GENOMIC CONSEQUENCES OF DNA OXIDATION BY PEROXYNITRITE

by

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ABSTRACT

The radicals nitric oxide and superoxide are produced endogenously by activated macrophages and neutrophils and combine in a diffusion-limited reaction to form peroxynitrite, a powerful oxidizing and nitrating agent capable of damaging a variety of biomolecules, including DNA. Of the four nucleobases of DNA, guanine has the lowest oxidation potential and thus considerable attention has been given to the study of the oxidation of this base by peroxynitrite. A variety of DNA lesions are generated from guanine including guanidinohydantoin, spiroiminodihydantoin, oxaluric acid, urea, 2-aminoimidazolone, and 5-guanidino-4-nitroimidazole. In order to assess the biological significance and consequences of peroxynitrite-damaged DNA, it is essential that these lesions be characterized for their genotoxic and mutagenic potential. This work focuses on the elucidation of those properties.

In the first study, the 2’-deoxynucleoside of 5-guanidino-4-nitroimidazole was chemically synthesized and incorporated into an oligonucleotide by the phosphoramidite method. In the second study, the genotoxic and mutational properties of 2-aminoimidazolone and 5-guanidino-4-nitroimidazole were determined in wild-type uninduced and SOS-induced E. coli. In the third study, oxaluric acid was found to hydrolyze to urea in a reaction catalyzed by magnesium cations and bicarbonate. The genotoxic and mutational properties of oxaluric acid and urea were determined in wild-type uninduced and SOS-induced E. coli. In the fourth study, the genotoxic and mutational properties of guanidinohydantoin and spiroiminodihydantoin were determined in wild-type uninduced E. coli. In the fifth study, the genotoxic and mutational properties of guanidinohydantoin, spiroiminodihydantoin, oxaluric acid, urea, 2-aminoimidazolone, and 5-guanidino-4-nitroimidazole were determined in wild-type, polymerase II deficient, polymerase IV deficient, polymerase V deficient, and polymerase II / polymerase IV / polymerase V deficient E. coli under both uninduced and SOS-induced conditions. All of the lesions studied were potent sources of mutations in vivo. Guanidinohydantoin, spiroiminodihydantoin, urea, and 5-guanidino-4-nitroimidazole were significant blocks to replication and were strongly dependent upon induction of the SOS system. Polymerase V was responsible for the majority of translesion synthesis.

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Cambridge, Massachusetts
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For Mom and Dad
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ABBREVIATIONS

AP, apurinic/apyrimidinic
Ca, cyanuric acid
Gh, guanidinohydantoin
HPLC, high-pressure liquid chromatography
IPTG, isopropyl-β-D-thiogalactoside
Iz, 2-aminoimidazolone
Kf, Klenow fragment of DNA polymerase I
Kf (exo⁻), Klenow fragment of DNA polymerase I without exonuclease activity
MALDI-TOF, matrix assisted laser desorption ionization-time of flight
8-MeOdG, 8-methoxy-2’-deoxyguanosine
MW, molecular weight
8-NO₂-G, 8-nitroguanine
NI, 5-guanidino-4-nitroimidazole
Oa, oxaluric acid
ODN, oligodeoxynucleotide
ONOΟ⁻, peroxynitrite

8-oxoG, 7,8-dihydro-8-oxoguanine
PAGE, polyacrylamide gel electrophoresis
PNK, polynucleotide kinase
pol α, calf thymus polymerase α
pol β, human polymerase β
pol I, DNA polymerase I
pol II, DNA polymerase II
pol IV, DNA polymerase IV
pol V, DNA polymerase V
REAP, restriction endonuclease and post-labeling analysis of mutation frequency
ss, single-stranded
Sp, spiroiminodihydantoin
THF, tetrahydrofuran
TLC, thin-layer chromatography
Ua, Urea
WT, wild-type
X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside
Z, oxazolone
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CHAPTER 1

Mechanisms of Formation, Genotoxicity, and Mutation of Guanine Oxidation Products: A Survey of the Literature
1.1. Introduction

Endogenous and exogenous oxidants continually assault the genome. A variety of deleterious processes and conditions \( (1) \) may result from DNA oxidation, including aging \( (2-5) \), carcinogenesis \( (6-8) \), and neurological syndromes such as Alzheimer’s disease \( (9) \) and amyotrophic lateral sclerosis (ALS) \( (10) \). DNA oxidation can result from its reaction with compounds of environmental origin such as transition metals \( (11) \) or from reactive oxygen species released during metabolism of exogenous organic compounds such as benzo(a)pyrene \( (12) \). DNA also can be oxidized by reactive oxygen and nitrogen species produced during cellular processes such as respiration and inflammation \( (13, 14) \).

Oxidation of DNA leads to the formation of an assortment of oxidation products \( (15) \). Relative to the other DNA nucleobases, oxidation of guanine (G) occurs most readily due to its low oxidation potential \( (16) \). Sequence context modulates the oxidation potential of G in DNA. For example, ab initio calculations and experimental data show that the 5’-G of stacked GG and GGG sequences oxidize more easily than a lone G in DNA and that the 5’-G of GGG oxidizes more easily than the 5’-G of GG \( (17, 18) \). The resultant radical cations (holes) generated in DNA from one electron abstraction can migrate long distances before being trapped \( (19-21) \). Consequently, there is speculation that GC rich domains outside the coding regions of genes may serve to protect the genome from mutagenesis by oxidation products derived from trapped holes \( (22) \). 7,8-Dihydro-8-oxoguanine (8-oxoG), an oxidation product of G and commonly used biomarker of oxidative stress, is more easily oxidized than G and can therefore serve as a better trap \( (23) \). Stacking with G improves the trapping ability (lowers the oxidation potential) of 8-oxoG in the sequence context 5’-(8-oxoG)G-3’. Interestingly, in the sequence context 5’-G(8-oxoG)-3’, G is preferentially oxidized with a ratio of about 1.5:1 demonstrating the strong influence of sequence context on oxidation potential \( (24) \).

Numerous studies show a predominance of GC→AT, GC→TA, and GC→CG mutations after replication of in vitro oxidized DNA in cells or upon exposure of cells to oxidants \( (25-42) \). Importantly, 8-oxoG does not induce GC→CG mutations, so these results suggest additional G-derived lesions are responsible for the observed mutagenesis.
Indeed, a multitude of products resulting from G oxidation has been reported (Figure 1.1, see Table 1.1 for the full names).

Many reports over the last several years have detailed the formation and properties of G oxidation products. This review will focus specifically on the products shown in Figure 1.1 and summarize the current understanding with regard to the mechanisms of formation, effect on DNA structure and stability, repair potential, and genotoxic and mutagenic prowess of these lesions. 8-OxoG was recently reviewed (15) and will not be discussed except in the context of its oxidation products. Formamidopyrimidine-G (FapyG) lesions have also been reviewed recently (43).
1.2. Chemistry of Nitric Oxide

A specific focus of the work in this thesis is the role of nitric oxide (\(\cdot\)NO) and its derivatives in carcinogenesis. Therefore, a brief discussion of the chemistry of \(\cdot\)NO is appropriate for placing the contents of this thesis in context.

\(\cdot\)NO is an important biological molecule that is involved in cellular signaling and the immune response. An enzyme, known as nitric oxide synthase (NOS), uses L-arginine, O\(_2\), and NADPH as substrates to generate \(\cdot\)NO, citrulline, and presumably H\(_2\)O (44). Three general isoforms of this enzyme exist and they can be divided into two groups. The first group consists of Ca\(^{2+}\)/calmodulin dependent isoforms, namely neuronal NOS (nNOS) (45) and endothelial NOS (eNOS) (46), whereas the second group is generally Ca\(^{2+}\)/calmodulin independent and consists of inducible NOS (iNOS) (47).

With regards to inflammation, iNOS is the most important isoform since it is induced in a variety of tissues including macrophages upon exposure of the tissues to signals such as γ-interferon and lipopolysaccharide (LPS) (48). Whereas small amounts of \(\cdot\)NO are produced by nNOS and eNOS for use as a signaling agent (pM-nM), iNOS produces much higher levels of \(\cdot\)NO (\(\mu\)M) for cytotoxic purposes (49).

The chemistry of \(\cdot\)NO has been reviewed (50, 51) and will be summarized here. The relatively unreactive \(\cdot\)NO reacts to form several reactive products including nitrous anhydride (N\(_2\)O\(_3\)) and peroxynitrite (ONOO\(^-\)). N\(_2\)O\(_3\) is the major nitrosating agent formed from \(\cdot\)NO, and the rate of formation is first order with respect to O\(_2\) and second order with respect to \(\cdot\)NO. The reaction of N\(_2\)O\(_3\) with DNA results in nitrosative deamination.

Formation of ONOO\(^-\) results from the combination of \(\cdot\)NO with O\(_2\)\(^{\cdot-}\), which react at a rate between 6.6 and 19 \(\times\) 10\(^9\) M\(^{-1}\) s\(^{-1}\). It is important to note that the consumption of O\(_2\)\(^{\cdot-}\) by superoxide dismutase occurs at a rate several fold slower than the rate of reaction with \(\cdot\)NO thereby allowing the formation of ONOO\(^-\) to compete with O\(_2\)\(^{\cdot-}\) dismutation. ONOO\(^-\) can protonate to form peroxynitrous acid (ONOOH), which has a \(pK_a\) of 6.8. In
the absence of CO₂ and at physiological pH, ONOOH decomposes with a $t_{1/2}$ of 1.9 s (52) to form •NO₂ and •OH in a solvent cage. Approximately 67% of the time, the two radicals recombine to form NO₃⁻ and H⁺; however about 33% of the time, the radicals escape the solvent cage and become free radicals that can participate in other chemistries. DNA damage by these ONOO⁻ decomposition products occurs primarily on the sugar moiety, leading to strand breaks and oxidized abasic site formation.

The addition of CO₂ to the reaction mixture profoundly changes the chemistry of ONOO⁻ and decreases the $t_{1/2}$ to about 50 ms. CO₂ reacts with ONOO⁻ at a rate of 3-6 × 10⁴ M⁻¹ s⁻¹ to form nitrosoperoxycarbonate (ONOOCO₂⁻). ONOOCO₂⁻ decomposes to CO₃•⁻ and •NO₂ that are initially in a solvent cage. As in the case of ONOOH decomposition, these radicals recombine about 67% of the time to form NO₃⁻ and CO₂ and about 33% of the time diffuse out of the solvent cage becoming free radicals. The major route of ONOO⁻ decomposition should occur via CO₂ since CO₂ exists at concentrations up to 1 mM physiologically. A difference of central importance caused by the presence of CO₂ is the shift in the predominant DNA damage from deoxyribose to guanine bases. In the following sections, the current understanding of guanine base damage is reviewed.
1.3. Mechanisms of Guanine Oxidation Product Formation in Vitro

Oxidation products of G are believed to result from two main pathways, the difference being the presence or absence of 8-oxoG as an intermediate. The first two sections deal with products formed primarily as a result of one- and two-electron oxidations of G and 8-oxoG. Each section is divided according to reactive species, and the products formed as a result of reaction with these species are discussed. The third section summarizes findings from $^1$O$_2$ oxidation of G and 8-oxoG. The major mechanistic pathways are summarized in Schemes 1.1-1.4.

1.3.1. Products derived directly from guanine

A variety of oxidants abstract a single electron from G such as HO$^\bullet$ (53, 54), radicals derived from ONOO$^-$ (55-58), Br$_2^-$ and SO$_4^-$ (59), light (photoionization) (60, 61) and riboflavin (type I photosensitization) (53, 62, 63). This reaction generates a guanine radical cation (G$^{•+}$) that, with a pK$_a$ of 3.9 at the nucleoside level (59), deprotonates at physiological pH to produce the neutral guanine radical (G($^-H$)$^•$). The deprotonation of G$^{•+}$ at the nucleoside level and in DNA occurs with a rate constant on the order of $10^7$ s$^{-1}$ at neutral pH (64). The lifetime of this radical depends on the presence of reactive species, but in the absence of these species, the lifetime can reach ~5 seconds (65).

The reaction of G($^-H$)$^•$ with radicals such as O$_2^•^-$, •NO$_2$, and CO$_3^-$ occurs rapidly and results in the formation of oxidative DNA lesions. A major product of this process is Iz and its hydrolysis product Z (reviewed in (66)). In the literature, these products are sometimes referred to together (Iz + Z) and at other times separately (Iz or Z). Combination of O$_2^•^-$ with G($^-H$)$^•$ occurs with a rate constant of $(4.7 \pm 1.0) \times 10^8$ M$^{-1}$ s$^{-1}$ in both single- and double-stranded oligonucleotides. This reaction results in the formation of Iz as the major product (Scheme 1.1) and 8-oxoG, Sp, and Gh as minor products (67), although it is likely Sp and Gh result from further oxidation of the 8-oxoG produced during the reaction. In contrast to single-stranded oligonucleotides, dGMP($^-H$)$^•$ and G($^-H$)$^•$ nucleoside react faster with O$_2^•^-$ at rates of $(1.3 \pm 0.3) \times 10^9$ M$^{-1}$
s$^{-1}$ (68) and about $3 \times 10^9$ M$^{-1}$ s$^{-1}$, respectively (69). Since these reactions occur in aerated solution, Cadet originally proposed that under $\gamma$-radiolysis conditions dG(−H)$^*$ (formed by reaction of dG with HO$^*$) reacts with $^3$O$_2$ (53). However, $\gamma$-radiolysis produces hydrated e$^-$ that are trapped by $^3$O$_2$ to form O$_2$$^•$ at a rate of $1.9 \times 10^{10}$ M$^{-1}$ s$^{-1}$ (70), and more recent experiments show that $^3$O$_2$ reacts with G(−H)$^*$ at least three orders of magnitude slower than does O$_2$$^•$, making the reaction with O$_2$$^•$ kinetically more favored (69). At the nucleoside level, Iz and its hydrolysis product Z are the major products of HO$^*$ and riboflavin (type I photosensitization) oxidation (71) and represent 80% of the products (53).

The combination of G(−H)$^*$ with *NO$_2$ (generated photochemically) has been studied, and the reaction proceeds with a rate of $\sim 4.3 \times 10^8$ M$^{-1}$ s$^{-1}$ in single- and double-stranded DNA, with the major products being NI and 8-NO$_2$-G (Scheme 1.1) (72). The formation of these products indicates reaction at the C5 (for NI) or C8 (for 8-NO$_2$-G) position of G(−H)$^*$. NI is a chemically stable lesion (73, 74), but 8-NO$_2$-G depurinates with a half-life of about 1 h at 37 °C (pH 7.2) at the nucleoside level (41). Half-lives of about 20 h at 23 °C (pH 7) (75) and 31 h at 25 °C (pH 8) (76) have been reported for 8-NO$_2$-G in single-stranded oligonucleotides, though a half-life as low as 4 h has also been reported for 8-NO$_2$-G in DNA at 37 °C (pH 7.4) (77). Joffe et al. demonstrated that the environment of the parent G affects the ratio of 8-NO$_2$-G to NI formation and found that the ratio decreases from 3.4 in 2',3',5'-tri-O-acetylguanosine to 2.1-2.6 in single-stranded oligonucleotides to 0.8-1.1 in double-stranded oligonucleotides. The maximum yield of these lesions in a single-stranded oligonucleotide containing one G residue is 28% (74).

Campbell and coworkers recently proposed a mechanism for dissociation of guanosine diphosphate (GDP) from Ras proteins (proteins involved in signal transduction) through oxidation of GDP to NI. *NO activates Ras through the exchange of bound GDP for bound GTP, and the study demonstrates that *NO$_2$ produced from the oxidation of *NO by O$_2$ reacts with the protein to form a thyl radical at a cysteine residue that oxidizes the bound GDP. Key interactions between the nucleotide and the protein...
are disrupted during this process and cause the nucleotide to dissociate from the protein and combine with a second equivalent of •NO₂ to form 5-NO₂-GDP, which collapses to form the nucleotide diphosphate of NI (78, 79).

Treatment of G with ONOO⁻ also generates 8-NO₂-G and NI (55). ONOO⁻ breaks down to form •NO₂ and either HO• or CO₃•⁻, depending on the absence or presence of CO₂, and therefore can cause a net oxidation and nitration of G (51). Reaction of CO₃•⁻ with dGMP occurs with a rate of \((6.6 \pm 0.7) \times 10^{7} \text{ M}^{-1} \text{ s}^{-1}\), which is about 10 times faster than the reaction with dATP, dCTP, or dTTP. CO₃•⁻ reacts with a G-containing oligonucleotide at a rate of \((1.9 \pm 0.2) \times 10^{7} \text{ M}^{-1} \text{ s}^{-1}\) (80). Treatment with a 50-fold excess of ONOO⁻ produces NI with a yield of 2.3 ± 0.2% in a single-stranded oligonucleotide and 0.3 ± 0.05% in a double-stranded oligonucleotide (73). The oxidation of 8-nitropurines by ONOO⁻ has been investigated. Oxidation of 9-ethyl-8-nitrooxanthine (a model compound of 8-NO₂-G) produces uric acid (a model compound of 8-oxoG), suggesting 8-oxoG may result from oxidation of 8-NO₂-G (81). \(N^2\)-NO₂-dG has been observed to form as a minor product when •NO and O₂ are bubbled through a solution of dG, and the yield of \(N^2\)-NO₂-dG increases with increasing levels of •NO or O₂ and with increasing pH. 8-NO₂-dG, 8-nitroxanthine, and xanthine also form during this reaction. Incubation at pH 7.4 and 37 °C for one week does not cause decomposition of \(N^2\)-NO₂-dG, indicating the lesion is a stable product (82). Another product from the reaction of ONOO⁻ with dG, nox-G, has been reported once (83).

Mn–TMPyP/KHSO₅ (a two electron oxidant of G) oxidizes dG to generate dG⁺. Presumed capture of the cation by HSO₅⁻ leads to the formation of Iz in 90% yield (84). Oxidation of Iz by Mn–TMPyP/KHSO₅ forms a putative N-oxide of Iz that has a half-life of 2 h at room temperature and pH 7 (84). Oxidation of a GpT dinucleotide by Mn–TMPyP/KHSO₅ generates both Iz and DGh (85). The half-life of DGh in an oligonucleotide is about 5 h at 37 °C in H₂O (86) and in a dinucleotide is 8 h at 0 °C in pH 6.5 buffer (87). In one study, hydrolysis of DGh was proposed to form Iz (88), but this transformation does not occur (87). Hydrolysis of DGh instead leads to Oa and, at
the dinucleotide level, NMR data show that Oa adopts a linear structure with a bond between N1 of Oa and C1’ of the sugar (85, 89) rather than a branched structure with a bond between N3 of Oa and the sugar. Experiments with ESR spectroscopy at 77 K reveal that further oxidation products of 8-oxoG that contain α-diketo groups, such as Oa, can trap electrons to produce anion radicals (90). Also, Oa hydrolyzes to form the tertiary lesion urea (Ua) which, historically, is more typically associated with pyrimidine oxidation (91). Divalent metal cations and HCO$_3^-$ catalyze the hydrolysis of Oa to Ua, and the half-life of Oa nucleoside under these conditions is about 40 h (92).

The structure of DGh was determined by reducing the putative DGh with NaBH$_4$ and then characterizing the resultant Gh product with NMR (87, 89). Other structures (3, 4, 5) (Figure 1.2) with molecular formulae identical to DGh and also leading to the formation of Oa have been proposed to form under other oxidation conditions (93-95); however, Meunier and coworkers have proposed that these other compounds are actually DGh.

Reaction of 3’,5’-di-O-acetyl-dG with HOCl at pH 7.4 generates many products including 8-Cl-dG, Sp, Gh, Iz, Z, and DiZ (96). At neutral pH, Sp is the major product, whereas at pH 5 Iz, DiZ, and Gh predominate. In accordance with observations from previous studies of pH dependence (97, 98), Gh is the principal product when the pH is less than 5 (96). The Sp and Gh lesions formed during the oxidation by HOCl originate from 8-oxoG and are discussed in more detail in the next section.

1.3.2. Products derived from 7,8-dihydro-8-oxoguanine

As with G, one electron oxidation of 8-oxoG forms a radical cation (8-oxoG$^{**}$). Additionally, due to the low oxidation potential of 8-oxoG ($E_7 = 0.74$ V/NHE) (23) relative to G ($E_7 = 1.29$ V/NHE) (16), 8-oxoG can serve as an effective hole trap in DNA. The oxidation of 8-oxodG by G(−H)$^*$ nucleoside occurs with a rate of $4.6 \times 10^8$ M$^{-1}$s$^{-1}$ (23). Upon oxidation, the p$K_a$ of the 8-oxoG nucleoside decreases from 8.6 (99) to 6.6 for 8-oxoG$^{**}$; therefore, 8-oxoG$^{**}$ and 8-oxoG(−H)$^*$ are present in similar proportions at neutral pH (23, 100).
Shafirovich and coworkers investigated the oxidation of 8-oxoG-containing oligonucleotides using kinetic laser spectroscopy and mass spectrometry (101). In this system, irradiation of a 2-aminopurine and 8-oxoG-containing oligonucleotide induces the one-electron oxidation of 8-oxoG. The irradiation also produces hydrated e\textsuperscript{−} that are captured by O\textsubscript{2} to form O\textsubscript{2}\textsuperscript{•−}. Reaction of 8-oxoG(−H)\textsuperscript{•} with O\textsubscript{2}\textsuperscript{•−} occurs with a rate constant of \( \approx 1 \times 10^8 \) M\textsuperscript{−1}s\textsuperscript{−1} in single- and double-stranded DNA and leads primarily to the formation of Oa through the intermediate DGh (Scheme 1.2). When Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is added to the reaction, the lifetime of 8-oxoG(−H)\textsuperscript{•} is 3-5 seconds in single-stranded DNA and 15-20 seconds in duplex DNA. 8-OxoG(−H)\textsuperscript{•} exhibits low reactivity with O\textsubscript{2} (<10\textsuperscript{2} M\textsuperscript{−1} s\textsuperscript{−1}) and, in the presence of Cu,Zn-SOD, instead combines with H\textsubscript{2}O to form Sp as the major product (101). If 8-oxoG(−H)\textsuperscript{•} is not oxidized prior to addition of H\textsubscript{2}O, then formation of Sp likely involves addition of H\textsubscript{2}O to 8-oxoG\textsuperscript{•+}. Provided the pK\textsubscript{a} of 8-oxoG\textsuperscript{•+} does not differ greatly in an oligonucleotide versus at the nucleoside level, a substantial amount of 8-oxoG\textsuperscript{•+} should be present in the pH range of 7-7.5 that is generally used experimentally in G and 8-oxoG oxidation mechanistic studies. For close to 20 years, the structure of Sp was misassigned as 4,8-dihydro-4-hydroxy-8-oxo-2'-deoxyguanosine (102-106). Evidence from HPLC, UV-vis spectroscopy, and ESI-MS/MS lead to the reassignment of the structure as Sp (107), and further proof of the structure was provided using SELINQUATE NMR (108).

The role of pH in the photooxidation of 2’,3’,5’-tri-O-acetyl-8-oxoG by riboflavin was investigated by Burrows and coworkers, and the amounts of each product formed were recorded (97). Riboflavin reacts primarily by a type I mechanism (103, 109), which leads to a one-electron oxidation of 8-oxoG. At pH 7, the predominant product is Sp, whereas Gh and Iz + Z are trace products. The second major product that makes up the remaining mass balance was assigned as Ia\textsuperscript{ox} (4), but another possible structure for this compound is DGh (Figure 1.2) (87). At pH 6, 4 makes up more than 50% of the product mixture and Sp and Gh balance the remaining mass in approximately equal proportions. Addition of superoxide dismutase (SOD) to the reaction decreases the amount of 4 formed to about 25% of the product mixture. Sp and Gh again form in similar
proportions and make up the remaining mass. At pH 8.6, Iz + Z and Sp are the major products and 4 is the minor product, but in the presence of SOD, Sp is the major product and Iz + Z are the minor products. Riboflavin can reduce O₂ to O₂•− (110), so the differences in product distribution at the various pH values are possibly due to a difference in reactivity of O₂•− with the 8-oxoG radical when it is protonated versus neutral and a competing reaction of O₂•− with protonated riboflavin at pH 7. An interesting difference in the chemistry of G versus 8-oxoG is that Iz + Z are only formed from 8-oxoG in substantial amounts under alkaline conditions (97).

There was controversy about whether Gh exists as a mixture of four isomers (diastereomers of two structural isomers, Gh and Ia) or two diastereomers (87, 95). The current consensus is that Gh is an equilibrating mixture of two diastereomers, although Ia may exist in very low quantities (below the detection limit of NMR spectroscopy) (111).

8-OxoG in a single-stranded oligonucleotide reacts with CO₃•− with a rate constant of \((3.2 \pm 0.4) \times 10^8\) M⁻¹ s⁻¹, which is about 10 times faster than reaction with the same oligonucleotide containing a single G instead of 8-oxoG \([2.4 \pm 0.3] \times 10^7\) M⁻¹ s⁻¹]. Reaction of a self-complementary oligonucleotide containing a single G residue reacts about half as fast as a single-stranded oligonucleotide containing a single G \([(1.4 \pm 0.2) \times 10^7\) M⁻¹ s⁻¹], and an oligonucleotide containing no G residues reacts even slower \([(5.7 \pm 0.6) \times 10^6\) M⁻¹ s⁻¹]. The major end product of CO₃•− oxidation of G and 8-oxoG is Sp in both single and double-stranded DNA, and the formation of Sp from G appears to go through 8-oxoG as an intermediate (Schemes 1.1 and 1.2) (112). Whereas reaction of CO₃•− with a G-containing single-stranded oligonucleotide generates Sp with a maximum yield of 60%, continued production of CO₃•− causes a slow degradation of Sp (112), presumably due to oxidation of the lesion. Interestingly, CO₃•− is the source of the oxygen atom incorporated into Sp when this lesion forms from 8-oxoG through this scheme. Oxidation of G to Sp proceeds through 8-oxoG as an intermediate and results in the incorporation of two oxygen atoms from two equivalents of CO₃•− (113).
Another mechanism of Sp formation (Scheme 1.2) involves $^\cdot$NO$_2$ addition to 8-oxoG(−H)$^\cdot$ and occurs with a rate constant of $\sim 4.3 \times 10^8$ M$^{-1}$s$^{-1}$ in single- and double-stranded DNA, yielding Sp as the major product at pH 7.5. The oxygen atom in Sp originates from water, not $^\cdot$NO$_2$ as determined isotopically (72). This result has significant mechanistic implications for ONOO$^-$-related chemistry since $^\cdot$NO$_2$ is a product of ONOO$^-$ breakdown.

Tannenbaum and coworkers have investigated the role of ONOO$^-$ flux in the identity and distribution of 8-oxoG oxidation products (98). Two groups of products were identified with the major mechanistic difference being the identity of the attacking nucleophile after the initial oxidation of 8-oxoG (Scheme 1.3). When a bolus of ONOO$^-$ is used (high initial concentration of ONOO$^-$), ONOO$^-$ can add nucleophilically to the oxidized 8-oxoG to form D Gh as the major product along with NO$_2$-D Gh and CAC as minor products. When ONOO$^-$ is infused (low flux), water is the source of exogenous oxygen atoms and the resultant products are Sp, Gh, and HICA (114). The ratio of Sp to Gh varies dramatically depending on the pH of the reaction solution. Below pH 5.8, Gh forms preferentially, whereas above pH 5.8 Sp predominates. It was therefore proposed that the $pK_a$ of the Sp and Gh precursor, 5-hydroxy-8-oxo-7,8-dihydroguanine (5-HO-8-oxoG), is 5.8. Initial studies of peroxynitrite oxidation of 8-oxoG revealed that at low [ONOO$^-$]/[DNA] ratios (<5), 3a-hydroxy-5-imino-3,3a,4,5-tetrahydro-1H-imidazo[4,5d]imidazol-2-one (5) is the major product and hydrolysis of this compound leads to Oa. A more recent study showed that 5 is likely DGh (87). At high [ONOO$^-$]/[DNA] ratios (>10), Ca and its precursor CAC are the major products (94).

Whereas the discussion above shows that the ratio of Sp to Gh formed from oxidation of 8-oxoG is highly dependent on pH, other factors are also important. Burrows and coworkers investigated the oxidation of 2’,3’,5’-tri-$O$-acetyl-8-oxoG by the one-electron oxidants Na$_2$IrCl$_6$, K$_3$Fe(CN)$_6$, and CoCl$_2$/KHSO$_5$ and found that at pH 7, the major product of the reaction is Sp regardless of the oxidant used (115). However, starting with unprotected 8-oxodG, Henderson and coworkers found that Gh is the major
oxidation product over the pH range 4.6 to 8.4 (116). In duplex DNA, oxidation of 8-oxoG by Na₂IrCl₆ also produces Gh as the major product (117, 118). These results reveal an underlying complexity to the mechanism of Sp and Gh formation. In the presence of peptides, crosslinks between Sp or Gh and a peptide form upon oxidation of 8-oxoG in DNA (119).

Oxidation of duplex DNA with Cr(VI)/ascorbate (ascorbate reduces Cr(VI) to Cr(IV)) at pH 7.0 converts 1.44 ± 0.06% of all guanines to Sp when 50 uM Cr(VI) is used and 0.315 ± 0.05% of guanines to Sp when 3.1 uM Cr(VI) is used, and the amount of Sp formed is about 20-fold higher than 8-oxoG (120). Reaction of peroxo-Cr(V) with calf thymus DNA at pH 7.2 yields Gh as a major product and 8-oxoG as a minor product, and reaction of 8-oxodG under the same conditions also yields Gh as the major product (121). These contrasting results with respect to which product forms predominantly suggest that at least in some cases the reaction pathways to Sp and Gh in duplex DNA are influenced by the nature of the oxidant.

Treatment of an oligonucleotide containing A, T, C, and G with 100 uM Cr(V)-Salen shows piperidine cleavable G specific oxidation, and the amount of piperidine cleavage does not increase upon posttreatment with Na₂IrCl₆ suggesting that any 8-oxoG formed is further oxidized. Using an oligonucleotide containing a site-specific 8-oxoG lesion, oxidation with Cr(V)-Salen occurs at the site of the 8-oxoG lesion with high selectivity and generates both Sp and Gh (122, 123).

Reaction of 8-oxodG with HOCl produces Sp as the major product, and the mechanism is believed to involve addition of the highly electrophilic Cl⁺ to the C4-C5 double bond. A solution of 1 mM 8-oxodG with 0.5 mM HOCl forms Sp in 76% yield with respect to the amount of HOCl present. For comparison, oxidation of 8-oxoG with CoCl₂/KHSO₅ or ONOO⁻/N-AcCys produces Sp with yields of 32% and 4%, respectively (124, 125). Certain biological compounds hinder the oxidation of dG and 8-oxodG. For example, the tea polyphenol epigallocatechin gallate (EGCG) inhibits the oxidation of dG and 8-oxodG by HOCl; however, the inhibition effect was greater for 8-oxodG (126).
Other biological compounds increase the efficiency of oxidation product formation. At low concentrations of HOCl (100 uM), Sp forms at levels 6.6 fold higher than 8-Cl-dG, and micromolar amounts of nicotine increase the yield. Nicotine apparently improves the reaction yield through the formation of a nicotine-Cl⁺ adduct that reacts with dG and 8-oxodG more efficiently than HOCl. Higher concentrations of nicotine (above 50 uM) cause an increase in the yield of 8-oxoG at the expense of Sp (125). Burrows and coworkers recently reported that oxidation of 8-oxoG in the presence of spermine produces Ua (127).

### 1.3.3. Type II photosensitized oxidation of guanine and 7,8-dihydro-8-oxoguanine

DNA is susceptible to oxidation by ¹⁰₂ (128-136). Photoexcitation of dGMP with UVB light generates ¹⁰₂ by type II photosensitization (energy transfer from a photoexcited sensitizer to ³O₂), and ¹⁰₂ then reacts with the nucleobase (137) to form the 5’-monophosphates of Sp and Iz + Z (138, 139). An earlier study noted that UV irradiation of the A, T, C, and U nucleobases produces ¹⁰₂; however, irradiation of G (free base), dGMP, and TpG do not cause ¹⁰₂ to accumulate (140), suggesting the reaction of ¹⁰₂ with the guanine nucleobase occurs quickly relative to the production of ¹⁰₂. The reaction of ¹⁰₂ with G is a [4+2] cycloaddition with the imidazole ring to form an endoperoxide (106, 141-145), which leads to the formation of 5-HO-8-oxoG (Scheme 1.4) (146). 5-HO-8-OxoG subsequently degrades to Sp or Gt, depending on the reaction conditions. As an aside, a previous study by Loeb and coworkers showed that methylene blue and white light treatment (a source of ¹⁰₂) of an M13mp2 single-stranded genome causes the formation of an unidentified DNA lesion that is both a block to replication and induces 66% G→C and 27% G→T mutations (31). Given what is now known about the mechanisms of G oxidation product formation and the mutational properties of these DNA lesions (see section 1.6 and Table 1.4), it is likely the DNA damage observed by Loeb and coworkers is Sp.

Two pathways to 5-HO-8-oxoG from the cycloaddition product have been proposed. One pathway proposes that two equivalents of ¹⁰₂ and two reduction steps result in the formation of 5-HO-8-oxoG, with no oxygen atom incorporation from H₂O.
This mechanism is inconsistent with a prior study where $^{18}$O from H$_2^{18}$O was found in the final Sp product. The proposed mechanism in the latter case involves only 1 equivalent of $^{1}$O$_2$ (Scheme 1.4, path B) (111). However, Foote and coworkers note that in their mechanism (path A) exchange of H$_2$O with the HOO- or HO- groups at C5 would account for the incorporation of oxygen from H$_2$O (146). Oxidation of dG by $^{1}$O$_2$ generates a minor product, DiZ, in addition to 8-oxodG, Iz + Z, and Sp. DiZ forms directly from dG, not from 8-oxodG, and hydrolyzes to form Iz + Z (147).

As shown in Scheme 1.4, path B, $^{1}$O$_2$ adds to 8-oxoG across the C4 and C5 bond by [2+2] cycloaddition to form a dioxetane intermediate (148), which opens to form 5-hydroperoxy-8-oxo-4,8-dihydroguanine (5-HOO-8-oxoG) and eventually 5-HO-8-oxoG (146). The structures of 5-HOO-8-oxoG and 5-HO-8-oxoG were initially misassigned as the respective 4-isomers (148). The distribution of end products from $^{1}$O$_2$ oxidation of 8-oxoG varies depending on the environment of the 8-oxoG base. At the nucleoside level, oxidation of the 8-oxoG base by methylene blue photosensitization generates Ca, Iz + Z, and Sp with yields of 50%, 35%, and <10%, respectively (149, 150), whereas Oa is by far the major product in single-stranded oligonucleotides (86). Methylene blue can participate in type I photosensitization to a small extent (151), so Cadet and coworkers investigated the oxidation of 8-oxoG by a “clean” source of $^{1}$O$_2$ (produced thermally, rather than photochemically) and found that essentially no Ca forms, suggesting other methylene blue-related reactions may be involved in the production of Ca (152). Iz and DGh appear to form from the intermediate 5-HOO-8-oxoG (Scheme 1.4). The photosensitized oxidation of 8-oxoG nucleoside by methylene blue in the presence of a thiol generates Sp as the major product. Presumably, the thiol reduces the 5-HOO-8-oxoG intermediate to 5-HO-8-oxoG, thereby allowing Sp to form (107). This result also suggests that Iz and DGh result from the 5-HOO-8-oxoG intermediate. Two additional compounds, 6-amino-2,5-diimino-2,5-dihydro-3H-pyrimidin-4-one (1) and 1,3,5-triazepane-2,4,6,7-tetrone (2), result from $^{1}$O$_2$ oxidation of 8-oxoG, but to date have only been observed in organic solvent (Figure 1.1) (144, 148).
1.4. Structural Aspects of Guanine Oxidation Products

Relatively few structural studies have been reported for G oxidation products. Helix melting temperature ($T_m$) experiments represent the majority of the investigations. DNA melting studies indicate duplexes containing Sp or Gh paired opposite A, T, C, or G are destabilized relative to duplexes containing a G:C pair at the same position. Additionally, duplexes consisting of one strand that contains an internally placed Sp or Gh and a complementary strand that occupies the region 3’ of the lesion with no base opposite the lesion experience a slight thermodynamic destabilization compared to when a G occupies the site of the lesion. The degree of thermodynamic destabilization generally increases as the length of the complementary strand increases (153). In a 14mer, the presence of a Ca:C base pair reduces the melting temperature by 15 °C to 40 ± 1 °C in comparison to an unmodified oligonucleotide containing a G:C pair at the same location (154). NI paired with A, T, C, or G decreases the melting temperature of a 19mer duplex by 10-12 °C (155).

A computational study of the Sp nucleoside reveals the rings of the diastereomeric lesions are essentially planar and perpendicular to each other (156). In comparison to G, the conformational freedom is restricted. The authors also compare the tautomeric forms that the lesions may adopt. Of the two possible amino forms, one was chosen to compare with the imino form and is 1 kcal/mol lower in energy (156). Uncertainty remains as to which of the two amino forms is preferred. Molecular dynamics simulations show that the Sp diastereomers generally are situated in the major groove of B-DNA and that intercalation structures severely disrupt duplex structure (157). Differences in duplex stability were noted for one diastereomer versus the other. For example, the lesions adopt both syn and anti conformations, but the opposing base and the stereochemistry of the lesion determine the preferred conformation. The lesions also form hydrogen bonds with the opposing base, and the $R$ isomer forms hydrogen bonds more favorably than the $S$ isomer.
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Ua exists as a mixture of $\alpha$- and $\beta$-anomers, and an NMR study showed that both anomers are present in a duplex 12mer. The $\beta$-anomer aligns interhelically with the base stack and shares at least one hydrogen bond with the complementary base T (158).
1.5. Repair of Guanine Oxidation Products

*E. coli* has three enzymes, MutM, MutY, and MutT, that belong to the GO system and protect the cell from mutagenesis by 8-oxoG (159, 160). Polymerases incorporate A opposite 8-oxoG, which leads to G→T mutations, but also incorporate C opposite the lesion, which produces no mutation. MutM excises 8-oxoG lesions when they are paired opposite C,T, and G, but shows weak activity towards 8-oxoG:A pairs (161). In contrast, MutY removes A when paired opposite 8-oxoG (162). Thus, the excision of A when paired with 8-oxoG by MutY gives the cellular replication machinery a second opportunity to insert a C opposite the lesion, which in turn allows the lesion to be excised by MutM. Endonuclease III (Endo III or Nth) (163) recognizes many oxidized pyrimidines (164, 165) and is of interest since most of the G oxidation products lack the two ring purine structural feature. Recently, endonuclease VIII (Nei) was found to possess activity for 8-oxoG paired with G, A, or C. Nei is hypothesized to prevent mutations resulting from 8-oxoG incorporation in the nascent strand opposite A in the template strand (166).

Several groups have tested 8-oxoG oxidation products as substrates for repair enzymes. Table 1.2 lists the kinetic properties of *E. coli* Nei, Nth, and MutM for the excision of Sp and Gh when paired opposite C, G, and A. Base pairs containing G are the preferred substrates for Nei, whereas base pairs containing C are preferred by MutM. Nth shows similar specificity for base pairs containing C or G. Significantly, only Nei can efficiently excise Sp and Gh when paired opposite A (167). Another study showed qualitatively that MutM excises Sp and Gh when paired with A, T, C, or G, although the rate of removal of the lesion opposite A is markedly lower (118). The ability of MutY to excise A, C, or G when paired with Sp or Gh has been investigated. Although MutY shows negligible glycosylase activity towards these base pairs, the enzyme does form stable complexes with the lesion-containing duplex oligonucleotides. Additionally, removal of Sp or Gh by MutM is inhibited by 10 nM MutY when the lesions are paired with G but not when they are paired with C, suggesting the two enzymes interact to prevent potentially mutagenic repair processes. Nth was inhibited in all cases by MutY,
whereas Nei was inhibited only when a higher concentration of MutY (100 nM) was used (118, 167).

Oa and Z paired opposite the four natural nucleobases are efficiently removed by MutM and Nth. Comparison of the catalytic efficiencies for the repair of Oa:C or Z:C versus 8-oxoG:C reveals that 8-oxoG is a better substrate by 2- and 4-fold, respectively. In contrast, Oa:C and Z:C are much better substrates for Nth than 5-hydroxycytosine:G by about 100-fold (168, 169). A separate study showed MutM excises Oa quantitatively, whereas 70% of Z and less than 10% of Ca are excised when these three lesions are paired opposite C (170). Gasparutto et al. found that neither MutM nor Nth could excise Ca from a duplex containing a Ca:C base pair (154). NI is a weak substrate for MutM and is not a substrate for Nth under the conditions tested (73).

Eukaryotic repair enzymes also show activity towards some of the lesions discussed in this review. Murine NEIL1 and NEIL2 efficiently excise Sp and Gh from single-stranded oligonucleotides. Both enzymes also excise Gh from double-stranded DNA opposite all four native bases with similar efficiency. NEIL1 shows activity for Sp in duplex DNA; however, NEIL2 shows little activity, but does bind to the Sp-containing oligonucleotide. The two enzymes show little to no activity for single- or double-stranded substrates containing 8-oxoG (171).

The eukaryotic homologues of MutM, known as OGG, have been screened for activity towards Sp- and Gh-containing oligonucleotides. The human homologue, hOGG1, shows activity only for 8-oxoG, with a 1000-fold preference for 8-oxoG paired with C over 8-oxoG paired with A. The yeast homologues, yOGG1 and yOGG2, remove Sp and Gh from duplexes, and the base paired with Sp or Gh does not significantly affect the rate. Whereas yOGG1 removes Sp and Gh at similar rates, yOGG2 removes Sp more efficiently than Gh. For comparison, the rate of removal of Sp and Gh by the yOGG enzymes is about 20-30 fold lower than the removal of 8-oxoG by hOGG1 or MutM. The ability of the yOGG proteins to excise Sp and Gh regardless of the identity of the
complementary base indicates the action of the yOGG proteins on these lesions is a mutagenic process except when the complementary base is C (172).

Human methylpurine DNA N-glycosylase (MPG) removes Ca when paired with any of the natural nucleobases, although Ca:C and Ca:T pairs are removed more efficiently than Ca:G and Ca:A pairs. Other enzymes tested in this study include MutM, Nth, AlkA, Ogg1, Ntg1, Ntg2, hOgg1, and hNth1, but these enzymes do not show activity for Ca-containing duplex substrates. Comparison of the rate of excision of 7-methylguanine:C versus Ca:C shows that the removal of 7-methylguanine is only 1.6-fold higher than that for the removal Ca indicating that Ca:C is a relatively good substrate for MPG (173).

Incorporation of 8-oxodGMP by DNA polymerases during replication induces AT\(\rightarrow\)CG mutations. The *E. coli* enzyme MutT hydrolyzes the triphosphate of 8-oxodGTP with a specificity 1000-fold higher than dGTP in order to prevent the incorporation of this lesion into the cellular genome (174, 175). A human homolog, denoted as hMTH1 (human MutT Homolog-1), exists and also possesses 8-oxodGTPase activity (176, 177). The ability of MutT or its homologs to hydrolyze triphosphates of the oxidation products discussed in this review has not been reported; however, given that the DNA repair enzymes discussed above often act on G oxidation products besides 8-oxoG, it would not be surprising if the triphosphates of these lesions are also substrates for MutT, its eukaryotic homologs, or another class of enzymes operating in a similar fashion to maintain the informational integrity of the nucleotide pool.
1.6. Genotoxic and Mutational Properties

The genotoxic and mutational properties of DNA lesions illustrate potentially deleterious aspects of lesion formation and therefore are critical metrics. The following sections review *in vitro* and *in vivo* mutagenesis studies involving G oxidation products. In the *in vitro* studies, the ability of a polymerase to extend a primer annealed to a template containing a single lesion at a defined position (known as a site-specifically modified template) is determined along with the identity of the base inserted opposite the lesion. These experiments are advantageous because the conditions can be precisely defined, the kinetic parameters for replication of a lesion by a specific polymerase can be determined, and the experiments can be completed relatively quickly. While critical for defining enzymatic properties, *in vitro* studies do not present a complete analysis of DNA lesion properties since they neglect the complexity of the cellular environment. *In vivo* experiments assess the genotoxic and mutagenic properties of a DNA lesion in the presence of all replication and repair components, or in the presence of these components minus specifically deleted or dysfunctional gene products, and thus provide a highly relevant appraisal of DNA lesion properties. The *in vivo* experiments discussed below use a site-specifically modified single-stranded M13 vector that can replicate in *E. coli* as a model for *in vivo* leading strand DNA replication (178). From these studies, the effect of the *E. coli* replication and repair machinery on a DNA lesion can be determined.

### 1.6.1. *In vitro* properties of guanine oxidation products

Sp and Gh are strong blocks to replication by Klenow fragment (Kf) and Klenow fragment exo⁻ [Kf (exo⁻)] (Table 1.3). In the presence of all four deoxynucleotides, the major product of standing start primer extension in the case of 8-oxoG-containing template is the full length product, but in the case of Sp- or Gh-containing template, only trace amounts of full length product are detected. For both Sp- and Gh-containing templates, the polymerase inserts predominantly A and G opposite the lesions, which would result in G→T and G→C mutations, respectively (117, 153). In a different sequence context, Sp and Gh are complete blocks to full length primer extension by Kf (exo⁻), and the polymerase inserts almost entirely A opposite the lesions (123). Under running start conditions where the primer must be extended by two bases prior to
insertion opposite the lesion, similar results are obtained as under standing start conditions with the exception of Gh, which is bypassed with much higher efficiency when the proceeding two bases in the template are G rather than A. This result suggests that the ability of Kf to bypass Gh is dependent on the stability of the duplex region upstream of the lesion (153).

Replication of Iz using DNA polymerase I (pol I) or the Kf shows that the lesion codes exclusively as a C, but it is inefficiently bypassed (62). Ab initio studies of Iz in vacuo and using a self-consistent reaction field method of solvation show that the lesion mimics the hydrogen bonding face of C, thereby providing a rationale for the G→C mutations induced by this lesion (62, 63). Ca does not block replication when Kf (exo−) is used. The polymerase inserts A most readily opposite Ca, but also G to a much smaller extent (154). Primer extension using Kf (exo−) reveals that A is incorporated opposite Oa and that full length extension results. When Taq DNA polymerase is used as the replicating polymerase, both A and G are inserted opposite the lesion, although Oa blocks synthesis to some degree. Oa inhibits replication by DNA polymerase β (pol β) in the presence of individual nucleotide triphosphates and all four triphosphates (168).

In a related study, Kf (exo−) inserts A opposite Z, and in the presence of all four nucleotide triphosphates, a small amount of full length product is observed. Z blocks replication by pol β, whereas Taq incorporates A and, to a smaller extent, C and G opposite the lesion; however, Taq does not fully extend the primer (169). Ua is a block to replication (179, 180). Kf (exo−), pol β, and T7 DNA polymerase can insert a nucleotide before and opposite Ua, but Ua inhibits full length extension of the primer. Kinetic analysis of insertion by Kf (exo−) reveals A and G are incorporated opposite the lesion (179).

The bypass and coding properties of DNA containing NI were previously studied using the purified DNA polymerases Kf (exo−), calf thymus polymerase α (pol α), and human pol β (73). All three polymerases could insert a nucleotide opposite the lesion and, although a kinetic analysis was not performed, the data suggest that extension of the
NI-containing base pair by Kf (exo⁻) and pol α is rate limiting and results in poor translesion synthesis. The nucleotides A and G are incorporated opposite NI by Kf (exo⁻) and pol α, with Kf (exo⁻) favoring insertion of C opposite the lesion. Interestingly, pol β efficiently bypasses the lesion and incorporates mostly C, rendering the lesion non-toxic and non-mutagenic.

The mutational properties of 8-NO2-G were investigated using mammalian DNA polymerases (76). Running start primer extension reactions in the presence of all four dNTPs show that 8-NO2-G inhibits DNA synthesis by pol α and pol β such that nucleotides were incorporated one base prior to the lesion and opposite the lesion, but negligible amounts of full length product result. Primarily, C is incorporated opposite the lesion resulting in no mutation, but to a lesser extent A and T are also inserted. Under similar conditions, pol η and pol κΔC fully extend the primer and experience only minor blocking by the lesion. Pol η inserts similar amounts of C and A preferentially and also inserts G and T. Pol κΔC mainly inserts A, but also inserts C, G, and T opposite the lesion.

1.6.2. In vivo properties of guanine oxidation products

The in vivo DNA polymerase bypass efficiencies of the oxidation products relative to G are given in Table 1.4. In general, the lesions can be separated into two groups based on the bypass efficiency. The first group consists of Oa, Ca, Iz, Z, and Gh, all of which are either well-bypassed or moderately well-bypassed. The second group includes Ua, Sp, and NI, which are poorly bypassed (92, 181-184). For comparison, the bypass efficiency of 8-oxoG in the same system is 85-90% (92, 181, 182). The bypass efficiencies of Ca, Oa, Ua, Iz, Z, and NI were also determined in SOS-induced E. coli. SOS induction upregulates the expression of more than 40 proteins in E. coli (185) including three DNA polymerases, pol II (186), pol IV (187), and pol V (188), which are known as translesion synthesis polymerases because of their ability to bypass DNA lesions (189). The bypass efficiency of NI increases 8-fold under these conditions, whereas no significant increase in the bypass efficiency occurs for the other 5 lesions (92, 181, 183, 184).
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The mutational properties for these lesions in single-stranded DNA as reported by Essigmann and coworkers are presented in Table 1.4 (92, 181-183). In comparison to 8-oxoG, which has a mutation frequency of about 3-7% in the same system, all of the lesions studied are highly mutagenic, suggesting their formation in vivo could be deleterious in the absence of effective repair mechanisms. SOS induction does not significantly affect the mutational signature of Ca, Oa, or Z (92, 181) but does affect the properties of Ua, Iz, and NI (92, 183). It is noted that repair systems that operate only on lesions in double-stranded DNA could represent defenses against these lesions. As indicated above, the replication properties of the lesions were evaluated in single-stranded genomes.

Interestingly, similar lesion structures do not infer similar in vivo bypass efficiencies or mutational signatures. For example, Gh and NI share much structural similarity but Gh is well-bypassed and codes almost exclusively as a C, whereas NI is poorly bypassed and codes mostly as G but also as A, T, and C. Iz and Z also are structurally similar, but Iz codes mostly as a C, whereas Z codes as an A. The mutational signatures of the Sp diastereomers remain qualitatively similar but differ significantly. These results clearly demonstrate that the identity of the lesion influences the rate of polymerase bypass and the choice of dNTP inserted opposite the lesion and suggest that in spite of the structural similarity the interactions between the lesion and the polymerase active site differ enough to cause significant shifts in mutational signature. Structural studies on polymerases in primer-template complexes with DNA lesions are needed to help develop an understanding of the aforementioned important issues.

Comparison of the bacterial in vivo mutagenesis data to the in vitro mutagenesis data with Kf indicates the two do not always correlate. This observation is not surprising given that in vitro conditions vary dramatically from in vivo conditions and that E. coli possesses four other known DNA polymerases besides pol I (Klenow fragment). The results for Ua, Iz, and NI from SOS-induced E. coli strongly suggest involvement of at least one of the SOS-inducible DNA polymerases, namely pol II, pol IV, and pol V. The
differences in mutagenesis data from normal cells and SOS-induced cells show that polymerases in SOS-induced cells do not necessarily insert dNTPs in the same ratio as do the polymerases in normal cells.

Many eukaryotic DNA polymerases have been identified (190) and five (pol η, pol δ, pol κ, pol ζ, and Rev1) are known to be involved in translesion DNA synthesis (TLS) (191). Although some of the in vitro studies discussed earlier report findings for eukaryotic polymerases, only one of these studies involves eukaryotic TLS polymerases and reports quantitative enzymatic data (76). Thus, numerous questions remain concerning the eukaryotic mechanisms for bypassing guanine oxidation products.
1.7. Biological Relevance

Of the lesions reviewed here, only 8-oxoG, 8-NO₂-G and Sp have been detected \textit{in vivo}. Numerous studies report the detection of 8-oxoG in human cells at levels of 1-100 lesions per $10^6$ bases (192). However, given the abundance of 8-oxoG in cellular DNA and its ease of oxidation relative to G, the \textit{in vivo} existence of many of the lesions discussed in this review is plausible. An important recent discovery is the \textit{in vivo} detection of Sp lesions by Sugden and coworkers (193). Previous studies showed that Sp forms in DNA treated with Cr(VI) (potassium dichromate) (120) and that the BER enzyme Nei excises Sp lesions (167). The authors treated Nei deficient \textit{E. coli} with Cr(VI), analyzed for Sp in the genomic DNA using mass spectrometry, and found the amount of Sp is ~20-fold greater than in wild-type cells when treated with 500 µM Cr(VI). The Nei deficient strain does not accumulate 8-oxoG lesions suggesting that Cr(VI) oxidation competes with the MutM/MutY repair system. Essentially no increase in the amount of Sp is observed for cells that are Nei proficient, including cells that are also MutM/MutY deficient (193).

The detection of 8-NO₂-G is useful as a biomarker of inflammation (194) and forms as a result of a variety of conditions such as chronic hepatitis C infection (195), intra-hepatic cholangiocarcinoma (196), \textit{Helicobacter pylori} infection (197), and inflammatory bowel disease (198). \textit{In vitro}, 8-NO₂-G and NI form in double-stranded DNA in approximately equal proportions (74), so it seems reasonable that this lesion should form under the same conditions that generate 8-NO₂-G \textit{in vivo}. Since NI is a chemically stable lesion, unlike 8-NO₂-G, this toxic and mutagenic lesion may accumulate in cells in the absence of an efficient repair system.

The potential discovery of very low amounts of each lesion in the cell relative to 8-oxoG could argue against their biological relevance. However, since the mutation frequency of many of these lesions is at least an order of magnitude higher than that for 8-oxoG, the amount of a well-bypassed lesion in the cell could be an order of magnitude lower than the amount of 8-oxoG and still induce a similar number of mutations. As suggested by the discovery of Sp in repair deficient bacterial cells, an efficient repair
system may have evolved to combat the potent mutagenicity of these DNA lesions. Consequently, discovery of more G oxidation products in cells may be contingent on elimination of the specific DNA repair activities for the lesion in question. As yet undiscovered repair activities may also preclude detection of other G oxidation products.
1.8. Concluding Remarks

Oxidation of G yields a diverse array of products, and many questions remain about the properties of these lesions. Recent studies have provided a much better understanding of the mechanisms of formation for these compounds, but additional studies are needed to explain numerous observations, such as the dependence of the ratio of Sp to Gh formation on the presence or absence of sugar protecting groups. Detailed experimental structural studies of the lesions in DNA and their interactions with repair proteins and DNA polymerases are also needed. Of note, NI, Ca, Sp, and Gh possess the stability necessary for NMR and X-ray structure studies, and phosphoramidite building blocks for automated DNA synthesis have been reported for NI (155) and Ca (154). Many in vitro studies demonstrate the ability of various repair enzymes and polymerases to act on G oxidation products. Further in vitro studies with TLS polymerases are essential since these polymerases likely have a major and critical role in the processing of the lesions. Studies in both prokaryotic and eukaryotic cells are needed to ascertain the true level of involvement of specific repair proteins and DNA polymerases on the oxidation products. Detection of the G oxidation products in vivo must occur to demonstrate the biological relevance of this chemistry. The recent discovery of Sp lesions in vivo should encourage further investigations in this area.
1.9. Acknowledgment

We thank the National Institutes of Health (CA26731 and CA080024) for financial support.
Figure 1.1. Guanine oxidation products discussed in Chapter 1.$^a$

$^a$ The names of these compounds are listed in Table 1.1.
Figure 1.2. Structure of DGh and other proposed precursors of Oa.
Scheme 1.1. Proposed mechanisms for formation of major oxidation products directly from G by one electron processes [adapted from Refs (55, 67, 113)].
Scheme 1.2. Proposed mechanisms for formation of major oxidation products directly from 8-oxoG by one electron processes [adapted from Refs (72, 92, 101, 113)].
Scheme 1.3. Proposed mechanisms for formation of major products of 8-oxoG oxidation by ONOO\(^-\) [adapted from Refs (98, 114)].
Scheme 1.4. $^1\text{O}_2$ oxidation of G and 8-oxoG [adapted from Refs (111, 146)].
### Table 1.1. Common names that appear in the literature for G oxidation products.

<table>
<thead>
<tr>
<th>abbr.</th>
<th>common names</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-oxoG</td>
<td>7,8-dihydro-8-oxoguanine</td>
</tr>
<tr>
<td>8-NO2-G</td>
<td>8-nitroguanine</td>
</tr>
<tr>
<td>N$^2$-NO2-G</td>
<td>N$^2$-nitroguanine</td>
</tr>
<tr>
<td>N</td>
<td>5-guanidino-4-nitroimidazole; nitroimidazole</td>
</tr>
<tr>
<td>NO2-DGh</td>
<td>N-NO$_2$-N'(2,4-dioxo-imidazolidin-5-ylidene)-guanidine</td>
</tr>
<tr>
<td>DGh</td>
<td>dehydroguanidinohydantoin</td>
</tr>
<tr>
<td>Pa</td>
<td>parabanic acid</td>
</tr>
<tr>
<td>Oa</td>
<td>oxaluric acid</td>
</tr>
<tr>
<td>Ua</td>
<td>urea</td>
</tr>
<tr>
<td>HICA</td>
<td>4-hydroxy-2,5-dioxo-imidazolidine-4-carboxylic acid</td>
</tr>
<tr>
<td>Sp1 + Sp2</td>
<td>spiroiminodihydantoin; 2-imino-5,5'-spirodihydantoin</td>
</tr>
<tr>
<td>Gh</td>
<td>guanidinohydantoin; 5-guanidinohydantoin</td>
</tr>
<tr>
<td>Ia</td>
<td>iminoallantoin</td>
</tr>
<tr>
<td>CAC</td>
<td>2,4,6-trioxo[1,3,5]triazinane-1-carboxamidine</td>
</tr>
<tr>
<td>Ca</td>
<td>cyanuric acid</td>
</tr>
<tr>
<td>DIz</td>
<td>diiminoimidazolone</td>
</tr>
<tr>
<td>Iz</td>
<td>imidazolone; 2-aminoimidazolone; 2,5-diaminoimidazolone</td>
</tr>
<tr>
<td>Z</td>
<td>oxazolone; 2,2,4-triamino-2H-oxazol-5-one</td>
</tr>
<tr>
<td>nox-G</td>
<td>4,5-dihydro-5-hydroxy-4-(nitrosooxy)-guanine</td>
</tr>
<tr>
<td>1</td>
<td>6-amino-2,5-diimino-2,5-dihydro-2H-pyrimidin-4-one</td>
</tr>
<tr>
<td>2</td>
<td>[1,3,5]triazepane-2,4,6,7-tetraone</td>
</tr>
</tbody>
</table>
### Table 1.2. Kinetic parameters for the excision of Gh and Sp in duplex oligonucleotides by Nei, Nth, and MutM (adapted from Ref (167)).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nei</th>
<th>Nth</th>
<th>MutM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_{m}^{a})</td>
<td>(k_{cat}^{b})</td>
<td>(k_{cat}^{b}/K_{m}^{c})</td>
</tr>
<tr>
<td>Sp(\cdot)C</td>
<td>30 ± 4.0</td>
<td>1.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Sp(\cdot)G</td>
<td>38 ± 5.2</td>
<td>5.8</td>
<td>0.15</td>
</tr>
<tr>
<td>Sp(\cdot)A</td>
<td>40 ± 6.0</td>
<td>2.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Gh(\cdot)C</td>
<td>40 ± 5.8</td>
<td>1.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Gh(\cdot)G</td>
<td>35 ± 3.8</td>
<td>6.1</td>
<td>0.17</td>
</tr>
<tr>
<td>Gh(\cdot)A</td>
<td>45 ± 5.0</td>
<td>3.0</td>
<td>0.07</td>
</tr>
</tbody>
</table>

\(a\) (nM). \(b\) (min\(^{-1}\)). \(c\) (min\(^{-1}\) nM\(^{-1}\)). N.D., not determined.
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**Table 1.3.** Summary of *in vitro* standing start primer extension results.

<table>
<thead>
<tr>
<th>guanine oxidation</th>
<th>pol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>opposing</th>
<th>insertion</th>
<th>extension</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp</td>
<td>Kf</td>
<td>A, G</td>
<td>+</td>
<td>–</td>
<td>(117, 123, 153)</td>
</tr>
<tr>
<td>Gh</td>
<td>Kf</td>
<td>A, G</td>
<td>+</td>
<td>–</td>
<td>(117, 123, 153)</td>
</tr>
<tr>
<td>Iz</td>
<td>Kf</td>
<td>C</td>
<td>+</td>
<td>–</td>
<td>(62)</td>
</tr>
<tr>
<td>Ca</td>
<td>Kf</td>
<td>A&gt;G</td>
<td>+</td>
<td>+</td>
<td>(154)</td>
</tr>
<tr>
<td>Oa</td>
<td>Kf</td>
<td>A</td>
<td>+</td>
<td>+</td>
<td>(168)</td>
</tr>
<tr>
<td>Taq</td>
<td></td>
<td>A, G</td>
<td>–</td>
<td>–</td>
<td>(168)</td>
</tr>
<tr>
<td>pol β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(168)</td>
</tr>
<tr>
<td>Z</td>
<td>Kf</td>
<td>A</td>
<td>+</td>
<td>–</td>
<td>(169)</td>
</tr>
<tr>
<td>Taq</td>
<td></td>
<td>A&gt;C,G</td>
<td>+</td>
<td>–</td>
<td>(169)</td>
</tr>
<tr>
<td>pol β</td>
<td></td>
<td>none detected</td>
<td>–</td>
<td>–</td>
<td>(169)</td>
</tr>
<tr>
<td>Ua</td>
<td>Kf</td>
<td>A, G</td>
<td>+</td>
<td>–</td>
<td>(179)</td>
</tr>
<tr>
<td>pol β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(179)</td>
</tr>
<tr>
<td>T7 pol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(179)</td>
</tr>
<tr>
<td>NI</td>
<td>Kf</td>
<td>C&gt;A, G</td>
<td>+</td>
<td>–</td>
<td>(73)</td>
</tr>
<tr>
<td>pol α</td>
<td></td>
<td>A, G</td>
<td>+</td>
<td>–</td>
<td>(73)</td>
</tr>
<tr>
<td>pol β</td>
<td></td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>(73)</td>
</tr>
<tr>
<td>8-NO&lt;sub&gt;2&lt;/sub&gt;-G</td>
<td>pol α</td>
<td>C&gt;A, T</td>
<td>+</td>
<td>–</td>
<td>(76)</td>
</tr>
<tr>
<td>pol β</td>
<td></td>
<td>C&gt;A, T</td>
<td>+</td>
<td>–</td>
<td>(76)</td>
</tr>
<tr>
<td>pol κΔC</td>
<td></td>
<td>A&gt;C,G, T</td>
<td>+</td>
<td>+</td>
<td>(76)</td>
</tr>
<tr>
<td>pol η</td>
<td></td>
<td>C, A&gt;G, T</td>
<td>+</td>
<td>+</td>
<td>(76)</td>
</tr>
</tbody>
</table>
### Table 1.4. Summary of bypass efficiencies and mutation frequencies for 8-oxoG oxidation products in wild-type *E. coli* (%).

<table>
<thead>
<tr>
<th>SOS property</th>
<th>8-oxoG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ca&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ni&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Iz&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Z&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gh&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Sp1&lt;sup&gt;c,e&lt;/sup&gt;</th>
<th>Sp2&lt;sup&gt;c,e&lt;/sup&gt;</th>
<th>Oa&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Ua&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>– bypass</td>
<td>~88</td>
<td>65 ± 8</td>
<td>7.0 ± 1.6</td>
<td>60 ± 5</td>
<td>57 ± 13</td>
<td>75 ± 5</td>
<td>9 ± 3</td>
<td>9 ± 4</td>
<td>51 ± 3</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>G→T&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7</td>
<td>97</td>
<td>22</td>
<td>1</td>
<td>86</td>
<td>1.4</td>
<td>27</td>
<td>41</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>G→C</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>8.9</td>
<td>88</td>
<td>&lt;1</td>
<td>98</td>
<td>72</td>
<td>57</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>G→A</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>19</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ bypass</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>57 ± 1</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
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<td>G→T</td>
<td>6.8</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>5.5</td>
<td>N.S.D.</td>
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<td>95</td>
<td>43</td>
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<td>G→C</td>
<td>1.3</td>
<td>N.S.D.</td>
<td>2.5</td>
<td>75</td>
<td>N.S.D.</td>
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<td>N.D.</td>
<td>3.8</td>
<td>46</td>
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<tr>
<td>G→A</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>13</td>
<td>3.4</td>
<td>N.S.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt;1</td>
<td>10</td>
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<sup>a</sup> Ref (181).  <sup>b</sup> Ref (183).  <sup>c</sup> Ref (182).  <sup>d</sup> Ref (92).  <sup>e</sup> Sp1 refers to the isomer eluting faster by HPLC, and Sp2 refers to the slower eluting isomer, see Ref (182).  <sup>f</sup> G→T implies a G*:A initial pairing, which would resolve to a G:C→T:A mutation upon further replication (*G* = guanine oxidation product under study).  N.S.D., no statistical difference.  N.D., not determined.
1.10. References


Chapter 1 – The Literature


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Chapter 1 – The Literature


CHAPTER 2

Efficient Synthesis of DNA Containing the Guanine Oxidation-Nitration Product 5-Guanidino-4-nitroimidazole: Generation by a Postsynthetic Substitution Reaction
Chapter 2 – Synthesis of 5-Guanidino-4-nitroimidazole

2.1. Abstract

A convertible nucleoside was synthesized and used to prepare the 2’-deoxynucleoside of 5-guanidino-4-nitroimidazole (NI), a putative in vivo product of the reaction of peroxynitrite with guanine (G). The convertible nucleoside was incorporated into an oligodeoxynucleotide (ODN) by the phosphoramidite method and converted post-synthetically to yield an ODN containing the NI lesion site-specifically. A melting temperature study revealed that duplexes containing NI are greatly destabilized relative to duplexes containing G at the same site.
2.2. Introduction

Of the four nucleobases of DNA, guanine has the lowest oxidation potential, and thus considerable attention has been given to the study of the oxidation of this base \((1)\). The DNA lesions 7,8-dihydro-8-oxoguanine (8-oxoG) and 8-nitroguanine (8-nitroG) are two major guanine (G) oxidation products that are formed by reaction with the endogenous oxidant peroxynitrite (ONOO\(^-\)) \((2)\). These lesions are also labile to oxidation and form a variety of secondary oxidation products \((3-5)\). These products may result from \textit{in vivo} oxidation by oxidants other than ONOO\(^-\) \((1, 6)\), and many are susceptible to hydrolysis or further oxidation. In contrast, 5-guanidino-4-nitroimidazole (NI) forms directly from the oxidation of G by ONOO\(^-\) and is both chemically stable and resistant to tested forms of enzymatic repair \textit{in vitro} \((7-9)\). Additionally, this lesion may be well-bypassed and mutagenic, as suggested by \textit{in vitro} primer extension assays \((7)\). NI therefore may contribute to the mutagenic spectrum of DNA damage induced by ONOO\(^-\), and its measurement may be useful as a specific biomarker of ONOO\(^-\)-mediated oxidation.

Evaluation of the chemical and biological properties of oxidative DNA lesions is facilitated by the availability of oligodeoxynucleotides (ODN) and, eventually, genomes containing these lesions site-specifically \((1)\). Most commonly, oxidative lesions are prepared site-specifically by damaging a specific base within an ODN \((10-12)\). This approach facilitates and accelerates the initial characterization of these lesions while circumventing the common problem of lesion instability, a major obstacle to incorporation of these damaged bases by automated DNA synthesis. However, low yields and the formation of multiple products that are often difficult to separate limit the use of ODN sequences to those containing only one G residue. The present methods for preparing NI-containing ODNs are hindered by both of these characteristics \((7, 8)\). In advance of biological evaluation of NI mutagenicity and genotoxicity, we sought to develop a synthetic method for the preparation of NI lesions site-specifically within ODNs that is high-yielding and independent of base sequence.
A convertible nucleoside phosphoramidite offers a convenient route to incorporating an unnatural nucleoside at a defined site within an ODN (13). This approach has been used successfully to prepare ODNs containing nucleosides that are unstable to DNA synthesis conditions (14, 15), containing structural analogs of a given base, and with additional functionality (16-22). In the present case, the use of the convertible nucleoside approach would avoid potential protection-deprotection schemes with the guanidino functionality of NI, enabling an efficient synthesis of a phosphoramidite building block. Herein, we describe the synthesis of a convertible nucleoside phosphoramidite for the site-specific incorporation of an NI lesion into an ODN by postsynthetic substitution and the effect of this lesion on duplex stability. Although we only present the synthesis of the biologically relevant NI lesion, the method allows access to other potentially useful alternative structures.
2.3. Experimental Procedures

Materials. Solvents were purchased anhydrous from Sigma-Aldrich, except for THF, which was distilled from CaH$_2$ prior to use. Reagents were purchased and used without further purification from Sigma-Aldrich, except for 3,5-di-O-toluoyl-α-1-chloro-2-deoxy-D-ribofuranose, which was purchased from Chemgenes Corporation. 5(4)-Bromo-4(5)-nitro-1H-imidazole (2) (NMR spectra are shown in Figures 2.1 and 2.2) was prepared as previously described from 5(4)-bromo-1H-imidazole (1) (Sigma-Aldrich) (23). All reactions were conducted under argon atmosphere. DNA synthesis reagents and standard phosphoramidites were purchased from Glen Research. Unmodified ODNs were purchased from IDT, Inc. and HPLC purified prior to use. SVPD was purchased from ICN Biomedical. Nuclease P1 and alkaline phosphatase were purchased from Roche Applied Science.

$^1$H NMR (500 MHz) and $^{13}$C NMR (125.8 MHz) spectra were obtained on a Varian Unity Inova spectrometer. $^{31}$P NMR (121.5 MHz) spectra were obtained on a Varian Mercury spectrometer and referenced to 85% H$_3$PO$_4$. HRMS was performed by the MIT Department of Chemistry Instrumentation Facility. ESI-MS was performed by the MIT Center for Cancer Research and Howard Hughes Medical Institute Biopolymers Laboratory. MALDI-TOF MS were acquired on a PerSeptive Biosystems Voyager-DE STR spectrometer (337 nm laser). Absorption spectra and $T_m$ curves were obtained on a Varian Cary 100 or 300 Bio UV-vis spectrophotometer equipped with a temperature controller unit.

5-Bromo-4-nitroimidazole-3',5'-di-O-toluoyl-β-2'-deoxy-D-ribofuranose (3) and 4-Bromo-5-nitroimidazole-3',5'-di-O-toluoyl-β-2'-deoxy-D-ribofuranose (3A)

Compound 2 (0.500 g, 2.60 mmol) was treated with NaH (60% dispersion in mineral oil, 0.115 g, 2.87 mmol) in 15 mL CH$_3$CN for 25 min with stirring. 3,5-Di-O-toluoyl-α-1-chloro-2-deoxy-D-ribofuranose (1.11 g, 2.87 mmol) was added in portions and the reaction stirred for 3.5 h. The mixture was then diluted with 100 mL of CH$_2$Cl$_2$ and washed with 100 mL of H$_2$O. The aqueous layer was back extracted twice with 50 mL of
CH$_2$Cl$_2$ and the combined organic layers were dried over Na$_2$SO$_4$, evaporated at room temperature on a rotary evaporator, suspended in a minimal amount of 30% hexanes in CH$_2$Cl$_2$, and loaded onto a silica column prepared in 30% hexanes in CH$_2$Cl$_2$. The first isomer was eluted with a step gradient of 17.5-0% hexanes:CH$_2$Cl$_2$ and the second isomer was eluted with a step gradient of 0-4% EtOAc:CH$_2$Cl$_2$. Fractions were pooled and concentrated to yield 3A (0.499 g, 35%) as a white solid and 3 (0.716 g, 50%) as a white foam. Isomer 3A: R$_f$ = 0.48 (1:2 EtOAc:hexanes). $^1$H NMR (CD$_2$Cl$_2$, 500 MHz) (Figure 2.3): $\delta$ = 8.05 (1H, d, $J$ = 0.8 Hz), 7.96 (2H, m), 7.81 (2H, m), 7.28 (4H, m), 6.68 (1H, t, $J$ = 6.2 Hz), 5.60 (1H, dt, $J$ = 3.1, 6.2 Hz), 4.76-4.66 (3H, m), 3.10 (1H, ddd, $J$ = 3.1, 6.0, 14.6 Hz), 2.56 (1H, dt, $J$ = 6.6, 14.6 Hz), 2.43 (3H, s), 2.41 (3H, s); $^{13}$C NMR (CD$_2$Cl$_2$, 125.8 MHz) (Figure 2.4): $\delta$ = 166.5, 166.3, 145.3, 145.2, 137.2, 130.25, 129.98, 129.91, 129.82, 126.93, 126.86, 122.3, 90.9, 84.7, 74.5, 64.2, 41.3, 22.02, 21.98; UV-vis (EtOH) $\lambda_{\text{max}}$ (Figure 2.5): 240, 315 nm; ESI-HRMS calc'd for C$_{24}$H$_{22}$N$_3$O$_7$Br [(M+Na$^+$)] 566.0533, found 566.0520. Isomer 3: R$_f$ = 0.30 (1:2 EtOAc:hexanes). $^1$H NMR (CD$_2$Cl$_2$, 500 MHz) (Figure 2.6): $\delta$ = 7.96 (2H, d, $J$ = 8.2 Hz), 7.88 (1H, s), 7.85 (2H, d, $J$ = 7.9 Hz), 7.28 (4H, dd, $J$ = 7.9, 21.1 Hz), 6.25 (1H, dd, $J$ = 6.1, 7.6 Hz), 5.68 (1H, m), 4.74-4.63 (3H, m), 2.96 (1H, ddd, $J$ = 2.2, 5.9, 14.3 Hz), 2.61 (1H, m), 2.43 (3H, s), 2.41 (3H, s); $^{13}$C NMR (CD$_2$Cl$_2$, 125.8 MHz) (Figure 2.7): $\delta$ = 166.5, 166.3, 145.4, 145.2, 134.1, 130.2, 129.95, 129.92, 129.83, 127.0, 126.8, 103.6, 88.4, 84.3, 75.0, 64.2, 40.1, 22.02, 21.96; UV-vis (EtOH) $\lambda_{\text{max}}$ (Figure 2.8): 241, 296 nm; ESI-HRMS calc’d for C$_{24}$H$_{22}$N$_3$O$_7$Br [(M+Na$^+$)] 566.0533, found 566.0552.

5-Bromo-4-nitro-6'-deoxy-D-ribofuranose (4)

Guanidine hydrochloride (0.175 g, 1.83 mmol) was treated with 0.5 M NaOMe in MeOH (3.53 mL, 1.76 mmol) in 36 mL MeOH and the solution stirred for 15 min in an ice bath. 3 (0.400 g, 0.735 mmol) was added and the suspension stirred in an ice bath for 3.75 h. The reaction was diluted with CH$_2$Cl$_2$ to form a 20% solution of MeOH in CH$_2$Cl$_2$ and then filtered through a bed of silica. The silica was washed with 100 mL of 20% MeOH in CH$_2$Cl$_2$, and the combined solutions evaporated. The mixture was adsorbed on to silica (1.5 g) by suspending in MeOH (5 mL) and then evaporating to dryness. The dry silica was suspended in CH$_2$Cl$_2$ and applied to a silica column prepared in CH$_2$Cl$_2$.
Elution with a step gradient of 3-5% MeOH in CH₂Cl₂ afforded 4 as a white foam (0.204 g, 90%). \( R_f = 0.28 \) (10% MeOH:CH₂Cl₂). \(^1\)H NMR (CD₃OD, 500 MHz) (Figure 2.9): \( \delta = 8.34 \) (1H, s, imidazolic H), 6.22 (1H, t, \( J = 6.1 \) Hz, 1’H), 4.50 (1H, m, 3’H), 4.03 (1H, ddd, \( J = 3.6, \) 3.6, 7.2 Hz, 4’H), 3.80 (1H, dd, \( J = 3.2, 12.1 \) Hz, 5’H), 3.73 (1H, dd, \( J = 3.7, 12.2 \) Hz, 5’H), 2.54 (2H, m, 2’H); \(^1^3\)C NMR (CD₃OD, 125.8 MHz) (Figure 2.10): \( \delta = 136.6, \) 105.4, 90.0, 89.2, 71.7, 62.4, 42.5; gCOSY NMR (CD₃OD, 500 MHz) (Figure 2.11); ESI-HRMS calc’d for C₈H₁₀N₃O₅Br [(M+Na⁺)] 329.9696, found 329.9698.

5-Guanidino-4-nitroimidazole-β-2’-deoxy-D-ribofuranose (5)

Guanidine hydrochloride (77.4 mg, 0.810 mmol) was dissolved in 1.62 mL of 0.5 M NaOMe in MeOH. The solution was stirred, evaporated to dryness, and redissolved in 5 mL of absolute EtOH. To this solution was added 50.0 mg (0.162 mmol) of 4, and the reaction refluxed for 2 h. The solvent was subsequently evaporated and the residue adsorbed onto silica (0.5 g) by suspending in MeOH and then evaporating to dryness. The dried silica was suspended in CHCl₃ and charged to a silica column prepared in CHCl₃. Elution with 15-30% MeOH in CHCl₃ gave 5 as a bright yellow, very hygroscopic solid (45 mg, 98%, by UV-vis spectrophotometry). \( R_f = 0.24 \) (30% MeOH:CH₂Cl₂). \(^1\)H NMR (D₂O, 500 MHz) (Figure 2.12): \( \delta = 7.73 \) (1H, s), 6.08 (1H, t, \( J = 6.7 \) Hz), 4.52 (1H, dt, \( J = 4.0, 6.3 \) Hz), 4.03 (1H, m), 3.77 (1H, dd, \( J = 3.7, 12.5 \) Hz), 3.69 (1H, dd, \( J = 5.2, 12.5 \) Hz), 2.60 (1H, m), 2.46 (1H, ddd, \( J = 4.3, 6.6, 14.1 \) Hz); \(^1^3\)C NMR (CD₃OD, 125.8 MHz) (Figure 2.13): \( \delta = 160.0, \) 143.4, 135.0, 132.2, 89.1, 85.5, 72.4, 63.1, 41.8; UV-vis (H₂O, pH 7) \( \lambda_{max} (\log \varepsilon) \) (Figure 2.14): 230 nm (4.13), 380 nm (3.82); ESI-HRMS calc’d for C₉H₁₄N₆O₅ [(M+Na⁺)] 309.0918, found 309.0917.

5’-O-(4,4’-Dimethoxytrityl)-5-bromo-4-nitroimidazole-β-2’-deoxy-D-ribofuranose (6)

Compound 4 (105 mg, 0.341 mmol) was dried by coevaporation twice with 2 mL of pyridine, once with 2 mL of toluene, and then by high-vacuum overnight. 4,4’-Dimethoxytritylchloride (DMT-Cl) (0.260 g, 0.767 mmol), 4-dimethylaminopyridine (DMAP) (4.2 mg, 10 mol %), and triethylamine (0.107 mL, 0.767 mmol) were combined
and dissolved in pyridine (3.4 mL). The solution was stirred for 5 min and then added to
a flask containing 4. The reaction was stirred for 3.5 h and then evaporated to dryness.
The mixture was dissolved in CH₂Cl₂ and applied to a column of neutral alumina,
Brockmann II, prepared in CH₂Cl₂ with several drops of pyridine. Elution with a step
gradient of 0-0.5% MeOH in CH₂Cl₂ yielded 0.179 g of a yellow mixture. This mixture
is unsuitable for the preparation of 7; thus, 0.208 g of the mixture were flashed through a
column of neutral alumina, Brockmann II, following the same procedure noted above.
Compound 6 was isolated as an off-white foam (0.181 g, 76%). R₇ = 0.46 (1:2
EtOAc:CH₂Cl₂). ¹H NMR (CD₂Cl₂, 500 MHz) (Figure 2.15): δ = 7.79 (1H, s), 7.42-
7.37 (2H, m), 7.33-7.21 (7H, m), 6.84 (4H, m), 6.13 (1H, t,  J = 6.1 Hz), 4.56 (1H, br m),
4.15 (1H, ddd,  J = 4.0, 4.0, 7.9 Hz), 3.78 (6H, s), 3.37 (2H, ddd,  J = 4.5, 10.9, 15.4 Hz),
2.61 (1H, ddd,  J = 4.4, 6.1, 13.6 Hz), 2.43 (2H, m); ¹³C NMR (CD₂Cl₂, 125.8 MHz)
(Figure 2.16): δ = 159.2, 145.1, 136.0, 135.8, 134.6, 130.52, 130.50, 128.46, 128.45,
127.5, 113.69, 113.68, 103.7, 88.2, 87.6, 87.2, 71.6, 63.8, 55.7, 42.2; ESI-HRMS calc’d
for C₂₉H₂₈N₃O₇Br [(M+Na⁺)] 632.1003, found 632.0995.

5'-O-(4,4'-Dimethoxytrityl)-5-bromo-4-nitroimidazole-β-2'-deoxy-D-ribofuranose-
3'-O-2-cyanoethyl-N,N-diisopropylphosphoramidite (7)

Compound 6 (50 mg, 0.082 mmol) was dried by coevaporation twice with 2 mL of
pyridine, once with 2 mL of toluene, and then by high-vacuum overnight. To the flask
containing 6 was added 0.8 mL of CH₂Cl₂ and diisopropylethylamine (0.043 mL, 0.25
mmol). 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (0.027 mL, 0.12 mmol)
was added dropwise by syringe and the reaction was stirred for 45 min at room
temperature. The reaction was poured into 20 mL of saturated aqueous NaHCO₃ and
extracted 3 times with 20 mL of CH₂Cl₂. The organic layers were combined, dried over
Na₂SO₄, and evaporated to an orange residue. The residue was applied to a column of
basic alumina, Brockman II-III, prepared in CH₂Cl₂. Elution with CH₂Cl₂ gave 7 as a
white solid (29 mg, 44%). R₇ = 0.49 and 0.58 (two diastereomers) (10% EtOAc:
CH₂Cl₂). ¹H NMR (CD₃CN, 500 MHz) (Figure 2.17): δ = 7.88 (1H, s), 7.42-7.34 (2H, m),
7.32-7.19 (7H, m), 6.84 (4H, m), 6.12 (1H, dddd,  J = 5.7, 5.7, 5.7, 11.2 Hz), 4.71
(1H, m), 4.18 (1H, d of br m, J = 21.2 Hz), 3.86-3.51 (10H, m including two s from –OCH₃ at 3.76 and 3.75 ppm), 3.37-3.20 (2H, m), 2.72-2.62 (3H, m), 2.53 (1H, t, J = 6.0 Hz), 1.19-1.04 (12H, m); ³¹P NMR (CD₃CN, 121.5 MHz) (Figure 2.18): δ = 150.2, 150.0 (two diastereomers); ESI-HRMS calc’d for C₃₈H₄₅BrN₅O₈P [(M+Na⁺)] 832.2081, found 832.2057.

**NOE-Difference NMR Spectroscopy.** The protons of 4 were assigned using a 2D gCOSY spectrum (Figure 2.11). Approximately 5 mg of 4 were dissolved in CD₃OD and transferred to an NMR tube fitted with a J. Young valve. The solution was degassed with four freeze-pump-thaw cycles, and then sealed. An NOE-difference experiment yielded the results in Table 2.1.

**Oligodeoxynucleotide Synthesis.** ODNs were synthesized on an Applied Biosystems 391 DNA synthesizer using PAC-protected phosphoramidites. The activator reagent was 0.25 M 4,5-dicyanoimidazole in CH₃CN, Cap A was 5% phenoxyacetic anhydride in THF/pyridine, Cap B was 10% 1-methylimidazole in THF, the deblocking reagent was 3% dichloroacetic acid in CH₂Cl₂, and the oxidizer was 0.02 M I₂ in pyridine/THF/H₂O. Syntheses were performed on a 1 µmol scale using 500-Å CPG supports and all coupling efficiencies, including that of the non-natural nucleoside, were greater than 95% as determined by trityl monitoring. The phosphoramidite 7 (16 mg) was dried by coevaporation twice with 2 mL of pyridine, once with 2 mL of toluene, and then by high-vacuum overnight. 7 was manually coupled by dissolving in 150 µL activator (0.25 M DCI in CH₃CN) and then pipetting directly on the CPG support. Coupling proceeded for 20 minutes.

Following DNA synthesis, ODNs containing 5-bromo-4-nitroimidazole were converted to ODNs containing NI by treating with 1 mL of a 0.5 M solution of guanidine in THF for 30 min at room temperature (8NI) or with 1 mL of a 0.5 M solution of guanidine in tert-butanol for 15 h at 55 °C (9NI). The guanidine solutions were prepared by treating 48 mg (0.5 mmol) of guanidine hydrochloride with 1 mL (0.5 mmol) of 0.5 M NaOMe in MeOH for 5 min, evaporating the MeOH, and redissolving in 1 mL of solvent. 8NI and 9NI were deprotected and cleaved from the solid support by treating with
concentrated NH\textsubscript{4}OH at room temperature for 4 hours and 2 hours, respectively. In the case of 8NI, the guanidine was first washed from the solid support with 10 mL of THF prior to treating with NH\textsubscript{4}OH. After treating 9NI with NH\textsubscript{4}OH, the solution was neutralized with triethylammonium acetate and acetic acid and then desalted using a NAP-10 column (Sephadex G-25, Amersham Biosciences).

ODNs containing NI were purified by reversed-phase HPLC on a Varian Microsorb-MV analytical C-18 column (4.6 mm × 25 cm, 5 µm), using 0.1 M triethylammonium acetate and CH\textsubscript{3}CN as solvents. The concentration of CH\textsubscript{3}CN was increased from 0-7% in 0.7 min, then 7-15% in 30 min with a 1.0 mL/min flow rate.

**ESI Mass Spectrometry.** Mass spectra (Figure 2.19) were acquired by direct infusion on a Sciex Model API 365 triple stage mass spectrometer.

**MALDI-TOF Mass Spectrometry.** Mass spectra (Figure 2.20) were acquired using a matrix consisting of anthranilic acid, nicotinic acid, and diammonium citrate (2:1:0.003) in H\textsubscript{2}O and CH\textsubscript{3}CN as described by Zhang and Gross.\textsuperscript{(24)} The instrument was operated in the linear mode with negative polarity. The accelerating voltage was 25,000 V and the grid voltage was 95% of the accelerating voltage. An extraction delay time of 150 ns was used.

**Enzymatic Digestion Analyses.** The purity and content of the ODNs 8NI and 9NI were established by HPLC analysis of the digested ODNs (Figure 2.21). The following conditions were used: 100 mM Tris-Cl, pH 8.8, 15 mM MgCl\textsubscript{2}, 500 mU SVPD, and 2 nmol ODN in 40 µL were incubated at 37 °C for 4.5 h, then 20 U of alkaline phosphatase were added and the mixture incubated for an additional 2 h.

ODN 9NI was digested with nuclease P1 using these conditions: 30 mM NaOAc, pH 5.3, 0.1 mM ZnSO\textsubscript{4}, 17 U nuclease P1, and 2 nmol 9NI in 50 µL were incubated at 37 °C for 2 h. Alkaline phosphatase (10 U, 10 µL) and 6.6 µL 10X buffer (500 mM Tris-Cl,
pH 8.5, 1 mM EDTA) were added and the solution incubated for an additional 2 h (Figure 2.22).

Digestion mixtures were analyzed at 254 nm by reversed-phase HPLC with a Supelco Supercosil C-18 analytical column (2.1 mm × 25 cm, 5 µm), using 150 mM NH₄OAc and CH₃CN as eluting solvents. The concentration of CH₃CN was increased from 0-15% over 40 min at a flow rate of 0.25 mL/min.

**Thermal Denaturation Studies.** Melting temperatures were determined in teflon-stoppered 1 cm pathlength quartz cells. Absorbances were monitored at 260 nm and the temperature was cycled between 20 and 85 °C at a rate of 0.5 °C per min with a 5 min delay at each temperature extreme. $T_m$ values and thermodynamic parameters were extracted from the melting curves (Figures 2.23 and 2.24) using the program MeltWin 3.5. Solutions consisted of 0.75 µM ODN in phosphate buffer (5 mM sodium phosphate, 50 mM NaCl, pH 7.0) in a total volume of 1.32 mL. All duplexes obeyed an apparent two state transition, and annealing curves matched melting curves.

**Semi-Empirical MO Calculations.** Calculations were performed using Hyperchem 6.03 on a Pentium IV 1.3 GHz personal computer. The geometry of the molecule was first optimized using the MM+ molecular mechanics method (Hyperchem’s version of MM2), and then optimized using the AM1 semi-empirical method without constraints. Several minima of similar energy in addition to that shown in Figure 2.25 were found (including syn conformations), and all depicted the guanidino group out of plane with respect to the imidazole ring. Geometries were optimized until a root-mean-square gradient of less than or equal to 0.01 kcal/(Å mol) was achieved.

**M13 Genome Constructions.** ODNs 8NI and 8G were inserted into an M13mp7(L2) bacteriophage genome using a previously reported procedure (Figure 2.26) (25). The desalted genomes (0.3 µg) were run on a 0.9% agarose gel in 0.5X TBE buffer. The gel was run for 5 h at 100 V, and then stained in a 1X solution of SYBR Gold (Molecular Probes, Inc.) in 1X TBE buffer (200 mL). The ligation efficiency of ODN insertion into
the genome was approximately 38% as determined by quantification of the stained DNA. Circular wild-type M13 DNA and linear M13 DNA (linearized by digestion with EcoRI restriction endonuclease) were used as markers.
2.4. Results and Discussion

The convertible nucleoside 4 was synthesized in three steps beginning with 5(4)-bromo-1H-imidazole (1) (Scheme 1). 5(4)-Bromo-4(5)-nitroimidazole (2) was prepared in 91% yield by nitrating 1 (23). Treatment of 2 with NaH in CH$_3$CN, followed by condensation with 3,5-di-O-toluoyl-α-1-chloro-2-deoxy-D-ribofuranose gave exclusively two β-nucleosides, the 5-bromo-4-nitroimidazole isomer 3 in 50% yield and the 4-bromo-5-nitroimidazole isomer in 35% yield. The structures of these isomers were assigned by comparison of the UV spectra with those of literature analogs (26, 27). Interestingly, 4-halo-5-nitroimidazoles have been reported to be resistant to nucleophilic displacement of the halo substituent (28, 29). In the present study, the 5-bromo-4-nitroimidazole isomer readily undergoes reaction at the site of the bromo substituent, while the 4-bromo-5-nitroimidazole isomer was resistant to reaction at the bromo position under the same conditions.

Saponification of the toluoyl esters of 3 was achieved in 90% yield by treatment with guanidine in MeOH for 3.75 hours at 0 °C. Initial attempts to deprotect 3 using reagents such as NaOMe in MeOH, NH$_4$OH, and K$_2$CO$_3$ in MeOH were unsuccessful because of decomposition of 3, presumably as a result of displacement of the bromo substituent. The β conformation of 4 was confirmed by NOE NMR spectroscopy. Irradiating at the 1’ proton, we observed an NOE enhancement at the 4’ proton, and by irradiating the imidazolic proton, NOE enhancements were observed at the 3’ proton and 5’ protons, thus confirming the structure. It is anticipated that 4 will serve as a branching point for generating libraries of 5-substituted-4-nitroimidazole nucleosides and, when incorporated into DNA, 5-substituted-4-nitroimidazole-containing ODNs, since previous studies have shown the bromo substituent of 5-bromo-4-nitroimidazoles to be displaced by carbon (27), sulfur and oxygen (29), and nitrogen (9) nucleophiles.

For later use as an analytical standard, the 2’-deoxynucleoside of NI (dNI, 5) was synthesized from 4 in nearly quantitative yield by treatment with guanidine in refluxing EtOH (Scheme 2). Having prepared 4 and 5, we then determined the stability of these compounds to DNA synthesis conditions. The half-life of 4 was found to be less than 15
Compound 4 was also stable for at least 24 h under standard oxidizing conditions used for DNA synthesis (0.02 M I₂ in pyridine/H₂O) and therefore was determined to have the stability necessary for automated DNA synthesis. Compound 5 was stable to treatment with concentrated NH₄OH for at least 4 h at room temperature. Under these conditions, phenoxyacetyl-protected ODNs can be cleaved from the solid support and deprotected.

Compound 4 was protected as the 5’-O-dimethoxytrityl ether using DMT-Cl, triethylamine, and a catalytic amount of DMAP in pyridine (Scheme 2). Two rounds of purification of the tritylated product by flash chromatography were necessary and gave the desired compound 6 in 76% yield. Compound 6 was subsequently treated with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and diisopropylethylamine to produce the phosphoramidite 7 in 44% yield. Alumina chromatography was necessary for the purification of 6 and 7, as both were unstable to purification on silica, even in the presence of triethylamine to reduce the acidity of the silica.

Compound 7 was incorporated into a 19mer ODN of mixed base sequence (8X, see Table 2.2 for structure and nomenclature) using standard automated procedures, with the exception that 3% dichloroacetic acid in CH₂Cl₂ was substituted for the deblocking reagent to minimize abasic site formation. The coupling efficiency after 20 min was greater than 95% as judged by trityl monitoring. Postsynthetic treatment of the DNA with 0.5 M guanidine in THF at room temperature caused a rapid color change of the solid support from white to bright yellow, indicating the production of NI. Deprotection of the DNA and cleavage from the support was achieved with concentrated NH₄OH for 4 h at room temperature. HPLC analysis of the crude 8NI (Figure 2.27) showed a single major peak with absorbance maxima at 260 and 380 nm. The purified 8NI was characterized by nuclease and phosphatase digestion followed by HPLC analysis (Figure 2.21) and by MALDI-TOF (Figure 2.20) and ESI mass spectrometry (Figure 2.19).
Digestion of 8NI with snake venom phosphodiesterase (SVPD) and alkaline phosphatase yielded the five expected nucleosides, with the dNI peak eluting with the same retention time and having the same UV-vis spectrum as the authentic standard 5. It has been reported that SVPD is unable to cleave the phosphodiester bond in the sequence context 5′-C[NI]-3′ (8). We did not observe this effect in our sequence context; however, in the sequence 5′-TT[NI]TTTTTTT-3′ (9NI), while SVPD could completely digest the ODN, nuclease P1 could not and left an NI-containing dinucleotide (Figure 2.22). As previously reported, MALDI mass spectrometry allowed detection of two degradation products, presumably of the nitro substituent induced by the MALDI laser (8). MALDI-induced degradation has been previously observed during MALDI analysis of aromatic nitro compounds (30, 31). Analysis by ESI mass spectrometry did not show these degradation products.

Thermal denaturation studies were performed to determine the stability of duplexes containing NI paired with each of the four natural bases (8NI:10Y) relative to the stability of unmodified duplexes (8G:10Y) (Table 2.2, which also defines nomenclature for 8 and 10). We found the NI lesion greatly destabilizes the duplex. The presence of the NI lesion results in a 10−12°C decrease in $T_m$ relative to an unmodified duplex containing a G-C base pair at the same position and a 4−6°C decrease in $T_m$ relative to the least thermally stable mismatch of the unmodified duplex (G-T). A comparison of all $T_m$ values and free energies shows a greater difference in magnitude among the unmodified duplexes than among the NI-containing duplexes, demonstrating that the presence of the lesion generally reduces the effect of the opposing base on duplex stability.

Given that a large reduction in thermal stability does not imply a large change in duplex structure and similarity among thermal stabilities does not necessitate structural similarities (32), it is not possible to draw conclusions from the thermal stabilities alone about the effect of the NI lesion on duplex DNA structure. In an effort to probe the origins of the observed duplex destabilization, a simple AM1 semiempirical method (33) was used to optimize the geometry of 5 (Figure 2.25). The calculation predicts that the
guanidino group will be rotated out of plane in relation to the imidazole ring and extend roughly 3 Å perpendicularly in either direction from the plane of the ring. Accommodation of such a structure into the helix would require at minimum a local distortion of the duplex and a disruption of the base π-stacking interactions.

We have site-specifically inserted 8NI into an M13 bacteriophage genome for mutagenesis and genotoxicity studies (Figure 2.26) (25). The ligation efficiency of the genome construction was similar to that observed for 8G. Mutagenesis and repair studies using this construct will be reported elsewhere.

In summary, we have developed a synthetic route for the preparation of 5 and provided a method for the incorporation of NI lesions into DNA by the phosphoramidite method utilizing the convertible nucleoside approach. Ready access to 5 will be beneficial for use as an analytical standard in future studies aimed at detecting the NI lesion in vivo, whereas the convertible nucleoside phosphoramidite will allow for the rapid preparation of NI-containing ODNs for mutagenesis and DNA repair studies, creation of libraries of 5-substituted-4-nitro analogs of NI (which may be useful as structural probes), and preparation of NI-containing ODNs in sufficient quantities for NMR and X-ray structure studies.
2.5. Acknowledgment

Paul T. Henderson (Lawrence Livermore National Laboratory) and John M. Essigmann (MIT) contributed to this work. We thank Dr. Uday Sharma for helpful discussions, the Sadighi laboratory at MIT for assistance with NOE NMR experiments, Prof. Catherine L. Drennan for the use of her UV-vis spectrophotometer, and Hector H. Hernandez for assistance with $T_m$ experiments. We also acknowledge the MIT Biological Engineering Division Mass Spectrometry Laboratory and the Department of Chemistry Instrumentation Facility. Financial support was provided by NCI award P01-CA26731 and by NSF awards DBI-9729592 and CHE-9808061.
Figure 2.1. $^1$H NMR spectrum of 2 in DMSO-d$_6$. 
Figure 2.2. $^{13}$C NMR spectrum of 2 in DMSO-$d_6$. 
Figure 2.3. $^1$H NMR spectrum of 3A in CD$_2$Cl$_2$. 
Figure 2.4. $^{13}$C NMR spectrum of 3A in CD$_2$Cl$_2$. 
Figure 2.5. UV-vis spectrum of 3A in CD$_2$Cl$_2$. 
Figure 2.6. $^1$H NMR spectrum of 3 in CD$_2$Cl$_2$. 
Figure 2.7. $^{13}$C NMR spectrum of 3 in CD$_2$Cl$_2$. 
Figure 2.8. UV-vis spectrum of 3 in CD$_2$Cl$_2$. 
Figure 2.9. $^1$H NMR spectrum of 4 in CD$_3$OD.
Figure 2.10. $^{13}$C NMR spectrum of 4 in CD$_3$OD.
Figure 2.11. gCOSY spectrum of 4 in CD$_3$OD.
Figure 2.12. $^1$H NMR spectrum of 5 in D$_2$O.
Figure 2.13. $^{13}$C NMR spectrum of 5 in CD$_3$OD.
Figure 2.14 UV-vis spectrum of 5 in H₂O, pH 7.
Figure 2.15. $^1$H NMR spectrum of 6 in CD$_2$Cl$_2$. 
Figure 2.16. $^{13}$C NMR spectrum of 6 in CD$_2$Cl$_2$. 
Figure 2.17. $^1$H NMR spectrum of 7 in CD$_3$CN.
Figure 2.18. $^{31}P$ NMR spectrum of 7 in CD$_3$CN.
Figure 2.19. (Top) ESI mass spectrum for 8NI: calc’d 5882.9, found 5884.0. (Bottom) ESI mass spectrum for 9NI: calc’d 3024.0, found 3024.6.
Figure 2.20. (Top) MALDI-TOF mass spectrum for **8NI**: calc’d 5882.9, found 5882.1. (Bottom) MALDI-TOF mass spectrum for **9NI**: calc’d 3024.0, found 3025.0.
Figure 2.21. Digestion analyses of 8NI (Top) and 9NI (Bottom) using SVPD and alkaline phosphatase. The insets show the UV-vis spectra of the dNI peaks.
Figure 2.22. Digestion analysis of 9NI using nuclease P1 and alkaline phosphatase. The inset shows the UV-vis spectrum of the dinucleotide peak.
Chapter 2 – Synthesis of 5-Guanidino-4-nitroimidazole

Figure 2.23. Representative melting curves for 8NI annealed with 10G (top left), 10A (top right), 10C (bottom left), and 10T (bottom right).
Chapter 2 – Synthesis of 5-Guanidino-4-nitroimidazole

Figure 2.24. Representative melting curves for 8G annealed with 10G (top left), 10A (top right), 10C (bottom left), and 10T (bottom right).
Figure 2.25. Energy-minimized (AM1) structure of dNI (5). The imidazole ring and nitro group are in the plane of the page, while the carbon and two of the nitrogen atoms of the guanidino group are projecting through the back of the page.
Figure 2.26. Agarose gel analysis of the M13 genome constructions. (1) Circular M13 DNA. (2) Linear M13 DNA. (3) Insertion of $8G$ into M13 DNA. (4) Insertion of $8NI$ into M13 DNA.
Figure 2.27. (Top) HPLC trace at 254 nm of the converted, deprotected 8NI and purified 8NI (inset). (Bottom) UV-vis spectrum of purified 8NI.

\[ \begin{align*}
&\text{1} \xrightarrow{\text{a)} HNO_3, \text{EtOH} \atop \text{b)} H_2SO_4} \text{2} \quad \text{a)} \text{NaH, CH}_3\text{CN} \atop \text{b)} p-\text{TolO} \quad \text{Cl} \\
&p-\text{TolO} \quad \text{3} \xrightarrow{\text{Guanidine} \atop \text{MeOH, } 0^\circ C} \text{4}
\end{align*} \]
Scheme 2.2. Synthesis of 5 and the convertible nucleoside phosphoramidite 7.
Table 2.1. NOE difference for 4 in CD$_3$OD (%).

<table>
<thead>
<tr>
<th>observed proton</th>
<th>1’H</th>
<th>imidazolic H</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidazolic H</td>
<td>1.41</td>
<td>___</td>
</tr>
<tr>
<td>1’H</td>
<td>___</td>
<td>1.73</td>
</tr>
<tr>
<td>2’H</td>
<td>6.43</td>
<td>3.27</td>
</tr>
<tr>
<td>3’H</td>
<td>0.28</td>
<td>2.65</td>
</tr>
<tr>
<td>4’H</td>
<td>2.20</td>
<td>0.49</td>
</tr>
<tr>
<td>5’H</td>
<td>0.10</td>
<td>1.47</td>
</tr>
</tbody>
</table>
**Table 2.2.** Melting Temperatures and Free Energies of Duplexes$^a$.

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
<th>$T_m$ (± 0.5 °C)</th>
<th>$-\Delta G_{37^\circ C}$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NI</td>
<td>G</td>
<td>57.0</td>
<td>16.8 ± 0.1</td>
</tr>
<tr>
<td>NI</td>
<td>A</td>
<td>56.4</td>
<td>16.7 ± 0.0</td>
</tr>
<tr>
<td>NI</td>
<td>C</td>
<td>55.8</td>
<td>16.7 ± 0.1</td>
</tr>
<tr>
<td>NI</td>
<td>T</td>
<td>54.8</td>
<td>16.0 ± 0.0</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>61.0</td>
<td>19.3 ± 0.0</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>62.4</td>
<td>20.4 ± 0.0</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>66.9</td>
<td>23.0 ± 0.1</td>
</tr>
<tr>
<td>G</td>
<td>T</td>
<td>60.6</td>
<td>19.4 ± 0.0</td>
</tr>
</tbody>
</table>

$^a$ Conditions: 50 mM sodium chloride, 5 mM sodium phosphate, pH 7.0. $T_m$ and $-\Delta G_{37^\circ C}$ values are the average of three melts.
2.6. References


Chapter 2 – Synthesis of 5-Guanidino-4-nitroimidazole


CHAPTER 3

*In Vivo* Bypass Efficiencies and Mutational Signatures of the Guanine Oxidation Products 2-Aminoimidazolone and 5-Guanidino-4-nitroimidazole
3.1. Abstract

The in vivo mutagenic properties of 2-aminoimidazolone and 5-guanidino-4-nitroimidazole, two products of peroxynitrite oxidation of guanine, are reported. Two oligodeoxynucleotides of identical sequence, but containing either 2-aminoimidazolone or 5-guanidino-4-nitroimidazole at a specific site, were ligated into single-stranded M13mp7L2 bacteriophage genomes. Wild-type AB1157 Escherichia coli cells were transformed with the site-specific 2-aminoimidazolone- and 5-guanidino-4-nitroimidazole-containing genomes, and analysis of the resulting progeny phage allowed determination of the in vivo bypass efficiencies and mutational signatures of the DNA lesions. 2-Aminoimidazolone was efficiently bypassed and 91% mutagenic, producing almost exclusively G to C transversion mutations. In contrast, 5-guanidino-4-nitroimidazole was a strong block to replication and 50% mutagenic, generating G to A, G to T and, to a lesser extent, G to C mutations. The G to A mutation elicited by 5-guanidino-4-nitroimidazole implicates this lesion as a novel source of peroxynitrite-induced transition mutations in vivo. For comparison, the error-prone bypass DNA polymerases were over-expressed in the cells by irradiation with UV light (SOS induction) prior to transformation. SOS induction caused little change in the efficiency of DNA polymerase bypass of 2-aminoimidazolone; however, bypass of 5-guanidino-4-nitroimidazole increased nearly 10-fold. Importantly, the mutation frequencies of both lesions decreased during replication in SOS-induced cells. These data suggest that 2-aminoimidazolone and 5-guanidino-4-nitroimidazole in DNA are substrates for one or more of the SOS-induced Y-family DNA polymerases and demonstrate that 2-aminoimidazolone and 5-guanidino-4-nitroimidazole are potent sources of mutations in vivo.
3.2. Introduction

Oxidative damage of DNA is implicated as a cause of aging (1-3), carcinogenesis (4-6), a variety of noncancerous diseases such as Alzheimer’s disease and cardiovascular disease (7), and the progression to acquired immunodeficiency syndrome in HIV-infected patients (8). The reactive species responsible for DNA damage are generated by common endogenous processes (9) such as respiration and inflammation (10, 11). During inflammation, an assortment of reactive oxygen and nitrogen intermediates are generated by activated immune system cells (11), and reaction of these molecules with DNA produces dozens of oxidized nucleobase derivatives (12).

The radicals nitric oxide (\(\cdot\)NO) and superoxide (\(\cdot\)O_2) are produced by macrophages and neutrophils (two types of inflammatory cells) upon immune response activation (13, 14). These radicals combine in a diffusion-limited reaction to form peroxynitrite (ONOO\(^-\)) (15), a powerful oxidizing and nitrating agent capable of damaging a variety of biomolecules (16-18), including DNA (19). Under physiological conditions, ONOO\(^-\) rapidly combines with CO_2 to form nitrosperoxycarbonate (ONOOCO_2\(^-\)), which subsequently undergoes homolysis to produce carbonate radical (CO_3\(^-\)) and nitrogen dioxide (\(\cdot\)NO_2) (20-22). These radicals are believed to be responsible for the oxidation and nitration of DNA caused by exposure to ONOO\(^-\) (23-25).

Since guanine possesses the lowest redox potential of the four DNA nucleobases (\(E_7 = 1.27\) V vs. NHE) (26), it is preferentially oxidized by ONOO\(^-\) compared to the other natural nucleobases (27). Several products are formed directly from guanine residues in DNA including 7,8-dihydro-8-oxoguanine (8-oxoG), 8-nitroguanine (8-NO_2-G), 2-aminoimidazolone (Iz), and 5-guanidino-4-nitroimidazole (NI) (Figure 3.1) (28). The lesions 8-oxoG and 8-NO_2-G are highly susceptible to further oxidation (27) and yield a variety of additional products (29, 30). Although many of these guanine-derived oxidation products have been characterized for their in vivo mutagenic potential (31, 32), Iz and NI have received little attention. In order to assess the biological significance and
consequences of oxidatively damaged DNA, it is essential that these lesions be characterized for their genotoxic and mutagenic potential.

In the work presented here, we report the in vivo genotoxic and mutagenic properties of Iz and NI. Oligodeoxynucleotides (ODNs), site-specifically modified with Iz or NI, were synthesized using a previously described method (33) and a procedure developed in our laboratory (34). The biological impact of unique Iz and NI lesions was addressed under normal and SOS-induced conditions in wild-type AB1157 E. coli cells using viral vectors containing the modified ODNs. Both lesions were bypassed by the E. coli replication machinery and were substrates for SOS-induced error-prone DNA polymerase bypass. Furthermore, each lesion was potently mutagenic during DNA replication.
3.3. Experimental Procedures

Oligodeoxynucleotides. DNA synthesis reagents were purchased from Glen Research. Unmodified ODNs were purchased from IDT, Inc. and were purified by polyacrylamide gel electrophoresis (PAGE). The 19mer ODN sequence used was 5’-GCG AAG ACC GX A GCG TCC G-3’, where X is G, Iz, NI, or a tetrahydrofuran (THF) abasic site. The 19mer containing the THF abasic site analog was prepared as described (31).

The 19mer containing Iz was prepared as previously described (33). Briefly, 8-methoxy-2’-deoxyguanosine (8-MeOdG) was incorporated into the 19mer ODN by the phosphoramidite method and the ODN was deprotected and cleaved from the solid support with concentrated NH₄OH at 55 °C for 15 h. The 8-MeOdG-containing ODN was purified by PAGE, and the 8-MeOdG subsequently converted to Iz by photoirradiation with 365 nm light in the presence of riboflavin. The Iz-containing 19mer was purified by anion exchange HPLC on a Dionex NucleoPac PA-100 (4 × 250 mm) analytical column using 10% CH₃CN in water (solvent A) and aqueous 1.5 M NH₄OAc (solvent B) as solvents. A flow rate of 1.0 mL/min was used and solvent B was increased from 10% to 25% over 2.5 min, then 25% to 100% over 30 min. The purified 19mer was characterized using MALDI-TOF mass spectrometry (calc’d MW: 5824.8; found: 5825.8) and by enzymatic digestion to nucleosides followed by HPLC analysis. The following conditions were used for the enzymatic digestion: 50 mM Tris-Cl, pH 7.0, 0.1 mM ZnSO₄, 18 units of nuclease P1, 12 units of alkaline phosphatase (both enzymes from Roche Applied Science), and 2 nmol of Iz-containing 19mer in 50 µL were incubated at room temperature for 30 min and then immediately analyzed by HPLC. For the HPLC analysis, a Supelco Supercosil LC-18-DB (250 × 2.1 mm, 5 µm) column was used with aqueous 150 mM NH₄OAc as solvent A and CH₃CN as solvent B. A flow rate of 0.25 mL/min was used and solvent B was increased from 0% to 15% over 40 min. Five peaks were observed with UV-vis spectra consistent with the nucleosides Iz, C, G, T, and A.

The ODN containing NI was prepared by incorporating 5-bromo-4-nitroimidazole into the 19mer sequence by the phosphoramidite method and subsequently treating the
Chapter 3 – Iz and NI are Mutagenic in Vivo

19mer with 0.5 M guanidine in THF and then concentrated NH₄OH to produce the 19mer containing NI (34). The NI-containing 19mer was purified by C18 reversed phase HPLC (34) and by anion exchange HPLC as described above, and characterized using MALDI-TOF mass spectrometry (calc’d MW: 5882.9; found: 5882.7) and by enzymatic digestion with snake venom phosphodiesterase (ICN Biomedical) and alkaline phosphatase (34) followed by HPLC analysis (same method as described for the 19mer containing Iz). For the HPLC analysis, five peaks were observed with UV-vis spectra consistent with the nucleosides NI, C, G, T, and A.

**Genome Construction.** Genomes were constructed in triplicate (Figure 3.2) on a 10 pmol scale, as previously described (31, 32, 35). Briefly, the ss M13 DNA was linearized by cleavage with EcoRI (30 U/pmol of DNA, 23 °C for 8 h) at a hairpin containing a single EcoRI site (35). The genome was recircularized by annealing in the presence of the 5'-phosphorylated 19mer insert and two “scaffold” ODNs (sequences that are partially complementary to the 5’ and 3’ sides of the insert and the genomic DNA termini) and incubating with 22.5 U/μL of T4 DNA ligase (NEB) for 2 h at 16 °C in a volume of 55 μL (31). Two short scaffolds are used in order to leave a ss gap at the site of the lesion, thereby facilitating efficient ligation regardless of the lesion structure. The scaffold DNA was removed using the exonuclease activity of T4 DNA polymerase (Amersham) by treating with 0.25 U/μL for 1 h at 16 °C in a volume of 65 μL (36, 37). Under these conditions, scaffold digestion was complete as determined by using a radiolabeled scaffold and analyzing the reaction with PAGE and phosphorimagery. The genome constructs were diluted to 115 μL with H₂O, extracted with 100 μL of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen), and desalted with Sephadex G50 fine resin (Amersham Biosciences).

The stability of the lesion-containing 19mers was assessed using MALDI-TOF mass spectrometry. The 19mers were exposed to the conditions used for genome construction, with the exception that M13 DNA, other ODNs, and enzymes were excluded. The mass spectra of the 19mers were essentially unchanged after exposure to the mock genome construction conditions. The amount of circular, 19mer insert-containing genome in each
sample was quantified by agarose gel electrophoresis and phosphorimagery. This was accomplished by annealing 1 pmol of a 5’-[32P]-labeled 30mer probe ODN (5’-TCC CAG TCA CGA CGT TGT AAA ACG ACG GCC-3’) to 0.1 pmol of each genome construct in a region of the genome that did not include the lesion-containing 19mer insert. The annealing solution consisted of 100 mM NaCl, 4.2% Ficoll, 0.042% bromophenol blue, and 0.042% xylene cyanol FF in a volume of 15.5 µL. The genome-probe mixtures (15.5 µL) were run on a 0.9% agarose gel in 1X TBE buffer for 4 h at 100 V, after which the free probe front was excised. The gel was run for an additional 3 h and then transferred onto a glass plate and dried under a box fan for 36 h. The amount of circular genome was quantified by phosphorimagery and the genome construct solution volumes adjusted such that each solution used for cell transformation contained an equal concentration of circular DNA.

_Preparation of Electrocompetent Cells._ 2 × 150 mL of LB media were each inoculated with 1.5 mL from separate overnight cultures of wild-type AB1157 _E. coli_ and grown on a shaker at 37 °C to an A600 of approximately 0.4. Each culture was centrifuged, resuspended in 25 mL of 10 mM MgSO4, and transferred to a large (150 × 15 mm) Petri dish. The SOS system was induced in the cells by irradiating with 254 nm light (45 J/m2 of energy), immediately transferring the cells to 2 × 125 mL of 2×YT media, and growing for 40 min at 37 °C with shaking. Uninduced cells were treated identically, except without exposure to UV light. The 2×YT cultures were centrifuged, combined, and washed with 2 × 175 mL of deionized water. The electrocompetent cells were resuspended in 4 mL of a 10% solution of glycerol in water, stored at 4 °C, and used the following day.

_Translesion Bypass Efficiency._ An equal amount of each circular genome construct was mixed with 0.025 pmol of internal standard (wild-type circular single-stranded M13mp7L2 DNA) and 100 µL of electrocompetent cells. The cell/genome mixtures were electroporated and transferred to 10 mL of LB, generating at least 10^5 independent transformed cells as determined by plating of an aliquot onto agar plates. The cultures were incubated at room temperature for at least 30 min, and then incubated on a roller
drum for 4 h at 37 °C to amplify the progeny phage. The cells were spun down and the progeny phage-containing supernatant retained. In this system, successful replication of the genomes by the *E. coli* host leads to the production of progeny phage. When plated on a lawn of NR9050 indicator *E. coli* in the presence of IPTG and X-gal (Gold Biotechnology), progeny phage derived from genomes containing the 19mer insert produce blue plaques if no mutation, a point mutation, or an in frame insertion or deletion mutation occurs at the lesion site, whereas progeny phage from WT M13mp7L2 genomes, genomes lacking an insert (genetic engineering mutants), and lesion induced out-of-frame insertions and deletions produce clear plaques (31). Thus, the amplified progeny phage were diluted and plated such that approximately 1000-2000 total plaques were produced per Petri dish, and the number of blue and clear plaques per plate were counted. The number of normalized blue plaques resulting from each lesion-containing genome relative to that of the normalized guanine control indicated the bypass efficiency of a lesion (see Results for details). The normalization allowed direct comparison of lesion bypass by DNA polymerase(s) between genomes containing different inserts, regardless of unavoidable variation in factors such as electroporation efficiency and the total number of plaques on each plate.

**Mutation Type and Frequency.** Electrocompetent cells were transformed with each genome construct, as described in the previous section, except that the internal standard was excluded. As before, the electroporations produced at least $10^5$ independent transformed cells. Amplified progeny phage (100 $\mu$L) and 10 $\mu$L of an overnight growth of SCS110 *E. coli* were added to 10 mL of LB media and grown for 6 h at 37 °C on a roller drum. The cultures were centrifuged and the phage-containing supernatant retained. Single-stranded phage DNA was isolated from 700 $\mu$L of each sample using a QIAPrep Spin M13 kit (Qiagen). The region containing the lesion site was amplified by PCR as previously described (31), except 0.7 $\mu$M of each primer was used, and the resultant 101mer PCR product was purified using a QIAPrep PCR purification kit (Qiagen). The REAP assay was used to determine the identity of the base at the site formerly occupied by the lesion (31, 32, 35, 38).
3.4. Results

**Genome construction.** Convertible nucleoside phosphoramidites were used to introduce each oxidized DNA lesion into a 19mer ODN at a defined site. Following incorporation into the 19mer by automated DNA synthesis, 8-methoxyguanine was converted to Iz by photoirradiation in the presence of riboflavin (33). Using a procedure recently developed in our laboratory, NI was formed within an ODN from the convertible nucleoside 5-bromo-4-nitroimidazole by treatment of the ODN with guanidine (34). Each ODN was characterized by MALDI-TOF mass spectrometry and by nuclease and phosphatase digestion followed by HPLC analysis. Single-stranded M13mp7L2 viral genomes containing Iz or NI at a specific site in the genome were constructed as shown in Figure 3.2. An aliquot of each genome construct, annealed to a radiolabeled ODN probe, was analyzed by agarose gel electrophoresis and phosphorimager to assess the yield of circular, insert-containing genome. Following quantification, the genome construct solutions were normalized such that each contained an equivalent amount of circular, insert-containing genome. The stability of each lesion to the genome construction conditions was confirmed by MALDI-TOF mass spectrometry after subjecting the lesion-containing ODNs to the same conditions.

**Translesion Bypass Efficiency.** A viral plaque assay based on lacZ α-complementation was employed for the determination of the lesion bypass efficiency (Figure 3.3). In this system, phage produced from genomes containing the 19mer insert cause the formation of blue plaques when plated on IPTG/X-gal indicator plates if either no mutation or a point mutation occurs at the lesion site, and phage produced from WT M13mp7L2 genomes produce clear plaques. The number of phage that form blue plaques varies with the efficiency of lesion bypass, and the rate of replication of the WT M13mp7L2 genomes is assumed to be independent of the identity of the insert-containing genomes. Thus, a genome containing a lesion that partially blocks replication yields a lower proportion of progeny phage relative to a genome containing a freely bypassed lesion. Since the same amount of internal standard (WT M13mp7L2 genome) was used in each mixture, the blue-to-clear ratio of each mixture can be directly compared after being normalized to reflect the identical amount of internal standard in each mixture. The
bypass efficiency of a lesion is scaled relative to guanine, which is defined as having a bypass efficiency of 100%. From this logic the equation given in Figure 3.3 results, where the term B(L)/B(G) is the definition of relative bypass efficiency and the term C(G)/C(L) is the normalization factor. A limitation of this assay is that lesion induced out-of-frame insertion and deletion mutations would also produce clear plaques and therefore not be counted as bypass events. However, as is discussed in the next section the lesions studied here induced negligible amounts of observable insertion and deletion mutations and so a correction for these events was not performed.

In normal WT AB1157 *E. coli*, the bypass efficiency of Iz was 60 ± 5% (Figure 3.4). By contrast, NI was a much stronger replication block and was bypassed with an efficiency of only 7.0 ± 1.6%. As a control, we also determined the bypass efficiency of a synthetic THF abasic site. The THF lesion was bypassed with an efficiency of 5.8 ± 0.7%, in agreement with previous work showing that this lesion is a block to DNA replication (31, 32, 39). The bypass efficiencies of the lesions were also determined in cells with the SOS system induced. Under these conditions, the bypass efficiency of Iz did not change significantly (71 ± 7%); however, the bypass efficiency of NI increased a remarkable 8-fold to 57 ± 1%. The bypass efficiency of the THF lesion increased to 30 ± 2%, demonstrating that the SOS system was indeed induced.

*Mutation type and frequency.* The mutational signature of each lesion in WT AB1157 *E. coli* was determined using the restriction endonuclease and post-labeling analysis of mutation frequency (REAP) assay (Figure 3.5). Insertion and deletion mutations which do not compromise the *Bbs*I recognition site or the PCR primer sites are detectable by the REAP assay during the PAGE purification step and were negligible in this work (for Iz, no detectable frameshifts were observed; for NI, 0.5-1% −1 frameshifts were observed, data not shown).

In normal cells, the mutation frequency of Iz was 91% versus 50% for NI (Figure 3.6). The mutations induced by Iz were essentially all G→C mutations, as predicted by molecular orbital calculations and *in vitro* primer extension experiments (40, 41). NI
caused 8.9% ± 0.5% G→C mutations, and a roughly equivalent amount of G→A and G→T mutations (19% ± 2% and 22 ± 3%, respectively). The mutation type and frequency for each lesion was also determined in SOS-induced cells to investigate the effect of the SOS response system on the coding properties of these lesions. Interestingly, Iz and NI had somewhat lower overall mutation frequencies of 84% and 33%, respectively. The coding properties of Iz were more degenerate under SOS-induced conditions. Whereas Iz caused 2.0 ± 0.1% G→A and 1.1 ± 0.2% G→T mutations without SOS induced, the lesion now induced 3.4 ± 0.5% G→A and 5.5 ± 0.8% G→T mutations. The mutational signature of NI also differed in SOS-induced cells, as the lesion induced many fewer G→A and G→C mutations (13 ± 2% and 2.5 ± 0.6%, respectively).
3.5. Discussion

In order to assess the biological fate of Iz and NI in DNA, we have determined the relative bypass efficiency and the mutation type and frequency of each lesion in vivo. The Iz derivative is a major and ubiquitous in vitro product of guanine oxidation. In addition to forming as a result of ONOO− oxidation (28), this lesion also forms when guanine is oxidized by other reactants including hydroxyl radical (42), Mn-TMPyp/KHSO5 (43-45), and riboflavin (photooxidation) (40-42, 46). Direct formation of Iz from 8-oxoG has also been observed (40, 47). On a biological timescale, Iz is stable ($t_{1/2}$ in single-stranded DNA ≈ 16 h at 37 °C) (33), but can hydrolyze to form an oxazolone derivative. Oxazolone is bypassed efficiently and exhibits a mutation frequency similar to that of Iz in vivo (86%), but preferentially generates G→T transversion mutations (31). In distinct contrast, NI has been observed only as a result of ONOO−-related chemistry (28, 48, 49). NI is a chemically stable lesion (when heated to 90 °C for 6 h at pH 7, ~10-15% decomposition of an ODN containing NI occurs) (49) and is refractory to repair by Fpg glycosylase (MutM) and endonuclease III in vitro (48). The chemical and biological properties of NI make this lesion a potential biomarker of ONOO−-mediated oxidation of DNA (28, 48).

In vitro studies have been conducted with Iz and NI, and the results suggest they would be both replication blocks and mutagenic in vivo. Ab initio studies of Iz in vacuo and using a self-consistent reaction field method of solvation indicate this lesion should give G→C mutations because of its apparent ability to mimic the hydrogen bonding face of C and to form a stable base pair with G (40, 41). Replication of the lesion using DNA polymerase I (pol I), and the Klenow fragment of pol I (Kf), showed that the lesion indeed codes exclusively as a cytosine, but is inefficiently bypassed (40). In contrast, the site-specific bacteriophage replication experiments presented here showed that the lesion was bypassed relatively efficiently and had a mutation frequency of 91%, suggesting one or more polymerases in addition to pol I may be responsible for the bypass of Iz under normal conditions.
The bypass and coding properties of DNA containing NI were previously studied using the purified DNA polymerases Kf (exo−), calf thymus polymerase α (pol α), and human polymerase β (pol β) (48). All three polymerases could insert a nucleotide opposite the lesion, and although a kinetic analysis was not performed, the data suggested that extension of the NI-containing base pair by Kf (exo−) and pol α is rate limiting and results in poor translesion synthesis. The nucleotides A and G were incorporated opposite NI by Kf (exo−) and pol α, with Kf (exo−) favoring insertion of C opposite the lesion. Interestingly, pol β efficiently bypassed the lesion and incorporated mostly C, rendering the lesion non-toxic and non-mutagenic. Our results differ from the in vitro data since substantial amounts of T were incorporated opposite NI, thereby producing G→A mutations in vivo. This observation indicates pol I is probably not solely responsible for the bypass of NI since this polymerase did not incorporate T opposite the lesion in vitro. The bypass efficiency of NI in WT cells shows that NI is a significant block to replication and has a bypass efficiency similar to that of the THF synthetic abasic site. This observation correlates with our recent thermal melting study that showed NI greatly destabilizes duplex DNA regardless of the identity of the base paired opposite the lesion. To explain this observation, we proposed that NI adopts a nonplanar and potentially DNA distorting conformation (34). Thus, the low bypass efficiency of NI may result from the inability of NI to fit within the tight active site of a replicative DNA polymerase (50). Alternatively, the large increase in NI bypass efficiency when replicated in SOS-induced cells may indicate the involvement of Y-family DNA lesion bypass polymerases, which have less sterically restrictive active sites and can bypass numerous types of lesions (51-58).

The differences in bypass efficiency and coding specificity of the lesions in normal versus SOS-induced cells indicate that SOS-induced proteins contribute to the processing of these lesions. In normal cells, the number of SOS-inducible DNA polymerase molecules per cell is approximately 50 for pol II (59), 250 for pol IV (60), and fewer than 15 for pol V (61). Upon SOS induction, the number of DNA polymerase molecules per cell increases to about 350 for pol II (59), 2500 for pol IV (60), and 200 for pol V (61). It has been demonstrated that one or more of these polymerases are required
to bypass certain DNA lesions, and the specific polymerases involved vary depending on the sequence context in which the lesion resides (62-64). Experiments using polymerase deficient cell lines would address which of the SOS-induced polymerases are involved in the bypass of Iz and NI.

The mutational signatures of Iz and NI determined here using site-specifically modified genomes correlate well with previous cell-based mutagenesis studies (19, 24). In one report, a pSP189 shuttle vector containing the supF gene was treated with ONOO$^-$ in the presence of NaHCO$_3$ (a physiological buffer component that facilitates the reaction of ONOO$^-$ with DNA by supplying CO$_2$), followed by replication in MBL50 E. coli (24). Essentially all mutations occurred at G-C base pairs and consisted of 55% G$\rightarrow$C, 31% G$\rightarrow$T, and 11% G$\rightarrow$A mutations. We previously showed in vivo that the 8-oxoG secondary oxidation products cyanuric acid, oxaluric acid, and oxazolone give exclusively G$\rightarrow$T mutations (31), whereas guanidinohydantoin generates exclusively G$\rightarrow$C mutations and the spiroiminodihydantoin diastereomers yield a mixture of G$\rightarrow$C and G$\rightarrow$T mutations (32). In both studies, 8-oxoG was slightly mutagenic and induced only G$\rightarrow$T mutations, so the discovery that guanidinohydantoin and the spiroiminodihydantoin diastereomers generate G$\rightarrow$C mutations implicated these lesions as the possible sources of the in vivo G$\rightarrow$C mutations found in the supF/pSP189 system. The results in the present work implicate Iz as an additional in vivo source of G$\rightarrow$C mutations. Importantly, prior to this work no ONOO$^-$-derived lesions had been found to give significant amounts of G$\rightarrow$A mutations. Our results implicate NI as a possible source of the G$\rightarrow$A mutations observed during the supF/pSP189 study.

Iz and NI are formed directly from guanine upon oxidation by ONOO$^-$. When replicated in vivo, these lesions are bypassed and potently mutagenic, collectively inducing G$\rightarrow$C, G$\rightarrow$T, and G$\rightarrow$A mutations. Our results support a role for Iz and NI as contributors to the mutational spectrum observed when ONOO$^-$-treated DNA is replicated in vivo. If these lesions persist in cells, they may be powerful sources of mutations.
3.6. Acknowledgment

James C. Delaney (MIT), Paul T. Henderson (Lawrence Livermore National Laboratory), and John M. Essigmann contributed to this work. We thank Dr. Yuriy O. Alekseyev for helpful discussions. We also acknowledge the MIT Biological Engineering Division Mass Spectrometry Laboratory and the Department of Chemistry Instrumentation Facility. Financial support was provided by NIH awards CA26731 and CA080024 and by NSF awards DBI-9729592 and CHE-9808061.
Figure 3.1. Primary products of ONOO⁻ oxidation and nitration of guanine.
Figure 3.2. Construction of genomes containing a guanine residue or DNA lesion at a specific site. A 5’-phosphorylated 19mer oligodeoxynucleotide insert containing a guanine residue or DNA lesion at a specific site (denoted by a solid circle) is positioned within a ss WT M13mp7L2 bacteriophage genome using two DNA scaffolds. The 19mer insert is ligated into the genome using T4 DNA ligase, and the scaffolds are digested using the exonuclease activity of T4 DNA polymerase to produce the genome construct.
**Figure 3.3. Assay for determining the efficiency of DNA polymerase bypass of a DNA lesion.** Genome constructs containing a site-specific guanine residue (G) or DNA lesion (X) are separately mixed with a known amount of internal standard (ss WT M13mp7L2 genome), and the equally proportioned mixtures are used to transform *E. coli* cells. Progeny phage (denoted by a circle within a hexagon) derived from a genome construct have a functional lacZ gene, whereas phage containing the WT M13mp7L2 genome do not. Thus, the composition of the progeny phage mixtures can be assayed by plating on a lawn of NR9050 indicator *E. coli* in the presence of IPTG and X-gal and counting the number of blue and clear plaques produced by the phage infections.

\[
\begin{align*}
\text{G-Control} \quad &\text{ + Internal Standard (WT M13mp7L2)} \\
\text{Yields Blue} \quad &\text{Yields Clear} \\
\text{Plaques} \quad &\text{Plaques} \\
\text{Transform } E. \text{ coli} \\
\text{G-Control} \quad &\text{ + Internal Standard (WT M13mp7L2)} \\
\text{Yields Blue} \quad &\text{Yields Clear} \\
\text{Plaques} \quad &\text{Plaques} \\
\text{Plate on lawn of} \quad &\text{Plate on lawn of} \\
\text{indicator } E. \text{ coli} \quad &\text{indicator } E. \text{ coli} \\
\# \text{ of Blue Plaques} = &\# \text{ of Blue Plaques} = B(G) \\
\# \text{ of Clear Plaques} = &\# \text{ of Clear Plaques} = C(G) \\
\# \text{ of Blue Plaques} = &\# \text{ of Blue Plaques} = B(L) \\
\# \text{ of Clear Plaques} = &\# \text{ of Clear Plaques} = C(L) \\
\text{Relative Bypass Efficiency} = \frac{B(L) \times C(G)}{B(G) \times C(L)} \times 100%
\end{align*}
\]
Figure 3.4. Bypass efficiency of Iz, NI, and a THF abasic site relative to G. The results shown are the average of three experiments, with the experimental error given as a 95% confidence interval of the mean.
**Figure 3.5.** *REAP assay for determining the mutation type and frequency of a DNA lesion.* *E. coli* cells are transformed with genome constructs site-specifically modified with a guanine residue or DNA lesion. Position N in the resultant progeny phage genomes represents the site formerly occupied by the site-specific modification. The region containing N is PCR amplified to yield a 101mer containing *Bbs*I and *Hae*III recognition sites. The *Bbs*I digestion product is radiolabeled and digested with *Hae*III to produce a 19mer with N radiolabeled. PAGE purification of the 19mer also allows insertion and deletion mutations (appearing as ODNs of longer or shorter length than the 19mer) to be quantified, if present. Digestion of the 19mer with nuclease P1 and subsequent partitioning of the nucleotide mixture by TLC allows the point mutation type and frequency to be quantified using phosphorimagery.

1. *Bbs*I, Phosphatase
2. [γ-32P]-ATP, Kinase
3. *Hae*III
4. PAGE

![Diagram of the REAP assay](image_url)

**Mutation Frequency =**

\[
\frac{C + T + A}{C + T + A + G} \times 100\%
\]
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Figure 3.6. REAP assay results for G, Iz, and NI. A. Representative polyethyleneimine TLC analysis of point mutation type and frequency. B. Quantification of the TLC data for G (top row), Iz (center row), and NI (bottom row). The graphical data shown are the average of three experiments, with the experimental error given as a 95% confidence interval of the mean.
3.7. References


Chapter 3 – Iz and NI are Mutagenic in Vivo


Chapter 3 – Iz and NI are Mutagenic in Vivo


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Chapter 3 – Iz and NI are Mutagenic in Vivo


CHAPTER 4

Urea Lesion Formation in DNA as a Consequence of 7,8-Dihydro-8-oxoguanine Oxidation and Hydrolysis Provides a Potent Source of Point Mutations
4.1. Abstract

The DNA oxidation product 7,8-dihydro-8-oxoguanine (8-oxoG) forms several mutagenic oxidation products, including a metastable oxaluric acid (Oa) derivative. We report here that a synthetic oligonucleotide containing Oa hydrolyzes under simulated “in vivo” conditions to form a mutagenic urea (Ua) lesion. Using the Oa 2'-deoxyribonucleoside as a model, the hydrolysis rate depended strongly upon the concentrations of bicarbonate and divalent magnesium. In buffered solutions containing physiologically relevant levels of these species, the half-life of Oa nucleoside was approximately 40 h at 37 °C. The mutagenic properties of Ua in DNA were investigated using an M13mp7L2 bacteriophage genome containing Ua at a specific site. Transfection of the lesion-containing genome into wild-type AB1157 Escherichia coli allowed determination of the mutation frequency and DNA polymerase bypass efficiency from the resulting progeny phage. Ua was bypassed with an efficiency of 11% compared to a guanine control and caused a 99% G→T mutation frequency, assuming the lesion originated from G, which is at least an order of magnitude higher than the mutation frequency of 8-oxoG under the same conditions. SOS induction of bypass DNA polymerase(s) in the bacteria prior to transfection caused the mutation frequency and type to shift to 43% G→T, 46% G→C and 10% G→A mutations. We suggest that Ua is instructional, meaning that the shape of the lesion and its interactions with DNA polymerases influence which nucleotide is inserted opposite the lesion during replication, and that the instructional nature of the lesion is modulated by the size of the binding pocket of the DNA polymerase. Replication past Ua, when formed by hydrolysis of the 8-oxoG oxidation product Oa, denotes a pathway that nearly quantitatively generates point mutations in vivo.
4.2. Introduction

DNA damage mediated by reactive oxygen and nitrogen species is believed to contribute to the etiology of human cancer, aging, and neurological disorders (1-3). Examining the mutagenicity and repair of DNA oxidation products and providing a structural basis for these effects are of fundamental importance for understanding their genotoxic properties. The lesion 7,8-dihydro-8-oxoguanine (8-oxoG) is regarded as perhaps the most critical product resulting from oxidative DNA damage due to its high steady-state concentration in cells and a capability to mispair with A, which leads to G→T transversion mutations (4-9). Recent studies in this laboratory and others have shown that 8-oxoG is an unstable species under a variety of oxidative conditions, owing to its low oxidation potential compared to the parent 2'-deoxyguanosine (10). A variety of DNA lesions is formed when 8-oxoG is exposed to oxidative conditions (11-21). The product distribution depends on the mechanism of action of the oxidant and the reaction conditions. For example, peroxynitrite (ONOO\(^-\)), a powerful oxidant that is formed \textit{in vivo} by the combination of nitric oxide with superoxide (22-24), reacts preferentially with 8-oxoG compared to the four canonical nucleobases to form several lesions including cyanuric acid (Ca), imidazolone (Iz) and its hydrolysis product oxazalone (Oz), guanidinohydantoin (Gh), spiroiminodihydantoin diastereomers (Sp1 and Sp2), and oxaluric acid (Oa) (2). The distribution of the 8-oxoG oxidation products is strongly dependent on the buffer composition and the concentration of ONOO\(^-\). For example, Oa, Oz, and Ca are predominantly formed in the presence of a molar excess of ONOO\(^-\) and 25 mM sodium bicarbonate in phosphate buffer (pH = 7.2) (20). In contrast, when ONOO\(^-\) is slowly infused over time the lesions Gh and Sp predominantly form (25). We recently reported that these secondary lesions are an order of magnitude more mutagenic than 8-oxoG, and that they are well-bypassed by DNA polymerase during replication in \textit{E. coli} (26-28) (Table 4.1).

In the course of our studies, we observed that Oa decomposes in aqueous solution to form a urea (Ua) derivative, a DNA base lesion that is usually ascribed to the presence of pyrimidine oxidation. To our knowledge, the formation of Ua during 8-oxoG oxidation has been reported only once (29); however, in that study a 1,3-diamine, such as
spermine or 1,3-diaminopropane, was required for the production of Ua. Herein, we describe the hydrolysis of Oa to Ua in 2'-deoxynucleoside and oligonucleotide models at physiologically relevant pH and salt concentrations (Scheme 4.1). The effect of normal and SOS-induced cellular conditions on the mutagenic potential of Oa and Ua in DNA was determined in an M13 bacteriophage system using the recently developed restriction endonuclease and postlabeling (REAP) assay (Figure 4.1) (30-32). The hydrolysis and mutation results support a novel potential pathway to mutagenesis in vivo from the oxidation of 8-oxoG.
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4.3. Experimental Procedures

Chemicals and Enzymes. Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. M13mp7L2 was a gift from C. W. Lawrence (University of Rochester). The E. coli cell strain used for transfection was AB1157, and for plating was NR9050 from R.M. Schaaper (NIEHS). The E. coli strain used for re-growth of phage was SCS110 from Stratagene. [γ-32P]ATP (6000 Ci/mmol) was from New England Nuclear, and ATP was from Pharmacia. Acetonitrile, β-mercaptoethanol, and ammonium acetate were from Aldrich and ammonium phosphate dibasic was from Mallinckrodt (AR grade). Polyethyleneimine coated TLC plates and phosphoric acid were from J.T. Baker. Sephadex G-25 spin columns were from Boehringer Mannheim.

Nucleosides. Treatment of 3',5'-di-O-Ac-8-oxoGuo with ONOO− followed by HPLC purification allowed isolation of the Oa nucleoside derivative, as previously described (33).

Hydrolysis of Oa. The decomposition of Oa was followed using a coupled enzymatic spectrophotometric oxalate assay (Sigma-Aldrich or Trinity Biotech), which allowed detection of oxalate in solution through the production of an indamine dye. The oxalate kit consisted of two reagents, A (active ingredients: 3 mM 3-(dimethylamino)-benzoic acid and 0.22 mM 3-methyl-2-benzothiazolinone hydrazone, pH 3.1) and B (active ingredients: 3000 U/L barley oxalate oxidase and 100,000 U/L horseradish peroxidase). The following general procedure was used for the decomposition reactions: Oa (1.5 nmol) in 30 µL of buffer solution was incubated at 37 °C for a prescribed period of time. The buffer solution was pH 7.4 and consisted of 150 mM potassium phosphate and, where indicated, 5 mM magnesium sulfate and/or 20 mM sodium bicarbonate. After the incubation, 30 µL of a 2X solution of reagent A was added, followed by 12 µL of reagent B. The solution was mixed, incubated at room temperature for 15 minutes, and the A590 of the resulting indamine dye was determined by spectrophotometry. The initial concentration of Oa was adjusted such that complete reaction to form oxalate resulted in an absorbance of 1.0 in a 1 cm path length cell.
Electrospray Ionization Mass Spectrometry. Oa 3',5'-di-O-Ac-2'-deoxynucleoside (0.2 mg, 0.6 µmol) in 12 µL of buffer solution was incubated at 37 °C for one day. The buffer solution was pH 7.4 and consisted of 150 mM potassium phosphate plus 5 mM magnesium sulfate and 20 mM sodium bicarbonate. The 3',5'-di-O-Ac-2'-deoxynucleosides were purified by HPLC using a 250 × 4.6 mm, Hypersil ODS column (Thermo Electron Corporation, Bellefonte, PA) (Figure 4.2). The mobile phases used were 60 mM aqueous potassium phosphate, pH 7.4 (A) and 100% acetonitrile (B). HPLC conditions used were: isocratic 5% B for 10 min, then 5% B to 27.4% B over 20 min. The flow rate was 1 mL/min, and products were monitored simultaneously at 213 nm and 260 nm. A Quattro Micro™ API mass spectrometer (Micromass UK Limited, Manchester, UK) was used for electrospray ionization (ESI)-MS experiments, and spectra were obtained in positive ion mode using a 49:40:10:1 water/methanol/acetonitrile/ammonium hydroxide or 78:20:2 water/2-propanol/ammonium hydroxide spraying solution (Figure 4.2).

Oligonucleotides. Oligonucleotides were from Research Genetics (Huntsville, AL), Integrated DNA Technologies (Coraville, IA), or synthesized on an Applied Biosystems model 391 DNA synthesizer. Oligonucleotides were deprotected with concentrated NH₃OH for 18 h at 55 °C. The strand containing 8-oxoG, to prevent degradation of the lesion, required 0.25 M β-mercaptoethanol in the deprotection solution. Oligonucleotides made by solid-phase synthesis were purified by PAGE and C18 reverse-phase HPLC before use (~99% purity) as described (26). MALDI-TOF mass-spectrometry verified the molecular weight of each strand after purification. The insert sequence was 5'-GCG AAG ACC GXA GCG TCC G-3' (X = G, 8-oxoG, Oa, Ua, or a tetrahydrofuran (THF) synthetic AP site).

ONOÖ− Treatment of 8-OxoG in DNA. ONOÖ− was prepared by ozonation of sodium azide in alkaline solution (0.1 M NaOH) and stored at −80 °C (34). Concentrations of ONOÖ− were determined by spectrophotometry (ε = 1670 M⁻¹ cm⁻¹, λ = 302 nm in 0.1 M
Reactions between ONOO$^-$ and oligonucleotides were performed in buffer containing 150 mM potassium phosphate and 25 mM sodium bicarbonate (pH = 7.2), as described (20). The purified Oa-containing oligonucleotide was hydrolyzed to Ua under well-defined conditions as described above for the Oa nucleoside.

**HPLC Purification of Modified Oligonucleotides.** The Oa- and Ua-containing oligonucleotides were purified by HPLC using a 250 mm x 2.1 mm, 5 µm Supelco Supelcosil LC-18 column with UV detection at 260 nm. The mobile phase was 150 mM aqueous ammonium acetate (A) and 100% acetonitrile (B) with a gradient of 7-12% B over 30 min at a flow rate of 0.25 mL/min. The quantities of each product were calculated by UV absorbance at 260 nm using the molar absorptivity of the G-containing oligomer (ε = 184700 M$^{-1}$ cm$^{-1}$) for all the modified oligonucleotides. The mass of each oligonucleotide was determined using MALDI-TOF mass spectrometry with Oa [MW = 5843.8 (calculated), 5844.7 (observed)] and Ua [MW = 5771.8 (calculated), 5771.5 (observed)] resulting in the expected mass. The mass of each oligonucleotide was calibrated using oligonucleotides that bracketed the molecular ion of each analyte, which allowed the measured mass to fall routinely within 1 atomic mass unit of the calculated mass for each lesion-containing oligonucleotide. Although a few percent of Ua contamination in the Oa sample was unavoidable, sample handling during the REAP assay either minimized or avoided additional Ua formation (26). Since there are relatively few products that form from 8-oxoG oxidation by ONOO$^-$, and these products are generally well-separated by HPLC purification, we are confident that the oligonucleotides were 95-99% pure.

**Construction of M13 Genomes Containing a Site-Specific Lesion.** Single-stranded (ss) M13 genomes containing a unique lesion were generated as previously described (Figure 4.1A) (26). Briefly, 1 pmol of ss M13mp7L2 was linearized by EcoRI and annealed to equimolar amounts of two oligonucleotide “scaffolds,” which are partially complementary to the 5'- and 3'-ends of the insert and the linearized genome. An equimolar amount of the 5'-phosphorylated 19-mer insert was added and covalently joined into the genome by incubation with T4 DNA ligase (16 °C, 1 h). The scaffolds
were removed by heating (50 °C, 5 min) in the presence of a 100-fold molar excess of “anti-scaffold” complement immediately prior to transfection.

Transfection and determination of translesion bypass efficiency. Cells, grown to an OD$_{600}$ = 0.4 in 100 mL of yeast tryptone (1X) medium (35), were made competent for transfection by the calcium chloride method as described previously (26, 36). For SOS induction of bypass DNA polymerases, the cells were irradiated with 254 nm light, at an intensity of 45 J/m$^2$ and transferred to an equivalent volume of 2×YT media and grown for 40 min at 37 °C before being made competent. Lesion bypass was determined by transfection with 5 ng of M13 DNA, and mutation frequency was determined using 50 ng of M13 DNA. The number of independent transformed cells was determined by adding 2.5 mL B-broth soft agar to the transfected cells and plating onto a lawn of NR9050 E. coli. The number of plaques on each plate was counted after incubation at 37 °C for 16 h. As a control to estimate the number of viable viral genomes that do not have an insert, a wild-type (WT) M13mp7L2 sample was exposed to the genome construction conditions, and the average number of resulting plaques was subtracted from the total formed by each insert-containing genomic construct. Transfections were performed in triplicate, and the bypass efficiency relative to the G control was calculated to within a 95% confidence interval of the mean for each lesion. A THF AP-site was used as a DNA polymerase blocking control, which provided significantly fewer plaques than the G-containing insert control.

Mutation Frequency Determination. The point mutations caused by each lesion in AB1157 E. coli were determined and quantified using the restriction endonuclease and post-labeling (REAP) assay as previously described (Figure 4.1B) (26). Briefly, transformed cells for determination of mutation frequency were added to 10 mL of LB media and incubated on a roller drum for 5 h at 37 °C (growth), followed by pelleting of the cells and decanting of the phage-containing supernatant into new 15 mL polypropylene tubes. A 100 µL aliquot of the suspension was added to 9 mL of LB and 1 mL of mid-log SCS110 E. coli. The cells were incubated for 4 h (regrowth), pelleted, and the phage-containing supernatant was decanted and stored at 4 °C. The growth and
regrowth steps served to amplify the amount of progeny phage while reducing the relative amount of non-transfected DNA to a negligible amount (<0.1%). The mutation frequencies were determined by performing the REAP assay on a DNA duplex produced by PCR amplification of the insert region of the progeny phage. Each 101-mer PCR product was cleaved by BbsI at the position in the template vector that originally contained the lesion, affording a 55-mer whose newly formed 5'-end contained the lesion site. The 55-mer was dephosphorylated with shrimp alkaline phosphatase, which exposed the lesion site at the 5'-OH for radiolabeling with PNK and [γ-32P]ATP. Incubation with HaeIII, to yield a 19-mer, allowed PAGE purification of the radiolabeled fragment of interest. Digestion of the desalted 19-mer to 5'-deoxynucleotide monophosphates (5'-dNMPs) with snake venom phosphodiesterase (SVPD) was followed by partitioning of the mixture on a polyethyleneimine TLC plate developed in saturated (NH₄)₂HPO₄ (adjusted to pH = 6.1 with H₃PO₄). The separated radiolabeled nucleotides were quantified by PhosphorImager analysis, which provided the point mutation frequency and type at the lesion site.
4.4. Results and Discussion

4.4.1. Hydrolysis of Oa to form Ua

We investigated the hydrolytic stability and mutagenic potential of the 8-oxoG-derived products, Oa and Ua. The specific oxidation of an 8-oxoG-containing oligonucleotide with ONOO\(^-\) followed by HPLC purification of the modified oligonucleotide was used to obtain pure Oa. The lesion Ua was observed to form via divalent metal cation and base catalyzed hydrolysis (Scheme 4.1), and the identity of the lesion was established from MALDI-TOF mass spectral data as previously reported \((20, 26)\). Incubation of Oa-containing oligonucleotide in phosphate buffered saline (pH = 7.4) at 37 °C caused no discernable conversion to Ua (data not shown). However, addition of Mg\(^{2+}\), Ca\(^{2+}\), and HCO\(_3^-\) to the solution at concentrations that are isotonic to mammalian cells, incubation in alkaline solution, or incubation in pure water at high temperature caused appreciable hydrolysis of the Oa oligonucleotide to form a new species, which was assigned as Ua (Figure 4.3). The decrease in mass by 72 amu corresponds to the expected loss of oxalate during Ua formation.

As a model system to study conveniently the hydrolysis reaction under a variety of conditions, the 3'-5'-di-O-acetyl-2'-deoxynucleoside of Oa was synthesized from 3',5'-di-O-acetyl-2'-deoxy-8-oxoguanosine \((26, 33)\). The Oa nucleoside was exposed to HCO\(_3^-\) and to various divalent alkaline earth metal cations in order to determine if these species can catalyze hydrolysis to Ua at or near in vivo concentrations. Exposure of the Oa nucleoside at 37 °C to a phosphate buffered solution containing 5 mM MgSO\(_4\) and 20 mM NaHCO\(_3\) (pH = 7.4) resulted in almost complete hydrolysis of the nucleoside (half-life ~40 h), as determined by a commercially available oxalate assay (see Materials and Methods), whereas exposure of the Oa nucleoside to similar conditions without a divalent metal cation (half-life ~52 h) and without HCO\(_3^-\) (half-life ~96 h) resulted in much slower hydrolysis of the nucleoside starting material, with the phosphate buffered control hydrolyzing at the slowest rate (half-life ~130 h) (Figure 4.4).

The addition of 5 mM MgSO\(_4\) to an aqueous potassium phosphate buffered solution (pH 7.4) increased the rate of hydrolysis of the Oa nucleoside (Figure 4.4),
implying that Mg\(^{2+}\) stabilizes the rate-determining transition state for the reaction. A previous study showed that oxalate binds Mg\(^{2+}\) effectively \((37)\), and Falvello et al. recently demonstrated that the divalent metal cations Co\(^{2+}\), Ni\(^{2+}\), and Cu\(^{2+}\) can form a complex with the oxalate moiety of oxalurate \((38)\). Thus, it is plausible that Oa also chelates Mg\(^{2+}\). The binding of a divalent metal cation to Oa could increase the rate of hydrolysis by several mechanisms, either alone or in concert. By binding to the oxalate portion of Oa, electron density could be reduced at the carbonyl of the amide, thereby facilitating nucleophilic addition at the amide carbon. The developing negative charge on the amide carbonyl oxygen during nucleophilic addition would also be stabilized by the presence of Mg\(^{2+}\). Divalent metal cations could also stabilize the electrostatic repulsion between the carboxylate of Oa and a negatively charged incoming nucleophile (HO\(^{-}\) or HCO\(_3\)^\(-\)). Alternatively, chelation of a divalent metal cation by the oxalate moiety could facilitate the elimination of this group in a manner similar to the commonly known Mg\(^{2+}\)-assisted elimination of inorganic phosphate or pyrophosphate during the hydrolysis of nucleotide triphosphates.

Surprisingly, HCO\(_3\)^\(-\) significantly increased the rate of hydrolysis of the Oa nucleoside (Figure 4.4). During the hydrolysis by water, a gem-diol tetrahedral intermediate is presumed to form at the amide carbon. The spontaneous hydrolysis of Oa may be explained through a comparison of the leaving group pK\(_a\) values. The pK\(_a\) of urea is approximately 14 \((39)\), whereas the pK\(_a\) of water is 15.7. Assuming a minimal effect on the Ua pK\(_a\) due to the 2-deoxyribose substituent, the reaction should favor elimination of Ua. If instead of hydroxide, HCO\(_3\)^\(-\) adds to the amide carbonyl of Oa, forming a tetrahedral intermediate, fragmentation would not occur directly from this structure. Collapse of the tetrahedral structure at this point should eliminate HCO\(_3\)^\(-\) (pK\(_a\) H\(_2\)CO\(_3\) = 6.35) about 10\(^7\) fold more frequently than Ua, making the fragmentation of Oa an unlikely event. However, the tetrahedral intermediate may follow a different reaction coordinate. The pK\(_a\) of HCO\(_3\)^\(-\) is 10.3; however esterification of HCO\(_3\)^\(-\) to produce carbonic acid monomethyl ester (CH\(_3\)OCOOH) results in a significant decrease of the pK\(_a\) of the acid to 2.92 \((40)\). Upon addition of HCO\(_3\)^\(-\) to Oa, it is likely that a large
decrease of the carbonic acid pK\textsubscript{a} also results. Deprotonation of the carbonic acid could conceivably initiate a decarboxylation event and subsequent fragmentation of Oa.

During the reaction of Oa with HCO\textsubscript{3}\textsuperscript{-}, several factors may contribute to the hydrolysis rate increase. We propose that the reaction proceeds by the addition of HCO\textsubscript{3}\textsuperscript{-} to Oa followed by decarboxylation and fragmentation (Scheme 4.2). Decarboxylation and fragmentation may occur in a concerted fashion or in several separate steps. Herschlag and Jencks investigated a Mg\textsuperscript{2+}-catalyzed reaction in which carboxylate ions react with phosphorylated pyridines in a nucleophilic fashion (41). They found that HCO\textsubscript{3}\textsuperscript{-} reacts much faster than CO\textsubscript{3}\textsuperscript{2-}, even though CO\textsubscript{3}\textsuperscript{2-} is more basic, because HCO\textsubscript{3}\textsuperscript{-} hydrogen bonds to a phosphoryl oxygen in the transition state, thereby avoiding unfavorable electrostatic interactions. They also note that Mg\textsuperscript{2+} may serve as a template for the reacting species. In the present case, intramolecular hydrogen bonding may contribute to the ability of HCO\textsubscript{3}\textsuperscript{-} to react with Oa. Given the stabilizing role Mg\textsuperscript{2+} appears to play during the hydrolysis of Oa in the absence of HCO\textsubscript{3}\textsuperscript{-}, it may also play a similar role in the addition of HCO\textsubscript{3}\textsuperscript{-} to Oa.

**4.4.2. In vivo mutagenesis by Oa and Ua under normal or SOS-induced conditions**

The mutagenic potential of Ua was tested in vivo by inserting oligonucleotides containing each defined lesion (8-oxoG, Oa, or Ua) into M13mp7L2 single-stranded viral genomes and then transfecting the genomes into wild-type *E. coli* (Figure 4.1). Each lesion was bypassed during replication in vivo efficiently enough to produce progeny phage for the determination of point mutation frequency and type using the REAP assay.

Since lesions in DNA may block or inhibit replication, the relative number of plaques formed from the immediate plating of the transformation mixture gives a measure of the efficiency of translesion DNA synthesis. A comparison of the number of plaques obtained after transformation of the site-specific lesion-containing phage genome with that from an identically constructed vector that did not carry any oxidative damage allowed calculation of the relative bypass efficiency (26, 27). As shown in Figure 4.5, vector survival for Oa is 51 ± 3%, which is in agreement with a previously published
value (26). The Ua lesion bypass efficiency was 11 ± 5%, which is significantly higher that that observed for a synthetic THF AP site employed as a control for DNA polymerase blocking and SOS-induced bypass (26, 27, 42, 43). The bypass efficiencies of the lesions were also determined in cells with the SOS system induced. Significant differences were not observed compared to uninduced cells using the assay described in Figure 4.1A, as Oa and Ua were bypassed with efficiencies of 56 ± 9% and 18 ± 4%, respectively. However, increased levels of insert-containing DNA after PCR amplification during the REAP protocol were observed for Ua and THF (Figure 4.6), which is consistent with an increased bypass efficiency. The error inherent in the bypass assay does not allow small increases in bypass efficiency to be observed with statistical significance. The inefficient bypass of Ua is in accord with previous studies (44). Successful induction of the SOS operon is supported by the change in the mutation specificity of Ua (see below) and the increased levels of insert-containing DNA after PCR amplification of Ua and the THF lesion compared to uninduced controls during the REAP assay (Figure 4.6).

As shown in Figure 4.7, in uninduced cells the mutation frequency and type for both Oa and Ua was 99% with nearly all of the mutations in the form of G→T transversions (based on the lesions originating from G). The mutation type and frequency for each lesion was also determined in SOS-induced cells in order to investigate the effect of inducible error-prone translesion DNA polymerase bypass on the coding properties of these lesions. Compared with normal cells, SOS induction caused Oa to have a somewhat similar mutation frequency and type, with 95% G→T and 4% G→C transversions. In contrast, the more blocking Ua had a dramatically different distribution of mutation types with 43% G→T, 46% G→C, and 10% G→A mutations and no measurable repair or correct coding. The observed insertion of G and A opposite Ua, regardless of SOS induction, is in general agreement with previous in vitro and in vivo studies (45-47). For example, Maccabee et al. reported that Ua produced from T oxidation directed insertion of G and T opposite the lesion with a frequency of 62% and 26%, respectively, compared to other point mutations (45). Direct comparison of these results to the REAP data is difficult because of sequence context variations and because
Ua was not introduced site-specifically into the DNA, which does not allow repair or correct coding to be scored. Studies in vitro demonstrate that Ua is excised by several DNA glycosylases involved in base excision repair in *E. coli* such as Endo III, Endo VIII, and their homologues in higher organisms (48). Additionally, another DNA glycosylase, MutM, also known as Fpg, removes Oa in duplex DNA when the lesion is situated opposite a C (20).

The increase in the degeneracy of the type of nucleoside inserted opposite Ua is consistent with the larger, more flexible binding pocket generally found in Y-family lesion-bypass polymerases (49). This binding pocket may accommodate atypical steric configurations and unfavorable electrostatic interactions induced by the lesion and allow promiscuous chain extension past the damaged DNA. Frameshifts and small deletions are assumed to be negligible in this work, since there was no detectable variation in the size of the *Bbs*I/*Hae*III restriction fragment (Figure 4.6). Clearly, the presence of Ua, like the precursor Oa, causes a higher mutation frequency in DNA than the parent 8-oxoG.

Since well-bypassed lesions (i.e., ~50% or greater relative to guanine) have essentially no change in bypass efficiency or mutation profile with SOS induction, they are likely to be successfully replicated in *E. coli* regardless of SOS status. The change in coding specificity observed for Ua during SOS induction is consistent with recent models describing the initiation of a “handoff” from the replicative DNA polymerase to an error-prone bypass DNA polymerase upon encountering a lesion that stalls the replication fork (49). Well-bypassed lesions include not only Oa, but also Ca, Iz, Oz, and Gh (Table 4.1). The Ua lesion exists as a mixture of α- and β-anomers, so it is intriguing to hypothesize the α-anomer may block DNA polymerase extension until β-anomerization permits limited translesion bypass. SOS induction likely allows error-prone DNA polymerases to bypass the lesion, perhaps regardless of the anomeric state of the sugar. Further experiments are needed to elucidate the structural basis of this possible effect. NMR analysis of a duplex dodecamer containing Ua showed evidence of both anomers when placed opposite T, and that the β-anomer was interhelical (aligned with the base stack) while sharing at least one hydrogen bond with the opposing nucleotide (50).
hydrogen bonding interaction indicates that the lesion may be instructional, rather than simply forcing the polymerase to obey the “A” rule, which states that A will be most likely incorporated opposite a noninstructional lesion (51). A recent paper by Kroeger et al. (52) discusses application of the REAP assay to 2'-deoxyribonolactone, an AP site analog, and proposes that even limited hydrogen bonding contributes to the lesion being instructional. We suggest that dA incorporation opposite Ua is, in part, attributable to hydrogen bonding between the carbonyl and/or distal hydrogen of the urea moiety and the respective N6-amino and/or N1 of dA. This explanation of Ua as an instructional lesion is speculative, and alternative hydrogen bonding patterns cannot be ruled out, particularly for SOS induced lesion bypass. Another explanation that is consistent with a recent report by Kobayashi et al. is that nucleotide insertion opposite Ua is inhibited, resulting in slippage and insertion of a second nucleotide opposite the base on the 3'-side of the template Ua (53). However, the local sequence used in our work [5'-G(Ua)A-3'] only allows for G→A mutations to occur by this mechanism, and these mutations are only observed to a modest degree during SOS induction compared to the predominant G→T and G→C mutations.

The 8-oxoG derived DNA lesions Oa and Ua are mutagenic under normal and SOS induced conditions in vivo. If these lesions form in cells, they clearly represent a threat to genomic integrity in the absence of an effective DNA repair mechanism.
4.5. Acknowledgment

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Figure 4.1. (A) Strategy for determination of translesion DNA polymerase bypass of base damage in vivo. A site-specifically modified oligonucleotide is ligated into ss viral M13 DNA. Transfection into host cells followed by translesion DNA synthesis ultimately affords infective progeny phage whose initial numbers are limited by the lethality of the site-specific lesion. Plating of the transformation mixture onto a lawn of indicator bacteria caused the formation of plaques (spots of infected cells), whose numbers allowed for the calculation of the efficiency of polymerase bypass when compared with the non-lesion control. (B) Detection of mutations using the REAP assay. PCR amplification of the region from the progeny phage that had originally contained the site-specific lesion was performed for each transfection. The resulting duplex DNA was cleaved with BbsI, dephosphorylated, radiolabeled at the 5'-end, and reduced in size with HaeIII to a 19-mer. Digestion of the radiolabeled and purified 19-mer to 5'-dNMPs, separation by TLC, and quantification of the radioactivity by PhosphorImager analysis allowed measurement of the fractional composition of each base to within 1%.

A. Lesion Bypass Efficiency Determination

B. Restriction Endonuclease and Postlabeling (REAP) Assay

Mutation Frequency = 
\[ \frac{T + C + A}{T + C + A + G} \times 100\% \]
Figure 4.2. ESI mass spectra and HPLC traces (insets) of 8-oxoG, Oa, and Ua 3',5'-di-O-Ac-2'-deoxynucleosides.
Figure 4.3. Hydrolysis of Oa to form Ua in an oligonucleotide. (Left) HPLC chromatogram of the Oa-containing 19-mer oligonucleotide after heating in neat H₂O for 20 min at 80 °C. (Right) MALDI-TOF mass spectra of the purified products.
Figure 4.4. Oxalate formation curves for the decomposition of Oa 2'-deoxynucleoside at 37 °C in 150 mM potassium phosphate buffer, pH 7.4. Where noted, the buffer also contained 5 mM MgSO₄ and/or 20 mM NaHCO₃. The error bars represent a 95% confidence interval of the mean for three independent experiments.
Figure 4.5. Relative bypass efficiency for Oa and Ua. Controls include G (well-bypassed) and a synthetic THF AP site (blocking). The error bars represent a 95% confidence interval of the mean for three independent experiments.
Figure 4.6. SOS induction allows preferential bypass of blocking lesions in vivo. PCR amplification of the insert region in M13 progeny phage, as described for the REAP assay in the text, yielded a binary distribution of DNA fragments; one contained the 19-mer insert that had originally borne a site-specific lesion (or a G or T control nucleotide) and another that is derived from insert-free phage DNA. Products were assayed by agarose gel electrophoresis, which included an unmodified (WT) phage control and a no template control (-phage). The band corresponding to a fragment ~80 bases long likely results from deletion of the hairpin in WT M13 DNA during PCR (54, 55). Additional controls were grouped according to bypass efficiency: G, T, and 8-oxoG (well-bypassed); Oa, Oz, and Ca (moderately-bypassed); whereas THF and Ua are nearly blocking. The ratio of the two PCR products varied according to the bypass efficiency of the site-specific lesion contained in the insert region. THF and Ua were the most blocking lesions, as determined by the plaque counting assay described in the text and showed the greatest increase in insert-containing PCR product after SOS induction by exposure to UV light. The insert-containing PCR products were digested with BbsI, 32P-radiolabeled, and digested with HaeIII to yield 19-mer oligonucleotides, which were purified by PAGE. Note that in both cases, the signal intensity of the bands in the THF and Ua lanes on the polyacrylamide gel correlates with findings from the agarose gel.

---

**Insert:**
- G
- T
- 8-oxoG
- Oa
- Oz
- Ca
- THF
- Ua
- -phage
- WT

**115-mer control**

**101-mer (+insert)**

**~80-mer (-insert)**

**SOS Induction**

(45 J/m² at 254 nm)

**3% Agarose, 100V, 4h**

THF

Ua

**20% PAGE**
Figure 4.7. TLC showing mutation frequency and type as determined by the REAP assay. Markers consisting of 5'-dNMPs were derived from digestion of a 13-mer oligonucleotide that contained a degenerate 5'-terminus.

<table>
<thead>
<tr>
<th>N = 8-oxoG</th>
<th>Oa</th>
<th>Ua</th>
<th>dNMPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS Induction:</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Elution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G → T:</td>
<td>7.0; 6.8</td>
<td>99; 95</td>
<td>99; 43</td>
</tr>
<tr>
<td>G → C:</td>
<td>&lt;1; 1.3</td>
<td>&lt;1; 3.8</td>
<td>&lt;1; 46</td>
</tr>
<tr>
<td>G → A:</td>
<td>&lt;1; &lt;1</td>
<td>&lt;1; &lt;1</td>
<td>&lt;1; 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>
Scheme 4.1. Oa hydrolyzes to Ua and oxalate.

\[
\text{oxalurate (Oa)} \xrightarrow{\text{Mg}^{2+} + \text{HCO}_3^- (\text{catalysts})} \text{urea (Ua)} + \text{oxalate}
\]

\[
R = \begin{array}{c}
\text{AcO} \\
\text{DNA}
\end{array}
\]

or

176
Scheme 4.2. Proposed mechanism for Mg$^{2+}$ and HCO$_3^-$ catalyzed hydrolysis of Oa.
Table 4.1. Summary of bypass efficiencies and mutation frequencies for 8-oxoG oxidation products in wild-type *E. coli*.

<table>
<thead>
<tr>
<th>8-oxoG oxidation product</th>
<th>Ca(^a)</th>
<th>Iz(^b)</th>
<th>Oz(^a)</th>
<th>Gh(^c)</th>
<th>Sp1(^c)</th>
<th>Sp2(^c)</th>
<th>On(^d)</th>
<th>Ua(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bypass efficiency (%)</td>
<td>65 ± 8</td>
<td>60 ± 5</td>
<td>57 ± 13</td>
<td>75 ± 5</td>
<td>9 ± 3</td>
<td>9 ± 4</td>
<td>51 ± 3</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>G→T mutations (%)</td>
<td>97</td>
<td>1</td>
<td>86</td>
<td>1.4</td>
<td>27</td>
<td>41</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>G→C mutations (%)</td>
<td>&lt;1</td>
<td>88</td>
<td>&lt;1</td>
<td>98</td>
<td>72</td>
<td>57</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>G→A mutations (%)</td>
<td>&lt;1</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

\(^a\) (26). \(^b\) (28). \(^c\) (27). \(^d\) This work.
4.6. References


Chapter 4 – Mutagenic Urea Lesion Formation From 8-OxoG Oxidation


Deletion mutagenesis during polymerase chain reaction: dependence on
CHAPTER 5

The Hydantoin Lesions Formed From Oxidation of 7,8-Dihydro-8-oxoguanine are Potent Sources of Replication Errors in Vivo
5.1. Abstract

Single-stranded DNA genomes have been constructed that site-specifically contain the 7,8-dihydro-8-oxo-2'-deoxyguanine (8-oxoG) oxidation products guanidino-hydantoin (Gh) and the two stable stereoisomers of spiroiminodihydantoin (Sp1 and Sp2). The circular viral genomes were transfected into wild-type AB1157 Escherichia coli and the efficiency of lesion bypass by DNA polymerase(s) was assessed. Viral progeny were analyzed for mutation frequency and type using the recently developed restriction endonuclease and post-labeling (REAP) assay. Gh was bypassed nearly as efficiently as the parent 8-oxoG but was highly mutagenic, causing almost exclusive GÆC transversions. The stereoisomers Sp1 and Sp2 were, in comparison, much stronger blocks to DNA polymerase extension, and caused a mixture of GÆT and GÆC transversions. The ratio of GÆT to GÆC mutations for each Sp lesion was dependent on the stereochemical configuration. All observed mutation frequencies were at least an order of magnitude higher than those caused by 8-oxoG. Were these lesions to be formed in vivo, our data show that they are absolutely miscoding and may be refractory to repair after translesion synthesis.
5.2. Introduction

Genotoxicity due to degradation of DNA by oxygen radicals is associated with aging and carcinogenesis (1). An understanding of the processes of spontaneous, oxidant-induced and ionizing radiation-induced mutagenesis requires knowledge of the exact types and biological significance of oxidative lesions formed in DNA (2). Both endogenous and exogenous agents have been implicated in the in vivo oxidation of DNA. The observation that oxidative damage to DNA naturally occurs in vivo was a major discovery because it demonstrated that DNA damage could occur endogenously as a consequence of normal metabolism (3-5). Currently, over 50 oxidative DNA lesions have been characterized (6). The 7,8-dihydro-8-oxo-2'-deoxyguanine (8-oxoG, Figure 5.1) lesion has been studied extensively because it is a major oxidative DNA lesion (7) and can be detected by a variety of assays (8). 8-oxoG is mutagenic because of its propensity to mispair with A during replication by DNA polymerases. Incorporation of A opposite 8-oxoG leads to a predominant G→T transversion mutation (9-11), which is the second most common somatic mutation in human carcinomas (12), and is especially frequent in the mutational spectrum of the p53 tumor suppressor gene (13).

Several studies have shown that 8-oxoG, which has a lower oxidation potential than any of the four normal DNA bases (14), is highly reactive towards oxidizing reagents. This suggests that 8-oxoG may also be sensitive to in vivo DNA oxidation. Peroxynitrite (ONOO\(^-\)), which is formed endogenously by the reaction of nitric oxide with superoxide, is a potent oxidant that decomposes into radical species capable of oxidizing DNA and other cellular constituents including thiols, lipids and proteins (15-17). Oxidation of DNA is known to have mutagenic consequences. For example, ONOO\(^-\) treatment of plasmid DNA containing the supF gene, followed by transfection into mammalian or bacterial cells, induces mainly G→T, and some G→C transversions (18). Reaction of ONOO\(^-\) with an oligonucleotide containing a portion of the supF gene followed by incubation with hot piperidine, which cleaves DNA preferentially at oxidized 8-oxoG nucleotides, causes strand scission at sites coinciding with the mutational “hot spots” observed after replication in bacterial cells (19). Since 8-oxoG is not a known
source of G→C transversions and 8-oxoG in DNA is refractory to cleavage by piperidine (20, 21), these experiments suggest that additional DNA lesions are forming and that these may contribute to ONOO⁻ mutagenicity.

Peroxynitrite is known to react preferentially with 8-oxoG in DNA to form several mutagenic 2′-deoxy-β-D-erythro-pentofuranosyl products, including cyanuric acid, oxaluric acid and oxazalone (22), which were recently reported to cause predominantly G→T transversion mutations in vitro using purified DNA polymerases (23-25), and in vivo by transfection of site-specifically adducted bacteriophage DNA into Escherichia coli (26). Determination of the in vivo mutagenic consequences of such lesions typically have taken the following course: (i) synthesis of the modified oligonucleotide containing the DNA adduct of interest, (ii) insertion of the oligonucleotide into the genome of a virus or plasmid, (iii) introduction of the site-specifically modified genome into a bacterial or mammalian host where replication, and possibly repair, occur, and (iv) enumeration and characterization of mutant progeny (27).

Several 8-oxoG oxidation products have yet to be analyzed for their in vivo mutagenic potential. Recently, two major products of 8-oxoG oxidation were identified: guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) (28-30), both of which exist as stereoisomers as shown in Figure 5.1. Although these lesions can be made by oxidation of 8-oxoG with ONOO⁻ (mimicking the chemistry of the in vivo inflammatory response) (31) or by oxidation of guanine with singlet oxygen or other oxidants, a difficult to separate mixture of products results when the reaction is carried out using oligonucleotides (unpublished data). Using a temperature-dependent method of synthesis, Burrows and co-workers developed selective methods that yield oligonucleotides containing Gh and Sp via oxidation of 8-oxoG using the one-electron oxidant Na₂IrCl₆, a compound whose redox potential is specific for 8-oxoG as compared with the four normal DNA bases (21, 32-34).
In view of the reported susceptibility of 8-oxoG to oxidation, the mutagenic potential of Gh and Sp was tested \textit{in vivo} by inserting oligonucleotides containing each well-defined lesion into bacteriophage DNA, followed by transfection into wild-type \textit{E. coli}. This report describes that each lesion was bypassed efficiently enough during replication \textit{in vivo} to yield progeny phage. The viral progeny was analyzed for mutation frequency and specificity, and it was shown that these oxidation products were much more mutagenic than the parent 8-oxoG.
5.3. Experimental Procedures

**Oligonucleotides.** The DNA base lesion 8-oxoG and tetrahydrofuran abasic (AP) site control 19mer oligonucleotides were made using phosphoramidite solid-phase methods and purified as described (26). Oligodeoxynucleotides containing Gh or Sp were prepared by reacting 12 µM 5'-GCGAAGACCXAGCGTCCG-3' (X = 8-oxoG) with the oxidizing agent Na$_2$IrCl$_6$ (175 µM final concentration) in 100 µL of 10 mM sodium phosphate and 100 mM NaCl for 1 h. Selective formation of Gh was accomplished by incubation of the 8-oxoG-containing oligonucleotide at pH 7.0 and 4 °C, while incubation at pH 6.5 and 60 °C afforded the Sp lesion. The reaction mixture was dialyzed against H$_2$O using 2000 molecular weight cutoff dialysis tubing for 24 h. The samples were analyzed by negative ion electrospray mass spectrometry (Micromass Quattro II), and their purity was estimated to be ~95% on the basis of the intensities of related molecular ions. Although both lesions were formed as a mixture of stereoisomers, the Sp-containing oligonucleotides were separable by anion-exchange HPLC. The pure stereoisomer-containing strands are termed Sp1 and Sp2, although their absolute stereochemistry has not been assigned. Purification of the Gh oligodeoxynucleotide was accomplished by HPLC using a Dionex DNA Pac PA-100 4 X 250mm column and an isocratic buffer system consisting of 50% solvent A (10% acetonitrile and 90% H$_2$O) and 50% solvent B [10% acetonitrile and 90% 1.5 M ammonium acetate (pH 7)] (Figure 5.2). The flow rate was 1.0 mL/min and UV spectra were recorded at 260 nm. Oligodeoxynucleotides containing the Sp isomers were purified in a similar manner, except that an isocratic buffer system consisting of 30% solvent A and 70% solvent B was used (Figure 5.3). Prior to use, the HPLC purified oligodeoxynucleotides were dialyzed against H$_2$O for 72 h, followed by dialysis against 1 mM NaCl for 72 h, to minimize the possibility of polynucleotide kinase inhibition by ammonium cations. The purity of the HPLC-purified oligodeoxynucleotides was determined by HPLC to be >99% using a Dionex DNA Pac PA-100 4 X 250mm column and a linear gradient of 35% solvent B to 100% solvent B in 30 min (Figures 5.2 and 5.3). MALDI-TOF mass spectrometry was used to verify the molecular weight (MW) of each strand after purification [Gh, MW = 5868.9 (calculated), 5869.2 (observed); Sp1 and Sp2, MW = 5894.9 (calculated), 5895.7 and 5895.7 (observed), respectively] (Figures
The synthetic AP site 19mer control and a 20mer oligonucleotide were used to calibrate the mass spectrometer, which allowed measurement of the MW to within 1 atomic mass unit of the calculated value. The stability of the lesions to the genome construction protocol was verified by MALDI-TOF mass spectrometry. Lesion-containing oligonucleotides were exposed to all reagents and conditions, with the omission of viral DNA and T4 polynucleotide kinase. After the incubations, mass spectrograms of the Gh, Sp1, and Sp2 were essentially identical to those of the purified strands prior to genome construction (Figures 5.4-5.6).

**DNA Polymerase Bypass Efficiency.** Lesion bypass experiments in wild-type AB1157 *E. coli* were performed as described (26, 35) in triplicate using genomes made from the purified oligodeoxynucleotides 5’-GCGAAGACCGXAGCGTCCG-3’ (X is the lesion or guanine for the unmodified insert control), as shown in Figure 5.7. Briefly, 1 pmol of ss M13mp7L2 was linearized by *Eco* RI digestion of a hairpin contained in the M13 polylinker region that interrupts the *lacZ* gene. Equimolar amounts of two “scaffolds” (oligonucleotides that are complementary to and span the 5’- and 3’-ends of both the vector and the lesion-containing insert) were annealed to the genome. Two scaffolds were used in order to leave a ss gap around the lesion that allowed the subsequent ligation efficiencies to be equal, regardless of the lesion employed. An equimolar amount of the 5’-phosphorylated 19mer insert was added and covalently joined into the genome by incubation with T4 DNA ligase (16 °C, 2 h). The ligation efficiencies were confirmed to be equal by electrophoresis of an aliquot of the ligation mixture on a 1% agarose gel, staining of the gel with ethidium bromide, and image analysis with digital photography (data not shown). AB1157 *E. coli* were made chemically competent with CaCl₂ and immediately plated after transfection using 20 ng of ss viral DNA and ~ 10⁸ cells as described (26). For each successful initial round of viral genome replication within each cell, viral progeny leaves a transfected AB1157 cell and infect nearby NR9050 *E. coli* (containing the F’ episome required for infection). The resulting localized infection slows the growth of the NR9050 cells, which is visible as a transparent spot (plaque) surrounded by a dense lawn of uninfected cells. A control for the presence of viable genomes that lacked an insert was made by constructing a genome containing a
nonphosphorylated AP-containing 19mer insert. The number of plaques arising from this control was subtracted from the number of plaques derived from genomes containing 5’-phosphorylated inserts; the latter inserts contained the lesion of interest. The number of plaques formed by each lesion relative to that of the guanine control genome is indicated as “relative bypass efficiency”, with the corrected guanine reference averaging 217 plaques per plate, as shown in Table 5.1.

**Mutation Frequency and Type.** Mutational analysis was performed using the recently developed restriction endonuclease and postlabeling (REAP) assay with triplicate genome constructions and transfections for each lesion (26, 35-37). Briefly, 100 μL of cells was transformed by electroporation (2.5 kV, 129 ohms, 2mm gap cuvette) of 50 ng (1 ng/μL) of the constructed genome, after which they were added to 10 mL of LB media and incubated on a roller drum for 6 h at 37 °C. This amplified the ~10⁴ initial events to ~10⁹ progeny. The cells were pelleted, and the supernatant was decanted into 15 mL polypropylene tubes and stored at 4 °C. To eliminate PCR artifacts due to the presence of nontransfected DNA, 50 μL of the phage suspension was added to 9 mL of LB and 50 μL of saturated SCS110 *E. coli*. The cells were grown an additional 4 h producing ~3 × 10¹¹ phage, thus reducing the amount of nontransfected DNA template to less than 0.1% with respect to the template from the progeny phage. We believe this subsequent infection will produce no change in the progeny population, since the genome was modified with the lesion in a nonessential gene, and reconstruction experiments in which progeny phage containing different mixtures of G and A at the lesion site retained their initial mixture composition after subsequent infection (36). Single-stranded viral template DNA was prepared using 700 μL of phage supernatant (QIAPrep spin M13 kit, Qiagen), from which 15% was used for each PCR reaction. As shown in Figure 5.8, the region that had contained the lesion in each progeny phage sample was PCR amplified to yield a 101 bp product whose purity was assayed by agarose gel electrophoresis. The resulting DNA duplex was cleaved with *BbsI* (a type II restriction endonuclease that cleaves a fixed numbers of bases away from its binding site) at the position that had originally contained the lesion in the template vector, thus affording a 55mer whose newly formed 5’-end contained the lesion site. The 55mer was dephosphorylated with
shrimp alkaline phosphatase, which allowed the lesion site at the 5'-overhang to be radiolabeled using PNK and [γ\(^{-32}\)P]ATP. Incubation with the restriction endonuclease HaeIII yielded a 19mer, allowing for PAGE purification of the radiolabeled fragment of interest. Digestion of the desalted 19mer to 5'-deoxynucleotide monophosphates (5'-dNMPs) was followed by the partitioning of the mixture on a 20 x 20 cm polyethyleneimine TLC plate developed in saturated (NH\(_4\))\(_2\)HPO\(_4\) (adjusted to pH 6.1 with H\(_3\)PO\(_4\)). The separated radiolabeled nucleotides were quantified by PhosphorImager analysis, thus providing the fractional base composition of each nucleotide at the lesion site, from which the mutation frequency and type were determined.
5.4. Results

The mutagenic potential of DNA lesions is described by two key metrics: (1) the ability of DNA polymerases to undergo translesion DNA synthesis (bypass efficiency) and (2) the resulting mutation frequency and type. Since the viral DNA used in this study was single-stranded, translesion DNA synthesis must have occurred in order for progeny phage to form in the *E. coli* cells. A comparison of the number of plaques (slow growing colonies of successfully transfected bacteria on a lawn of infectable cells) formed by viral replication of lesion-bearing versus control genomes provided a measure of translesion bypass by DNA polymerases in the cell. The REAP assay was used to determine the identity of the base in the progeny DNA at the position that had contained the lesion, which gave the mutation frequency and type.

Translesion Bypass Efficiency. Since lesions in DNA may block or inhibit replication, the relative number of plaques formed from the immediate plating of the transformation mixture gives a measure of the efficiency of translesion DNA synthesis. A comparison of the number of plaques obtained after transformation of the damaged phage genome to that from an identically constructed vector that did not carry any oxidative damage allowed calculation of the bypass efficiency for each lesion. Transfections were performed in triplicate, and the bypass efficiency relative to the G control was calculated to within a 95% confidence interval of the mean for each lesion. As shown in Figure 5.7, vector survival for the 8-oxoG control was 87 ± 4% with respect to the G control, which clearly demonstrated that 8-oxoG only weakly inhibited replication. This result agrees, within experimental error, with previously reported values, both in *E. coli* and in mammalian cells (10, 11, 38-41). Synthesis past the secondary oxidative lesion Gh was effective for 75 ± 5% of the vectors, which was nearly the same as for 8-oxoG, and slightly less efficient than the G control. Sp1 and Sp2 were clearly more blocking to DNA polymerase bypass than Gh, with survival percentages of 9 ± 3% and 9 ± 4%, respectively. All of the lesion-containing genomes afforded enough progeny phage for mutation analysis (*vide infra*). As a control demonstrating DNA replication blockage, lesion survival for a synthetic tetrahydrofuran AP site was 4 ± 2%, which is somewhat higher than that reported for the natural aldehydic AP site placed site-
specifically in a nearly identical bacteriophage system (42). In contrast, the bypass efficiency measured for the AP site is an order of magnitude lower than that reported for transfection of a plasmid carrying the AP site in a gapped region into cultured human cells (43), which may be due to ubiquitous expression of Y-family DNA error-prone bypass polymerases in mammalian cells whose homologues are present at negligible concentrations in uninduced E. coli (44). Since the 8-oxoG oxidation products are appreciably bypassed (especially the Gh lesion) by polymerases in E. coli, they are potential contributors to mutagenesis.

*Mutation Frequency and Type.* As shown in Figure 5.8, the mutation frequencies were determined alongside an 8-oxoG control, with radiolabeled 5'-dNMPs as markers for the relative migration of each nucleotide. The mutation frequency of 8-oxoG was modest, providing a 3% G→T transversion. In contrast to the low mutation frequency of 8-oxoG, the mutation frequencies were essentially 100% for Gh, Sp1, and Sp2. The well-bypassed lesion Gh caused 98% G→C and 2% G→T mutations. Interestingly, the same mutation frequencies were observed for the Sp stereoisomers, but the mutation types were distributed differently, with G→C being the predominant mutation for both Sp1 and Sp2 stereoisomers. We observed 72% G→C and 27% G→T transversions for Sp1 and 57% G→C and 41% G→T transversions for Sp2.
5.5. Discussion

8-OxoG is one of the most common oxidative DNA base lesions due to the low oxidation potential of guanine (14). The further oxidation of 8-oxoG after its formation from guanine is reasonable, since the adduct has a lower oxidation potential than the four normal nucleotides. Additionally, base radical cations (the initial products of one-electron oxidation of DNA bases) can migrate over long distances (>55 bp) in duplex DNA to become trapped preferentially at 8-oxoG sites (45-48), resulting in oxidation of the lesion (47). Oxidation of 8-oxoG results in the formation of the hydantoin derivatives described in the present study, as well as a variety of products such as cyanuric acid, oxaluric acid and a 2,5-diaminoimidazalone, which undergoes slow hydrolysis to form oxazalone (49, 50). The hydantoin products may also be obtained directly from guanine by use of four-electron oxidants such as singlet oxygen or type I photochemistry involving superoxide (29, 49), which implicates them as general products of guanine oxidation in DNA.

We investigated the mutagenic potential of three significant products of peroxynitrite oxidized 8-oxoG in DNA: Gh, Sp1, and Sp2. Convenient synthesis of each defined lesion was enabled by reaction of an 8-oxoG-containing oligonucleotide with the oxidant Na2IrCl6 under conditions that favored nearly exclusive formation of either Gh or Sp from 8-oxoG. Each lesion was placed site-specifically into a bacteriophage genome and assayed for bypass efficiency and mutation type following transfection into E. coli. A modest mutation frequency (3%) was observed for 8-oxoG, as expected on the basis of literature values (10, 11, 26, 39, 41). The predominant mutation for this lesion was G→T. It is known that 8-oxoG, placed site-specifically in a ss DNA vector, is subject to repair in E. coli, since the mutation frequency increases approximately 10-fold in cells deficient in the base-excision repair glycosylases MutM (which catalyzes cleavage of a variety of oxidized guanine lesions from duplex DNA) and MutY (predominantly removes A opposite 8-oxoG) (11, 51). Presumably, the repair occurs after the first round of replication, since both MutM and MutY require duplex substrates (11, 52-54). In E. coli, it appears that no mechanism exists to restore the original G-C pair from the secondary oxidation products of 8-oxoG after translesion DNA synthesis, since there
were negligible amounts of G in the position of the bacteriophage that had contained Gh or Sp, as measured by the REAP assay. Additionally, the lesions Gh, Sp1, and Sp2 are well-bypassed by the E. coli DNA polymerase(s). This combination of translesion bypass and miscoding properties apparently contributes to potent mutagenicity of the guanine oxidation products presented in this report. The ~100% mutation frequencies observed may implicate these lesions collectively as a primary source for the frequently observed G\rightarrow T and G\rightarrow C transversions caused by the endogenous oxidation of DNA \((2, 13, 18, 51, 55)\).

It is not known if the sequence context surrounding the lesions influences the mutagenesis of the adducts nor if nontargeted mutations can arise from Gh and Sp. Such a sequence context effect on mutation frequency is observed for 8-oxoG \((56, 57)\). Studies of context effects on Gh and Sp mutagenesis and toxicity would be useful. In this regard, the present work may shed light on the context-dependent mutagenesis induced by methylene blue plus UV light in studies by Loeb and co-workers \((58)\). They observed frequent G\rightarrow C mutations under conditions now known to produce abundant amounts of Sp \((31)\).

Significant frequencies of small deletions or insertions would have been detected by the REAP assay, since the \textit{BbSI-HaeIII} cleavage products would vary detectably in length. The homogeneity of the resulting 19mer, as assayed by PAGE, indicated that point, rather than frame-shift, mutations predominated in the sequence context evaluated (data not shown).

Lesions derived from 8-oxoG have yet to be detected in cells, but there is indirect evidence that their formation in DNA is relevant in cell-based mutagenesis studies. Juedes and Wogan investigated the mutagenicity of peroxynitrite using the \textit{supF} gene of the pSP189 shuttle vector as a mutation target in bacterial and mammalian cells \((18)\). Exposure to ONOO\(^-\) caused the mutation frequency to increase 21-fold in the vector when replicated in \textit{E. coli} MBL50 cells, and 9-fold upon replication in human Ad293 cells compared to untreated vector. In both systems, mutations occurred at G-C base
pairs, predominantly involving G→T transversions (65% when replication was in bacteria and 63% when in human cells). However, G→C transversions were observed but at a lower frequency (28% in MBL50 and 11% in Ad293 cells). In a related study, Termini and co-workers reported that, upon transfection of peroxy radical exposed bacteriophage DNA into *E. coli* (55), approximately 88% of the of the mutant progeny consisted of nearly equal numbers of G→T and G→C transversions. The authors could not detect 8-oxoG by HPLC/electrochemical analysis, suggesting that the G→T transversions were not caused by this base lesion. The work presented here provides at least a partial explanation for the mutation spectrum observed in the *supF* and bacteriophage mutagenesis studies. Other lesions that may be responsible for G→C transversions include the 2-amino-5-[(2'-deoxy-β-D-erythro-pentofuranosyl)amino]-4H-imidazol-4-one derivative (Iz), which is formed from the oxidation of guanine and 8-oxoG (59). Iz is known to pair preferentially with G during primer extension with Pol I and, therefore, may be a contributor to G→C transversions *in vivo* (60).

Single nucleotide primer extension studies carried out using the Klenow fragment (exo⁻) recently showed that both Gh- and Sp-containing DNA templates directed insertion of dAMP and dGMP opposite the lesions (61). Both Gh and Sp showed nearly the same pattern of insertion of nucleotides opposite the lesions, always with an approximately 2:1 preference for dAMP over dGMP. It is noteworthy that, *in vivo*, nearly exclusive dGMP incorporation occurs opposite Gh, while Sp lesions exhibit a mixture of dAMP and dGMP insertion opposite the lesion, but with an opposite preference of dGMP incorporation with respect to the *in vitro* primer extension work. Perhaps there is an unknown repair pathway operating on Gh·A or Sp·A base pairs, since recent studies in the David group have indicated that the adenine opposite the lesions is not a substrate for MutY (34). An alternative explanation is that the replicative DNA polymerase(s) in *E. coli* has (have) different dNTP incorporation preferences opposite Gh and Sp than the Klenow fragment. It would be interesting to determine the molecular basis for the dGMP nucleotide insertion preference for the hydantoin lesions with respect to the many oxidative lesions such as Oa, Ca, and Oz, which prefer dAMP incorporation (nearly exclusively for Oa and Ca) *in vivo* (26).
Guanine oxidation results predominantly in the formation of 8-oxoG, Gh and Sp, as well as a variety of other products. Indeed, oxidation of 8-oxoG has been shown to be a chemically favorable event, and its oxidation products also include Gh and Sp. We report that the hydantoin products Gh, Sp1, and Sp2 are much more mutagenic in vivo than 8-oxoG, thus demonstrating that lesions arising from facile oxidation of dG and 8-oxoG may contribute to the observed G→T and G→C transversions upon replication of oxidized DNA in *E. coli* and mammalian cells.
5.6. Acknowledgment

Paul T. Henderson (MIT) was the lead author of this work. James C. Delaney (MIT), James G. Muller (U. of Utah), Steven R. Tannenbaum (MIT), Cynthia J. Burrows (U. of Utah), and John M. Essigmann (MIT) also contributed. Financial support was provided by grants from the NIH (CA86489, CA80024, CA90689, ES07020, CA26731, CA55861, and ES04705).
**Figure 5.1.** Structures of 8-oxoG, Gh, Sp1, Sp2 and the oligonucleotide sequence used for genome construction.

\[
5'-\text{GCGAAGACCG} - \text{P} - \text{AGCGTCCG}-3' \\
\text{X} = 8\text{-oxoG, Gh, Sp1, and Sp2} \\
\text{19mer insert}
\]
Figure 5.2. HPLC chromatogram for crude (A) vs. purified (B) oligodeoxynucleotide 5’-GCG AAG ACC GXA GCG TCC G-3’ (X = Gh).
Figure 5.3. HPLC chromatogram for crude (A) vs. purified (B) oligodeoxynucleotide 5’-GCG AAG ACC GXA GCG TCC G-3’ (X = Sp1 or Sp2).
Figure 5.4. MALDI-TOF mass spectra for purified (A) vs. exposure to mock genome construction conditions (B) of oligodeoxynucleotide 5’-GCG AAG ACC GXA GCG TCC G-3’ (X = Gh).
Figure 5.5. MALDI-TOF mass spectra for purified (A) vs. exposure to mock genome construction conditions (B) of oligodeoxynucleotide 5’-GCG AAG ACC GXA GCG TCC G-3’ (X = Sp1).

![MALDI-TOF mass spectra](image_url)
Figure 5.6. MALDI-TOF mass spectra for purified (A) vs. exposure to mock genome construction conditions (B) of oligodeoxynucleotide 5’-GCG AAG ACC GXA GCG TCC G-3’ (X = Sp2).
Figure 5.7. (A) Strategy for determination of \textit{in vivo} translesion DNA polymerase bypass of site-specific lesions in a ss bacteriophage DNA (M13mp7L2). A site-specifically adducted ss viral DNA was transfected into the host cell, followed by translesion DNA synthesis to afford a ds replicative form intermediate. Expression of gene products from the ds intermediate enabled the formation of infective progeny phage. Plating of the transformation mixture onto a lawn of generic bacteria immediately after transfection caused formation of plaques, whose number allowed for the calculation of the efficiency of polymerase bypass for each lesion relative to a guanine control. The base composition (N) at the site that had contained the lesion prior to transfection gave the mutation frequency and type. (B) Relative bypass efficiency data for Gh, Sp1, and Sp2. Controls for DNA polymerase bypass efficiency include G and 8-oxoG (well-bypassed) and a synthetic THF AP site (blocking). The error bars represent a 95% confidence interval of the mean based on individual transfections from three independently constructed genomes.
Figure 5.8. (A) Detection of mutations using the REAP assay. (B) TLC showing mutation frequency analysis by the REAP assay for Gh, Sp1, and Sp2. Nucleotide insertion frequencies were determined in triplicate experiments, except for Sp2, which was performed in duplicate. The 8-oxoG lesion was included as a control of known mutation frequency and type, and 5'-dNMP markers were derived from digestion of a 13-mer oligonucleotide that contained a radiolabeled degenerate 5'-end.

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### Table 5.1

Bypass data for G, Gh, Sp1, Sp2, and AP. The number of plaques per plate produced from a cut control (using a nonphosphorylated AP-containing insert that cannot ligate) was subtracted from each measurement to correct for the presence of noninsert-containing circular ss DNA. The assay was performed in triplicate (one construct was transfected into three pools of cells). The G-control was defined as 100% bypass. The square root of the sum of the variances for the lesion (or G control) and the cut control divided by the average number of plaques for the G control was used to calculate the standard deviation.

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Chapter 5 – The Hydantoin Lesions Produce G-to-T and G-to-C Transversions in Vivo

5.7. References


Chapter 5 – The Hydantoin Lesions Produce G-to-T and G-to-C Transversions in Vivo


Chapter 5 – The Hydantoin Lesions Produce G-to-T and G-to-C Transversions in Vivo


lack of involvement of 8-oxo-dG1 and/or abasic site formation. *Biochemistry* **37**, 7030-7038.


CHAPTER 6

Effect of Escherichia coli Polymerases II, IV, and V on the Bypass Efficiencies and Mutational Signatures of Guanine Oxidation Products
6.1. Abstract

The role of the *E. coli* SOS inducible polymerases II, IV, and V in the translesion synthesis of guanine oxidation products *in vivo* was determined. M13 viral genomes containing 7,8,-dihydro-8-oxoguanine (8-oxoG), guanidinohydantoin (Gh), spiroiminodihydantoin (Sp) diastereomers, oxaluric acid (Oa), urea (Ua), and 5-guanidino-4-nitroimidazole (NI) were constructed and replicated in wild-type, pol II deficient, pol IV deficient, pol V deficient, and pol II / pol IV / pol V deficient AB1157 *E. coli*. The bypass efficiency and mutational properties of each lesion was determined in each strain under uninduced and SOS induced conditions. In uninduced wild-type cells, Gh, Sp, Ua, and NI were poorly bypassed. They were well-bypassed, however, in SOS induced wild-type cells. Elimination of pol V completely abrogated the increase in bypass efficiency upon SOS induction demonstrating the importance of this polymerase in alleviating the genotoxicity of these lesions. Removal of pol II and pol IV activity only affected the bypass efficiency of Sp1. With the exception of 8-oxoG, all of the lesions studied were potent sources of mutations in all strains. Pol V had a strong effect on the identity of the bases inserted opposite Sp2, Ua, and NI. Additionally, pol II and pol V were shown to be involved in the translesion replication of NI, and this lesion was the only damage product for which multiple polymerases were involved in translesion synthesis.
6.2. Introduction

Induction of the SOS system in *E. coli* occurs in response to DNA damage and causes the upregulation of at least 43 genes (1). Three of the protein products of these genes have been identified as DNA polymerases and are known as polB (pol II) (2, 3), dinB (pol IV) (4), and umuD2’C (pol V) (5-7). Extensive research on the structure and function of these polymerases and their homologues suggests that these polymerases allow replication to progress in the presence of DNA lesions that are strongly inhibitory to the replicative polymerase, pol III.

Pol II is a B-family DNA polymerase and possesses a 3’→5’ exonuclease activity. Consequently, this enzyme replicates normal DNA with high fidelity and has an error rate less than $10^{-6}$ (8). Pol II participates in error-free replication restart (9, 10) and can also carry out translesion synthesis of lesions such as abasic sites (2, 11), 3,N4-ethenocytosine (12), and N-2-acetylaminofluorene (13). Pol IV and pol V are members of the Y-family of DNA polymerases, which have less sterically restrictive active sites than normal replicative polymerases (14, 15) and lack exonuclease activity (16, 17). The error rates of these polymerases are much higher than the other *E. coli* polymerases and are on the order of $10^{-3}$ to $10^{-4}$ for pol V (18, 19) and $10^{-4}$ to $10^{-5}$ for pol IV (19) *in vitro*. Pol IV does not appear to affect the chromosomal mutation rate but rather operates mostly on extrachromosomal DNA (20) and functions in adaptive mutagenesis by inducing −1 frameshifts (21). Pol IV can also participate in translesion synthesis but does so in a pol V dependent manner (22, 23). Pol V is the major translesion synthesis polymerase (24) and has been shown to bypass lesions such as abasic sites and UV photoproducts of DNA (5, 19, 25) and also is involved in untargeted mutagenesis (18).

In an effort to explore potential mechanisms of carcinogenesis as a result of inflammation, we have examined the role of the SOS-inducible *E. coli* polymerases in the bypass of DNA lesions generated by peroxynitrite (ONOO−) oxidation of DNA. Activation of the immune response causes the secretion of nitric oxide (•NO) and superoxide (O2•−) by macrophages (26) and neutrophils (27) (two types of inflammatory
cells). These radicals combine in a diffusion-limited reaction to form \( \text{ONOO}^- \) (28), a powerful oxidizing and nitrating agent capable of damaging DNA (29). Possessing the lowest redox potential of the four DNA nucleobases \( (E_\text{7} = 1.27 \text{ V vs. NHE}) \) (30), guanine (G) is preferentially oxidized by agents such as \( \text{ONOO}^- \) as compared to the other natural nucleobases (31). A variety of DNA oxidation products results from guanine including guanidinohydantoin (Gh), spiroliminodihydantoin diastereomers (Sp1 and Sp2), cyanuric acid (Ca), oxaluric acid (Oa), urea (Ua), 2-aminoimidazolone (Iz), oxazalone (Z), and 5-guanidino-4-nitroimidazole (NI) (32), and these oxidation products are potently mutagenic \textit{in vivo} as determined by M13 viral-based mutagenesis studies (33-36).

Previous studies in our laboratory showed that several of the \( \text{ONOO}^- \)-derived DNA lesions are substrates for the SOS response system in \textit{E. coli} (35, 36). These findings strongly suggested involvement of the \textit{E. coli} polymerases II, IV, and/or V and prompted us to investigate here the roles of these polymerases in the bypass of these lesions. In the present work, we have examined the effect of the SOS inducible DNA polymerases on the bypass and mutagenicity of Gh, Sp1, Sp2, Oa, Ua, and NI. The results of these experiments will serve to define further the properties of these polymerases while examining in greater detail the mechanisms at the molecular level for how \( \text{ONOO}^- \) causes genotoxicity and mutagenesis.
6.3. Experimental Procedures

**Oligodeoxynucleotides.** DNA synthesis reagents were purchased from Glen Research. Unmodified ODNs were purchased from IDT, Inc. and were purified by polyacrylamide gel electrophoresis (PAGE). The 16mer ODN sequence used was 5'-GAA GAC CTX GGC GTC C-3', where X is G, 8-oxoG, Gh, Sp1, Sp2, Oa, Ua, NI, or a tetrahydrofuran (THF) abasic site. The 16mers containing 8-oxoG or the THF abasic site analog were prepared on an Applied Biosystems 391 DNA synthesizer and purified by PAGE (HPLC and MALDI-TOF data for 8-oxoG are given in Figure 6.2). The 16mers containing 8-oxoG, Gh, Sp1, Sp2, Oa, Ua, and NI used for genome construction were purified by anion exchange HPLC on a Dionex NucleoPac PA-100 (4 × 250 mm) analytical column using 10% CH₃CN in water (solvent A) and aqueous 1.5 M NH₄OAc (solvent B). A flow rate of 1.0 mL/min was used and solvent B was increased from 10% to 50% over 5 min, then 50% to 75% over 25 min. The effluent was monitored at 254 nm. The purified oligonucleotides were desalted twice with NAP-10 (Amersham Biosciences) columns (eluting with H₂O) and lyophilized. ODNs were characterized by MALDI-TOF mass spectrometry on a PerSeptive Biosystems (Framingham, MA) Voyager-DE STR spectrometer. The instrument was operated in the negative ion linear mode with the accelerating voltage set at 25,000 V, the grid voltage set at 95%, and the guide wire set at 0.28%. A delayed extraction time between 110 and 220 ns and a manual laser intensity between 1983 and 2083 were used to collect data. The lesion-containing ODN (15-30 pmol) was mixed with 4 pmol of 5'-GAA GAC TGG GCG TC-3' ([M−H⁺]: 4327.9) and 1.2 pmol 5'-T₁₈-3' ([M−H⁺]: 5412.6). These additional unmodified ODNs serve as internal standards for instrument calibration and have masses that bracket the mass of the lesion-containing ODN. A C18-ZipTip (Millipore) was used for sample preparation to exchange metal cations for triethylammonium cations, and the DNA was eluted with approximately 2 µL of matrix, which was prepared as described (37).

The 16mer containing Gh was prepared as previously described (38) using 5 nmol of 8-oxoG-containing 16mer in a reaction volume of 416 µL and purified by anion exchange HPLC on a Dionex NucleoPac PA-100 (4 × 250 mm) analytical column using 10% CH₃CN in water (solvent A) and aqueous 1.5 M NH₄OAc (solvent B). A flow rate of 1.0 mL/min was used and solvent B was increased from 10% to 50% over 5 min, then 50% to 75% over 25 min. The effluent was monitored at 254 nm. The purified oligonucleotides were desalted twice with NAP-10 (Amersham Biosciences) columns (eluting with H₂O) and lyophilized. ODNs were characterized by MALDI-TOF mass spectrometry on a PerSeptive Biosystems (Framingham, MA) Voyager-DE STR spectrometer. The instrument was operated in the negative ion linear mode with the accelerating voltage set at 25,000 V, the grid voltage set at 95%, and the guide wire set at 0.28%. A delayed extraction time between 110 and 220 ns and a manual laser intensity between 1983 and 2083 were used to collect data. The lesion-containing ODN (15-30 pmol) was mixed with 4 pmol of 5'-GAA GAC TGG GCG TC-3' ([M−H⁺]: 4327.9) and 1.2 pmol 5'-T₁₈-3' ([M−H⁺]: 5412.6). These additional unmodified ODNs serve as internal standards for instrument calibration and have masses that bracket the mass of the lesion-containing ODN. A C18-ZipTip (Millipore) was used for sample preparation to exchange metal cations for triethylammonium cations, and the DNA was eluted with approximately 2 µL of matrix, which was prepared as described (37).
exchange HPLC as described above. \([M-\text{H}^+](\text{calculated}): 4912.2; [M-\text{H}^+](\text{observed}): 4912.5\) (Figure 6.3).

The 16mer containing Sp1 or Sp2 was prepared as previously described (38) using 5 nmol of 8-oxoG-containing 16mer in a reaction volume of 416 µL. Following the reaction, the Sp isomers were purified and separated by anion exchange HPLC as described above. Sp1 \([M-\text{H}^+](\text{calculated}): 4938.2; [M-\text{H}^+](\text{observed}): 4939.0\) Sp2 \([M-\text{H}^+](\text{calculated}): 4938.2; [M-\text{H}^+](\text{observed}): 4939.0\) (Figures 6.4 and 6.5).

The 16mer containing Oa was prepared as previously described (39) by mixing 2.5 nmol of 8-oxoG-containing 16mer with ~130 nmol of ONOO\(^-\) (a gift of Prof. P.C. Dedon, MIT) in 50 µL of buffer (125 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), pH 7.2). Following the addition of ONOO\(^-\), the solution was incubated for 18 h at 37 °C and then purified by anion exchange HPLC as described above. \([M-\text{H}^+](\text{calculated}): 4887.2; [M-\text{H}^+](\text{observed}): 4887.5\) (Figure 6.6).

The 16mer containing Ua was prepared by hydrolysis of the unpurified 16mer containing Oa. Following incubation for 18 h at 37 °C, the 50 µL reaction solution was adjusted to 750 µL and 0.1 M NaOH, incubated for 6.5 hr at 37 °C, and purified by anion exchange HPLC as described above. \([M-\text{H}^+](\text{calculated}): 4815.1; [M-\text{H}^+](\text{observed}): 4815.7\) (Figure 6.7).

The 16mer containing NI was prepared as previously described (40) and purified by anion exchange HPLC as described above. \([M-\text{H}^+](\text{calculated}): 4925.2; [M-\text{H}^+](\text{observed}): 4926.0\) (Figure 6.8).

**Genome Construction.** Genomes were constructed on a 50 pmol scale. Single-stranded M13 DNA (0.5 pmol/µL) was linearized by cleavage with EcoRI (1 U/µL, 23 °C for 8 h) (New England Biolabs) at a hairpin containing a single EcoRI site (41). The linearized M13 DNA was annealed with 1.25 equivalents each of scaffolds 5'-GGT CTT CCA CTG
AAT CAT GGT CAT AGC-3' and 5'-AAA ACG ACG GCC AGT GAA TTG GAC GC-3' (sequences that are partially complementary to the 5’ and 3’ sides of the insert and the genomic DNA termini) by heating the mixture to 50 °C for 5 min and cooling linearly to 0 °C over 50 min (41). Insert ODNs (75 pmol) were phosphorylated on the 5’-terminus with 1 U/µL T4 polynucleotide kinase (U.S. Biochemical) in the supplier’s buffer supplemented with 1 mM ATP and 5 mM DTT (final volume 75 µL, 15 min at room temperature). The phosphorylated ODN (75 pmol in 75 µL) and the annealed scaffold-M13 mixture (50 pmol M13 in 100 µL) were mixed, and the solution was adjusted to 1 mM ATP, 10 mM DTT, 32 µg/mL BSA, and 22.5 U/µL T4 DNA ligase (New England Biolabs) (final volume 190 µL) and incubated for 2 h at 16 °C to recircularize the genome (33, 34, 41). The scaffold DNA was removed using the exonuclease activity of T4 DNA polymerase (New England Biolabs) (42, 43). The ligation solution was adjusted to 9.85 mM MgCl₂, 50 µg/mL BSA, and 0.25 U/µL T4 DNA polymerase (final volume 210 µL) and incubated for 1 h at 16 °C. The genome constructs were reduced to ~115 µL in vacuo, extracted with 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen), and desalted with Sephadex G50 fine resin (Amersham Biosciences). The desalting procedure also removes residual organics.

**Normalization of the Genomes.** The amount of circular, 16mer insert-containing genome in each sample was quantified by PAGE and phosphorimaging by the procedure detailed below. The desalted, insert-containing genomes were diluted to ~100 µL and the concentration of genome in each sample was estimated by spectrophotometry using the conversion factor 14 pmol/mL = 1 A₂₆₀ unit. Approximately 0.5 pmol of genome (5 µL) was mixed with 3.5 pmol each of the scaffolds used for genome construction and incubated with 1.2 U/µL HinF1 (New England Biolabs) and 0.12 U/µL shrimp alkaline phosphatase (Roche) in 1X Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9) (New England Biolabs) for 1 h at 37 °C, followed by 5 min at 80 °C and cooling to 20 °C at 0.2 °C/s (final volume 8.12 µL). This reaction linearizes the genome 5’ of the insert and exposes an M13 5’-terminus for radiolabeling. The genome/scaffold mixture was supplemented with 5.6 mM DTT, 14 µM ATP, 0.16 µM γ-
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$^{32}$P-ATP (6,000 Ci/mmol, PerkinElmer), 0.47 U/µL T4 polynucleotide kinase, and 0.94 U/µL HaeIII (New England Biolabs) (final volume 10.7 µL) and incubated for 2 h at 37 °C. This reaction releases a radiolabeled 34mer fragment from the M13 genomes constructed with a 16mer insert and a 37mer fragment from the M13 genome constructed with a 19mer insert (referred to as the “+3 control”). The reactions were quenched by the addition of 10 µL of 2X formamide/EDTA gel loading buffer, and 20 µL of each solution was analyzed by 20% denaturing PAGE. Dilutions corresponding to 5, 10, 20, 30, 40, and 50% of the +3 control were also loaded in order to assess how much of the +3 control to add as an internal standard to the normalized genomes (see Translesion Bypass Efficiency). The gel was run at 550 V for 2 hr and visualized by phosphorimagery. The bands corresponding to the 34mer fragments were quantified and the solutions normalized such that each contained identical amounts of the 34mer fragment. An $R^2$ value of 0.99 was achieved for the dilutions of +3 control (Figure 6.9).

Cell Strains. The cell strains used in this study were gifts of D.F. Jarosz and G.C. Walker (MIT) (Table 6.1). All strains were characterized by sequencing of PCR products of the gene regions of interest. E. coli genomes were isolated from 1 mL of an overnight LB culture grown in the presence of 25 µg/mL chloramphenicol (pol IV$^+$ and GW8017 strains), 70 µg/mL spectinomycin (pol II$^+$ strain), or both chloramphenicol and spectinomycin (triple knockout strain). Cells were pelleted by centrifugation at 13,200 RPM for 3 min and resuspended in 570 µL TE buffer, pH 8.0. To this solution was added 30 µL of 10% SDS and 3 µL of proteinase K (Roche), and the mixture was incubated for 1 h at 37 °C. Following the incubation, 100 µL of 5 M NaCl and 80 µL of 10% CTAC/0.7 M NaCl were added and the solution was mixed gently. After incubation at 65 °C for 10 min, the solution was extracted twice with 750 µL phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen). Approximately 700 µL of aqueous solution remained after extraction. Isopropanol was added (420 µL), and the solution was mixed thoroughly and then centrifuged for 5 min at 13,200 RPM. The supernatant was removed and 70% ethanol (250 µL) was added. The solution was centrifuged for 5 min at 13,200 RPM and the supernatant removed. The DNA pellet was
dried in vacuo for 10 min and resuspended in 100 µL TE, pH 8.0. PCR amplification of the E. coli genomic DNA was carried out using Taq DNA polymerase (Invitrogen). The 50 µL PCR mix for wild-type polB, dinB, and umuDC gene regions and ΔdinB and ΔumuDC gene regions included 25 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.5 mM MgCl₂, 2% DMSO, 0.5 µM each primer, 0.05 mM each dNTP, 0.05 unit/µL Taq, and 2 µg of genomic DNA. The amplification cycle consisted of denaturation at 94 °C for 0.5 min, annealing at 56 °C for 0.5 min, and extension at 72 °C for 2 min. After 30 cycles, samples were incubated at 72 °C for 10 min and stored at 4 °C until further use. The 50 µL PCR mix for the polBΔ1 gene region included 25 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 2% DMSO, 0.5 µM each primer, 0.05 mM each dNTP, 0.05 unit/µL Taq, and 0.5 µg of genomic DNA. The amplification cycle consisted of denaturation at 94 °C for 0.5 min, annealing at 56 °C for 0.5 min, and extension at 72 °C for 3.5 min. After 30 cycles, samples were incubated at 72 °C for 10 min and stored at 4 °C until further use. The primers used amplification of the polB and polBΔ1 regions were 5’-CTG GAA CGA AGT GTA TTA CGG GTT TCG-3’ and 5’-TAT TAT GGT ACT GGA TGG CAA AGC ATT CG-3’. The primers used amplification of the dinB region were 5’-CAT GGG GAT AAA GTG GTG CAG C-3’ and 5’-CTG GCA CTT AAG AGA TAT CCT GCG G-3’. The primers used amplification of the umuDC region were 5’-CCA CGT GAG CC AAG ATA AGA GAA CG-3’ and 5’-TAC CTG ATT GTC GCA GTG CTG G-3’. The PCR products were analyzed by 1% agarose gel electrophoresis in 1X TBE buffer. To 5 µL of each PCR product solution was added 5 µL of H₂O and 2 µL of 6X Ficoll loading buffer (25% Ficoll), and 10 µL were loaded into each well. The gel was run for 130 V for 1.40 h and stained with 1X SYBR Gold (Molecular Probes) in 1X TBE for 1 h. The PCR products were prepared for DNA sequencing using a Qiagen PCR Purification Kit, eluting with a 1:10 dilution of buffer EB (final concentration 1 mM Tris-HCl, pH 8.5). For strains containing wild-type polB, 5’-TAT CGA TGC AGC GCA AAT GCC-3’, 5’-CGA TCA TCC GCA CCT TTC TGA TTG-3’, 5’-CAA TCA GAA AGG TGC GGA TGA TCG-3’, and 5’-TAT GGT ACT GGA TGG CAA AGC ATT CG-3’ were used as primers for DNA sequencing. For strains containing polBΔ1, 5’-CTG GAA CGA AGT GTA TTA CGG GTT TCG-3’ was used in place of 5’-TAT CGA TGC AGC GCA AAT GCC-3’. For strains containing wild-type dinB and ΔdinB, the
same primers used for PCR were used for DNA sequencing. For strains containing wild-type \textit{umuDC} and \textit{∆umuDC}, 5'-GAA AGT TGG AAC CTC TTA CGT GCC-3' and the same primers used for PCR were used for DNA sequencing.

\textit{Preparation of Electrocompetent Cells.} LB media (4 × 150 mL) was inoculated with 1.5 mL each from separate overnight cultures of \textit{E. coli}. The culture was grown on a shaker at 37 °C to an A\textsubscript{600} of approximately 0.4. The overnight cultures were grown in the presence of antibiotics (25 µg/mL chloramphenicol and/or 70 µg/mL spectinomycin, see \textit{Cell Strains}). Each culture was centrifuged, resuspended in 25 mL of 10 mM MgSO\textsubscript{4}, and transferred to a large (150 × 15 mm) Petri dish. The SOS system was induced in two of the cultures by irradiating with 254 nm light (45 J/m\textsuperscript{2} of energy), immediately transferring the cells to 2 × 125 mL of 2×YT media, and growing for 40 min at 37 °C with shaking. Uninduced cells were treated identically, except without exposure to UV light. Each set of 2×YT cultures was centrifuged, combined, and washed with 2 × 175 mL of deionized water. Each set of electrocompetent cells was resuspended in 4 mL of a 10% solution of glycerol in water, stored at 4 °C and used the following day.

\textit{Production and Isolation of Phage Populations.} The normalized genomes were split into three portions (for triplicate analysis) and +3 control was mixed with each genome solution to serve as an internal standard for the bypass assay. An aliquot of genome/internal standard solution was added to 100 µL of electrocompetent cells. The cell-genome mixtures were electroporated (2.5 kV, 129 ohms in a 0.2-cm cuvette) and transferred to 10 mL of LB, generating at least 10\textsuperscript{3} independent, transformed cells ("infective centers") as determined by plating of an aliquot onto agar plates immediately following transformation. The remaining balance of the cultures was incubated at room temperature for at least 30 min, and then incubated on a roller drum for 4 h at 37 °C to amplify the progeny phage. The cells were spun down and the progeny phage-containing supernatant retained. In this system, successful replication of the genomes by the \textit{E. coli} host leads to the production of progeny phage. Amplified progeny phage (100 µL) and
10 µL of an overnight growth of SCS110 *E. coli* were added to 10 mL of LB media and grown for 6 h at 37 °C on a roller drum. The cultures were centrifuged and the phage-containing supernatant retained. Single-stranded phage DNA was isolated from 700 µL of each sample using a QIAprep Spin M13 kit (Qiagen) and used for the determination of translesion bypass efficiency and mutation frequency.

**PCR Amplification.** PCR amplification of the isolated M13 DNA was carried out using Turbo Pfu polymerase (Stratagene). The 25 µL PCR mix included 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 0.1% Triton X-100, 100 µg/mL BSA, 1.0 µM each primer, 0.2 mM each dNTP, 0.05 unit/µL Pfu, and 10 µL of Qiagen isolated M13 DNA. The amplification cycle consisted of denaturation at 94°C for 0.5 min, annealing at 67°C for 1 min, and extension at 72°C for 1 min. After 30 cycles, samples were incubated at 72°C for 5 min and stored at 4°C until further use. The primers used to generate the 84 bp (from 16mer-insert-containing genomes) or 87 bp (from the internal standard 19mer-insert-containing genome) PCR products for determination of translesion bypass efficiency were 5’-Y CAG CTA TGA CCA TGA TTC AGT GGA AGA C-3’ and 5’-Y CAG GGT TTT CCC AGT CAC GAC GTT GTA A-3’ [Y is an aminoethoxyethyl ether group from a “5’-amino-modifier 5” phosphoramidite (Glen Research, Sterling, VA) or an aminohexyl group from a “5’-amino-modifier C6” phosphoramidite (IDT, Inc.), which prevent ³²P-labeling of the 5’-termini]. The primers used to generate the 61 bp PCR products for determination of mutation frequency were 5’-Y CAG CTA TGA CCA TGA TTC AGT GGA AGA C-3’ and 5’-Y TGT AAA ACG ACG GCC AGT GAA TTG GAC G-3’. Following PCR, each reaction was diluted to 115 µl with H₂O, extracted with 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1), and desalted with Sephadex G50 fine resin.

**Restriction Endonuclease Digestion.** An aliquot of the PCR product (10 µL) was incubated in 20 µL of final volume containing 1X Buffer 2, 5 U BbsI (New England Biolabs), and 1 U of shrimp alkaline phosphatase (Roche) for 4 h at 37 °C, followed by 5 min at 80 °C and cooling to 20°C at 0.2°C/s. The cut, dephosphorylated PCR product (4
µL) was incubated in a 6 µL final volume containing 1X Buffer 2, 10 mM DTT, 3.33 µM cold ATP, 0.278 µM γ-32P-ATP (6,000 Ci/mmol, PerkinElmer), and 5 U of T4 polynucleotide kinase for 15 min at 37°C, followed by 20 min at 65°C and cooling to 23°C at 0.1°C/s. To the radiolabeled PCR product was added 4 µL of 1X Buffer 2 containing 10 units of HaeIII. The resultant solution was incubated for 2 h at 37 °C, and the reaction was quenched by the addition of 10 µL of 2X formamide/EDTA loading buffer.

Translesion Bypass Efficiency. The samples (20 µL) were analyzed by 20% denaturing PAGE (550 V for 3-3.5 h) and visualized by phosphorimagery. Two bands of interest appear on the gel. The faster migrating band results from the genome that contains a 16mer insert, and the slower migrating band results from the internal standard genome that contains a 19mer insert. The translesion bypass efficiency was calculated as described (36, 44).

Mutation Type and Frequency. The REAP assay was used to determine the identity of the base at the site formerly occupied by the lesion (33, 34, 41, 45).
6.4 Results

6.4.1. Translesion bypass efficiency

Given the number of samples to be analyzed in this study, an improved version of the bypass assay described in Chapter 3 was used (44). Genomes were constructed as described by inserting a 16mer G- or lesion-containing oligonucleotide into M13mp7L2 single-stranded DNA. In the improved assay, restriction endonucleases and polynucleotide kinase were employed to excise and radiolabel a 34mer fragment from the genome construct. Because the 34mer includes the 16mer insert, quantification of the fragments by PAGE and phosphorimagery allows by deduction the amount of circular, 16mer insert-containing genome to be determined. A genome containing a 19mer insert was also constructed for use as an internal standard in the bypass assay. When subjected to the same quantification procedure, a 37mer oligonucleotide results and quantification of this band allows specification of the proper ratio of 16mer insert-containing genome and internal standard. To this end, the quantified 16mer insert-containing genomes were normalized to each other and mixed with the internal standard to produce an approximate ratio of 75% 16mer insert-containing genome to 25% 19mer insert-containing genome. Passage of these mixtures through *E. coli* causes a change in the ratio that reflects the efficiency of bypass of a DNA lesion since genomes containing poorly bypassed lesions exhibit poor survivability and genomes containing well-bypassed lesions exhibit high survivability relative to the internal standard genome. The harvested progeny phage are PCR amplified, digested and radiolabeled, and the resultant DNA fragments analyzed by PAGE and phosphorimagery. An 18mer oligonucleotide results from the 16mer insert-containing genome, whereas a 21mer oligonucleotide results from the internal standard. The values of the quantified bands were translated into percent bypass efficiency as described (44). The bypass efficiency of a lesion is scaled relative to guanine, which is defined as having a bypass efficiency of 100%. A limitation of this assay is that +3 frameshifts overlap with the signal from the internal standard. All increases and decreases noted below are statistically significant.

The bypass efficiencies of 8-oxoG, Oa, Gh, Sp1, Sp2, Ua, and NI were determined in wild-type, pol II deficient, pol IV deficient, pol V deficient, and pol II / pol
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IV / pol V deficient *E. coli* strains under normal and SOS-induced conditions for a total of ten cellular experimental systems. The bypass efficiencies of 8-oxoG and Oa were identical within experimental error to G (100%) and did not differ among the ten cellular systems. The remaining bypass results therefore will focus exclusively on the remaining lesions. In the wild-type strain, these lesions exhibited poor bypass efficiencies and were strong substrates for the SOS system (Figure 6.10). The Sp diastereomers showed a significant difference in bypass efficiency under both normal and SOS-induced conditions, with the Sp1 diastereomer bypassed more efficiently. As a control demonstrating a blocking lesion and showing induction of the SOS system, the bypass efficiency of a tetrahydrofuran (THF) synthetic abasic site was determined and found to increase from 1.0 ± 0.4% to 19 ± 9%, or about 20 fold. In contrast, the bypass efficiency increased about 3 fold from 19 ± 4% to 65 ± 8% for Gh, increased about 4 fold from 16 ± 2% to 69 ± 5% for Sp1, increased about 6 fold from 8.2 ± 2.3 to 50 ± 8 for Sp2, increased about 6 fold from 5.2 ± 1.7% to 34 ± 10% for Ua, and increased about 8.5 fold from 4.5 ± 1.8% to 38 ± 7% for NI.

Deletion of pol II had no significant effect on the bypass efficiencies of Gh, Sp2, Ua, NI, and THF, but caused a reduction in the bypass efficiency of Sp1 from 16 ± 2% to 8.2 ± 2.3% (Figure 6.11). No significant differences were observed upon SOS induction as compared to SOS induced wild-type cells. In comparison to results in wild-type cells, deletion of pol IV produced a significant effect only for Sp1, decreasing the bypass efficiency from 16 ± 2% to 10 ± 1% and 69 ± 5% to 60 ± 1% in uninduced and SOS-induced cells, respectively (Figure 6.12).

Deletion of pol V produced dramatic changes in the bypass efficiencies (Figure 6.13). With the exception of 8-oxoG and Oa, which exhibited no differences upon inactivation of pol V, the bypass efficiencies were lower in uninduced pol V deficient cells as compared to uninduced wild-type cells and essentially did not increase for any of the lesions upon induction of the SOS system. In the pol V deficient strain, Gh was bypassed with efficiencies of 8.7 ± 3.0% and 9.7 ± 4.0% in uninduced and SOS-induced pol V deficient cells, respectively. The bypass efficiency of Sp1 decreased to 0.44 ±
0.16% and 0.39 ± 0.16% in uninduced and SOS-induced cells, respectively, whereas essentially no bypass was observed for Sp2. In uninduced cells, the bypass efficiency of Ua decreased to 2.3 ± 0.6% and in SOS induced cells decreased to 1.9 ± 0.2%. The bypass efficiency of NI decreased to 1.7 ± 0.5% and 2.7 ± 0.4% in normal and SOS-induced cells, respectively. Results from the pol II / pol IV / pol V deficient strain mirrored those from the pol V deficient strain (Figure 6.14).

6.4.2. Mutation type and frequency

The mutational signature of each lesion in WT AB1157 E. coli was determined using the restriction endonuclease and post-labeling analysis of mutation frequency (REAP) assay (46). As for the bypass assay results, all increases and decreases noted below are statistically significant.

Frameshift mutations. In wild type cells, both uninduced and SOS-induced, frameshift mutations were negligible (less than 1%). Frameshifts (−1) were observed in the pol V deficient strain and the triple knockout strain for Sp1 and Sp2 at levels of about 2% and 7.5%, respectively, in uninduced cells. Sp1 also generated roughly 1.5% −2 frameshifts, whereas Sp2 produced about 6%. When these strains were SOS induced, Sp1 generated about 15% and 4% −1 frameshifts in the pol V deficient and triple knockout strains, respectively. Sp2 produced about 10% and 12% −1 frameshifts in the SOS induced pol V deficient and triple knockout strains, respectively. In uninduced cells, NI produced about 2% −1 frameshifts in the pol II and pol IV deficient strains, about 7% in the pol V deficient strain, and about 10% in the triple knockout strain. A low amount (~1%) of −2 frameshifts were also observed in the pol V deficient strain. When the SOS system was induced, NI caused about 6% −1 frameshifts in the pol V deficient strain and about 16% in the triple knockout strain.

Point mutations in wild-type cells (Figure 6.15). In uninduced cells, replication of 8-oxoG was 1.72% mutagenic, and the mutations were primarily G→T. Gh, Sp1, Sp2, Oa, and Ua were all about 99% mutagenic, but the mutational signatures differed greatly among this group of lesions. Gh induced 37% G→T and 59% G→C mutations, whereas
Sp1 caused 83% G\(\rightarrow\)T and 15% G\(\rightarrow\)C mutations and Sp2 caused 43% G\(\rightarrow\)T and 54% G\(\rightarrow\)C mutations. Oa generated 99% G\(\rightarrow\)T mutations, and Ua produced 9% G\(\rightarrow\)A, 55% G\(\rightarrow\)T, and 35% G\(\rightarrow\)C mutations. NI was 64% mutagenic and induced 16% G\(\rightarrow\)A, 30% G\(\rightarrow\)T, and 18% G\(\rightarrow\)C mutations. In SOS-induced cells, a small increase in G\(\rightarrow\)A mutations was observed for Gh (1.9% to 3.6%). No difference in mutational signature was observed for Sp1, but G\(\rightarrow\)T mutations induced by Sp2 increased to 58% and G\(\rightarrow\)C mutations decreased to 39%. The mutational signature of Oa changed slightly and the lesion induced 1.0% G\(\rightarrow\)A, 97% G\(\rightarrow\)T, and 1.7% G\(\rightarrow\)C mutations. The mutation frequency and signature of Ua was essentially unchanged. The mutation frequency of NI decreased to 48% and the mutational signature shifted to fewer G\(\rightarrow\)A mutations (9.0%) and G\(\rightarrow\)C mutations (4.7%).

**Point mutagenesis in polymerase deficient cells.** Figure 6.16 shows the mutation frequency of 8-oxoG as a function of cell type under both uninduced and SOS-induced conditions. The mutation frequency remains constant within experimental error in uninduced cells. Comparison of the mutation frequencies in SOS induced cells reveals a small decrease in the amount G\(\rightarrow\)T mutations in pol V deficient and triple knockout strains as compared to wild-type.

The results for Gh are shown in Figure 6.17. In uninduced cells, statistically significant differences were only observed for G\(\rightarrow\)A mutations, which decreased from 1-2% in wild-type, pol II deficient, and pol IV deficient cells to about 0.1% in pol V deficient and triple knockout cells. Induction of the SOS system caused a decrease in G\(\rightarrow\)C mutations from 62% to 52% in pol II deficient cells. G\(\rightarrow\)A mutations increased to 3-5% in pol II and pol IV deficient cells upon SOS induction. In the pol V deficient strain, SOS induction decreased the G\(\rightarrow\)A mutations to 0.13%. Relative to SOS induced wild-type cells, G\(\rightarrow\)T mutations decreased to 35%, whereas G\(\rightarrow\)C mutations increased to 64% in SOS induced pol V deficient cells.

The general mutational signature for Sp1 did not differ among the pol V proficient strains; however, small differences were noted for the specific mutations
observed (Figure 6.18). In the pol II deficient strain, SOS induction caused an increase from 79% to 87% in G\(\rightarrow\)T mutations and a decrease from 19% to 11% in G\(\rightarrow\)C mutations. Induction of the SOS system in the pol IV deficient strain increased G\(\rightarrow\)T mutations from 80% to 85% and decreased G\(\rightarrow\)C mutations from 18% to 13%. The overall mutation frequency decreased from 99% in uninduced and SOS induced pol V proficient cells to 93% in uninduced and 94% in SOS induced pol V deficient cells. G\(\rightarrow\)T mutations increased from 79-83% in uninduced and 85-87% in SOS induced pol V proficient cells to 88% in uninduced and 91% in SOS induced pol V deficient cells. Fewer G\(\rightarrow\)C mutations resulted and were 5% in uninduced and 3% in SOS induced pol V deficient cells versus 15-19% in uninduced and 11-13% in pol V proficient cells.

Comparison of the results for wild-type and pol II deficient strains indicate pol II is not involved in Sp2 induced mutagenesis (Figure 6.19). Elimination of pol IV does not affect the mutational signature of Sp2 uninduced cells. In SOS-induced cells, elimination of this polymerase reduces the amount of G\(\rightarrow\)T mutations from 57% to 49% and the amount of G\(\rightarrow\)C mutations from 38% to 48% as compared to SOS induced pol II deficient cells. These differences may also apply in comparison to SOS induced wild-type cells, but statistical error precludes this determination. Dramatic differences in mutational signature were observed upon elimination of pol V. In both uninduced and SOS induced cells, the overall mutation frequency decreased from 99% to 86%. G\(\rightarrow\)T mutations increased from 43-46% to 79% and from 49-58% to 74% in uninduced and SOS induced cells, respectively. G\(\rightarrow\)C mutations decreased from 51-54% to 6% in uninduced cells and 38-48% to 10% in SOS induced cells.

The mutation frequency of Oa remained constant at greater than 99% across all strains and under both uninduced and SOS induced conditions (Figure 6.20). By far, the predominant mutation induced was G\(\rightarrow\)T. In SOS induced pol II and pol IV deficient strains, G\(\rightarrow\)C mutations were higher than in uninduced wild-type cells, and removal of pol V lowered the G\(\rightarrow\)C mutations from 1.6-2.6% to less than 0.5% in SOS induced cells.
The mutational signature of Ua was similar in wild-type, pol II deficient, and pol IV deficient strains and was drastically different in the pol V deficient strain under both uninduced and SOS induced conditions (Figure 6.21). G→T mutations were the major mutations in the pol V deficient strains. These mutations increased from 55-69% to 93% in uninduced cells and from 47-53% to 94% in SOS induced cells. G→C transversions accounted for the remaining mutations.

Arguably the most interesting results came from NI (Figure 6.22). In uninduced cells, the overall mutation frequency of the lesion increased from 64% to 81% upon removal of pol II, but decreased to 57% in the pol IV deficient strain, 40% in the pol V deficient strain, and 51% in the triple knockout strain. In SOS induced cells, the mutation frequency increased to 82% from 48% when pol II was removed, increased slightly to 49% when pol IV was removed, decreased to 21% when pol V was removed and decreased slightly to 45% when all three SOS inducible polymerases were removed. These results suggest the involvement of both pol II and pol V in the bypass of NI, and the differences in mutational signature among the cell strains tested support this conclusion. Removal of pol II did not affect the amount of G→A and G→C mutations induced but caused an increase in the amount of G→T mutations to 45% and 62% in uninduced and SOS induced cells, respectively, with a concomitant increase in the overall point mutation frequency. This result suggests pol II preferentially inserts a C opposite the lesion. Removal of pol IV did not affect the mutational signature of the lesion, whereas removal of pol V caused a large decrease in the amount of G→T mutations with a corresponding decrease in mutation frequency. In uninduced cells, the amount of G→T mutations decreased from 30% to 7% and in SOS induced cells decreased from 35% to 4% relative to wild-type cells. This result shows involvement of pol V in generating G→T mutations from NI. Results from the triple knockout strain resemble those from the pol V single knockout strain.
6.5. Discussion

All of the lesions studied in this work were previously examined in wild type *E. coli* cells in the sequence context GXA, where X is the site of the lesion (34-36). Results from the present TXG sequence context differ dramatically in some cases. For example, in the GXA context Gh is bypassed with 75% efficiency and induces 98% G→C mutations. In the context TXG, bypass of Gh occurred with only 19% efficiency, and the mutational signature shifted to 59% G→C and 37% G→T. In the case of Sp1, the bypass efficiency in the TXG context was 16% versus 9% for the GXA context, and the mutational signature changed from 72% G→C and 27% G→T to 15% G→C and 83% G→T. The bypass efficiency and mutational signature of Sp2 did not differ between the two sequence contexts. The bypass properties of the hydantoin lesions, Gh and Sp, have shown sequence context dependence previously. Burrows and coworkers found that the bypass of Gh and Sp by Klenow fragment occurs more readily in the sequence context XGG as compared to XAA and proposed the differences were due either to primer misalignment or to stabilization of the primer/template complex by the GG pair 3’ of the lesion (38). In the first case, simple primer misalignment should yield frameshift mutations in our system; however, these mutations are not observed upon replication in wild-type cells. A complex primer misalignment in which a bulge in the template forms initially to allow synthesis beyond the lesion followed by slippage of the template to remove the bulge could account for the large increase in G→T mutations observed by us for Gh and Sp1 in the TXG context without concomitant frameshift mutations. As for the argument about the sequence 3’ of the template, the present sequence is TXGG, whereas the sequence we used previously was GXAG. According to the hypothesis put forth by Burrows and coworkers, the TXGG sequence should produce a more stable duplex structure prior to translesion synthesis as a result of the more stable GG pair and facilitate lesion bypass. Whereas the bypass results for Sp1 are as predicted by this hypothesis, the results for Gh disagree. Significant differences in mutational signature were also observed for Ua. In the GXA context, Ua induces 99% G→T mutations in uninduced cells; however, in the present study, Ua induced 9% G→A, 55% G→T and 35% G→C mutations. The mutational signature of NI also changed although the overall mutation pattern remained consistent. Clearly, the sequence context effect is complicated, and
further experiments are needed to determine the reason for the large changes observed for the bypass and coding properties of these DNA lesions.

Comparison of the data in Figures 6.10 and 6.13 shows that pol V was responsible for the vast majority of SOS dependent translesion synthesis for the oxidation products in this study. Pol II and pol IV appeared to have a small role in the bypass efficiency of Sp1. By contrast, pol V was essential for bypass. The small effect of pol IV existed in both uninduced and SOS-induced cells, whereas the reduction in bypass efficiency as a result of pol II deficiency was rescued in SOS-induced cells. In its simplest form, the bypass of a DNA lesion consists of two parts, the first part is insertion of a nucleotide opposite the lesion and the second part is extension of the nascent strand (47). One possible explanation for the Sp1 bypass observations is that pol V is required for the extension step and pol II and/or pol IV participate in, but are not required for, the insertion step. A second explanation is that pol II and/or pol IV may participate in the extension step with pol V being required for the insertion step. As a third possibility, pol II and pol IV may increase the rate of translesion synthesis without the use of their inherent polymerase activity, for instance by interacting with the replication complex or with pol V.

The bypass results from wild type, pol V deficient, and triple knockout cells demonstrated that pol I and/or pol III can bypass Gh, Ua, and NI to a small extent and Sp1 and Sp2 to a negligible (but still measurable in terms of determining mutation frequency) extent. Experiments with Klenow fragment suggest that the extension step is rate limiting for Gh (38, 48, 49), Ua (50), and NI (51). Thus, it is possible that the role of pol V in the bypass of these lesions is more for extension rather than insertion. However, pol V undoubtedly has a significant role in the insertion step for Ua and NI based on the large differences in mutational signature that occurred with these lesions upon inactivation of pol V.

NI was the only lesion for which involvement of more than one SOS inducible polymerase could be concluded from the mutagenesis data. Inactivation of pol II caused
a significant increase in the amount of A inserted opposite the lesion and a significant
decrease in the amount of C incorporated opposite the lesion, while causing no decrease
in the bypass efficiency. This result suggests pol II is involved principally during the
insertion step of translesion synthesis and preferably inserts C opposite the lesion, which
leads to no mutation. Inactivation of pol V causes a large increase in the amount of C
inserted opposite the lesion and a significant decrease in the amount of A inserted
opposite the lesion. This result demonstrates that pol V preferentially inserts A opposite
the lesion leading to G→T mutations. Since the wild type mutational signature consists
of roughly equivalent amounts of no mutation and G→T mutations, pol II and pol V
appear to have comparable rates of insertion opposite NI. This result coupled with the
strong dependence of NI bypass efficiency on pol V suggests pol V is responsible for
extension of the primer after incorporation of a nucleotide opposite the lesion by pol II or
pol V. The involvement of two polymerases for the bypass of a lesion has been
demonstrated previously in the case of benzo[a]pyrene and N-2-acetylaminofluorene (22,
52, 53).

The Sp diastereomers exhibited markedly different mutational signatures and
responded differently to the knockout of pol V. Sp1 induced more than 85% G→T and
about 10% G→C mutations in SOS induced pol V proficient strains. The elimination of
pol V activity caused a small decrease in the amount of G→C mutations and a
corresponding increase in the amount of G→T mutations. This result contrasts with that
from Sp2 where the inactivation of pol V caused the mutational signature to shift from
similar amounts of G→T and G→C mutations to primarily G→T mutations. This result
indicates that in the case of Sp2, pol V induces principally G→C mutations. As with the
other lesions studied, it is not possible to conclude from these data in the case of Sp1 if
pol V is more important for extension or insertion. For Sp2, pol V has a significant role
in insertion; however, the significance of this polymerase for extension after the insertion
step is unclear. The stereochemical difference between Sp1 and Sp2 is the logical
explanation for the observed differences in bypass and mutational signature. Jia et al.
performed a computational study of duplexes containing the Sp diastereomers (54). The
authors note that the orientation in the duplex of one isomer is opposite that of the other
isomer and that the duplex containing the $R$ isomer is always lower in energy than the duplex containing the $S$ isomer. Furthermore, the A ring of both isomers mimics the hydrogen bonding face of thymine, whereas the $R$ isomer forms a more stable base pair with $G$ than the $S$ isomer. While these results do not allow prediction of bypass efficiencies or specific coding properties, they do set a precedent for differences based on stereochemistry.

In this study, we have examined the role of the E. coli SOS inducible polymerases in the bypass and mutagenicity of selected $G$ oxidation products. Our results demonstrate a clear requirement of the $umuDC$ gene product for the bypass of Sp, Ua, and NI, and strong involvement of this gene product in the bypass of Gh. These results expand the substrate repertoire of pol V to include ring opened $G$ oxidation products and provide mechanistic details for how these oxidation products may cause mutations and ultimately cancer in inflamed tissues.
6.6. Acknowledgment

Sarah Delaney (MIT), Yuriy O. Alekseyev (MIT), James C. Delaney (MIT), and John M. Essigmann (MIT) contributed to this work. We thank Daniel Jarosz and Graham Walker (MIT) for providing the cell strains used in this work. We also thank Peter T. Rye for assistance with cell strain characterization. We acknowledge the MIT Biological Engineering Division Mass Spectrometry Laboratory and the MIT Biopolymers Laboratory for their services. Financial support was provided by NIH awards CA26731 and CA080024 and by NSF awards DBI-9729592 and CHE-9808061.
Figure 6.1. Products of ONOO$^-$ oxidation and nitration of G.

![Diagram of oxidation and nitration products](image-url)
Figure 6.2. (Top) HPLC trace of purified 8-oxoG oligonucleotide. (Bottom) MALDI-TOF spectrum of purified 8-oxoG oligonucleotide.
Figure 6.3. (Top) HPLC trace of purified Gh oligonucleotide. (Bottom) MALDI-TOF spectrum of purified Gh oligonucleotide.
Figure 6.4. (Top) HPLC trace of purified Sp1 oligonucleotide. (Bottom) MALDI-TOF spectrum of purified Sp1 oligonucleotide.
Figure 6.5. (Top) HPLC trace of purified Sp2 oligonucleotide. (Bottom) MALDI-TOF spectrum of purified Sp2 oligonucleotide.
Figure 6.6. (Top) HPLC trace of purified Oa oligonucleotide. (Bottom) MALDI-TOF spectrum of purified Oa oligonucleotide.
**Figure 6.7.** (Top) HPLC trace of purified Ua oligonucleotide. (Bottom) MALDI-TOF spectrum of purified Ua oligonucleotide.
Figure 6.8. (Top) HPLC trace of purified NI oligonucleotide. (Bottom) MALDI-TOF spectrum of purified NI oligonucleotide.
Chapter 6 – Effects of Bypass Polymerases on Guanine Oxidation Products

Figure 6.9. Genome quantification gel and standard curve (see Experimental Procedures for details). (Left) PAGE of digested and radiolabeled competitor and genome constructs (lanes 1-11). G (lane 1), 8-oxoG (lane 2), Gh (lane 3), Sp1 (lane 4), Sp2 (lane 5), Oa (lane 6), Ua (lane 7), NI (lane 8), THF (lane 9). The unlabeled lanes are not relevant to the present work. (Right) Plot of quantified competitor lanes, $R^2 = 0.99$. 

% Competitor: 5 10 20 30 40 50
Figure 6.10. Bypass efficiency of DNA lesions relative to G in wild-type cells.
Figure 6.11. Bypass efficiency of DNA lesions relative to G in pol II deficient cells.
Figure 6.12. Bypass efficiency of DNA lesions relative to G in pol IV deficient cells.
Figure 6.13. Bypass efficiency of DNA lesions relative to G in pol V deficient cells.
Figure 6.14. Bypass efficiency of DNA lesions relative to G in triple knockout cells.
Figure 6.15. (A) REAP assay results for uninduced wild-type cells. (B) REAP assay results for SOS-induced wild-type cells.
Figure 6.16. (A) REAP assay results for 8-oxoG in uninduced cells. (B) REAP assay results for 8-oxoG in SOS-induced cells.
Figure 6.17. (A) REAP assay results for Gh in uninduced cells. (B) REAP assay results for Gh in SOS-induced cells.
Figure 6.18. (A) REAP assay results for Sp1 in uninduced cells. (B) REAP assay results for Sp1 in SOS-induced cells.
Figure 6.19. (A) REAP assay results for Sp2 in uninduced cells. (B) REAP assay results for Sp2 in SOS-induced cells.
Figure 6.20. (A) REAP assay results for Oa in uninduced cells. (B) REAP assay results for Oa in SOS-induced cells.
Figure 6.21. (A) REAP assay results for Ua in uninduced cells. (B) REAP assay results for Ua in SOS-induced cells.
Figure 6.22. (A) REAP assay results for NI in uninduced cells. (B) REAP assay results for NI in SOS-induced cells.
Table 6.1. *E. coli* strains used in this work.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>F− thr−1 araC14 leuB6(Am) Δ(gpt-proA)62 lacY1 tsx−33 supE44(AS) galK2(Oc) hisG4(Oc) rfbD1 mgl−51 rpoS396(Am) rpsL31(Str6) kgdK51 xylA5 mtl−1 argE3(Oc) thi−1</td>
<td>G. Walker</td>
</tr>
<tr>
<td>pol II−</td>
<td>As AB1157 but <em>polBΔ1::Δ</em> Sm−Sp</td>
<td>G. Walker</td>
</tr>
<tr>
<td>pol IV−</td>
<td>As AB1157 but <em>dinB::cat</em></td>
<td>G. Walker</td>
</tr>
<tr>
<td>GW8017</td>
<td>As AB1157 but <em>umuDC595::cat</em></td>
<td>G. Walker</td>
</tr>
<tr>
<td>triple k.o.</td>
<td>As AB1157 but <em>polBΔ1::Ω</em> Sm−Sp <em>ΔdinB umuDC595::cat</em></td>
<td>G. Walker</td>
</tr>
</tbody>
</table>
6.7 References


Chapter 6 – Effects of Bypass Polymerases on Guanine Oxidation Products


FUTURE DIRECTIONS
Future Directions

Introduction

The work in this thesis describes the genotoxic and mutational properties for the majority of the end products of guanine oxidation by peroxynitrite. Additionally, the significant involvement of DNA polymerase V in the replication of several of these products was demonstrated. Potential future studies are outlined below.

Mutational Properties of Other Guanine Oxidation Products

Thus far, the mutagenesis studies of guanine oxidation products have focused on the most chemically stable products due to the availability of procedures for the preparation of these lesions and their ability to survive current genome construction conditions. Several compounds discussed in Chapter 1, notably 8-nitroguanine (8-NO₂-G), dehydroguanidinohydantoin (DGh), and 4-hydroxy-2,5-dioxo-imidazolidine-4-carboxylic acid (HICA) are of particular interest. Suzuki et al. have developed a method for the preparation of 8-NO₂-G in oligonucleotides and found that the lesion is more stable ($t_{1/2} = 31$ h, $25$ °C) than previously reported (1). The stability exhibited by this lesion is sufficient for use in our M13 system. The stability of DGh is too low for current genome construction conditions; however, the conditions could be improved to allow study of this lesion. For example, the ligation step is currently $2$ h at $16$ °C, but ligation reactions may also be carried out at $4$ °C. Also, differences in stability as a result of sequence context have been noted for 8-NO₂-G, perhaps in the appropriate sequence context the stability of DGh will improve. Study of HICA is precluded by the lack of a method for preparing oligonucleotides containing the lesion in amounts sufficient for study. At this point, we have successfully synthesized a protected 2’-deoxynucleoside precursor of HICA. Completion of the synthesis is dependent on successful removal of the protecting groups.

Structural Studies

Little is known about the mechanisms by which polymerases bypass DNA lesions. NI and Ca both have the stability necessary for X-ray crystal studies, and a study of a translesion synthesis polymerase cocrystallized with a primer/template containing...
one of these lesions would provide valuable mechanistic data. We are currently collaborating with Dinshaw Patel at Memorial Sloan-Kettering to obtain a structure of a primer/template containing NI complexed with the bypass polymerase Dpo4.

**Repair of Guanine Oxidation Products**

Quantitative *in vitro* repair studies of the lesions discussed in this thesis are lacking and only one *in vivo* study has been reported (2). The effect of repair enzymes and their complexes on the *in vivo* mutational properties of guanine oxidation products are unknown. Given the availability of methods for preparing oligonucleotides containing guanine oxidation products and for purifying many repair enzymes, *in vitro* studies easily could be completed. Use of a double-stranded vector containing a site-specific DNA lesion could allow determination of the effect of repair enzymes *in vivo*.

**In Vivo Detection**

The relevance of guanine oxidation products ultimately depends on the detection of these lesions *in vivo*. So far, 8-oxoG, 8-NO₂-G, and Sp have been detected in cells. The recent discovery of Sp was facilitated by knocking out the repair enzyme, Nei (2). Discovery of other guanine oxidation products may be dependent on more sensitive detection methods or on the identification of enzymes that show activity for specific lesions. The detection of Sp proves that at least some of the chemistry responsible for guanine oxidation product formation does occur *in vivo*. Strictly from a chemical perspective, the formation of the other lesions *in vivo* is plausible. Given the results presented in this thesis, an efficient repair system for these oxidation products may exist to combat the toxic and mutagenic effects and likely would need to be inactivated to allow detection and quantification of the DNA lesions.

**References**

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RESEARCH EXPERIENCE
2002 – Present Graduate Research Assistant, Department of Chemistry, MIT, Cambridge, MA
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• Developing chemical methods for the synthesis and incorporation into DNA of nucleosides produced by peroxynitrite oxidation.
• Evaluating the genotoxicity and mutagenic potential of DNA lesions in vivo resulting from peroxynitrite oxidation.
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2000 – 2001 Undergraduate Research Assistant, Department of Chemistry, Washington University, St. Louis, MO
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• Prepared four novel photoproduct analogs within oligodeoxynucleotides.
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PUBLICATIONS

SELECTED ORAL PRESENTATIONS

SELECTED POSTER PRESENTATIONS