this conformation of the lesion may result in misreplication by a DNA polymerase. Previously, the minor imino tautomers of DNA bases were believed to be relatively unimportant in lesion mutagenesis (von Borstel, 1994), however, more recent evidence is beginning to mount that this may not be the case (Fazakerley et al., 1993). Theoretically, the imino tautomer of 5-OH-dC can form a stable base pair with dA, resulting in a C→T transition, or it may pair with dC, resulting in a C→G transversion. Interestingly, both of these mutations were observed in the current mutagenesis study (Kreutzer and Essigmann, 1998), as well as in the *in vitro* polymerase bypass studies from the Wallace

laboratory (Purmal et al., 1994b; Purmal et al., 1994a).

4. Mutation Frequencies of 5-OH-dU and dUg

In contrast to the low mutation frequency of 5-OH-dC, the mutagenicities of 5-OH-dU and dUg were strikingly high (Table 4). The mutation frequencies of 5-OH-dU and dUg were determined to be 83% and 80%, respectively, with 100% of the sequenced mutations derived from these two lesions being C→T transitions.

5-OH-dU and dUg are oxidative deamination products of cytidine, and the extremely high mutation frequencies observed probably result from deamination of the 4-amino group of the oxidized dC, altering the base-pairing properties of the pyrimidine from dC to dT. Given this argument about the mutagenicity of 5-OH-dU and dUg, an interesting question to ask is why are these lesions *only* 80% mutagenic in this system? That is, 80% of the time 5-OH-dU and dUg preferentially base pair with dA, but why do these lesions pair with dG 20% of the time?

A significant body of literature exists focusing on the mutagenic properties of 5-substituted uridine derivatives, particularly their ability to base pair with dG. Based primarily on the mutagenicity of 5-halogenated dU lesions, the hydrogen bonding properties of a dU:dG base pair have been investigated, and it has been suggested that three factors may be involved (Figure 20): (1) formation of the dU enol tautomer (Freese, 1959), (2) ionization of the dU base (Lawley and Brookes, 1962), and (3) formation of a dU:dG wobble base pair (Crick, 1966; Patel et al., 1984) Each of these scenarios may contribute to the oxidized dU:dG base pairing indirectly observed in the current

mutagenesis study; however, the extent to which 5-OH-dU and dUg adopt either imino or ionized conformations or participate in wobble base pairing remains to be investigated.

5. Repair of Lesions Resulting From Cytidine Oxidation

The primary defense against oxidized pyrimidines was initially attributed to the glycosylase activity of endonuclease III, primarily based on its broad substrate specificity. Experiments *in vitro* indicate that dUg, 5-OH-dC, and 5-OH-dU, as well as many other modified pyrimidines, are all excised by endonuclease III (Breimer and Lindahl, 1984; Dizdaroglu et al., 1993; Hatahet et al., 1994; Purmal et al., 1998; Wagner et al., 1996; Wang and Essigmann, 1997). However, *nth* mutants, which lack endonuclease III, 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 some overlapping substrate specificity (Jiang et al., 1997b; Melamede et al., 1994). Interestingly, analysis of its amino acid sequence reveals that endonuclease VIII shares homology with MutM rather than with endonuclease III (Jiang et al., 1997a). 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., 1997a). Further experimentation is required to reconcile the observed differences. Homologues of endonuclease III have been cloned from yeast (Augeri et al., 1997; Eide et al., 1996; Roldán-Arjona et al., 1996) 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 in yeast (Eide et al., 1996). In addition to endonuclease III and endonuclease VIII, Wallace and coworkers report that MutM from *E. coli* excises 5-OH-dC, 5-OH-dU and dUg from oligonucleotides *in vitro* (Hatahet et al., 1994; Purmal et al., 1998). Furthermore, it is possible that UDG from *E. coli* and humans may repair 5-OH-dU (Dizdaroglu et al., 1996; Hatahet et al., 1994) although others have found otherwise (Fujimoto et al., 1997; Zastawny et al., 1995).

Another glycosylase, whose function in the repair of oxidized uridine lesions in *E. coli* warrants further investigation, is the MUG (mismatch-specific uracil DNA glycosylase) protein (Gallinari and Jiricny, 1996). This enzyme is believed to function in the excision of uracil and thymine from mispairs with dG. A recent crystal structure of MUG revealed structural and functional homology to UDGs despite low sequence homology, and suggested that the enzyme's specificity may result from direct recognition of dG in the complementary DNA strand (Barrett et al., 1998). Much effort is currently focused on determining the true substrate specificity of this enzyme; oxidized uridines are attractive candidate lesions since productive repair depends upon removal of the lesions when they are situated opposite dG. If the lesions were removed from DNA when situated opposite dA, repair synthesis would result in incorporation of dT, thus fixing a

mutation.

The adverse role that oxidized, deaminated cytidines may play in mammalian cells remains to be investigated. 5-OH-dU and dUg have both been detected in mammalian tissues and in human cells at levels comparable to that of 8-OXO-dG (Wagner et al., 1992). For example, the levels of 5-OH-dU, dUg, and 8-OXO-dG in human leukocytes have been determined to be 7, 20 and 12 adducts per 10^7 bases, respectively. If mammalian polymerases misincorporate nucleotides opposite 5-OH-dU and dUg with similar frequency to that of *E. coli* polymerases, these lesions pose a serious threat to genomic integrity. Proteins that may be involved in diminishing the cellular effects of oxidized cytidines in mammalian cells include functional homologues of endonuclease III, and TDG, the human homologues of MUG (Wiebauer and Jiricny, 1989; Neddermann and Jiricny, 1994).

D. CONCLUSION

Replication in *E. coli* of a single stranded vector containing a unique 5-OH-dU or dUg shows that these lesions exclusively induce C→T transitions at a frequencies of 83% and 80%, respectively (Kreutzer and Essigmann, 1998). By contrast, 5-OH-dC is much less mutagenic (0.05%) in the same experimental system, and in addition to C→T transitions, some C→G transversions are induced (Kreutzer and Essigmann, 1998). Although a previous study suggested that 5-OH-dC was more significantly mutagenic in *E. coli* (2.5% mutation frequency), the technique used to generate 5-OH-dC in that study also allowed for the generation of other oxidized products (Feig et al., 1994); the possibility that a small amount of a highly mutagenic oxidized dU or readily deaminated oxidized dC species confounded the measurement of the mutation frequency of 5-OH-dC cannot be excluded. Since the levels of 5-OH-dC, 5-OH-dU and dUg experimentally found in cellular DNA are fairly comparable (Wagner et al., 1992), the biological significance of 5-OH-dC relative to 5-OH-dU and dUg may be minimal when one considers that the mutation frequency of 5-OH-dC is three orders of magnitude smaller than that of 5-OH-dU and dUg.

The presence of 5-OH-dU and dUg in oxidatively damaged DNA (Douki et al., 1996b; Wagner et al., 1992) taken together with the 83% and 80% mutation frequencies observed in this work for these lesions implicate 5-OH-dU and dUg as likely sources of the frequently observed C→T transitions in the mutational spectrum of oxidatively damaged DNA. However, the relative contributions of these lesions to the overall

observed spectra of C→T mutations cannot be assessed until further experiments addressing the rates of formation of these lesions are performed. More specifically, it would be of interest to examine the rates of cytidine glycol deamination and/or dehydration that lead to the formation of 5-OH-dC, 5-OH-dU and dUg in cellular DNA. In addition, the relative contribution of these lesions compared with that of dU arising from deamination of dC should be addressed.

A primarily goal of this laboratory has been to understand the chemical nature and mutagenic properties of DNA lesions arising from oxidative DNA damage. When viewed *en masse*, the spectrum of spontaneous and oxidant-induced mutations is primarily dominated by two base substitutions: GC→TA transversions and GC→AT transitions. Which lesions are responsible for these mutations? Clearly, dG and dC oxidation products are logical candidates for investigation. In studies from the early 1990s, it became evident that 8-OXO-dG caused significant levels of G→T transversions. Moreover, the close interrelationship between the repair of 8-OXO-dG and mutagenicity provided a paradigm of nature's efficiency in her ability to minimize risks to the genome. Subsequently, efforts were made to study dC derived lesions. As demonstrated by the work of this thesis, the determination of the mutational properties of 5-OH-dU, dUg, and 5-OH-dC offers several explanations for the preponderance of C→T mutations. In addition, thanks to studies of other purine and pyrimidine adducts in many laboratories, lesions sharing mutational specificity with all six possible base pair substitutions have been identified (Table 5).

Current accomplishments are by no means exhaustive and many more oxidative

adducts remain to be evaluated; it is likely that there are additional lesions capable of inducing the same type of mutations. Moreover, of the twelve possible single base substitutions, some that have been observed in mutational spectra have not yet been assigned to any specific DNA lesion. For example, G→C mutations are observed after treatment of DNA with Fe^{2+} (McBride et al., 1991), yet the nature of the dG derived lesion causing this mutation has not been determined.

Nevertheless, much progress has been made toward the goal of understanding the chemical progenitors to genetic change. This progress is a consequence of the convergence of experts in many fields: organic synthesis, mutagenesis, DNA repair, structural biology, and analytical chemistry. The synthesis of defined DNA adducts has permitted extensive studies of the biological properties of individual adducts, including mutational specificity. Moreover, a chemical rationale for some of these observations has been buttressed by NMR and X-ray crystallographic structural data. From studies of repair enzymes, the critical role of DNA repair in mitigating the effects of DNA adducts has also been established. As improvements in analytical techniques permit the detection of rarer oxidative species, and chemical synthetic means advance, more pieces of the puzzle will fall into place. Second order variables such as sequence context effects on repair and replication will also be analyzed. Eventually, it may be possible to realize the ultimate goal of site-specific studies on the mutagenic potential of DNA adducts: attributing every observed mutation in the reported mutational spectrum to a specific DNA adduct and understanding the factors contributing to the non-random distribution of mutations.

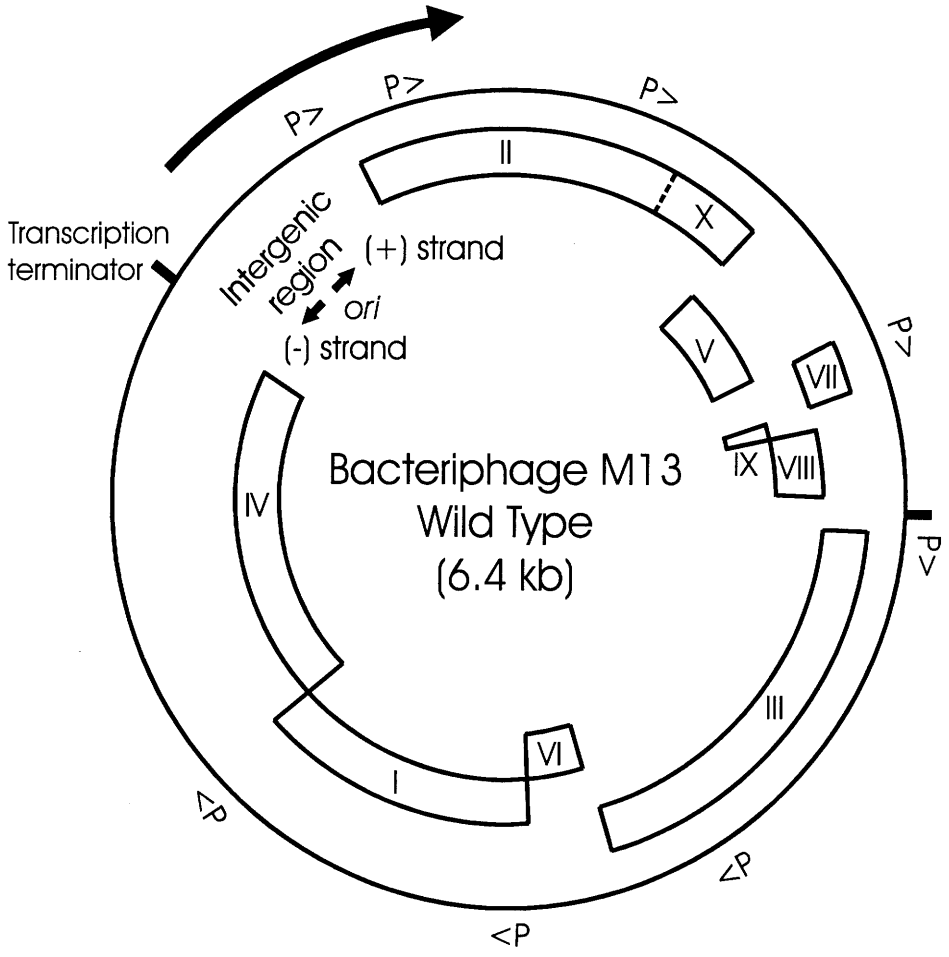


Figure 15: The genome of wild-type bacteriophage M13 is a ss circular DNA molecule approximately 6400 nucleotides in length. The figure shows the approximate locations of the genes (Roman numerals), the positions of the major promoters (P>), and the terminators of transcription. Between genes II and IV exists an intergenic region into which foreign DNA may be inserted. Adapted from Maniatis, 1989.

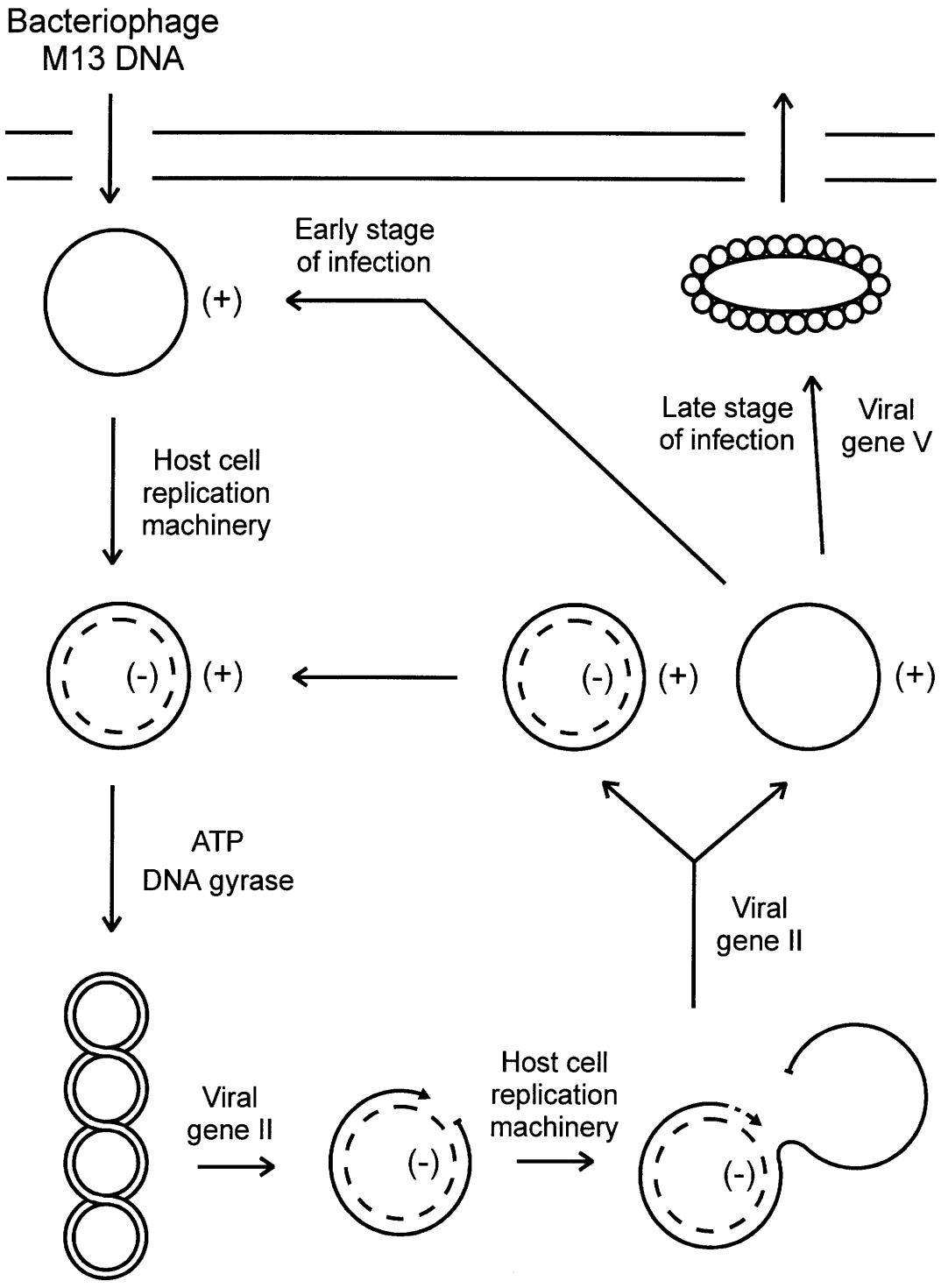


Figure 16: The life cycle of the filamentous bacteriophage, M13. (See text for details.)

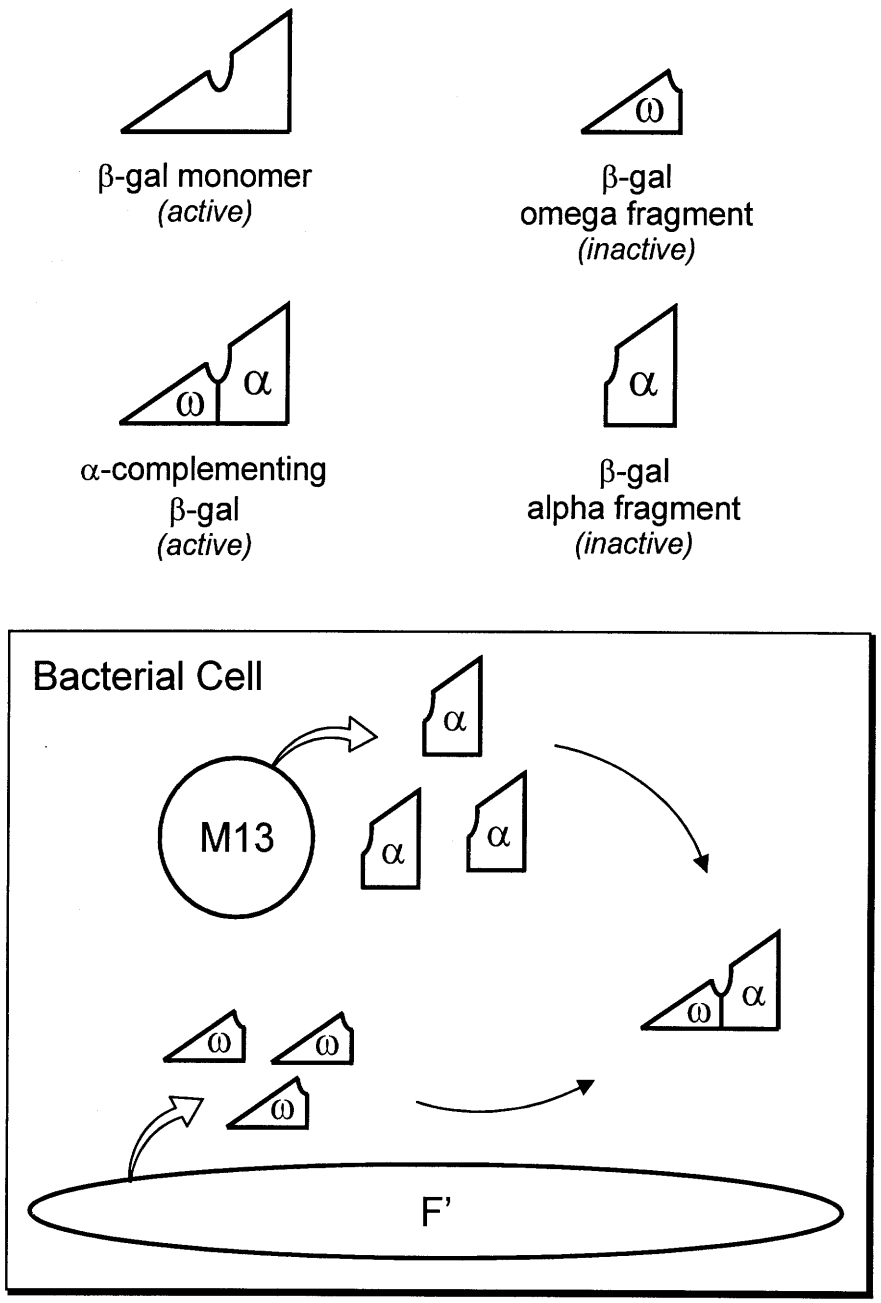


Figure 17: Schematic representation of the α-complementation assay. The α-fragment of the *lacZ* gene carried on a vector can complement the ω-fragment of the gene carried on the F' episome of *E. coli* to generate a fully functional β-galactosidase (β-gal) protein.

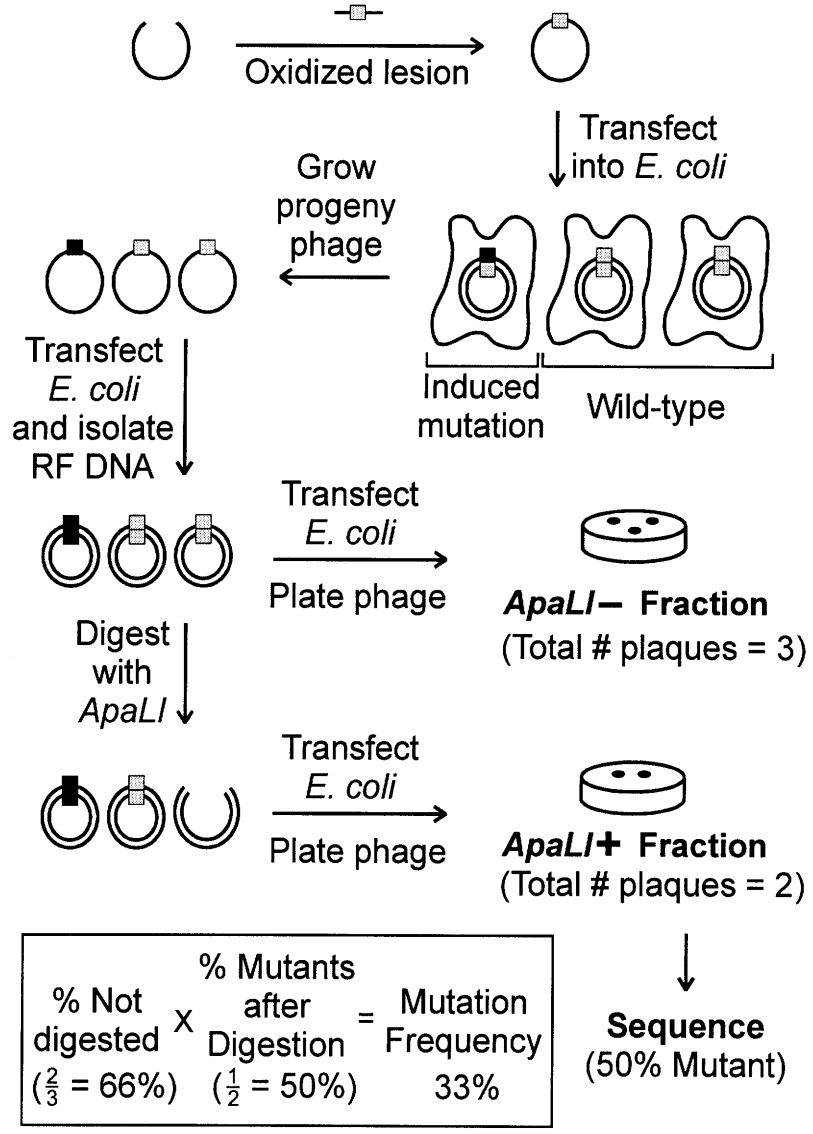


Figure 18: Experimental design to evaluate the mutation frequency of 5-OH-dC, 5-OH-dU and dUg. Mutation frequency was determined in two steps. First, a preliminary mutation frequency was determined by measuring the ratio of phage produced from RF DNA that was either treated with or without *ApaLI*. That ratio, or preliminary mutation frequency, was next adjusted by multiplying it by the fraction of phage in the *ApaLI*-resistant population that was verified to be mutant by DNA sequencing. This adjustment is necessary because some wild-type RF DNA escapes *ApaLI* selection. In the example shown, 66% of the progeny were in the pool that was resistant to digestion by *ApaLI*. Of these, one-half were true mutants as determined by sequencing. Thus, as shown, the mutation frequency was (66%)(50%) = 33%.

Table 4: Mutation frequencies and specificities of the unmodified control (dC), 5-OH-dC, 5-OH-dU and dUg.

Lesion	Not Digested by *ApaLI*, %	Mutants in *ApaLI*+, %	Lesion Mutation Frequency, %	Mutation Specificity (# of mutants)
dC	0.08	4 (3/79)	0.003	C→T (3)
5-OH-dC	0.09	57 (79/139)	0.05	C→T (77) C→G (2)
5-OH-dU	83	100 (95/95)	83	C→T (95)
dUg	80	100 (92/92)	80	C→T (92)

Data represent a total of three independent genome constructions and a total of nine transfections per lesion. The progeny phage from these transfections were combined. *ApaLI* digest data for dC, 5-OH-dC, and 5-OH-dU represent one experiment and for dUg represent three pooled digests. Sequence analysis was performed once.

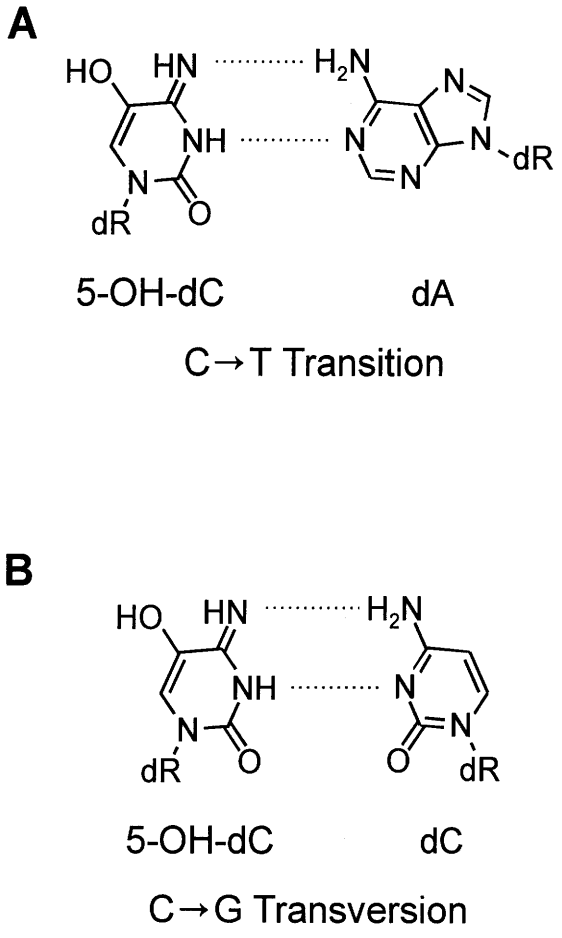


Figure 19: Proposed mechanism of mutagenesis by 5-OH-dC. If 5-OH-dC adopts an imino tautomeric conformation, base pairs with dA (**A**) and dC (**B**) are possible; these mispairs would subsequently result in C to T and C to G mutations, respectively.

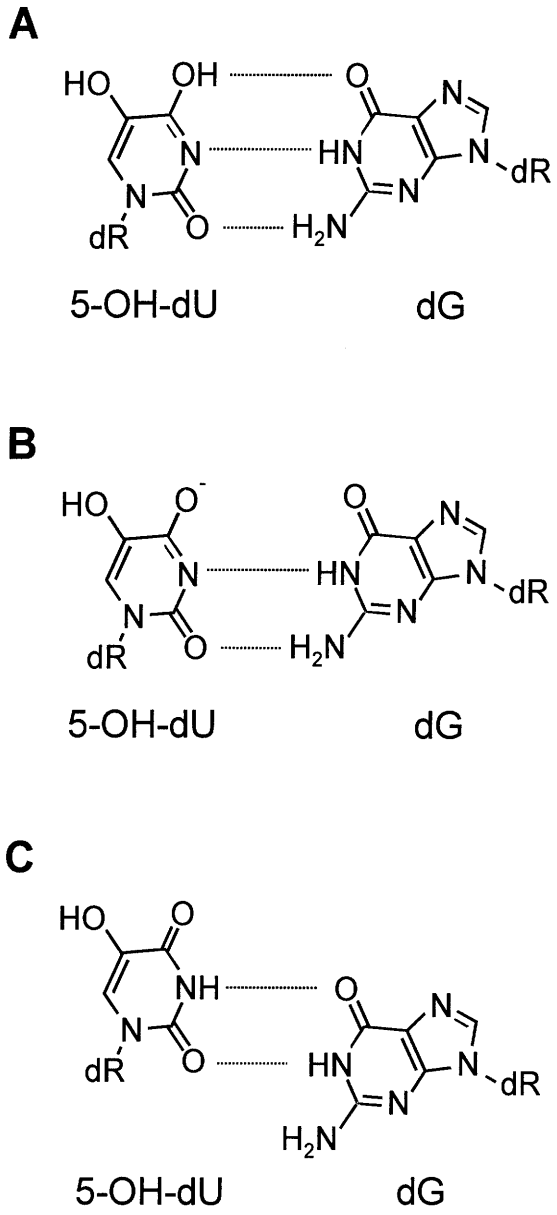


Figure 20: Hypothetical base pairing of an oxidized uridine lesion, as represented by 5-OH-dU, and dG. (**A**) 5-OH-dU in the minor enol tautomeric form; (**B**) 5-OH-dU in an ionized form; (**C**) 5-OH-dU: dG wobble base pair.

Table 5: Potential correlation between mutations observed in spontaneous and oxidant-induced mutational spectra and oxidative DNA lesions

Observed base substitution	DNA adducts of that mutational specificity[a]
GC→AT	5-OH-dC, 5-OH-dU, dUg
GC→TA	8-OXO-dG
GC→CG	5-OH-dC
AT→CG	8-OXO-dA, 8-OXO-dG[b]
AT→GC	dTg, 8-OXO-dA, 2-OH-dA
AT→TA	2-OH-dA

[a] As determined by replication of a single adduct *in vivo*.
[b] Mutation induced if the lesion is incorporated into DNA from the nucleotide pool.

CHAPTER IV:

Future Program of *In Vivo* Repair Studies

A. INTRODUCTION

Since previous work has shown that oxidized cytidines and uridines are present in cells (Wagner et al., 1992), and since the work of this dissertation has shown that at least two of these lesions, 5-OH-dU and dUg, are extremely mutagenic in *E. coli* (Kreutzer and Essigmann, 1998), it follows that there is a high probability that organisms maintain a means of removing these lesion from DNA. *In vitro*, it has been established that a number of repair enzymes can excise oxidized dC and dU lesions from DNA including endonuclease III, endonuclease VIII and MutM (Hatahet et al., 1994; Jiang et al., 1997b; Wang and Essigmann, 1997). Although each of these enzymes is capable of removing said lesions from DNA, none of them do so with high efficiency, and thus it is unclear whether any of these enzymes is involved in the excision of the aforementioned lesions from DNA *in vivo*. Moreover, cells deficient in these enzymes display only a minor, if any, increase it C→T transitions (Cabrera et al., 1988; Cunningham and Weiss, 1985; Jiang et al., 1997a), suggesting that overlapping repair systems operate on these lesions. This chapter proposes a method as well as the experimental groundwork by which several known repair systems may be tested for their efficacy in removing lesions from DNA *in vivo*.

The idea underlying the proposed experimental work of this chapter is that there exists in a repair deficient cell strain an increased level of DNA adducts as compared to a wild-type cell strain, and that this differential is measurable. More specifically, a cell line deficient in a repair protein known to excise a particular adduct from DNA will possess a larger number of that lesion in its genome as compared to a repair proficient strain. Let it

be noted at the outset that there is no direct experimental evidence supporting this hypothesis, although indirect mutagenesis studies appear to bolster this claim; the case of 8-OXO-dG is a perfect illustration of this point. The bacterial repair system responsible for mediating the detrimental effects of 8-OXO-dG is comprised mainly of three proteins, MutM, MutY and MutT (Michaels and Miller, 1992b), the roles of which have been described earlier in this dissertation. In bacteria, 8-OXO-dG induces GC→TA transversions (Wood et al., 1990), and the frequency by which this mutation occurs is significantly higher when the lesion is replicated in a *mutM*, *mutY* cell line (Michaels et al., 1992a; Moriya and Grollman, 1993b). Interestingly, *E. coli* cells deficient in either *mutM*, *mutY*, or *mutT* display an increase in the frequency of GC→TA transversion mutations (Michaels and Miller, 1992b), thus leading to the assumption that an increased level of 8-OXO-dG lesions exists in these cells as well.

To test the idea experimentally that an elevated level of DNA adducts exists in repair deficient as compared to repair proficient cells, two criteria must be fulfilled. First, a series of cell strains deficient in the repair proteins believed to act on the particular adduct of interest must be created. For the work proposed here, *E. coli* cells are a logical choice since most knowledge about the repair of oxidative dC and dU adducts has been garnered through the use of bacterial proteins, and since genetic manipulation of bacteria is fairly straightforward enabling the creation of cell strains deficient in more than one repair protein. Second, a sensitive and reproducible method to measure the level of adducts in DNA must be used. Two methods of analysis are put forward below. The first making use of gas chromatography-mass spectrometry (GC-MS) (Watson, 1997) and the

second using a site-specific genetic approach.

GC-MS is a combination of two analytical techniques: GC, a separation technique, and MS, an identification technique (Watson, 1997). Compared to HPLC-EC, GC-MS is a more versatile technique because it is less dependent on the chemical properties of the compound to be measured (for a review, see Dizdaroglu, 1990; Dizdaroglu, 1994). The method, however, requires hydrolysis of DNA followed by derivatization of the corresponding bases or nucleosides to make them volatile for GC analysis. For accurate quantitation, a known amount of an internal standard can be added to each sample. The internal standard, usually a compound that has a similar GC retention time to the analyte, but a different mass, is used to compensate for any loss of the sample during the hydrolysis and derivatization steps, as well as to offset the lack of reproducibility between sample injections. The ideal internal standard is a stable isotope-enriched analogue of the analyte that differs by at least three mass units. To increase the sensitivity of detection, the mass spectrometer can be set in the selected-ion-monitoring (SIM) mode in which only a few selected mass values for each analyte are monitored during the time in which they elute from the GC (for a review, see Watson, 1990). This provides a specific and sensitive detection, which is on par with that of HPLC-EC.

GC-MS has been used to successfully measure the levels of oxidative adducts from DNA treated *in vitro* with an oxidizing agent, and from DNA isolated from treated mammalian cells. However, to date, these techniques have not been used to assess the levels of DNA adducts generated in *E. coli*. In this chapter, a proposed method enabling the measurement of adduct levels in the genomic DNA of *E. coli* is presented. In

addition, the way in which this method can be used to evaluate the *in vivo* repair of oxidized dC and dU lesions is detailed.

B. GC-MS Approach

1. Characterization of Isotopically-Labeled 5-OH-C, 5-OH-U and 8-OXO-G by RP-HPLC

Isotopically-labeled 5-OH-C (^{13}C, $^{15}N_2$), 5-OH-U (^{13}C, $^{15}N_2$) and 8-OXO-G (^{13}C, $^{15}N_3$) were synthesized and generously provided by Dr. Victor Nelson at NCI-Frederick Cancer Research and Development Center. The isotopically labeled DNA bases were characterized and quantitated by UV (Moschel and Behrman, 1974; Singer and Grunberger, 1983). Typically, a small aliquot of compound was dissolved in approximately 1 ml H_2O and 10-100 µl NH_4OH. For quantitation, a portion of each sample was diluted into the appropriate buffer and analyzed by UV (Table 6).

The purity of the 5-OH-C, 5-OH-U and 8-OXO-G isotope standards was assessed by RP-HPLC. Each standard (20-30 nmol) was analyzed by using a gradient of isocratic A for 2 min followed by a gradient of 0-33% B over 33 min (A: 10 mM $(NH_4)_2HPO_4$ and 10 mM tetrabutylammonium hydroxide pH 7.4, and B: 10 mM $(NH_4)_2HPO_4$, 10 mM tetrabutylammonium hydroxide, CH_3CN (3:1) pH 7.4). The chromatograms for these samples are shown in Figure 21.

2. Characterization of DNA bases by GC-MS

Prior to analysis of DNA samples, it was necessary to determine the GC retention

times and characteristic ions of the normal and isotope-labeled DNA bases (C, A, T, G, U, 5-OH-U and 8-OXO-G were purchased from Sigma). A small amount of each base was dissolved and quantitated as described (Table 6) (Maniatis et al., 1989).

Samples were derivatized by mixing 2 μg of each base with 15 μl CH_3CN, 10 μl pyridine and 25 μl *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) (silylation grade reagents, including CH_3CN, pyridine and MTBSTFA, were from Pierce and were stored under argon) in a silanized reaction vial. (One ml conical reaction vials were from Wheaton. To silanize the vials, glassware was filled with a 5% solution of dimethyldichlorosilane in toluene for 15 min, rinsed with toluene, rinsed with CH_3OH, and then dried in an oven. Following an experiment, the vials were rinsed with H_2O, sonicated in CH_3CN for 30 min and rinsed.) The samples were vortexed, capped with argon, and then heated at 130°C for 30 min.

After cooling, 1 μl of the sample (40 ng) was directly analyzed by GC-MS (Hewlitt-Packard [HP] 5890 Series II GC and an HP Model 5898A MS) in the scan mode under the following conditions: GC injector temp at 240°C, GC detector temp at 280°C, oven temp at 100°C with a gradient of 20°C/min, solvent delay at 2-3 min, electron multiplier at 2200 V, MS temp at 250°C and MS quadrapole at 100°C. The GC column was an HP-5 column #19091J-112 370 from HP and consisted of a cross-linked 5% phenyl-methyl silicone gum phase with a 0.2 mm internal diameter and a 0.33 μm film thickness. The purchased column was 25 m long, but it was cut to 12.5 m for use in these experiments. Other GC equipment, including column ferrules, glass liner and gold seal were purchased from HP. Septas for the GC injector and the reaction vials were from

Supelco.

Structures of the MTBSTFA-derivatized modified bases are shown in Figure 22. The bases were easily separated under the GC conditions employed, and the retention times, state of derivatization and characteristic ions for the DNA bases are listed in Table 7.

3. Construction of Standard Curves for Selected Ion Monitoring

For successful quantitative analysis, the MS response must be calibrated with standard quantities of the analyte to ensure that the instrument responds linearly. The way in which this is often done is by mixing varying quantities of the analyte and internal standard. The response of the GC-MS to the isotope standards was assessed by constructing calibration curves for 5-OH-U and 8-OXO-G. Samples containing 5 ng of the isotope compound and 0, 2.5, 5, 10 and 20 ng of the standard were derivatized and analyzed by GC-MS-SIM. Current at 452 and 456 was monitored for 8-OXO-G, and current at 414 and 417 was monitored for 5-OH-U. (Subsequently, current at 413 and 416 was monitored for 5-OH-U, and this change was shown to have no effect on the linearity of the calibration curve.) The ratios of corresponding ion currents were measured and plotted against the sample-composition ratio to give the calibration plot (Figure 23).

The graph obtained from these plots is linear and passes through the origin. Since the presence of a non-zero intercept is indicative of contamination with unlabeled material in the isotopically-labeled sample, it appears that the isotope compounds

obtained for use as internal standards for these experiments is pure. A calibration curve for 5-OH-C was not constructed due to the lack of pure non-isotopically-labeled compound. It was assumed that the GC-MS response to the isotopically-labeled 5-OH-C would be similar to that of 5-OH-U and 8-OXO-G. Also, when the isotope standard of 5-OH-C was analyzed by GC-MS in the scan mode, no unlabeled compound was observed by extracting the chromatogram for current at 412.

4. GC-MS Method

To assess the accuracy of GC-MS for quantitating the amount of adduct present in a DNA sample, a mock experiment was performed in which the amount of 5-OH-U in the singly modified oligonucleotide 5'-CA(5-OH-U)GCAG-3' was measured. To hydrolyze the oligonucleotide to its corresponding bases, 190 μl H_2O and 310 μl 98% formic acid were added to 1 μg of the oligonucleotide and 5 ng of the 5-OH-U and 8-OXO-G isotope standards. Argon was blown over the sample, and then the sealed vial was heated at 100°C for 1 hr. Following acid hydrolysis, the sample was lyophilized by using a Speed-Vac and derivatized with MTBSTFA as previously described. After cooling, 1 μl of the sample (20 ng of oligonucleotide and 100 pg of standard) was directly analyzed by GC-MS-SIM, with current monitored at 413 and 416 for 5-OH-U and at 452 and 456 for 8-OXO-G.

The amount of 5-OH-C in the sample was determined through comparison of the GC peak area for 5-OH-U and the 5-OH-U isotopically-labeled internal standard. Using the value, a back calculation was performed to confirm the amount of oligonucleotide in

the original sample (Figure 24). Interestingly, following hydrolysis and derivatization of the oligonucleotide, no detectable levels of 8-OXO-G were observed in this sample.

5. Isolation of Genomic DNA from *E. coli*

Since this experimental method requires measuring the level of adducts in cellular DNA, a technique was developed to isolate genomic DNA successfully from *E. coli* cells. Liquid cultures of cells were grown in K medium (1% glucose, 1% Casamino acids, 1 μg/ml thiamine hydrochloride, 1 mM $MgSO_4 \bullet 7H_20$, 0.1 mM $CaCl_2$, M9 salts [Miller, 1992]); saturated overnight cultures (5 ml) were diluted to a density of 1 x 10^7 cells/ml (approximately 4 ml of the overnight culture into 500 ml K medium), and the cultures were grown at 37°C with 195 rpm of shaking. Genomic DNA was isolated by using a Qiagen Genomic DNA isolation kit on the Midi scale. The isolation was performed as directed by the kit except that the lysozyme/Proteinase K digestion was for 1 hr at 37°C, and the Buffer B2 (3 M guanidine•HCl and 20% Tween-20) digestion was for 1 hr at 50°C. The DNA pellet was resuspended in 500 μl TE by overnight incubation at 37°C, and the typical DNA yield was approximately 75 μg.

6. Repair Deficient Cell Strains

As previously discussed, it is of interest to measure the level of oxidative dC and dU lesions in cell lines deficient for enzymes known to repair these adducts. Cell lines deficient in all possible combinations of the *nth*, *nei* and *mutM* genes have been constructed by Professor Michael R. Volkert (UMass Medical Center, Worcester, MA).

In addition, it may also be interesting to look at the effects, if any, of the *mug* gene.

7. Induction of Oxidative Stress in *E. coli*

If the difference between steady-state adduct levels of repair proficient and deficient cells can not detectable by the method described, it may be useful to increase the overall number of adducts in order to enhance any differences that may exist. It may, therefore, be necessary to treat the cells with an agent that induces oxidative DNA damage, such as ionizing radiation, H_2O_2, or an O_2^- generating chemical. For simplicity, initial treatment experiments were performed with H_2O_2.

Cytotoxicity induced by H_2O_2 appears to progress by two pathways (Imlay et al., 1988; Imlay and Linn, 1986). At low concentrations (1-3 mM), H_2O_2-mediated cell killing is characterized by DNA damage and requires active cellular metabolism during exposure. In contrast, at higher concentrations of H_2O_2 (up to 50 mM), the mechanism of cell killing is less well defined, but appears to be independent of cellular metabolism. Since the purpose of treating cells in this experiment is to increase the number of DNA adducts in the cell, it is of interest to treat cells under conditions that would induce DNA damage. Thus, it is necessary to treat cells with a low concentration of H_2O_2 at a dilute culture density to ensure active metabolism, while simultaneously culturing enough cells from which to isolate 50 μg DNA. Based on a protocol from the Linn laboratory (Imlay and Linn, 1986), innoculation of overnight cultures as previously described, challenging the cells at a density of 4×10^7 cells/ml with 1.5 mM H_2O_2 (84 μl of a 30% solution from Sigma) for 15 min at 37°C with 195 rpm shaking, and terminating the challenge by

addition of 20 μg of beef liver catalase (1 μl of a 20 μg/ml, 65,000 U/mg suspension from Boehringer Mannheim) should result in H_2O_2-mediated DNA damage. Under these treatment conditions, a 500 ml cell culture should produce 50 μg of isolated DNA.

8. Discussion

Although GC-MS analysis is a widely used technique for the detection of oxidative DNA bases, until recently, the yields of 8-OXO-dG by using this method were much higher (approximately 50-fold) than those obtained by RP-HPLC with electrochemical detection (HPLC-EC) (Halliwell and Dizdaroglu, 1992). The HPLC-EC method most commonly used for analysis of DNA samples is based on the measurement of current resulting from oxidation of analyte molecules (Rocklin, 1993). Thus, in work with DNA adducts, this method is useful only in the detection of oxidized lesions with a low oxidation potential. Initially developed for the measurement of 8-OXO-dG (Floyd et al., 1986; Kasai et al., 1986), HPLC-EC has been extended to the detection of other oxidized lesions including 8-OXO-dA, 5-OH-dC, and 5-OH-dU (Berger et al., 1990; Wagner et al., 1992).

Observation of an artifactual oxidation of dG or G during the silylation step of the GC-MS analysis is the likely cause of these discrepancies (Hamberg and Zhang, 1995; Ravanat et al., 1995). More recently, this observation has been extended to other oxidized bases including 5-OH-C and 8-OXO-A (Douki et al., 1996a). Thus, artifactual oxidation may confound the measurement of adducts in this experimental system.

A number of analytical steps has been suggested to reduce the levels of undesired

oxidation. First, during the DNA isolation step, addition of an antioxidant prior to cell lysis may be useful (Kvam and Tyrrell, 1997). In addition, since it is believed that oxidation of the normal bases is the source of the observed artifactual oxidation, inclusion of a pre-purification step to separate the oxidized bases away from their normal constituents prior to sample derivatization may be helpful. In fact, it has been recently demonstrated that addition of this purification procedure lowers the adduct levels detected by the GC-MS method to those observed by HPLC-EC (Ravanat et al., 1995).

Another stage at which error may be introduced into the experiment is during the release of the bases or nucleosides from DNA, since incomplete hydrolysis of the DNA or degradation of the analytes will result in inaccurate and inconsistent measurements (Cadet et al., 1997). Thus, conditions should be established such that the bases or nucleosides will be liberated from DNA while the chemical integrity of the adducts is maintained. Compared to formic acid hydrolysis, gentler methods of releasing a base or nucleoside from DNA include enzymatic hydrolysis by nuclease P1 (Shigenaga et al., 1994) and incubation with HF/pyridine (Douki et al., 1996b).

If after optimization of the DNA extraction and preparation methods, still no difference in adduct levels between repair deficient and proficient cells is detected, then two possibilities remain: (1) the appropriate repair deficient cell lines were not selected, implying that further efforts should be directed toward identifying and isolating new repair proteins that have activity toward oxidized dC and dU lesions, or (2) the analytical methodology is not yet sensitive enough to detect the differential adduct levels. If (2) is found to be the case, a more sensitive genetic method for assaying repair deficient cells is

available as described below.

C. Mutagenesis Approach

An alternative method that could be used to probe the putative effects of a given repair protein involves a similar procedure to that described in Chapter 2 of this dissertation, and has been previously used to confirm the role of MutM and MutY proteins in mediating the mutagenicity of 8-OXO-dG *in vivo* (Michaels et al., 1992a).

Briefly, a double-stranded bacteriophage genome containing either 5-OH-dC, 5-OH-dU or dUg is constructed such that replication of both the (+) and (-) strands can be monitored (Figure 25). The basis of this method involves manipulation of the α-complementation assay described in Chapter 2 of this dissertation. The (-) strand of the genome is built such that the sequence of the *lacZ* gene is out of frame. This may be accomplished, for instance, by either the insertion or deletion of nucleotides. Thus, progeny phage derived from this strand would lead to the formation of colorless plaques.

The oxidized lesion is incorporated into the (+) strand such that the sequence of the *lacZ* gene is in frame, and progeny derived from this strand would give rise to blue plaques. Since a mismatch is required in order to create a color selection for the (+) and (-) strands of the proposed genome, transfection into a mismatch repair deficient strain may be required. Following transfection and replication of the genomes in *E. coli*, the experiment could proceed as described in Chapter 2: (1) blue plaques are selected, (2) if the lesion is situated within a restriction enzyme sequence, then a mutant enrichment

procedure can be performed, and (3) the mutation frequencies and specificites can be determined by sequencing.

As shown in Chapter 2, the predominant mutation induced by 5-OH-dC, 5-OH-dU and dUg is a C→T transition. Thus, if only this mutation is examined, a second method of analysis may be used that will eliminate the requirement of excess DNA sequencing for mutation frequency determination. Use of this method requires placement of the oxidized lesion in the (+) strand within the nucleotide sequence C*AG. Thus, if a C→T mutation occurs, the nucleotide sequence would be altered to TAG, which is read by RNA polymerase as a stop codon resulting in truncation of protein synthesis. The resulting β-gal protein product would not be functional in an α-complementation assay, however, if plated in the presence of an amber suppressor *E. coli* strain, a low level of transcription beyond the stop codon will occur. Thus, a mixture of functional and nonfunctional protein will be transcribed, resulting in the formation of a light blue plaque. The mutation frequency of the lesion can be calculating by determining the ratio of light blue to light plus dark blue plaques.

The mutagenic experiment outlined above using either sequencing analysis or color analysis in an amber supressor strain is designed to measure the fluctuation of adduct-induced mutagenesis in repair deficient cell strains, and would provide suggestive evidence of which repair enzymes are responsible for protecting a cell against the detrimental effects of oxidized dC and dU lesions *in vivo*. While not as direct as the GC-MS method described previously in this chapter, assay of individual plaques provides maximum possible sensitivity to the mutational event in question, and would resolve the

issue even if the use of GC-MS was ineffective in measuring increased adduct levels over background oxidation. Thus, through either the use of whole cell oxidation and GC-MS measurement of DNA lesion or an adducted DNA construct, the question of adduct repair by a given protein or set of proteins can be positively answered.

Either or both of these methods provide a set of experiments for the continued exploration of *in vivo* pyrimidine oxidation and repair. It is hoped that this dissertation provides both a waypoint in the field of oxidative DNA damage and a springboard to the future understanding of mutagenesis and ultimately cancer.

Table 6: Spectral characteristics of normal and oxidized DNA bases.

Base	pH[a]	λ_{max} (nm)	ε_{max} (M^{-1})
C[b]	7.0	267	6130
A	7.0	260.5	13350
T	7.0	264.5	7890
G	7.0	246	10700
		275.5	8150
U	7.0	259.5	8200
5-OH-C[c]	6.8	288	5000
5-OH-U[c]	7.0	282	6200
8-OXO-G[d]	9.5	283	8140

[a] Samples were quantitated in 0.1 M KH_2PO_4/K_2HPO_4 buffer, except for 8-OXO-G which was in 0.02 M NH_4Cl/NH_4OH buffer.

[b] (Maniatis et al., 1989)

[c] (Moschel and Behrman, 1974)

[d] (Singer and Grunberger, 1983)

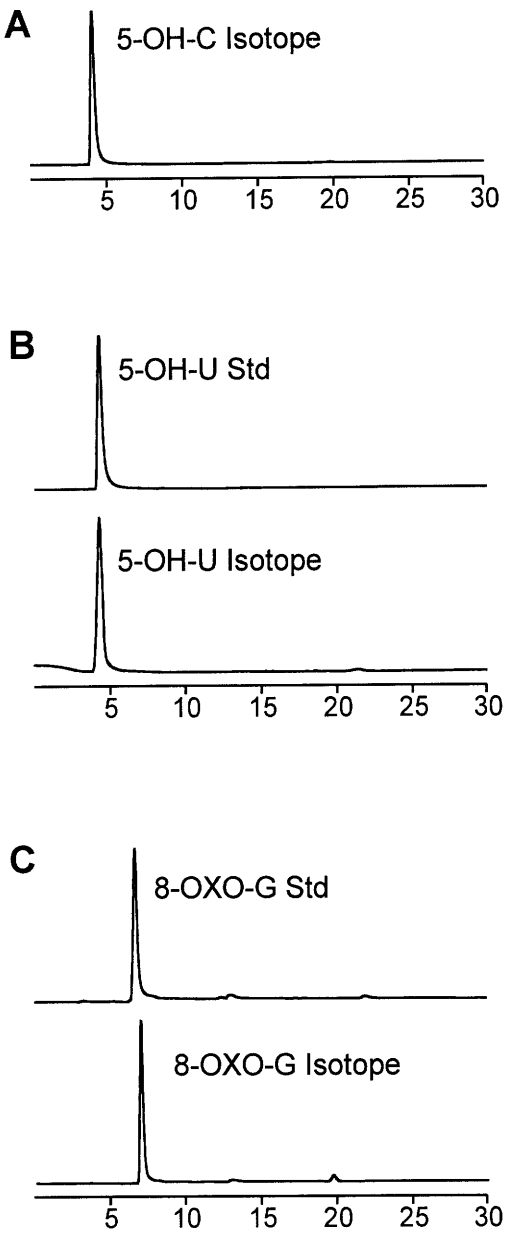


Figure 21: HPLC chromatograms (280 nm) of base standards and isotopically labeled compounds. (**A**) 5-OH-U; (**B**) 5-OH-C; (**C**) 8-OXO-G.

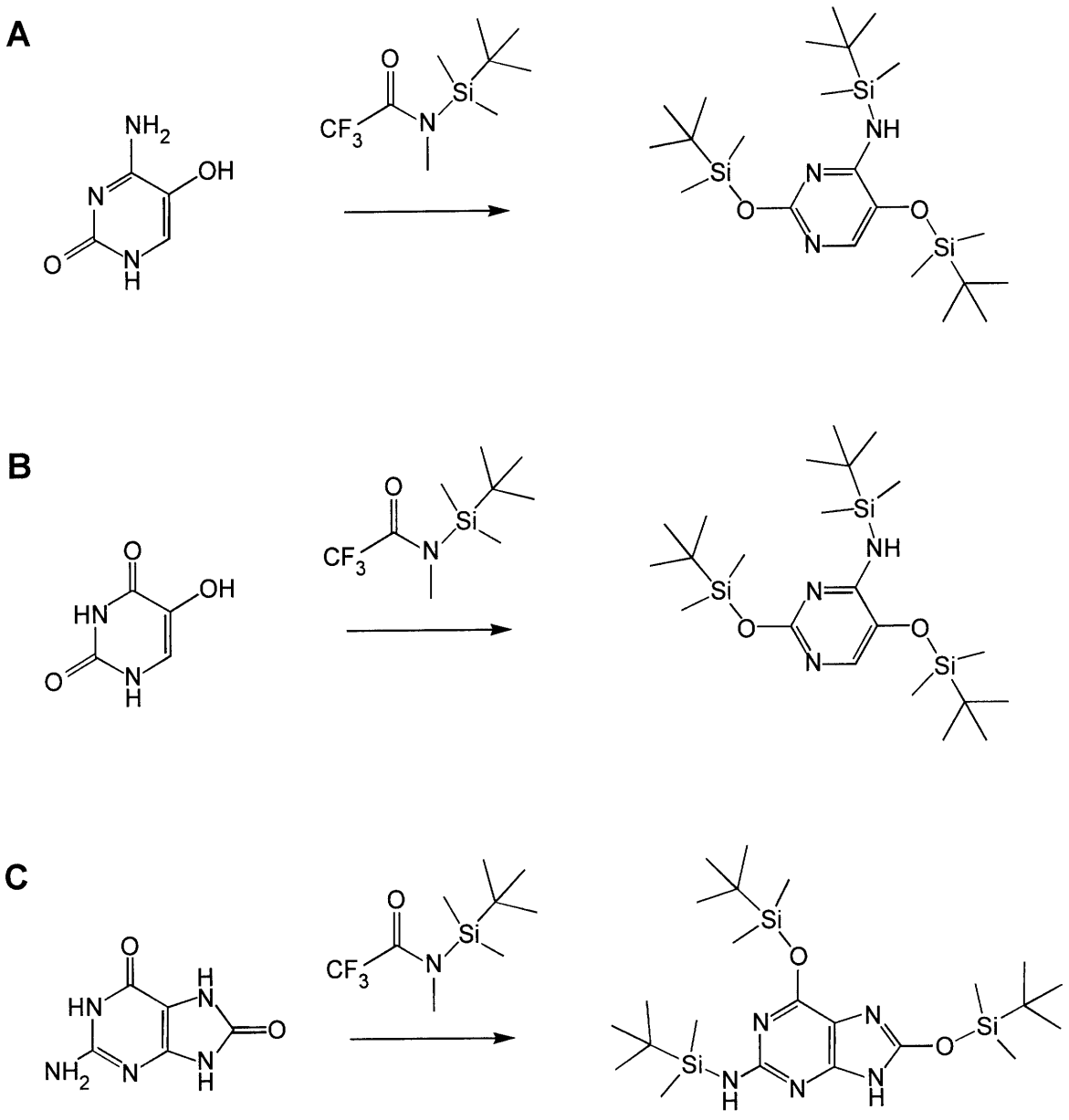


Figure 22: Derivatization for GC-MS analysis: Silylation of 5-OH-C (**A**), 5-OH-U (**B**) and 8-OXO-G (**C**) by *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA).

Table 7: GC-MS characterization of normal and oxidized DNA bases.

Base	Retention time, (min)	Theoretical Derivatization Sites	Actual Derivatization State	Characteristic ion[a]
C	5.48	2	2 TBDMS[b] - 1 TB[c]	282
A	7.13	2	2 TBDMS - 1 TB	306
T	4.89	2	2 TBDMS - 1 TB	297
G	7.5	3	1 TBDMS - 1 TB	208
	8.78		3 TBDMS - 1 TB	436
U	4.48	2	2 TBDMS - 1 TB	283
5-OH-C (^{13}C, $^{15}N_2$)	7.25	3	3 TBDMS - 1 TB	415
5-OH-U	6.84	3	3 TBDMS - 1 TB	413
5-OH-U (^{13}C, $^{15}N_2$)	6.84	3	3 TBDMS - 1 TB	416
8-OXO-G	8.65	4	3 TBDMS - 1 TB	452
8-OXO-G (^{13}C, $^{15}N_3$)	8.65	4	3 TBDMS - 1 TB	456

[a] Characteristic ions corresponded to those mass values that gave the strongest signal.
[b] Samples were derivatized by addition of *tert*-butyldimethylsilyl groups, TBDMS, MW = 115.27.
[c] TBDMS groups are prone to loss of the *tert*-butyl (TB) moiety, MW = 57.

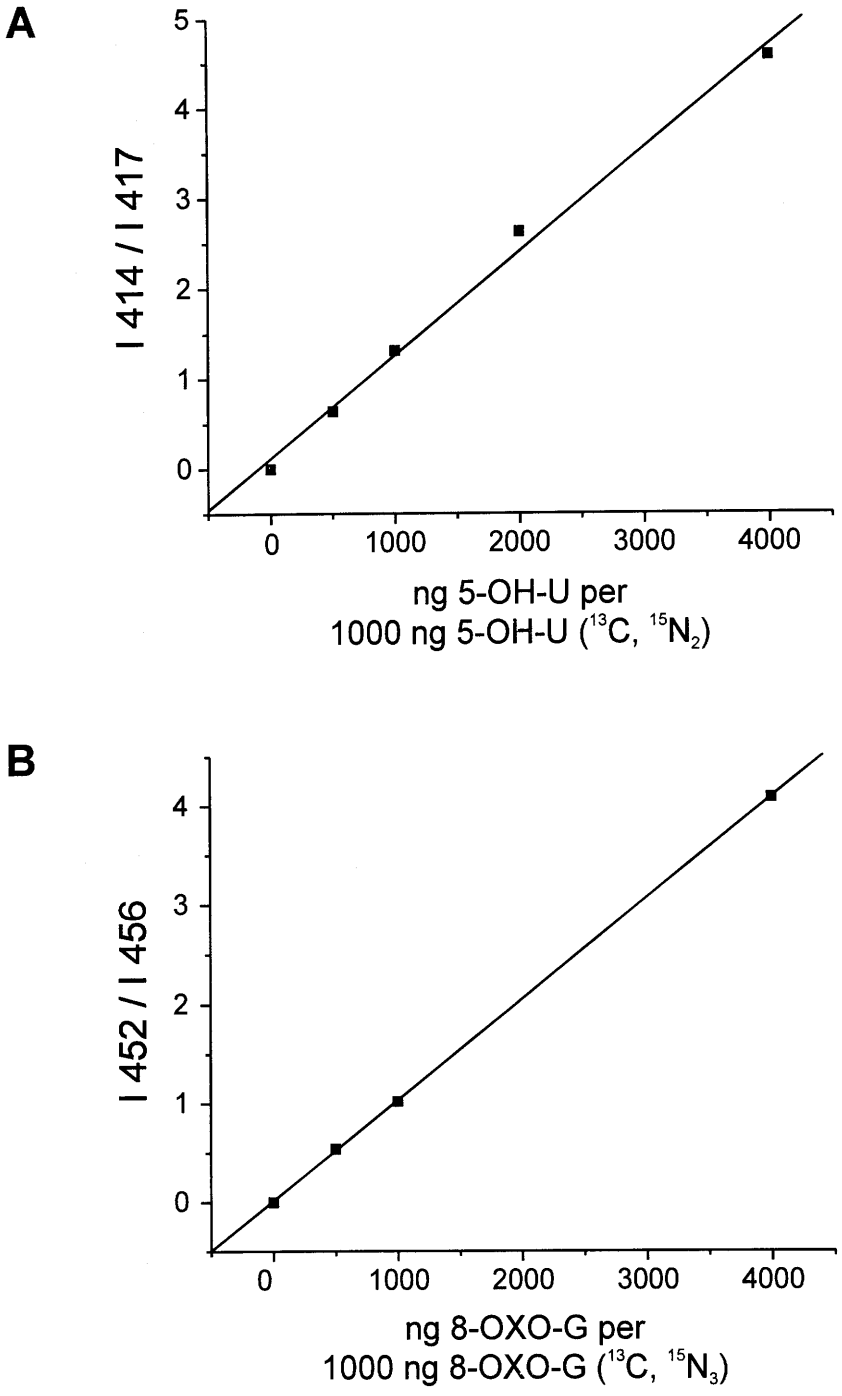


Figure 23: Calibration plots for (**A**) 5-OH-U with current monitored at 414 and 417 and (**B**) 8-OXO-G with current monitored at 452 and 456. Samples containing 5 ng of the isotope standard and 0, 2.5, 5, 10 and 20 ng of the appropriate base were derivatized and analyzed by GC-MS-SIM. The response of the MS increased linearly with increasing amounts of analyte.

1 μg 5'-CA(5-OH-U)GCAG-3' + 5 ng 5-OH-U isotope std

↓

Acid hydrolyze to bases
(formic acid, 100°C, 1 hr in sealed vial)

↓

Derivatize
(MTBSTFA, CH_3CN, pyridine, 130°C, 30 min)

↓

Inject 1 μl onto GCMS

Run #	5-OH-U in Sample*	100 pg Std*	pg 5-OH-U in Sample
1	347,000	27,000	1280
2	311,000	23,000	1350
3	392,000	29,900	1310
4	1,457,000	136,000	1070
			1260

1260 pg 5-OH-U in 1 μl = 63 ng per 50 μl (0.49 nmol)

0.49 nmol 5-OH-U = 0.49 nmol oligo or 1.0 μg

Figure 24: Mock experiment to test the accuracy of the GC-MS method for determining the level of adducts in a DNA sample. Briefly, the amount of 5-OH-U in a sample containing a known amount of singly modified oligonucleotide was measured. (*) Values were obtained by integration of the GC peak corresponding to the 5-OH-U and 5-OH-U isotopically-labeled compounds.

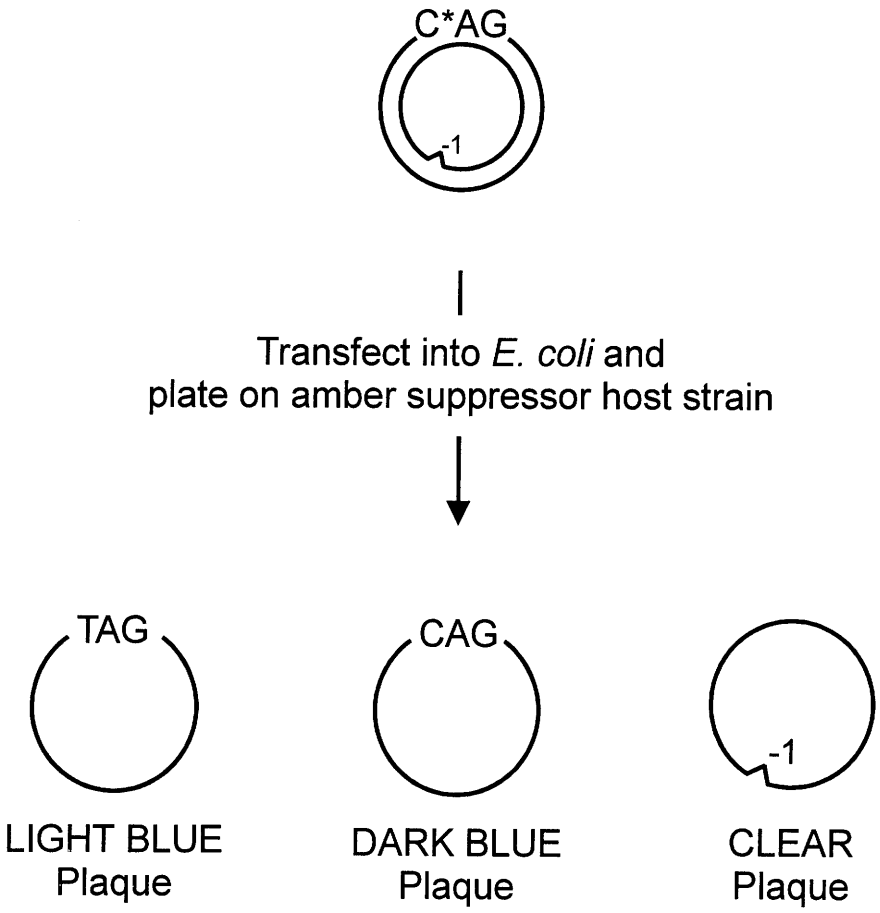


Figure 25: An alternative genetic method to determine the repair proteins removing oxidized dC and dU lesions from DNA *in vivo*. Briefly, ds M13mp7L2 genomes containing either 5-OH-dC, 5-OH-dU and dUg are constructed such that ligation of a modified oligonucleotide into the (+) strand puts the nucleotide sequence of the α-fragment of the *lacZ* gene in frame. Moreover, the lesion is situated within the sequence, C*AG, and thus, a C to T mutation would result in the formation of the stop codon, TAG, in the *lacZ* gene. If plated in the presence of an amber suppressing *E. coli* strain, plaques derived from replication of this strand would be light blue. In contrast, if no mutation is induced, replication of the (+) strand of the genome would yield dark blue plaques. In addition, the (-) strand of the genome is constructed such that the *lacZ* gene is shifted out of frame. Thus, if replication of the (-) strand occurs, clear plaques would be formed. The frequency of C to T mutations can be assessed by determining the ratio of light blue to light plus dark blue plaques.

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