BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 7833–7841 7833
DOI: 10.1021/bi900927d

The Mutagenicity of Thymidine Glycol in *Escherichia coli* Is Increased When It Is Part of a Tandem Lesion†

Haidong Huang, Shuhei Imoto, and Marc M. Greenberg*

Department of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218

Received June 2, 2009; Revised Manuscript Received July 8, 2009

ABSTRACT: Tandem lesions are comprised of two contiguously damaged nucleotides. Tandem lesions make up the major family of reaction products generated from a pyrimidine nucleobase radical, which are formed in large amounts by ionizing radiation. One of these tandem lesions contains a thymidine glycol lesion flanked on its 5′-side by 2-deoxyribonolactone (LTg). The replication of this tandem lesion was investigated in *Escherichia coli* using single-stranded genomes. LTg is a much more potent replication block than thymidine glycol and is bypassed only under SOS-induced conditions. The adjacent thymidine glycol does not significantly affect nucleotide incorporation opposite 2-deoxyribonolactone in wild-type cells. In contrast, the misinsertion frequency opposite thymidine glycol, which is negligible in the absence of 2-deoxyribonolactone, increases to 10% in wild-type cells when LTg is flanked by a 3′-dG. Experiments in which the flanking nucleotides are varied and in cells lacking one of the SOS-induced bypass polymerases indicate that the mutations are due to a mechanism in which the primer misaligns prior to bypassing the lesion, which allows for an additional nucleotide to be incorporated across from the 3′-flanking nucleotide. Subsequent realignment and extension results in the observed mutations. DNA polymerases II and IV are responsible for misalignment induced mutations and compete with DNA polymerase V which reads through the tandem lesion. These experiments reveal that incorporation of the thymidine glycol into a tandem lesion indirectly induces increases in mutations by blocking replication, which enables the misalignment−realignment mechanism to compete with direct bypass by DNA polymerase V.

More than 50 individual lesions have been identified in DNA that is exposed to oxidative conditions (*1*−*3*). If not repaired, these lesions can block replication and/or produce mutations. A great deal has been learned about how specific lesions affect the activities of polymerase and repair enzymes through the combined efforts of organic chemistry, biochemistry, and molecular and cellular biology (*3*−*5*). There is a growing appreciation for the importance of a family of lesions known as clustered lesions whose biochemical effects are less well understood (*6*). Clustered lesions are defined as two or more damage sites within one or two turns of DNA. They are often associated with ionizing radiation because of the high energy flux and correspondingly high localized concentration of hydroxyl radical to which DNA is exposed by this method (*7*−*9*). Clustered lesions have been shown to affect DNA repair and replication in a manner that is strongly dependent upon their proximity to one another (*10*−*16*). Tandem lesions, defined as two contiguously damaged nucleotides, make up a subset of clustered lesions. Although tandem lesions were first observed in DNA samples that were exposed to ionizing radiation, their formation is not dependent on this means of inducing damage. Tandem lesions can form via a single initial reaction in which a reactive intermediate or lesion reacts with another nucleotide. Less research has been conducted on the effects of tandem lesions than on clustered lesions as a whole. However, available data indicate that tandem lesions exert significant detrimental effects on DNA repair and replication (*17*−*20*). In this work, we describe how the mutagenicity in *Escherichia coli* of a tandem lesion that is derived from a single chemical event is different from that of either lesion alone.

Nucleobase radicals make up the major family of reactive intermediates formed when pyrimidines are exposed to hydroxyl radical, which is produced by ionizing radiation and some metal complexes (*21*). These radicals result from addition of hydroxyl radical to the pyrimidine double bond, which occurs preferentially at the more electron rich C5 position of the pyrimidine. The respective peroxyl radicals are produced under aerobic conditions. Analysis of short oligonucleotides exposed to ionizing radiation revealed tandem lesions whose formation was consistent with the reaction of a nucleobase (peroxyl) radical with an adjacent nucleotide (*22*−*24*). Unambiguous evidence for the formation of tandem lesions from nucleobase radicals has been obtained by using organic chemistry to independently generate the reactive intermediates in synthetic oligonucleotides (*25*−*30*). The nucleobase radicals and their respective peroxyl radicals add to the double bonds of the adjacent 5′- and 3′-nucleotides. In at least some instances, the peroxyl radicals of the nucleobase radical adducts also selectively abstract the C1′ hydrogen atom from the 5′-adjacent nucleotide, ultimately resulting in the formation of 2-deoxyribonolactone (L)[1] (Scheme 1) (*28*−*32*). In one system, the L-containing lesion was found to account for

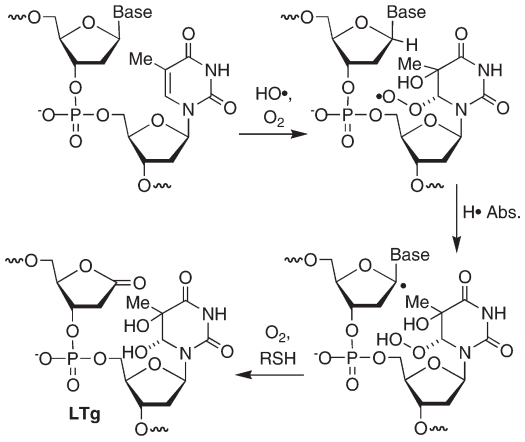
†We are grateful for support of this research by the National Institute of General Medical Sciences (GM- 063028).

*To whom correspondence should be addressed: Department of Chemistry, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. Telephone: (410) 516-8095. Fax: (410) 516-7044. E-mail: mgreenberg@jhu.edu.

[1]Abbreviations: AP, abasic site; L, 2-deoxyribonolactone; C2-AP, C2′-oxidized abasic site; C4-AP, C4′-oxidized abasic site; Tg, thymidine glycol; BER, base excision repair; OxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; 5′-LTg, 5′-(2-deoxyribonolactone)−thymidine glycol; Pol II, DNA polymerase II; Pol IV, DNA polymerase IV; Pol V, DNA polymerase V; REAP, restriction endonuclease and postlabeling.

Published on Web 07/20/2009

Scheme 1: Postulated Hydroxyl Radical-Mediated Formation of the 5′-LTg Tandem Lesion



more than 10% of the lesions produced from the original nucleobase radical (*28*).

The replication and repair of a tandem lesion containing 2-deoxyribonolactone were of particular interest because of this oxidized abasic site's distinctive biochemical effects. The lactone (L) irreversibly inhibits proteins involved in base excision repair of abasic sites by forming cross-links with the lysine side chains that are involved in Schiff base formation of endonuclease III and DNA polymerase β (*33*, *34*). In addition, L impacts replication in *E. coli* by inducing incorporation of dG opposite it instead of following the "A-rule" (*35*−*37*). Studies on the 5′-LTg tandem lesion showed that its repair is distinct from that of either isolated lesion (*38*). For instance, endonuclease III is not cross-linked by the tandem lesion, but the base excision repair (BER) protein is also unable to excise the thymine glycol when it is part of the 5′-LTg tandem lesion. Instead, the tandem lesion is repaired by nucleotide excision repair and long patch BER. Herein, we describe the replication of single-stranded plasmids containing the 5′-LTg in *E. coli*.

MATERIALS AND METHODS

Materials and General Methods. Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer. Commercially available DNA synthesis reagents, including the (5*R*,6*S*)-thymine glycol phosphoramidite, were obtained from Glen Research Inc. Oligonucleotides containing the photolabile 2-deoxyribonolactone precursor were synthesized as previously described (*39*). Oligonucleotides containing Tg were synthesized using standard cycles as described by Iwai (*40*) and deprotected as described below. All others were synthesized and deprotected using standard protocols. Synthetic oligonucleotides containing the photochemical precursor to L and/or Tg were characterized by ESI-MS, which are included in the Supporting Information. T4 polynucleotide kinase, T4 DNA ligase, BbsI, and HaeIII were obtained from New England Biolabs. Shrimp alkaline phosphatase was from Roche. Nuclease P1 was from Sigma. T4 polymerase was from Promega, and Pfu Turbo was from Stratagene. Radionuclides were obtained from Perkin-Elmer. Analysis of radiolabeled nucleotides was conducted using a Storm 840 Phosphorimager and ImageQuant version 5.1. The data presented in Tables 1−3 and Figures 1−6 are the average of two or three experiments. Each experiment consists of three replicates.

Deprotection Method for Oligonucleotides Containing Thymine Glycol (*Tg*). The resin was suspended in ammonia at room temperature for 3 h. The resin was spun to the bottom, and the supernatant was transferred to another tube. The resin was washed with water (2 × 100 μL). The wash was combined with the supernatant and concentrated. The pellet was resuspended in 250 μL of a 1.4 M hydrofluoric acid (HF) solution [1.5 mL of *N*-methylpyrrolidinone, 750 μL of triethylamine (TEA), and 1.0 mL of TEA·HF] at 65 °C for 3 h. The solution was quenched with 3 M NaOAc (25 μL) and EtOH (1.0 mL), and the solution was kept at −80 °C for 1 h. The solution was spun at 13,200 rpm and 4 °C for 30 min. The supernatant was decanted and the pellet dried. The pellet was resuspended in 100 μL of formamide loading buffer (95% formamide and 10 mM EDTA) and loaded on a 20% denaturing PAGE gel (1.5 mm thick).

Chart 1: Oligonucleotides Used To Create Genomes

1	5′-d(GAA GAC CC**Tg** GGC GTC C)
2	5′-d(GAA GAC CC**L TgG**C GTC C)
3	5′-d(GAA GAC C**LTg** GGC GTC C)
4	5′-d(GAA GAC CT**Tg** GGC GTC C)
5	5′-d(GAA GAC CT**L TgG**C GTC C)
6	5′-d(GAA GAC T**LTg** GGC GTC C)
7	5′-d(GAA GAC CT**Tg** AGC GTC C)
8	5′-d(GAA GAC CT**L TgA**C GTC C)
9	5′-d(GAA GAC T**LTg** AGC GTC C)
10	5′-d(GAA GAC CC**L** GGC GTC C)
11	5′-d(GAA GAC CTT GGC GTC C)

M13 Genome Construction and Replication in SOS-Induced E. coli Cells. The synthetic DNA insert was cloned into the M13mp7L2 vector in triplicate as previously described (*36*, *41*). Briefly, the insert (15 pmol) was phosphorylated (12 units of PNK, 37 °C, 1 h) and ligated (1200 units, 16 °C, 2 h) into 10 pmol of EcoRI-digested plasmid using a complementary scaffold (15 pmol). After digestion of the scaffolds with T4 DNA polymerase (16 units, 16 °C, 1 h), the vectors were purified by phenol extraction and G-25 Sephadex filtration. When the inserts containing 2-deoxyribonolactone were introduced, the oligonucleotide containing the photochemical precursor was phosphorylated and then irradiated at 350 nm for 1 h in a transparent Eppendorf tube using a Rayonet Photochemical Reactor, followed by ligation into the plasmid.

To examine the bypass of plasmids containing different lesions in SOS-induced *E. coli* cells, wild-type (AB1157), polymerase II (STL1336), polymerase IV (Xs-1), polymerase V (SR1157U), and triple-knockout (SF2108) cells were grown to an OD_{600} of 0.3, pelleted, and resuspended in 10 mM $MgSO_4$. The cells were irradiated at 45 J/m^2, added to 25 mL of 2×YT, and incubated at 37 °C for 45 min. The cells were pelleted, washed with cold water, and resuspended in 10% glycerol. The prepared cells (100 μL) were electroporated with 1 pmol of the vector (2.5 kV, 4.74 ms) and plated with X-Gal and IPTG.

REAP Assay To Determine Mutation Frequency. Mutation analysis was conducted using the restriction endonuclease and postlabeling (REAP) assay, which has previously been described (*42*,*43*). Briefly, viral DNA was recovered from the growth medium and PCR amplified. Following digestion with BbsI and shrimp alkaline phosphatase, the DNA was labeled with ^{32}P and further digested with HaeIII. The desired product(s) was purified using 20% denaturing PAGE and desalted using a G25-Sephadex column. Finally, the samples were digested with nuclease P1 and nucleotides separated on a PEI-cellulose TLC plate which was run with saturated $(NH_4)_2HPO_4$ and H_3PO_4 (pH 5.8).

RESULTS

Oligonucleotide Synthesis and Genome Construction. The restriction endonuclease and postlabeling (REAP) procedure is a powerful method for determining the mutagenicity of DNA lesions (*42*, *43*). REAP enables one to monitor millions of replication events in a single sample via straightforward analysis of thin layer chromatography, providing statistically meaningful incorporation frequencies. It also enables one to quantify full-length replication, as well as deletion and insertion products in a single experiment. Utilization of REAP requires construction of a single-stranded plasmid containing the lesion of interest at the cleavage site of a restriction enzyme that binds at an invariant neighboring sequence. In these studies, we used the restriction enzyme (BbsI) employed by Essigmann and Delaney in their pioneering work (*42*, *43*). BbsI binds to 5′-d(GAA GAC) and hydrolyzes the 5′-phosphate two nucleotides further downstream. It was shown in this original report that BbsI incision is independent of the identity of the nucleotide at the cleavage site. Determining the outcome of tandem lesion replication requires the preparation of separate genomes for the analysis of each nucleotide component because the REAP method detects the outcome of lesion bypass at a single position. Consequently, 16-nucleotide oligonucleotide inserts (Chart 1) were prepared in which the position of the tandem lesion with respect to the 5′-terminus of the insert varied by one nucleotide depending upon whether one wanted to determine the outcome of bypassing 2-deoxyribonolactone (**2**, **5**, **8**, and **10**) or thymidine glycol (**1**, **3**, **4**, **6**, **7**, and **9**). The tandem lesions were flanked on the 5′-side by either dT or dC and on the 3′-side by dG or dA. Although the 5′- and 3′-flanking nucleotides were identical when L or Tg was probed (e.g., **2** vs **3**) in a given sequence context, there were slight nucleotide differences beyond this point. Genomes containing an isolated Tg (**1**, **4**, and **7**) and native nucleotides (**11**) were prepared as controls.

A variety of oligonucleotides (Chart 1) were synthesized using previously established methods to prepare the respective genomes. Oligonucleotides containing thymidine glycol were prepared using a commercially available phosphoramidite originally described by Iwai (*40*). Although the Tg phosphoramidite consists of a single diastereomer (5*R*,6*S*), the 6-positions of 6-hydroxy-5,6-dihydropyrimidines epimerize in water following deprotection (*44*, *45*). Hence, the oligonucleotides containing Tg effectively consist of a mixture of 5*R*,6*S* and 5*R*,6*R* stereoisomers. Oligonucleotides containing L were prepared from the photolabile nitroindole derivative, which were synthesized as previously described (*39*). The 2-deoxyribonolactone was freshly prepared via photolysis following 5′-phosphorylation of the respective oligonucleotide and immediately before ligation into the linearized M13 plasmid, as previously described (*35*).

Bypass Efficiency. The bypass efficiency was determined by comparing the number of colonies that grew on agar plates when genomes containing Tg or LTg (prepared using inserts **1**−**9**) were plated to those produced when a genome containing only native nucleotides (prepared using insert **11**). When cells that were not exposed to SOS induction (UV irradiation) were transfected with genomes constructed from oligonucleotides in Chart 1, colonies were produced in quantities >50% relative to undamaged genomes from individual thymidine glycol (Tg, **1**, and **4**) lesions (data not shown). Tandem lesions were bypassed less than 1% as efficiently as undamaged DNA (genome prepared from **11**) in non-SOS-induced cells. The bypass efficiency of isolated Tg lesions did not increase significantly, if at all, in SOS-induced cells (Table 1). However, the bypass efficiency of tandem lesions increased to between 6 and 9% in wild-type cells. The necessity for bypass polymerases when replicating LTg was confirmed in cells lacking Pol II, Pol IV, and Pol V. Less than 1% bypass of LTg was detected in the triple-knockout cells. However, the bypass of Tg (**1**) was 26%. In addition, removing any one of the SOS-induced polymerases reduced but did not eliminate bypass of the tandem lesion (Table 1).

Table 1: Bypass Efficiencies in SOS-Induced *E. coli*

	% bypass (cell type)[a]			
sequence[b]	wild type	Pol II$^-$	Pol IV$^-$	Pol V$^-$
CC**Tg**GG (**1**)	56.6 ± 1.7	65.6 ± 5.3	57.7 ± 19.5	52.4 ± 5.9
CC**L**TgG (**2**)	8.7 ± 0.8	3.9 ± 1.5	2.9 ± 0.9	4.9 ± 0.4
CL**Tg**GG (**3**)	6.6 ± 0.3	1.4 ± 0.1	2.2 ± 0.7	4.0 ± 0.4
CT**Tg**GG (**4**)	58.9 ± 4.7	nd[d]	nd[d]	nd[d]
CT**L**TgG (**5**)	9.0 ± 0.4	nd[d]	nd[d]	nd[d]
TL**Tg**GG (**6**)	7.7 ± 0.6	nd[d]	nd[d]	nd[d]
CT**Tg**AG (**7**)	56.1 ± 1.8	nd[d]	nd[d]	46.1 ± 2.1
CT**L**TgA (**8**)	7.2 ± 1.7	nd[d]	nd[d]	3.8 ± 0.3
TL**Tg**AG (**9**)	8.3 ± 0.6	nd[d]	nd[d]	3.0 ± 0.1
CC**L**GG (**10**)[c]	13.2 ± 2.4	7.8 ± 1.0	6.1 ± 0.6	9.3 ± 1.0

[a] Determined by comparing the number of colonies formed to those from a genome constructed from **11**. [b] Local sequence shown and oligonucleotide insert number in parentheses. A bold nucleotide indicates the position probed in the REAP assay. [c] Data taken from ref *35*. [d] Not determined.

Table 2: Single-Nucleotide Deletions as a Function of Cell Type

	% single-nucleotide deletions			
sequence[a]	wild type	Pol II$^-$	Pol IV$^-$	Pol V$^-$
CC**Tg**GG (**1**)	0	0	0	0
CC**L**TgG (**2**)	8.6 ± 0.4	8.8 ± 2.7	10.6 ± 2.2	8.9 ± 0.9
CL**Tg**GG (**3**)	19.6 ± 5.2	21.9 ± 6.0	22.3 ± 9.0	71.4 ± 8.2
CT**Tg**GG (**4**)	0	nd[b]	nd[b]	nd[b]
CT**L**TgG (**5**)	1.2 ± 0.8	nd[b]	nd[b]	nd[b]
TL**Tg**GG (**6**)	13.4 ± 2.3	nd[b]	nd[b]	nd[b]
CT**Tg**AG (**7**)	0	nd[b]	nd[b]	0
CT**L**TgA (**8**)	2.8 ± 1.4	nd[b]	nd[b]	81.5 ± 0.9
TL**Tg**AG (**9**)	4.1 ± 2.2	nd[b]	nd[b]	65.8 ± 9.1

[a] Local sequence shown and oligonucleotide insert number in parentheses. A bold nucleotide indicates the position probed in the REAP assay. [b] Not determined.

Formation of Single-Nucleotide and Three-Nucleotide Deletions. No deletions are detected when Tg is bypassed in wild-type or polymerase deficient cells. In contrast, single-nucleotide deletions are observed when the tandem lesion (LTg) is bypassed in all cell types that were examined (Table 2), as they are when only L is present (*35*). When the tandem lesion is flanked on the 3′-side by dG, single-nucleotide deletions are greater in number when the 5′-flanking nucleotide is dC than when it is dT. The amount of single-nucleotide deletions is also always greater when inserts designed to probe nucleotide incorporation opposite Tg (**3**, **6**, and **9**) are examined. These constructs shift the tandem lesion one nucleotide closer to the 5′-terminus of the insert than do inserts designed to probe incorporation of a nucleotide opposite L (**2**, **4**, and **8**). Removing Pol II or Pol IV had no effect on the amounts of single-nucleotide deletions produced in the tandem lesions flanked by 5′-dC and 3′-dG. However, in three of the four sequences examined, large increases

Table 3: Three-Nucleotide Deletions as a Function of Cell Type

	% three-nucleotide deletions			
sequence[a]	wild type	Pol II$^-$	Pol IV$^-$	Pol V$^-$
CC**T**gGG (**1**)	0	0	0	0
CC**L**TgG (**2**)	0	9.9 ± 4.9	6.9 ± 4.3	73.1 ± 8.1
CL**T**gGG (**3**)	0	2.1 ± 1.5	0	11.1 ± 2.2
CT**T**gGG (**4**)	0	0	0	0
CTLTgG (**5**)	0	nd[b]	nd[b]	nd[b]
TL**T**gGG (**6**)	0	nd[b]	nd[b]	nd[b]
CT**T**gAG (**7**)	0	0	0	0
CTLTgA (**8**)	0	nd[b]	nd[b]	0
TL**T**gAG (**9**)	0	nd[b]	nd[b]	10.2 ± 0.6

[a] Local sequence shown and oligonucleotide insert number in parentheses. A bold nucleotide indicates the position probed in the REAP assay. [b] Not determined.

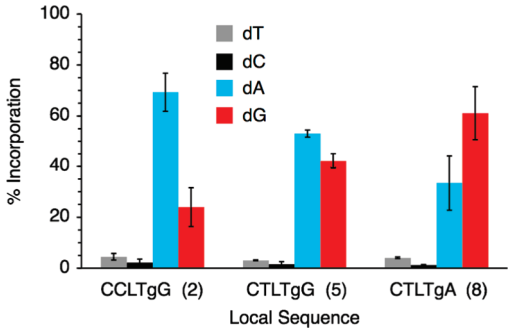


FIGURE 1: Nucleotide incorporation opposite 2-deoxyribonolactone (L) in the full-length product in wild-type cells as a function of local sequence. The oligonucleotide insert used to prepare each respective genome is indicated in parentheses (see Chart 1).

in the single-nucleotide deletion level were observed when Pol V was removed.

Three-nucleotide deletions are not commonly observed when isolated lesions are replicated in *E. coli* (*46*). However, varying amounts of three-nucleotide deletions are observed when single-stranded plasmid containing LTg is replicated in bypass polymerase deficient cells (Table 3). With one exception, the level of three-nucleotide deletions is ≤11% in all cell types. The genome produced from insert **2** was the exception. Translesion synthesis in this genome yielded a high level of three-nucleotide deletions in Pol V deficient cells that was typically observed for −1 frameshift products (Table 2). However, the sum total levels of deletion products from Pol V deficient cells were comparable for all four sequences.

Nucleotide Incorporation opposite 2-Deoxyribonolactone and Thymidine Glycol within the LTg Tandem Lesion in Wild-Type Cells. Translesion synthesis of an abasic site results in predominant incorporation of dA opposite it, consistent with its adherence to the A-rule (*37*, *47*, *48*). However, 2-deoxyribonolactone and analogues containing a carbonyl hydrogen bond acceptor induce DNA polymerase to incorporate significantly higher levels of dG opposite it (*35*, *36*). The presence of thymidine glycol on the 3′-side of L does not alter this general phenomenon in wild-type cells (Figure 1). dG incorporation opposite L ranges from ~25% in the genome constructed from **2** to more than 60% in the 5′-d(CLTgA) sequence (**8**). The combined incorporation frequency of pyrimidines opposite 2-deoxyribonolactone was less than 7% in all sequence contexts examined.

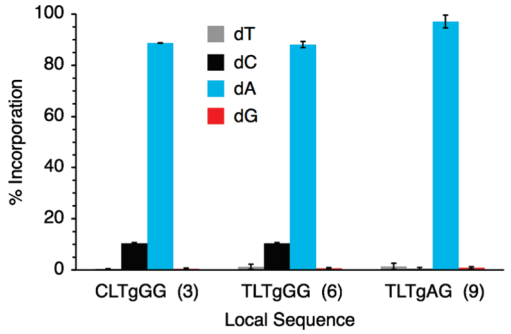


FIGURE 2: Nucleotide incorporation opposite thymidine glycol (Tg) in the full-length product in wild-type cells as a function of local sequence. The oligonucleotide insert used to prepare each respective genome is indicated in parentheses (see Chart 1).

In contrast, the presence of L in the tandem lesion has a significant effect on replication of thymidine glycol in wild-type cells. Mutations cannot be detected using the REAP assay in genomes constructed from inserts containing an isolated Tg (**1**, **4**, and **7**). However, dC is misincorporated opposite Tg ~10% of the time when the lesion is flanked on its 5′-side by L and is bonded via its 3′-phosphate to dG (Figure 2). The combined misincorporation of dT and dG opposite thymidine glycol when plasmids derived from **3** and **6** are bypassed is less than 4%. Consideration was given to the possibility that dC misincorporation opposite Tg was an artifact introduced during the REAP procedure that arose due to the presence of a single-nucleotide deletion impurity in the product that is excised from the denaturing polyacrylamide gel and digested prior to analysis of the nucleotide monophosphates by thin layer chromatography. However, analysis of the excised product by analytical denaturing gel electrophoresis showed that the product was not contaminated by any shorter oligonucleotide (data not shown). In addition, changing the 3′-flanking nucleotide from dG (**3** and **6**) to dA (**9**) eliminated the significant level of dC misincorporation (Figure 2). The level of incorporation of dA opposite thymidine glycol increased from ~88 to >97% when the plasmid constructed from **9** is bypassed in wild-type cells.

Nucleotide incorporation in −1 frameshifts produced in wild-type cells were characterized using genomes constructed from **2** and **3**, which were designed to probe the effects of L and Tg, respectively. A single nucleotide was incorporated in each instance. These were dA in the genome obtained from **2** and dC from the genome designed to report on nucleotide incorporation opposite Tg.

Nucleotide Incorporation opposite 2-Deoxyribonolactone and Thymidine Glycol within the LTg Tandem Lesion in Polymerase Deficient Cells. The effects of DNA polymerase II and polymerase IV on the replication of a genome containing the LTg tandem lesion were examined in a single sequence context. Bypass of the tandem lesion flanked by 5′-dC and 3′-dG showed no change in the level of dC or dT incorporation opposite Tg compared to what was observed in wild-type cells (Figures 2 and 3). Removing Pol II or Pol IV from the cell had a more pronounced effect on nucleotide incorporation opposite 2-deoxyribonolactone (Figure 4). The percent of dG incorporation opposite L increased to more than 40% in both polymerase deficient cell lines from ~25% in the wild-type cells. A modest increase in the level of dT incorporation opposite the oxidized abasic site was observed, but the levels were still less than 10%.

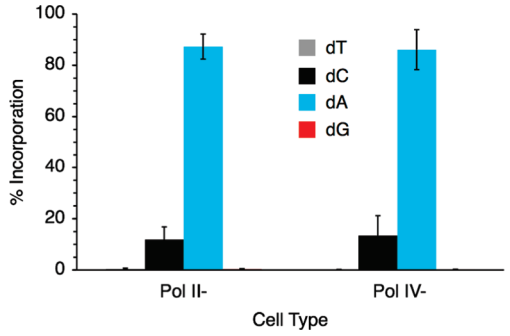


FIGURE 3: Nucleotide incorporation opposite thymidine glycol (Tg) in Pol II$^-$ and Pol IV$^-$ cells in the full-length product. Oligonucleotide **3** was used to prepare the genome.

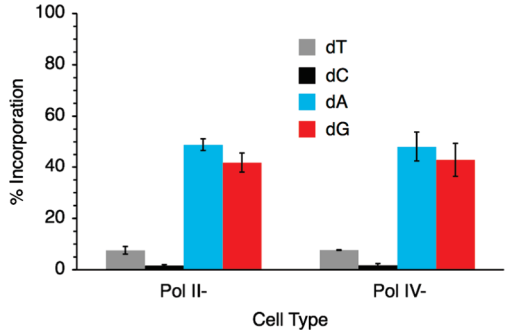


FIGURE 4: Nucleotide incorporation opposite 2-deoxyribonolactone (L) in Pol II$^-$ and Pol IV$^-$ cells in the full-length product. Oligonucleotide **2** was used to prepare the genome.

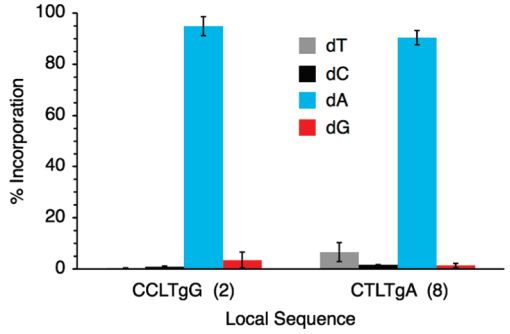


FIGURE 5: Nucleotide incorporation opposite 2-deoxyribonolactone (L) in the full-length product produced in Pol V$^-$ cells. The oligonucleotide insert used to prepare each respective genome is indicated in parentheses (see Chart 1).

Initially, the effect of removing Pol V from the *E. coli* was examined in the same sequence context as described above (5′-dC and 3′-dG). Although as described above, the overall amount of full-length product formed is significantly reduced in Pol V deficient cells, the effect on nucleotide incorporation opposite 2-deoxyribonolactone was dramatic (Figure 5). The incorporation of dA opposite L increased to almost 95%. The magnitude of the effect on nucleotide incorporation opposite Tg was comparable (Figure 6). The level of misincorporation of dC opposite thymidine glycol increased from ~10% in wild-type and Pol II or Pol IV deficient cells to 90% in *E. coli* lacking Pol V. This dramatic change led us to investigate the effect of removing Pol V on the replication of DNA containing the LTg tandem lesion in which dA is the 3′-flanking nucleotide (Figure 6). Again, the effects on nucleotide incorporation were profound. The level of incorporation of 2′-deoxyadenosine opposite L increased to

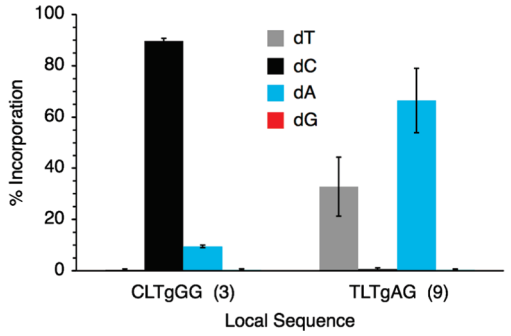


FIGURE 6: Nucleotide incorporation opposite thymidine glycol (Tg) in the full-length product produced in Pol V$^-$ cells. The oligonucleotide insert used to prepare each respective genome is indicated in parentheses (see Chart 1).

90%. The change in nucleotide incorporation opposite Tg was not as large as when the tandem lesion is flanked by 5′-dC and 3′-dG, but is still very large. However, instead of large amounts of dC misincorporation opposite Tg, thymidine was inserted opposite the glycol more than 30% of the time (Figure 6).

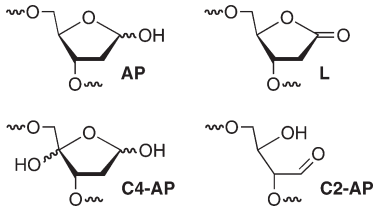
REAP analysis of the three-nucleotide deletions produced in Pol V deficient cells was conducted for the three genomes that produced this lesion (Table 3). 2′-Deoxyguanosine incorporation opposite the template nucleotide analyzed was exclusively detected in all three instances.

DISCUSSION

Recently, the effects of clustered lesions and, to a lesser extent, the subset of tandem lesions have been the subject of numerous studies concerning their effects on DNA in cells (*18*, *20*, *49*−*52*). We investigated the effects of the LTg tandem lesion on replication in *E. coli*. LTg is of interest because it is a member of a family of lesions that are derived from the reactions under aerobic conditions of nucleobase radicals, which themselves account for the majority of reactions between hydroxyl radical and nucleic acids (*21*). In addition, LTg was previously shown to require alternative DNA repair pathways from those used to excise thymidine glycol and 2-deoxyribonolactone individually (*38*). The effects of isolated thymidine glycol and 2-deoxyribonolactone on replication in *E. coli* have also been characterized. Although Tg blocks replication, it is weakly mutagenic (<1% promutagenic lesions are formed) and is bypassed in cells in which a SOS response has not been induced (*53*, *54*). The oxidized abasic site, L, is a more potent replication block than Tg and gives rise to an unusual nucleotide incorporation pattern in which the A-rule is not followed and significant quantities of dG are inserted opposite it (*35*, *36*). Placing these two lesions in tandem had unanticipated effects on replication that are not observed when either individual lesion is bypassed.

The tandem lesion is bypassed much less efficiently than an isolated Tg. As is the case for 2-deoxyribonolactone bypass, SOS induction is required to produce detectable levels of replication products in wild-type cells. Overall, LTg is bypassed slightly less efficiently than an isolated L in wild-type cells (Table 1) (*35*). The modest reduction in LTg bypass efficiency (Table 1) upon removal of any single SOS-induced polymerase is also similar to the effects that these changes had on replication of an isolated 2-deoxyribonolactone (*35*). Since, Tg is bypassed (~50%) without SOS induction, it is not surprising that tandem

lesion bypass parallels that of the more potent blocking lesion, 2-deoxyribonolactone.



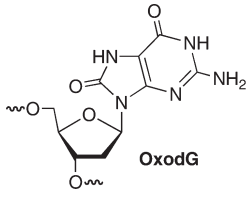
The REAP process enables one to analyze incorporation opposite a single nucleotide (*42*). Hence, separate genomes are prepared for examining bypass of each component of LTg, and slight modifications are required in the neighboring sequences of the inserts for examination of nucleotide incorporation opposite L and Tg with the same flanking nucleotides (e.g., **2** vs **3** and **5** vs **6**). These changes did not significantly impact the bypass efficiency. However, the distribution of full-length and deletion products was affected by these proximal but nonadjacent nucleotides. Without exception, sequences designed to identify the nucleotide incorporated opposite Tg (**3**, **6**, and **9**) by REAP gave rise to higher levels of single-nucleotide deletion products (Table 2) than did the respective inserts that probed the mutagenicity of 2-deoxyribonolactone (**2**, **5**, and **8**). This was true in the wild type, as well as the three varieties of bypass polymerase deficient cells. Although the reason for this is not evident, subtle effects of local sequence on AP site bypass by DNA polymerase η have also been observed (*55*). The effect of the 5′-flanking nucleotide on the level of single-nucleotide deletions was reminiscent of that observed in the bypass of an isolated L. Larger amounts of −1 frameshift products were observed when the oxidized abasic site was flanked by a 5′-dC than by a 5′-dT. In wild-type cells, dA and dC were exclusively detected in single-nucleotide deletion products using genomes designed to probe L and Tg bypass, respectively. The simplest explanation for these observations is that the single-nucleotide deletions result from looping out of L and incorporation of dA opposite Tg. This would then result in dC incorporation in the position that is opposite the original site of Tg in the genome. It is well-known that bypass of the lactone and other abasic sites yields single-nucleotide deletions (*35*, *36*, *46*, *47*, *56*), and in our hands, −1 frameshifts are not observed upon thymidine glycol bypass (Table 2). Furthermore, bypass of isolated Tg yields only dA incorporation, whereas L does not.

In addition to −1 frameshift products, LTg bypass in cells lacking one of the SOS-induced polymerases gave rise to less common three-nucleotide deletions (Table 3). Neither Tg nor 2-deoxyribonolactone when present by themselves in the genome gives rise to three-nucleotide deletions (*35*, *36*). C4-AP is the only abasic lesion whose bypass results in three-nucleotide deletions (*46*). Assuming that the entire tandem lesion is part of the three nucleotides deleted, there are two possible stretches of nucleotides that can be deleted. The LTg deletion can be flanked on either its 5′-side (5′-NLTg) or its 3′-side (5′-LTgN) by the third nucleotide. REAP analysis does not distinguish between these. In either case, the opposing nucleotide detected in the REAP assay is dG, which is the exclusive nucleotide observed.

Overall, Pol V was crucial for minimizing the total number of deletions observed upon LTg bypass (Tables 2 and 3). This is consistent with studies of the replication of other abasic lesions, in which Pol V was essential for producing full-length products (*35*, *46*, *56*). However, unlike C4-AP replication, removing Pol V does not result in exclusive formation of the three-nucleotide deletion product. Instead, the distribution of one- and three-nucleotide deletions produced in Pol V minus cells varies with respect to the LTg flanking sequence.

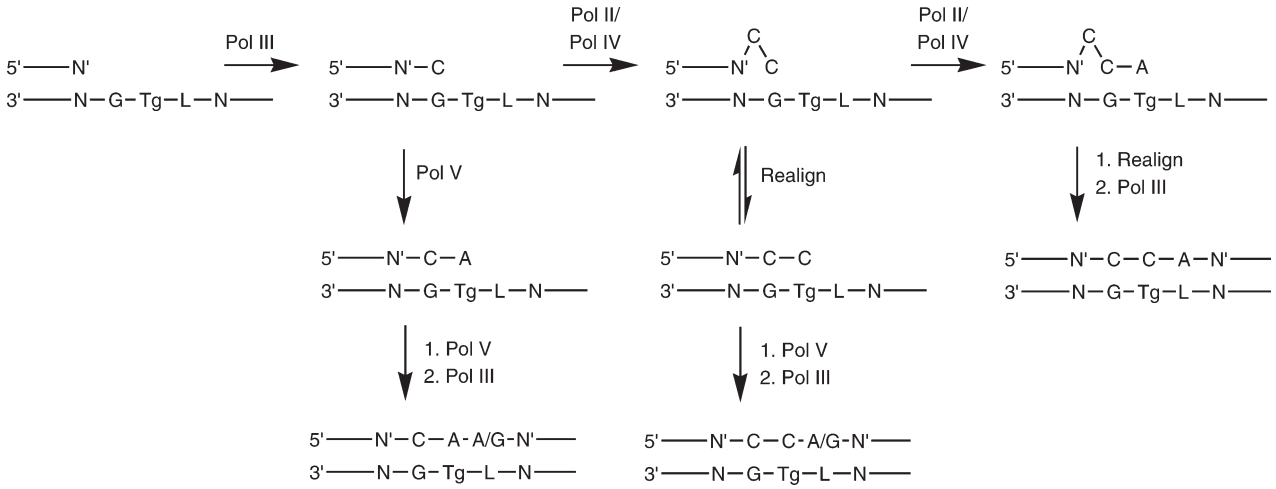
In full-length products, the flanking sequence also affected nucleotide incorporation opposite the damaged nucleotides in the tandem lesion. In wild-type cells, high levels of dG were incorporated opposite 2-deoxyribonolactone, as they are when genomes containing the isolated lesion are bypassed (*35*, *36*). A complete comparison of the effects of the flanking sequence on nucleotide incorporation opposite the isolated lesions and when they were part of tandem lesions was not possible. However, the effect of the 5′-flanking pyrimidine on nucleotide incorporation opposite L was comparable (*35*). A 5′-dT gave rise to greater amounts of dG incorporation opposite L in LTg than did a 5′-dC (Figure 1). In wild-type cells, the decreased level of incorporation of dG opposite L when the tandem lesion was flanked by a 5′-dC correlated with an increase in the amount of single-nucleotide deletion products (Table 2). As was proposed for replication of an isolated L, the single-nucleotide deletion is ascribed to a nucleotide insertion−misalignment mechanism (*36*). The correlation between the larger amount of single-nucleotide deletion and lower level of incorporation of dG opposite L in the tandem lesion replication product is attributed to a more favorable thermodynamic driving force for misalignment provided by a potential dG·dC base pair.

The modest effect of removing Pol II or Pol IV on nucleotide incorporation opposite an isolated 2-deoxyribonolactone was also evident in LTg bypass (Figures 3 and 4) (*35*). There was a modest increase in the level of incorporation of dG opposite the lactone at the expense of dA. In contrast, deleting Pol V had a dramatic effect on nucleotide incorporation opposite 2-deoxyribonolactone in LTg (Figure 5). The two sequences examined differed in 5′- and 3′-flanking nucleotides, yet in each instance, dA was incorporated opposite L more than 90% of the time in Pol V deficient cells. Although one might be tempted to ascribe this to a change to the adherence to the A-rule, the associated observations discussed below regarding nucleotide incorporation opposite Tg in the tandem lesion indicate otherwise.



The effect of a 5′-adjacent 2-deoxyribonolactone on nucleotide incorporation opposite Tg was more obvious. An isolated thymidine glycol blocks replication but is weakly mutagenic (*53*, *54*). Our experiments using the REAP assay were consistent with these findings. We could not detect any misincorporation above background. However, when the tandem lesion was flanked by a 3′-dG, ~10% dC was incorporated opposite the thymidine glycol in wild-type cells (Figure 2). This was a striking result, as the misincorporation frequency opposite the glycol in LTg is greater than that typically observed when OxodG is bypassed in repair proficient *E. coli* (*57*−*60*). Incorporation of dC opposite the thymidine glycol is attributed to a misalignment−insertion−realignment mechanism (Scheme 2) in which the upstream dG directs promutagenic base pair formation. This type of an effect by an upstream nucleotide was previously observed in studies on

Scheme 2: Postulated Mechanism for Bypass of the 5′-LTg Tandem Lesion



C2-AP replication in *E. coli* (*56*). We speculate that misalignment competes with direct bypass because the tandem lesion is a potent replication block.

Using the observations described above regarding nucleotide incorporation opposite L in the tandem lesion, we expected that Pol V would be critical to any effect on nucleotide incorporation opposite Tg. Indeed, removing Pol II or Pol IV had no effect on nucleotide incorporation opposite Tg when the tandem lesion was flanked by 3′-dG (Figure 3). However, removing Pol V resulted in a dramatic increase in the level of incorporation of dC opposite Tg to almost 90% (Figure 6). This indicated that Pol II and Pol IV are responsible for the misalignment mechanism that results in misincorporation of dC opposite Tg in the tandem lesion, and that Pol V competes with these enzymes to prevent this process. The same division of responsibilities between the bypass polymerases was observed in the studies of C2-AP mentioned above (*56*). A genome in which LTg was flanked by a 3′-dA was constructed to test this mechanism. Although dC incorporation opposite Tg was reduced to background levels, the anticipated dT incorporation was not observed in wild-type cells (Figure 2). However, dT was incorporated opposite thymidine glycol more than 30% of the time in Pol V deficient cells (Figure 6). Although the 3′-dA flanking sequence is less prone to the misalignment mechanism than that containing 3′-dG, the overall trend is consistent with the proposal. Finally, the misalignment mechanism also helps to explain the preponderance of dA incorporation opposite L of the tandem lesion in Pol V deficient cells (Scheme 2). If realignment occurs after nucleotide incorporation opposite Tg, then faithful bypass of the glycol will result in dA incorporation opposite it, which will shift to the position opposite 2-deoxyribonolactone upon realignment.

The effects of removing individual bypass polymerases illustrate the competition between them for bypassing the LTg tandem lesion. Overall, Pol V is the most proficient at producing full-length products, as well as the least error prone when bypassing thymidine glycol. Pol II and Pol IV behave similarly to one another, and they are more likely to bypass the tandem lesion via a misalignment−realignment mechanism (Scheme 2).

CONCLUSIONS

The repair and replication of various clusters of two or more DNA lesions have been shown to be different from the respective isolated forms of damage. Thus far, tandem lesions have been investigated to a lesser extent than clustered DNA damage as a whole. The mutagenicity of tandem lesions compared to the same isolated damaged nucleotides is variable and depends upon the nature of the modifications as well as their relative orientation. In one instance, inclusion of OxodG in a tandem lesion with an AP site actually reduces the mutagenicity of the former in repair deficient cells (*18*). In this study, we showed that a tandem lesion that results from initial addition of hydroxyl radical to a pyrimidine double bond increases the mutagenicity of thymidine glycol to levels that are greater than that of OxodG. Importantly, the change in mutagenicity is dependent on the 3′-flanking nucleotide and is attributed to the involvement of a misalignment−realignment process that determines the identity of the nucleotide that is ultimately misincorporated opposite thymidine glycol and to some extent 2-deoxyribonolactone. The mutagenicity of the thymidine glycol when part of the tandem lesion is indirect in that it is the upstream nucleotide that directs the bypass polymerase. This is the second example in which a strongly blocking nucleotide gives rise to mutations via a mechanism that involves a misalignment−realignment mechanism. It is possible that other strongly blocking (tandem) lesions may give rise to mutations through a similar mechanism.

ACKNOWLEDGMENT

We thank Professors Steven Frankel and Myron Goodman for providing the bypass polymerase deficient cells.

SUPPORTING INFORMATION AVAILABLE

ESI-MS of oligonucleotides used to prepare genomes. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Dizdaroglu, M., Jaruga, P., Birincioglu, M., and Rodriguez, H. (2002) Free radical-induced damage to DNA: Mechanisms and measurements. *Free Radical Biol. Med. 32*, 1102–1115.
2. Cadet, J., Douki, T., Gasparutto, D., and Ravanat, J. L. (2003) Oxidative Damage to DNA: Formation, Measurement and Biochemical Features. *Mutat. Res. 531*, 5–23.
3. Wang, Y. (2008) Bulky DNA Lesions Induced by Reactive Oxygen Species. *Chem. Res. Toxicol. 21*, 276–281.
4. Delaney, J. C., and Essigmann, J. M. (2008) Biological Properties of Single Chemical-DNA Adducts: A Twenty Year Perspective. *Chem. Res. Toxicol. 21*, 232–252.

5. Neeley, W. L., and Essigmann, J. M. (2006) Mechanisms of Formation, Genotoxicity, and Mutation of Guanine Oxidation Products. *Chem. Res. Toxicol. 19*, 491–505.
6. Dianov, G. L., O'Neill, P., and Goodhead, D. T. (2001) Securing Genome Stability by Orchestrating DNA Repair: Removal of Radiation-Induced Clustered Lesions in DNA. *BioEssays 23*, 745–749.
7. Purkayastha, S., Milligan, J. R., and Bernhard, W. A. (2007) On the Chemical Yield of Base Lesions, Strand Breaks, and Clustered Damage Generated in Plasmid DNA by the Direct Effect of X-rays. *Radiat. Res. 168*, 357–366.
8. Hada, M., and Sutherland, B. M. (2006) Spectrum of complex DNA damages depends on the incident radiation. *Radiat. Res. 165*, 223–230.
9. Lomax, M. E., Gulston, M. K., and O'Neill, P. (2002) Chemical Aspects of Clustered DNA Damage Induction by Ionizing Radiation. *Radiat. Prot. Dosim. 99*, 63–68.
10. Georgakilas, A. (2008) Processing of DNA Damage Clusters in Human Cells: Current Status of Knowledge. *Mol. BioSyst. 4*, 30–35.
11. Kozmin, S. G., Sedletska, Y., Reynaud-Angelin, A., Gasparutto, D., and Sage, E. (2009) The Formation of Double-strand Breaks at Multiply Damaged Sites is Driven by the Kinetics of Excision/Incision at Base Damage in Eukaryotic Cells. *Nucleic Acids Res. 37*, 1767–1777.
12. Paap, B., Wilson, D. M.III, and Sutherland, B. M. (2008) Human Abasic Endonuclease Action on Multilesion Abasic Clusters: Implications for Radiation-induced Biological Damage. *Nucleic Acids Res. 36*, 2717–2727.
13. Lomax, M. E., Salje, H., Cunniffe, S., and O'Neill, P. (2005) 8-OxoA Inhibits the Incision of an AP Site by the DNA Glycosylases Fpg. Nth and the AP Endonuclease HAP1. *Radiat. Res. 163*, 79–84.
14. Budworth, H., Matthewman, G., O'Neill, P., and Dianov, G. L. (2005) Repair of Tandem Base Lesions in DNA by Human Cell Extracts Generates Persisting Single-strand Breaks. *J. Mol. Biol. 351*, 1020–1029.
15. Pearson, C. G., Shikazono, N., Thacker, J., and O'Neill, P. (2004) Enhanced Mutagenic Potential of 8-Oxo-7,8-dihydroguanine When Present Within a Clustered DNA Damage Site. *Nucleic Acids Res. 32*, 263–270.
16. Blaisdell, J. O., and Wallace, S. S. (2001) Abortive base-excision repair of radiation-induced clustered DNA lesions in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 98*, 7426–7430.
17. Jiang, Y., Wang, Y., and Wang, Y. (2009) In vitro replication and repair studies of tandem lesions containing neighboring thymidine glycol and 8-oxo-7,8-dihydro-2′-deoxyguanosine. *Chem. Res. Toxicol. 22*, 574–583.
18. Cunniffe, S. M. T., Lomax, M. E., and O'Neill, P. (2007) An AP Site Can Protect Against the Mutagenic Potential of 8-oxoG When Present Within a Tandem Clustered Site in *E. coli*. *DNA Repair 6*, 1839–1849.
19. Jiang, Y., Hong, H., Cao, H., and Wang, Y. (2007) In Vivo Formation and in Vitro Replication of a Guanine-Thymine Intrastrand Cross-Link Lesion. *Biochemistry 46*, 12757–12763.
20. Kalam, M. A., and Basu, A. K. (2005) Mutagenesis of 8-Oxoguanine Adjacent to an Abasic Site in Simian Kidney Cells: Tandem Mutations and Enhancement of G to T Transversions. *Chem. Res. Toxicol. 18*, 1187–1192.
21. von Sonntag, C. (1987) The Chemical Basis of Radiation Biology , Taylor & Francis, London.
22. Box, H. C., Patrzyc, H. B., Dawidzik, J. B., Wallace, J. C., Freund, H. G., Iijima, H., and Budzinski, E. E. (2000) Double base lesions in DNA X-irradiated in the presence or absence of oxygen. *Radiat. Res. 153*, 442–446.
23. Douki, T., Riviere, J., and Cadet, J. (2002) DNA Tandem Lesions Containing 8-Oxo-7,8-dihydroguanine and Formamido Residues Arise from Intramolecular Addition of Thymine Peroxyl Radical to Guanine. *Chem. Res. Toxicol. 15*, 445–454.
24. Bellon, S., Ravanat, J.-L., Gasparutto, D., and Cadet, J. (2002) Cross-Linked Thymine-Purine Base Tandem Lesions: Synthesis, Characterization, and Measurement in γ-Irradiated Isolated DNA. *Chem. Res. Toxicol. 15*, 598–606.
25. Zhang, Q., and Wang, Y. (2005) Generation of 5-(2′-deoxycytidyl)methyl radical and the formation of intrastrand cross-link lesions in oligodeoxyribonucleotides. *Nucleic Acids Res. 33*, 1593–1603.
26. Romieu, A., Bellon, S., Gasparutto, D., and Cadet, J. (2000) Synthesis and UV Photolysis of Oligodeoxynucleotides That Contain 5-(Phenylthiomethyl)-2′-deoxyuridine: A Specific Photolabile Precursor of 5-(2′-Deoxyuridilyl)methyl Radical. *Org. Lett. 2*, 1085–1088.
27. Greenberg, M. M., Barvian, M. R., Cook, G. P., Goodman, B. K., Matray, T. J., Tronche, C., and Venkatesan, H. (1997) DNA Damage Induced via 5,6-Dihydrothymidin-5-yl in Single-Stranded Oligonucleotides. *J. Am. Chem. Soc. 119*, 1828–1839.
28. Hong, I. S., Carter, K. N., Sato, K., and Greenberg, M. M. (2007) Characterization and Mechanism of Formation of Tandem Lesions in DNA by a Nucleobase Peroxyl Radical. *J. Am. Chem. Soc. 129*, 4089–4098.
29. Carter, K. N., and Greenberg, M. M. (2003) Tandem Lesions are the Major Products Resulting from a Pyrimidine Nucleobase Radical. *J. Am. Chem. Soc. 125*, 13376–13378.
30. Tallman, K. A., and Greenberg, M. M. (2001) Oxygen-Dependent DNA Damage Amplification Involving 5,6-Dihydrothymidin-5-yl in a Structurally Minimal System. *J. Am. Chem. Soc. 123*, 5181–5187.
31. Tallman, K. A., Tronche, C., Yoo, D. J., and Greenberg, M. M. (1998) Release of Superoxide from Nucleoside Peroxyl Radicals, a Double-Edged Sword?. *J. Am. Chem. Soc. 120*, 4903–4909.
32. Emanuel, C. J., Newcomb, M., Ferreri, C., and Chatgilialoglu, C. (1999) Kinetics of 2′-Deoxyuridin-1′-yl Radical Reactions. *J. Am. Chem. Soc. 121*, 2927–2928.
33. Hashimoto, M., Greenberg, M. M., Kow, Y. W., Hwang, J.-T., and Cunningham, R. P. (2001) The 2-Deoxyribonolactone Lesion Produced in DNA by Neocarzinostatin and Other DNA Damaging Agents Forms Cross-links with the Base-Excision Repair Enzyme Endonuclease III. *J. Am. Chem. Soc. 123*, 3161–3162.
34. DeMott, M. S., Beyret, E., Wong, D., Bales, B. C., Hwang, J.-T., Greenberg, M. M., and Demple, B. (2002) Covalent Trapping of Human DNA Polymerase β by the Oxidative DNA Lesion 2-Deoxyribonolactone. *J. Biol. Chem. 277*, 7637–7640.
35. Huang, H., and Greenberg, M. M. (2008) Hydrogen Bonding Contributes to the Selectivity of Nucleotide Incorporation Opposite an Oxidized Abasic Lesion. *J. Am. Chem. Soc. 130*, 6080–6081.
36. Kroeger, K. M., Jiang, Y. L., Kow, Y. W., Goodman, M. F., and Greenberg, M. M. (2004) Mutagenic Effects of 2-Deoxyribonolactone in *Escherichia coli*. An Abasic Lesion That Disobeys the A-Rule. *Biochemistry 43*, 6723–6733.
37. Taylor, J.-S. (2002) New Structural and Mechanistic Insight Into the A-Rule and the Instructional and Non-Instructional Behavior of DNA Photoproducts and Other Lesions. *Mutat. Res. 510*, 55–70.
38. Imoto, S., Bransfield, L. A., Croteau, D. L., Van Houten, B., and Greenberg, M. M. (2008) DNA Tandem Lesion Repair by Strand Displacement Synthesis and Nucleotide Excision Repair. *Biochemistry 47*, 4306–4316.
39. Kotera, M., Roupioz, Y., Defrancq, E., Bourdat, A.-G., Garcia, J., Coulombeau, C., and Lhomme, J. (2000) The 7-nitroindole nucleoside as a photochemical precursor of 2′-deoxyribonolactone: Access to DNA fragments containing this oxidative abasic lesion. *Chem.—Eur. J. 6*, 4163–4169.
40. Iwai, S. (2001) Synthesis and Thermodynamic Studies of Oligonucleotides Containing the Two Isomers of Thymine Glycol. *Chem.—Eur. J. 7*, 4343–4351.
41. Neeley, W. L., Delaney, J. C., Henderson, P. T., and Essigmann, J. M. (2004) In vivo Bypass Efficiencies and Mutational Signatures of the Guanine Oxidation Products 2-Aminoimidazolone and 5-Guanidino-4-nitroimidazole. *J. Biol. Chem. 279*, 43568–43573.
42. Delaney, J. C., and Essigmann, J. M. (1999) Context-Dependent Mutagenesis by DNA Lesions. *Chem. Biol. 6*, 743–753.
43. Delaney, J. C., and Essigmann, J. M. (2006) Assays for Determining Lesion Bypass Efficiency and Mutagenicity of Site-Specific DNA Lesions In Vivo. *Methods Enzymol. 408*, 1–15.
44. Carter, K. N., and Greenberg, M. M. (2001) Direct Measurement of Pyrimidine C6-hydrate Stability. *Bioorg. Med. Chem. 9*, 2341–2346.
45. Brown, K. L., Adams, T., Jasti, V. P., Basu, A. K., and Stone, M. P. (2008) Interconversion of the cis-5R,6S- and trans-5R,6R-Thymine Glycol Lesions in Duplex DNA. *J. Am. Chem. Soc. 130*, 11701–11710.
46. Kroeger, K. M., Kim, J., Goodman, M. F., and Greenberg, M. M. (2004) Effects of the C4′-Oxidized Abasic Site on Replication in *Escherichia coli*. An Unusually Large Deletion Is Induced by a Small Lesion. *Biochemistry 43*, 13621–13627.
47. Kroeger, K. M., Goodman, M. F., and Greenberg, M. M. (2004) A Comprehensive Comparison of DNA Replication Past 2-Deoxyribose and its Tetrahydrofuran Analog in *Escherichia coli*. *Nucleic Acids Res. 32*, 5480–5485.
48. Strauss, B. S. (1991) The 'A Rule' of Mutagen Specificity: A Consequence of DNA Polymerase Bypass of Non-Instructional Lesions?. *BioEssays 13*, 79–84.
49. Radford, I. R., and Lobachevsky, P. N. (2008) Clustered DNA lesion sites as a source of mutations during human colorectal tumourigenesis. *Mutat. Res., Fundam. Mol. Mech. Mutagen. 646*, 60–68.
50. Shikazono, N., Pearson, C., O'Neill, P., and Thacker, J. (2006) The Roles of Specific Glycosylases in Determining the Mutagenic

Consequences of Clustered DNA Base Damage. *Nucleic Acids Res. 34*, 3722–3730.
51. Colis, L. C., Raychaudhury, P., and Basu, A. K. (2008) Mutational specificity of γ-radiation-induced guanine-thymine and thymine-guanine intrastrand cross-links in mammalian cells and translesion synthesis past the guanine-thymine lesion by human DNA polymerase η. *Biochemistry 47*, 8070–8079.
52. Hong, H., Cao, H., and Wang, Y. (2007) Formation and Genotoxicity of a Guanine-cytosine Intrastrand Cross-link Lesion *In Vivo*. *Nucleic Acids Res. 35*, 7118–7127.
53. Basu, A. K., Loechler, E. L., Leadon, S. A., and Essigmann, J. M. (1989) Genetic Effects of Thymine Glycol: Site-Specific Mutagenesis and Molecular Modeling Studies. *Proc. Natl. Acad. Sci. U.S.A. 86*, 7677–7681.
54. Hayes, R. C., Petrullo, L. A., Huang, H., Wallace, S. S., and LeClerc, J. E. (1988) Oxidative damage in DNA. Lack of mutagenicity by thymine glycol lesions. *J. Mol. Biol. 201*, 239–246.
55. Fang, H., and Taylor, J.-S. (2008) Serial analysis of mutation spectra (SAMS): A new approach for the determination of mutation spectra of site-specific DNA damage and their sequence dependence. *Nucleic Acids Res. 36*, 6004–6012.
56. Kroeger, K. M., Kim, J., Goodman, M. F., and Greenberg, M. M. (2006) Replication of an Oxidized Abasic Site in *Escherichia coli* by a dNTP-Stabilized Misalignment Mechanism that Reads Upstream and Downstream Nucleotides. *Biochemistry 45*, 5048–5056.
57. Wood, M. L., Esteve, A., Morningstar, M. L., Kuziemko, G. M., and Essigmann, J. M. (1992) Genetic Effects of Oxidative DNA Damage: Comparative Mutagenesis of 7,8-Dihydro-8-oxoguanine and 7,8-Dihydro-8-oxoadenine in *Escherichia coli*. *Nucleic Acids Res. 20*, 6023–6032.
58. Henderson, P. T., Delaney, J. C., Gu, F., Tannenbaum, S. R., and Essigmann, J. M. (2002) Oxidation of 7,8-Dihydro-8-oxoguanine Affords Lesions That Are Potent Sources of Replication Errors in Vivo. *Biochemistry 41*, 914–921.
59. Henderson, P. T., Delaney, J. C., Muller, J. G., Neeley, W. L., Tannenbaum, S. R., Burrows, C. J., and Essigmann, J. M. (2003) The Hydantoin Lesions Formed From Oxidation of 7,8-Dihydro-8-oxoguanine Are Potent Sources of Replication Errors in Vivo. *Biochemistry 42*, 9257–9262.
60. Patro, J. N., Wiederholt, C. J., Jiang, Y. L., Delaney, J. C., Essigmann, J. M., and Greenberg, M. M. (2007) Studies on the Replication of the Ring Opened Formamidopyrimidine, Fapy·dG in *Escherichia coli*. *Biochemistry 46*, 10202–10212.