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The Mutagenicity of Thymidine Glycol in *E. coli* is Increased When Part of a Tandem Lesion &

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Abstract

Tandem lesions are comprised of two contiguously damaged nucleotides. Tandem lesions are 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 *E. 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 Pol V.

Keywords

DNA damage; tandem lesions; clustered lesions; mutagenesis

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 of the importance of a family of lesions known as clustered lesions whose biochemical effects are less well understood (6). Clustered lesions are defined as 2 or more damage sites within 1-2 turns of DNA. They are often associated with ionizing radiation because of the high energy flux and correspondingly large localized concentration of hydroxyl radical that DNA is exposed to 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, are a subset of clustered lesions.

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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 carried out 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 manuscript we describe how the mutagenicity in *E. coli* of a tandem lesion that is derived from a single chemical event is different than that of either lesion alone.

Nucleobase radicals are 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 hydroxyl radical addition to the pyrimidine double bond, which occurs preferentially at the more electron rich C5-position of the pyrimidine. The respective peroxy 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 (peroxy) 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 peroxy radicals add to the double bonds of the adjacent 5'- and 3'-nucleotides. In at least some instances the peroxy 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) (Scheme 1) (28-32). In one system, the L containing lesion was found to account for 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 dG incorporation 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 5'-LTg. Instead, the tandem lesion is repaired by nucleotide excision repair and long patch BER. Herein, we describe the replication of single stranded plasmids containing 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, Bbs1, 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 carried out using a Storm

840 Phosphorimager and ImageQuant 5.1 software. The data presented in Tables 1 - 3 and Figures 1-6 are the average of 2-3 experiments. Each experiment consists of 3 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 (100 μ L, 2 $\times$). The wash was combined with the supernatant and concentrated. The pellet was resuspended in 250 μ L of a 1.4 M hydrofluoric acid (HF) solution (1.5 mL N-methylpyrrolidinone, 750 μ L triethylamine (TEA), 1.0 mL TEA-HF) at 65 $^{\circ}$ C for 3 h. The solution was quenched with 3 M NaOAc (25 μ L), EtOH (1.0 mL), and the solution was kept at -80 $^{\circ}$ C for 1 h. The solution was spun at 13.2 rcf at 4 $^{\circ}$ C for 30 min. The supernatant was decanted and the pellet dried. The pellet was resuspended in 100 μ L formamide loading buffer (95% formamide, 10 mM EDTA) and loaded on a 20 % denaturing PAGE gel (1.5 mm thick).

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 U PNK, 37 $^{\circ}$ C, 1 h) and ligated (1200 U, 16 $^{\circ}$ C, 2 h) into 10 pmol of *EcoRI*-digested plasmid using complementary scaffold (15 pmol). After digestion of the scaffolds with T4 DNA polymerase (16 U, 16 $^{\circ}$ C, 1 h), the vectors were purified by phenol extraction and G-25 Sephadex filtration. When introducing the inserts containing 2-deoxyribonolactone, 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.

In order to examine the bypass rate of plasmids containing different lesions in SOS-induced *E. coli* cell, wild-type (AB1157), polymerase II (STL1336), polymerase IV (Xs-1), polymerase V (SR1157U) and triple knockout cells (SF2108) were grown to an OD₆₀₀ of 0.3, pelleted, and resuspended in 10 mM MgSO₄. The cells were irradiated at 45 J/m², added to 25 mL 2 $\times$ YT, and incubated at 37 $^{\circ}$ C for 45 min. The cells were pelleted, washed with cold water, and resuspended in 10% glycerol. The prepared cells (100 μ L) 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 carried out using the restriction endonuclease and postlabeling (REAP) assay, which has previously been described.(36) Briefly, viral DNA was recovered from the growth medium and PCR amplified. Following digestion with *BbsI* and shrimp alkaline phosphatase, the DNA was ³²P-labeled and further digested with *HaeIII*. The desired 18mer product 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 (NH₄)₂HPO₄ and H₃PO₄, 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 (*Bbs*I) employed by Essigmann and Delaney in their pioneering work (42,43). *Bbs*I binds to 5'-d(GAA GAC) and hydrolyzes the 5'-phosphate 2 nucleotides further downstream. It was shown in this original report that *Bbs*I incision is independent of the identity of the nucleotide at the cleavage site. Determining the outcome of tandem lesion replication requires preparing 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 long oligonucleotide inserts 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**) or thymidine glycol (**1**, **3**, **4**, **6**, **7**, **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 probing L or Tg (e.g. **2** versus **3**) in a given sequence context, there were slight nucleotide differences beyond this point. Genomes containing an isolated Tg (**1**, **4**, **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**, **4**) lesions (data not shown). Tandem lesions were bypassed less than 1% as efficiently than 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).

Formation of single nucleotide and 3-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 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**) examined. These constructs shift the tandem lesion one nucleotide closer to the

5'-terminus of the insert than do inserts designed to probe nucleotide incorporation 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 3 of the 4 sequences examined large increases 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 3 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 3 nucleotide deletions is $\leq 11\%$ in all cell types. The genome produced from insert **2** was the exception. Translesion synthesis in this genome yielded a high level of 3 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 4 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 of 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 5'6d(CLTTgA) sequence (**8**). The combined incorporation frequency of pyrimidines opposite 2-deoxyribonolactone was less than 7% in all sequence contexts examined.

In contrast, the presence of L in the tandem lesion has a significant effect on replication of thymidine glycol in wild type cells. Mutations are not detectable using the REAP assay in genomes constructed from inserts containing an isolated Tg (**1**, **4**, **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**, **6**) to dA (**9**) eliminated the significant level of dC misincorporation (Figure 2). Incorporation of dA opposite thymidine glycol increased from ~88% to greater than 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 relative to what was observed in wild type cells (Figures 2 and 4). Removing Pol II or Pol IV from the cell had a more pronounced effect on nucleotide incorporation opposite 2-deoxyribonolactone (Figure 3). 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 dT incorporation opposite the oxidized abasic site was observed, but the levels were still less than 10%.

Initially, the effect of removing Pol V from the *E. coli* was examined in the same sequence context as above (5'-dC, 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). 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. 2'-Deoxyadenosine incorporation opposite L increased to 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 was carried out of the 3-nucleotide deletions produced in Pol V deficient cells for the 3 genomes that produced this lesion (Table 3). 2'-Deoxyguanosine incorporation opposite the template nucleotide analyzed for was exclusively detected in all 3 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 in order 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 in order to examine nucleotide incorporation opposite L and Tg with the same flanking nucleotides (e.g. **2** versus **3** and **5** versus **6**). These changes did not significantly impact the bypass efficiency. However, the distribution of full-length and deletion products were affected by these proximal but nonadjacent nucleotides. Without exception, sequences designed to identify the nucleotide incorporated opposite Tg (**3**, **6**, **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**, **8**). This was true in 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 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 3-nucleotide deletions (Table 3). Neither Tg nor 2-deoxyribonolactone when present by themselves in the genome gives rise to 3-nucleotide deletions (35,36). C4-AP is the only abasic lesion whose bypass results in 3-nucleotide deletions (46). Assuming that the entire tandem lesion is part of the 3 nucleotides deleted there are two possible stretches of nucleotides that can be deleted. The LTg deletion can be flanked on either its 5'-(5'-NLTg) or 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, 3). This is consistent with studies on 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 3-nucleotide deletion product. Instead, the distribution of 1- and 3-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 dG incorporation 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 greater amount of single nucleotide deletion and lower dG incorporation 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, 4) (35). There was a modest increase in dG incorporation 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 change to an 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 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 4). However, removing Pol V resulted in a dramatic increase of dC incorporation 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 on 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 is 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, 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 2 or more DNA lesions have been shown to behave differently than 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 hydroxyl radical addition to a pyrimidine double bond increases the mutagenicity of thymidine glycol to levels that are greater than 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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
 LTg, 5'-(2-deoxyribonolactone)-thymidine glycol tandem lesion
 Nth, endonuclease III
 Pol β , DNA polymerase β
 REAP, restriction endonuclease and postlabeling

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Figure 1. Nucleotide incorporation opposite 2-deoxyribonolactone (L) in 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.)

Figure 2. Nucleotide incorporation opposite thymidine glycol (Tg) in 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.)

Figure 3. Nucleotide incorporation opposite 2-deoxyribonolactone (L) in Pol II⁻ and Pol IV⁻ cells in full-length product. Oligonucleotide **2** was used to prepare the genome.

Figure 4. Nucleotide incorporation opposite thymidine glycol (Tg) in Pol II⁻ and Pol IV⁻ cells in full-length product. Oligonucleotide **3** was used to prepare the genome.

Figure 5. Nucleotide incorporation opposite 2-deoxyribonolactone (L) in full-length product produced in Pol V⁻ cells. The oligonucleotide insert used to prepare each respective genome is indicated in parentheses. (See Chart 1.)

Figure 6. Nucleotide incorporation opposite thymidine glycol (Tg) in full-length product produced in Pol V⁻ cells. The oligonucleotide insert used to prepare each respective genome is indicated in parentheses. (See Chart 1.)

Scheme 1.

Postulated hydroxyl radical mediated formation of the 5'-LTg tandem lesion.

Scheme 2.

Postulated mechanism for bypass of the 5'-LTg tandem lesion.

- 1** 5'-d(GAA GAC CCT**g** GGC GTC C)
- 2** 5'-d(GAA GAC CCL **Tg**GC GTC C)
- 3** 5'-d(GAA GAC CL**Tg** GGC GTC C)
- 4** 5'-d(GAA GAC CTT**g** GGC GTC C)
- 5** 5'-d(GAA GAC CTL **Tg**GC GTC C)
- 6** 5'-d(GAA GAC TL**Tg** GGC GTC C)
- 7** 5'-d(GAA GAC CTT**g** AGC GTC C)
- 8** 5'-d(GAA GAC CTL **Tg**AC GTC C)
- 9** 5'-d(GAA GAC TL**Tg** AGC GTC C)
- 10** 5'-d(GAA GAC CCL GGC GTC C)
- 11** 5'-d(GAA GAC CTT GGC GTC C)

Chart 1.
Oligonucleotides used to create genomes.

Structures of AP, L, C4-AP, and C2-AP

Structure of OxodG

Table 1Bypass efficiency in SOS-Induced *E. coli*

Sequence ^b	% Bypass (cell type) ^a			
	wild type	Pol II ⁻	Pol IV ⁻	Pol V ⁻
CCTgGG (1)	56.6 ± 1.7	65.6 ± 5.3	57.7 ± 19.5	52.4 ± 5.9
CCLTgG (2)	8.7 ± 0.8	3.9 ± 1.5	2.9 ± 0.9	4.9 ± 0.4
CCTgGG (3)	6.6 ± 0.3	1.4 ± 0.1	2.2 ± 0.7	4.0 ± 0.4
CTTgGG (4)	58.9 ± 4.7	n.d.	n.d.	n.d.
CTLTgG (5)	9.0 ± 0.4	n.d.	n.d.	n.d.
TLTgGG (6)	7.7 ± 0.6	n.d.	n.d.	n.d.
CTTgAG (7)	56.1 ± 1.8	n.d.	n.d.	46.1 ± 2.1
CTLTgA (8)	7.2 ± 1.7	n.d.	n.d.	3.8 ± 0.3
TLTgAG (9)	8.3 ± 0.6	n.d.	n.d.	3.0 ± 0.1
CCLGG (10) ^c	13.2 ± 2.4	7.8 ± 1.0	6.1 ± 0.6	9.3 ± 1.0

^aDetermined by comparing the number of colonies formed to those from a genome constructed from **11**.^bLocal sequence shown and oligonucleotide insert number in parentheses. Bold nucleotide indicates position probed in REAP assay. n.d. = not determined.^cData taken from reference 35.

Table 2

Single nucleotide deletions as a function of cell type

Sequence ^a	% Single nucleotide deletions			
	wild type	Pol II ⁻	Pol IV ⁻	Pol V ⁻
CCTgGG (1)	0	0	0	0
CCLTgG (2)	8.6 ± 0.4	8.8 ± 2.7	10.6 ± 2.2	8.9 ± 0.9
CLTgGG (3)	19.6 ± 5.2	21.9 ± 6.0	22.3 ± 9.0	71.4 ± 8.2
CTTgGG (4)	0	n.d.	n.d.	n.d.
CTLTgG (5)	1.2 ± 0.8	n.d.	n.d.	n.d.
TLTgGG (6)	13.4 ± 2.3	n.d.	n.d.	n.d.
CTTgAG (7)	0	n.d.	n.d.	0
CTLTgA (8)	2.8 ± 1.4	n.d.	n.d.	81.5 ± 0.9
TLTgAG (9)	4.1 ± 2.2	n.d.	n.d.	65.8 ± 9.1

^aLocal sequence shown and oligonucleotide insert number in parentheses. Bold nucleotide indicates position probed in REAP assay. n.d. = not determined

Table 3

Three nucleotide deletions as a function of cell type

Sequence ^a	% Three nucleotide deletions			
	wild type	Pol II ⁻	Pol IV ⁻	Pol V ⁻
CCTgGG (1)	0	0	0	0
CCLTgG (2)	0	9.9 ± 4.9	6.9 ± 4.3	73.1 ± 8.1
CLTgGG (3)	0	2.1 ± 1.5	0	11.1 ± 2.2
CTTgGG (4)	0	0	0	0
CTLTgG (5)	0	n.d.	n.d.	n.d.
TLTgGG (6)	0	n.d.	n.d.	n.d.
CTTgAG (7)	0	0	0	0
CTLTgA (8)	0	n.d.	n.d.	0
TLTgAG (9)	0	n.d.	n.d.	10.2 ± 0.6

^aLocal sequence shown and oligonucleotide insert number in parentheses. Bold nucleotide indicates position probed in REAP assay. n.d. = not determined