Mutagenic Effects of 2-Deoxyribonolactone in *Escherichia coli*. An Abasic Lesion That Disobeys the A-Rule†

Kelly M. Kroeger,‡ Yu Lin Jiang,‡ Yoke Wah Kow,§ Myron F. Goodman,|| and Marc M. Greenberg*,‡

Department of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, Division of Cancer Biology, Department of Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30335, and Department of Biological Sciences and Chemistry, Hedco Molecular Biology Laboratories, University of Southern California, Los Angeles, California 90089-1340

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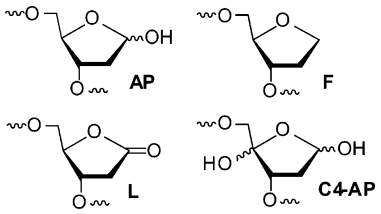
ABSTRACT: Abasic sites are often referred to as noninstructive lesions. The C1′-oxidized abasic site (2-deoxyribonolactone, L) is produced by several DNA damaging agents, including γ-radiolysis and the neocarzinostatin chromophore (NCS). The effects of a C1′-oxidized abasic site incorporated at a defined site in single-stranded plasmid were examined in SOS polymerase-proficient and -deficient *Escherichia coli*. For comparison, experiments utilizing plasmids containing an abasic site (AP) were carried out side by side. In contrast to plasmid containing AP, dA and dG were incorporated most often when plasmid containing L was replicated. The ratio of dG:dA incorporation depended upon local sequence and varied from 0.9 to 2.2. High levels of translesion incorporation of dA are consistent with previous observations that treatment of DNA with the neocarzinostatin chromophore resulted in large amounts of G·C → A·T transitions [Povirk and Goldberg (1986) *Nucleic Acids Res. 14*, 1417] and support the proposal that L is the source of these mutations. Both abasic lesions were 100% lethal in triple knockout cells lacking pol II, pol IV, and pol V. Analysis of translesion synthesis in repair-deficient cells revealed that pol V played a significant role in replication of L and AP. Significant levels of −1 frameshifts were formed in 5′-d(CL) sequences in the presence of pol V and were the exclusive product in pol V-deficient cells. Frameshift products were not formed when the nucleotide on the 5′-side of L was either dT or dG. Deleting pol II or pol IV had only modest effects on replication of L-containing plasmid but significantly decreased the amount of −1 frameshift product formed from an AP lesion. Experiments carried out side by side using otherwise identical plasmids containing an AP site illustrate the distinct properties of these two abasic lesions and that neither should be thought of as noninstructive.

Apurinic/apyrimidinic sites (AP),[1] which can be mutagenic and/or cytotoxic, are produced thousands of times per day in individual cells as a direct consequence of spontaneous hydrolysis and indirectly as a result of chemical modification of nucleobases (*1*, *2*). Oxidized abasic sites (e.g., L and C4-AP) are produced in smaller amounts as a result of reactive



oxygen species and other oxidants. Less is known about the in vivo effects of oxidized abasic lesions (*3*). One of these, 2-deoxyribonolactone (L), is produced during oxidative DNA damage by a variety of agents (Scheme 1) (*4*). For instance, redox-active metal complexes effect the requisite two-electron oxidation via direct hydride abstraction (*5*, *6*). Two sequential one-electron oxidation steps, in which the C1′ radical is formed as an intermediate, are more common. Oxidation can be initiated indirectly by one-electron nucleobase oxidation followed by C1′ deprotonation but more commonly occurs via initial C1′ hydrogen atom abstraction (*7*−*9*). The C1′ hydrogen lies deep within the minor groove and is relatively inaccessible to diffusible species, such as hydroxyl radical (*10*). For this reason, 2-deoxyribonolactone (L) was believed formed in small quantities when DNA is exposed to γ-radiolysis (*3*). However, recent studies have shown that L is formed as the 5′-component of tandem lesions that emanate from nucleobase radicals, which are the major family of species initially formed when DNA is exposed to γ-radiolysis (*11*−*13*). Consequently, the lactone lesion (L) may be formed in larger quantities than previously thought by this very common means of DNA damage.

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* To whom correspondence should be addressed. Phone: 410-516-8095. Fax: 410-516-044. E-mail: mgreenberg@jhu.edu.

‡ Johns Hopkins University.

§ Emory University School of Medicine.

|| University of Southern California.

[1] Abbreviations: C4-AP, C4′-oxidized abasic site; AP, abasic site; L, 2-deoxyribonolactone; F, tetrahydrofuran abasic site model; PAGE, polyacrylamide gel electrophoresis; NCS, neocarzinostatin chromophore; pol II, DNA polymerase II; pol IV, DNA polymerase IV; pol V, DNA polymerase V.

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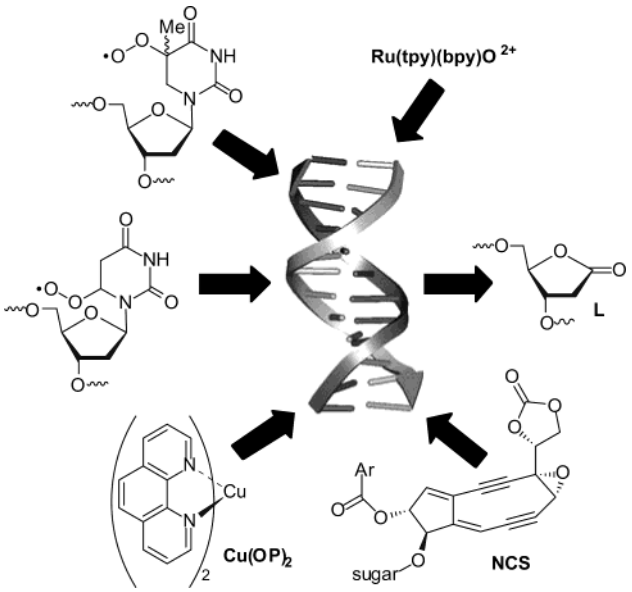
Scheme 1: Examples of DNA Damage Processes That Produce 2′-Deoxyribonolactone (L)



Various antitumor agents, such as the neocarzinostatin chromophore (NCS), overcome the inaccessibility of the C1′ hydrogen by binding in the minor groove (*14*). NCS produces 2-deoxyribonolactone as part of bistranded lesions. Despite the frequent occurrence of L, information regarding its effects in vivo has only been obtained indirectly (*15*, *16*). We now report the results of shuttle vector experiments in which 2-deoxyribonolactone is incorporated in a single-stranded plasmid at a defined site. These experiments reveal distinct differences between the lactone lesion and a regular AP site.

Recently developed methods for the independent synthesis of oligonucleotides containing 2-deoxyribonolactone have made possible significant advances in our understanding of the lesion's effects on DNA structure, reactivity, and in vitro interaction with enzymes (*17*−*20*). Although duplex DNA containing L is comparable in structure to that containing an AP site, the lactone lesion blocks translesion synthesis by Klenow more strongly (*21*, *22*). The C1′-oxidized abasic site follows the "A-rule" established in studies on AP sites (*23*, *24*). However, the preference for incorporation of dA over dG opposite L is smaller than for a template containing AP (or analogue F). Another similarity between 2-deoxyribonolactone (L) and AP is the formation of frameshift products in vitro that are dependent upon the template sequence downstream from the lesion, presumably through a misalignment mechanism (*21*, *22*). The lactone is also one of two DNA lesions known to cross-link to DNA repair enzymes (*25*−*27*). 2-Deoxyribonolactone cross-links to the lysine residue of *Escherichia coli* Nth involved in the lyase step of AP repair (Lys_{120}). Similarly, pol β cross-links 2-deoxyribonolactone following its incision by *Ape*I. Here, too, the dRPase activity is associated with the lysine residue that is involved in Schiff base formation with an AP site, and a mutant protein lacking Lys_{72} is unable to cross-link the lactone.

How these observations translate into what happens in cells containing L is uncertain. Exposure of λ phage to the neocarzinostatin chromophore (NCS), followed by plating on *E. coli*, yielded mutant colonies containing a higher than expected level (based upon random reaction) of G·C → A·T transitions at the deoxycytidine position in 5′-dAGC sequences (*15*). This sequence was known to be a hot spot for neocarzinostatin-induced depyrimidination. The structure of the abasic site formed at the site of deoxycytidine oxidation was uncertain at the time of the phage experiments but was later determined to be 2-deoxyribonolactone (*28*). The observed G·C → A·T mutations are consistent with in vitro studies in which Klenow preferentially incorporates dA opposite the lesion (*21*). Furthermore, exposure of SOS-induced cells to NCS-treated phage indicated that the SOS polymerases now known to be associated with this response played a role in 2-deoxyribonolactone's cellular effects. Although activation of the SOS response had little effect on survival, the mutation frequency increased by at least 1 order of magnitude (*15*). Using these leading results as a guide, we have taken advantage of site-specific generation of 2-deoxyribonolactone in oligonucleotides to unambiguously examine the lesion's mutagenicity in *E. coli* using the M13 shuttle vector.

MATERIALS AND METHODS

General Procedures. Oligonucleotide synthesis was carried out on an Applied Biosystems Inc. 394 DNA synthesizer using standard protocols. Oligonucleotides containing 2-deoxyribonolactone (L) were synthesized as described (*20*, *29*, *30*). Oligonucleotides containing AP sites were generated from 2′-deoxyuridine by UDG treatment, and those containing the THF analogue (F) of an AP site were prepared from commercial reagents (*22*). Commercially available oligonucleotide synthesis reagents were obtained from Glen Research. Purified oligonucleotides were characterized by ESI-MS or MALDI-TOF MS. Electrospray and MALDI-TOF mass spectrometry samples were prepared by ethanol precipitating from NH_4OAc. Electrospray mass spectra were obtained on an LCQ-Duo. MALDI-TOF mass spectra were obtained on a Kratos Seq 5. The oligonucleotides used in these experiments are shown in Table 1. DNA manipulation, including enzymatic labeling, was carried out using standard procedures (*31*). Preparative and analytical oligonucleotide separations were carried out on 20% polyacrylamide denaturing gel electrophoresis [5% cross-link, 45% urea (by weight)]. T4 polynucleotide kinase, *Bbs*I, *Hae*III, and uracil DNA glycosylase (UDG) were obtained from New England Biolabs. T4 DNA polymerase was obtained from USB. *E. coli* cells containing single SOS polymerase knockouts were obtained as previously described (*32*). [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Radioactive samples were quantitated by Cerenkov counting using a Beckman LS6500 liquid scintillation counter. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Storm 840 phosphorimager equipped with ImageQuant version 5.1 software. Oligonucleotide photolyses were carried out in Pyrex tubes (0.25 in. i.d.) using a Rayonet photoreactor (RPR-100) equipped with 16 lamps having a maximum output at 350 nm.

Construction of M13 Genomes. M13 genomes were generated in triplicate for each oligonucleotide insert (**2b**,**d**−**f**, **3b**,**d**,**e**, **4b**,**c**, **5b**,**c**, **6b**,**c**, **7b**,**c**) using a modified version of a previously reported procedure (*33*, *34*). The oligonucleotide insert (6 pmol) was phosphorylated by reacting with ATP (1 mM) and T4 polynucleotide kinase (6 units) in kinase

Table 1: Oligonucleotides Employed in Experiments

5′-d(GAA GAC CT**X** GGC GTC C)
2a-f
a X = **1** d X = **AP**
b X = **L** e X = **T**
c X = **dU** f X = **F**
5′-d(GAA GAC CC**X** GGC GTC C)
3a-e
a X = **1** d X = **AP**
b X = **L** e X = **T**
c X = **dU**
5′-d(GAA GAC CC**X** **Y**GC GTC C)
4-6 (**a-c**)
4 Y = **dA** a X = **1**
5 Y = **dC** b X = **L**
6 Y = **dT** c X = **T**
5′-d(GAA GAC AG**X** GGC GTC C)
7a-c
a X = **1**
b X = **L**
5′-d(GGT CTT CCA CTG AAT CAT GGT CAT AGC)
8
5′-d(TGT CTT CCA CTG AAT CAT GGT CAT AGC)
9
5′-d(AAA ACG ACG GCC AGT GAA TTG GAC GC)
10
5′-H_2N-d(TTTCAC ACA GGA AAC AGC TAT GAC CAT G)
11
5′-H_2N-d(CAG GGT TTT CCC AGT CAC GAC GTT GTA A)
12

buffer (50 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 5 mM DTT, and 1 μg/mL) for 1 h at 37 °C.

M13mp7(L2) (6 pmol for each insert) was incubated with *Eco*RI (12 units) in reaction buffer (100 mM MOPS, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL) for 4 h at 37 °C. The final plasmid concentration was 500 nM. The plasmid was then annealed to scaffolds **8** and **10** (6 pmol each for each insert) by heating at 80 °C for 5 min, followed by slow cooling to 25 °C. Scaffold **9** was substituted for **8** when preparing plasmid for insert **7b**. ATP (100 mM) and T4 DNA ligase (480 units) were added to the annealed vector. This mixture was then added to the respective oligonucleotide insert (6 pmol) in ligase buffer (50 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 110 mM DTT, 1 mM ATP, 25 μg/mL BSA). The samples were incubated at 16 °C for 12 h. After ligation, the scaffolds were digested by adding T4 DNA polymerase (1.6 units) and the appropriate amount of the above buffer in order to maintain concentration of these components. The mixture was incubated for 4 h at 37 °C, at which time the polymerase was deactivated by phenol extraction, and the DNA was purified by Centricon 100 filtration using H_2O to recover the plasmid from the membrane.

To determine the ligation efficiency, 0.5 pmol of plasmid ligation was added to 3% glycerol loading buffer and loaded on a 1% TBE agarose gel. Uncut and linearized M13 vectors (0.5 pmol) were used as markers. After being electrophoresed for 9 h at 100 V, the gel was stained with Sybr Green II for 30 min and then visualized using an 840 Storm Imager.

Preparation of E. coli for Electroporation. Wild-type (K16), pol II minus [STL1336 (SpcR)], pol IV minus [Xs-1 (KanR)], pol V minus [SR1157U (CamR)], and SOS polymerase triple knockout cells [SF2108 (SpcR, KanR, CamR)] were grown overnight from a genetic stock in 10 mL of LB at 37 °C with 270 rpm orbital shaking. A portion of each overnight culture (2.5 mL) was added to LB (250 mL) in a 1 L baffled flask and shaken at 270 rpm (37 °C) until the OD_{600} reached 0.5. The bacteria (175 mL) were decanted into centrifuge bottles, placed on ice for 15−90 min, and spun at 5500 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was placed on ice. The pellet was resuspended in 175 mL of ice-cold H_2O and spun at 8000 rpm for 15 min at 4 °C. The supernatant was discarded, and the pellet was placed on ice and resuspended in 2 mL of ice-cold 10% glycerol.

Electroporation of M13 Genomes into E. coli Cells. The prepared cells (100 μL) were mixed with 1 pmol of ligated M13 plasmid genome on ice and then electroporated (~2.5 kV, 4.74 ms). The electroporated cells were then transferred to a tube (15 mL) containing LB (10 mL). An aliquot (20 μL for the T control plasmid and 100 μL for lesion-containing plasmids) of the solution was added to plating bacteria [375 μL of NR9050 cells, 2.7 mM isopropyl β-D-thiogalactopyranoside (IPTG), 0.07% thiamin, and 4.3 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)] and soft agar (2 mL). This was poured onto B broth plates and incubated overnight at 37 °C. Colonies containing a full-length replicated insert appear blue.

The tubes of electroporated cells in LB were incubated at 37 °C with 270 rpm orbital shaking for 7 h and then centrifuged at 9500 rpm for 10 min at 4 °C. The supernatant, which contained the viral progeny, was decanted into fresh tubes and stored at 4 °C.

Lethality of AP and L in E. coli. The percent survival based on colony count was determined by dividing the number of blue colonies produced from electroporated M13 plasmid containing AP, or L, by the number of blue colonies derived from M13 containing dT instead of a lesion. The 18-nucleotide deletion produced in AP, and L, samples also generated blue colonies. To determine the amount of 18-nucleotide deletion in each sample, the REAP assay was carried out through the PCR step (see below). PCR products were 3′-end labeled using [α-^{32}P]ddATP and terminal deoxynucleotidyl transferase. Aliquots (5 μL) from each sample were mixed with formamide loading buffer (5 μL). Samples were analyzed by 20% denaturing PAGE. The relative amount of the full-length PCR product to the 18-nucleotide deletion PCR product was quantified using ImageQuant 5.1. Lethality was then adjusted to account for the 18-nucleotide deletion and, if present, single-nucleotide deletion which would not produce blue colonies (see Supporting Information).

REAP Assay. The M13 progeny phage (100 μL) was used to infect SCS110 cells (10 mL, 1:10 dilution of an overnight culture) in LB. The samples were incubated at 37 °C with 270 rpm orbital shaking for 7 h and were then centrifuged at 9500 rpm for 10 min at 4 °C. The supernatant (containing

the progeny) was decanted to a clean tube and stored at 4 °C. To isolate the viral progeny from the regrowth, a QIAprep Spin M13 kit was used following the manufacturer's protocol.

The DNA was then amplified by PCR. QIAprep purified DNA (15 μL) was added to 10 μL of the following solution: 5 mM dNTPs, 2.5 μM forward (**11**) and reverse amino modified primers (**12**), and 1.25 units of Turbo PFU in 2.5 × PCR buffer [25 mM KCl, 25 mM $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 8.75, 5 mM $MgSO_4$, 0.25% Triton X-100, and 0.25 mg/mL BSA]. The DNA was amplified using the following protocol: (1) heat to 94 °C for 1 min, (2) 94 °C for 30 s, (3) 58 °C for 1 min, (4) 72 °C for 1 min, (5) repeat steps 2−4 59 times, (6) 72 °C for 5 min, and (7) hold at 4 °C.

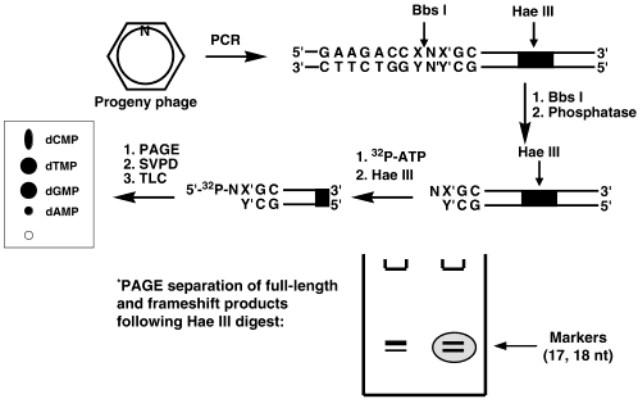
PCR products were phenol extracted to inactivate the exonuclease activity of the polymerase and remove any dNTPs. The aqueous layer (450 μL) was purified using Microcon filters according to the manufacturer's protocol, making a final sample volume of 50 μL.

Purified DNA (10 μL) was then added to 10 μL of reaction buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing *Bbs*I (5 units) and shrimp alkaline phosphatase (1 unit). The samples were incubated at 37 °C for 4 h, heated at 80 °C for 5 min to inactivate the phosphatase, then cooled to 20 °C at a rate of 0.2 °C/s (in a thermal cycler), and stored at 4 °C. The digested DNA (4 μL) was radiolabeled with 2 μL of the following solution: 1.67 μM [γ-^{32}P]ATP, 10 μM cold ATP, and T4 polynucleotide kinase (5 units) in buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). The samples were incubated at 37 °C for 45 min, heated at 65 °C for 20 min, cooled to 23 °C at 0.1 °C/s, and stored at 4 °C. The DNA was then digested with *Hae*III by the addition of 4 μL of buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing *Hae*III (10 units). The solutions were incubated at 37 °C for 2 h and stored at 4 °C. The products were separated in a 20% denaturing PAGE. This enabled the separation of full-length (18 nt) from single-nucleotide deletion (17 nt) products.

Formamide loading buffer (10 μL) was added to each sample (10 μL), and the samples were run on a 20% denaturing gel (0.8 mm thick) for 2.5 h at 550 V. The gels were then exposed to a phosphorimaging screen for 15 min and then visualized using ImageQuant 5.1. The bands corresponding to the full-length and/or deletion products were cut out of the gel and transferred to a small Eppendorf tube. The gel was eluted (200 μL of H_2O) via the crush and soak method for 2 h. The eluted solutions were desalted using Sephadex G25 columns that were freshly equilibrated with H_2O, and the eluent was evaporated to dryness.

DNA was resuspended in 5 μL of 0.05 mg/mL P1 nuclease in 25.5 mM NaOAc, pH 5.3, and 10 mM $ZnCl_2$. The reaction mixture was incubated at 50 °C for 1 h and then stored at 4 °C. TLC plates were soaked in distilled H_2O for 2 min and dried overnight at room temperature before being spotted with 0.5 μL of each sample. After spotting, the plates were air-dried for 50 min before being placed in a tank with buffer [180 mL of saturated $(NH_4)_2HPO_4$ and 18 mL of H_3PO_4]. The TLC plates were run for 10 h and then air-dried for 2 h. The plates were exposed to a phosphorimaging screen for ~24 h, and the relative amounts of each nucleotide were calculated using ImageQuant 5.1.

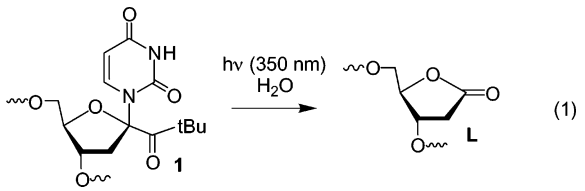
Scheme 2: Description of the REAP Analysis of Mutations in a Single-Stranded Shuttle Vector Showing Separation of Full-Length and Frameshift Products



RESULTS

Plasmid Preparation. Shuttle vectors were generated in M13mp7(L2) plasmid using the design previously reported by Essigmann for use with the restriction endonuclease and postlabeling (REAP) mutation detection system (Scheme 2) (*33*, *34*). The REAP method was attractive to us because it rapidly provides a statistically meaningful readout of nucleotide incorporation across from a lesion. Furthermore, PCR amplification of progeny phage enables mutation frequency analysis to be carried out when survival rates are low. The stringent requirements that progeny contain *Bbs*I and *Hae*III restriction enzyme sites help to reduce introducing artifacts due to foreign sources of DNA. Frameshift products are separated prior to mutation analysis and can be quantitated in the same experiment. The lesion was positioned so that the restriction digest of the duplex PCR product with *Bbs*I yields linearized material containing the nucleotide incorporated in place of the original lesion at the 5′-terminus of one of the strands. Radiolabeling is achieved at the 5′-termini of both strands using kinase, following dephosphorylation of the PCR product. The radiolabeled nucleotide of interest is liberated following a second restriction digest (*Hae*III), isolation of the shorter (18mer) single-stranded oligonucleotide produced, and phosphodiesterase digestion. The identity of this nucleotide in a collection of progeny plasmid is ultimately determined by thin-layer chromatography (TLC) analysis of ^{32}P-labeled nucleotide monophosphates.

Lesions were introduced into the plasmid using 16mers in which the labile 2-deoxyribonolactone (L) or an abasic site (AP) was generated from the appropriate precursor prior to ligation. 2-Deoxyribonolactone was produced from one of three known photochemical precursors (**1**, eq 1). The AP



(1)

site was produced from UDG treatment of an oligonucleotide containing 2′-deoxyuridine (*22*). The respective control

oligonucleotides containing F or thymidine were synthesized directly using commercially available reagents. Ligation was effected after hybridizing 5′-phosphorylated inserts with the appropriate scaffolds. Yields of fully ligated circular plasmid were determined by agarose gel electrophoresis. Ligation yields of several plasmids prepared simultaneously were within a few percent. Control experiments using incompletely ligated plasmid did not yield any product resulting from the insert (see below for details).

Lethality of Abasic Lesions. The percent survival following transfection, which is based upon comparison with a control containing thymidine instead of a lesion in an otherwise identical plasmid, was determined in two steps. The first step involved counting colonies using a blue/white screen. Those colonies containing the insert appear blue. However, frameshifts that do not differ by multiples of three nucleotides (e.g., −1 nucleotide) are undetectable using this screen. These replication products were quantitated using denaturing PAGE following *Hae*III digestion. Separate PAGE analysis of PCR-amplified product revealed an unexpected 18-nucleotide deletion product, which was observable in the progeny phage of all three lesions but not in the thymidine-containing control or a comparable plasmid containing 7,8-dihydro-8-hydroxyguanine (data not shown). The source of this deletion product was investigated using the plasmid containing the chemically stable abasic site F (derived from **2f**) and was found to result from incompletely circularized plasmid. Transfection of *E. coli* with plasmid in which either the 5′- or 3′-terminus or both termini of the insert (**2f**) containing F were unligated produced the 18-nucleotide deletion product but no full-length or −1 frameshift products. The phage lacking 18 nucleotides produced blue colonies, which were accounted for when determining the percent survival of the abasic lesions (L, AP).

The survival of cells transfected with one of the abasic lesions was analyzed in two (AP) or more (L) sequence contexts in wild-type *E. coli* and a variety of repair-deficient cells. The values reported are the average of three replicates carried out simultaneously (Figure 1). Survival of cells transfected with plasmids containing abasic sites is much lower than when thymidine is present in the plasmid. The effects of the abasic lesions on survival of wild-type cells were comparable to one another. A more varied set of plasmids containing L also showed no effect of local sequence on survival in wild-type *E. coli* (Table 2). Although all three abasic lesions were completely lethal to triple knockout cells lacking pol II, pol IV, and pol V (data not shown), there were subtle differences between the lesions in cells lacking a single bypass polymerase. Lesion lethality increased significantly in cells deficient in pol V (Figure 1). However, removing pol II or pol IV had a more modest effect.

Effects of Sequence and Bypass Polymerases on the Relative Yields of Substitution and −1 Frameshift Products. Frameshift products were separated from substitution (full-length) products by PAGE (Figure 2) following restriction with *Hae*III. The identity of the 5′-adjacent nucleotide had a significant effect on whether frameshift products were observed. No frameshift product was detected during replication of 2-deoxyribonolactone (L) plasmids containing 5′-dT or 5′-dG in wild-type *E. coli* (Figure 3, Table 2). However, plasmids containing the sequence 5′-d(CLX), where X is one

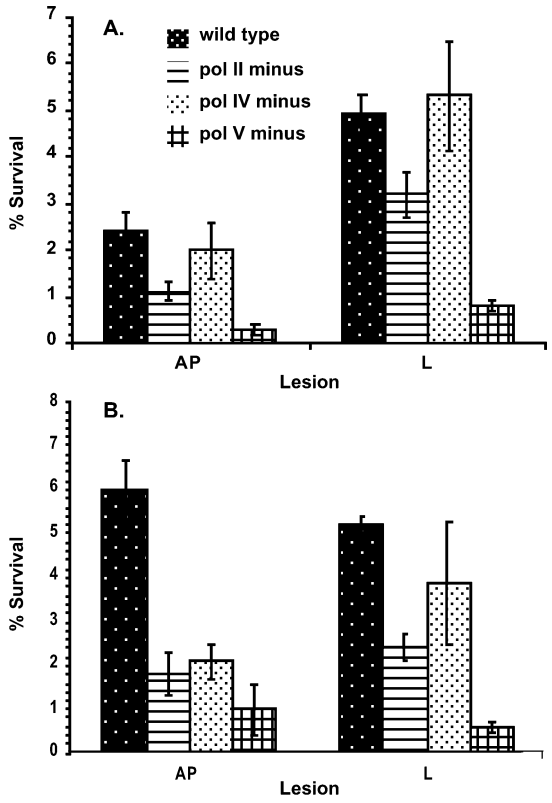


FIGURE 1: Percent survival of wild-type and bypass polymerase-deficient *E. coli* transfected with plasmids containing an abasic site (AP) or 2-deoxyribonolactone (L) when the 5′-adjacent nucleotide is dT (A) or dC (B).

Table 2: Effect of Local Sequence on Survival and Percent Full-Length Product Obtained during Replication of 2-Deoxyribonolactone (L) Plasmid in Wild-Type *E. coli*

local sequence	% survival	% full-length[a]
C-T-L-G	4.9 ± 0.4	100
C-C-L-G	5.2 ± 0.2	85.9 ± 2.1
C-C-L-C	5.6 ± 0.8	78.6 ± 3.0
C-C-L-A	4.9 ± 1.3	79.8 ± 3.4
C-C-L-T	3.9 ± 0.4	65.8 ± 4.2
A-G-L-G (NCS)	5.2 ± 0.8	100

[a] Percent full length is defined in terms of full-length and −1 frameshift products only.

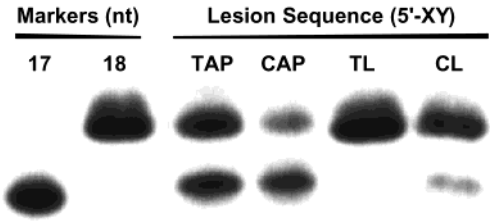


FIGURE 2: Detection of the full-length replication product and single-nucleotide deletion from AP and L replication in wild-type *E. coli*.

of the four native nucleotides, produced between 14% and 34% −1 frameshift product. Abasic sites (AP) produced the largest amount of deletion product in wild-type *E. coli*, and a modest increase in deletion product was detected when the downstream nucleotide was changed from dT to dC.

The fraction of full-length product was affected by deleting individual SOS polymerases (Figure 3). AP and 2-deoxyribonolactone (L) lesions responded differently to individual

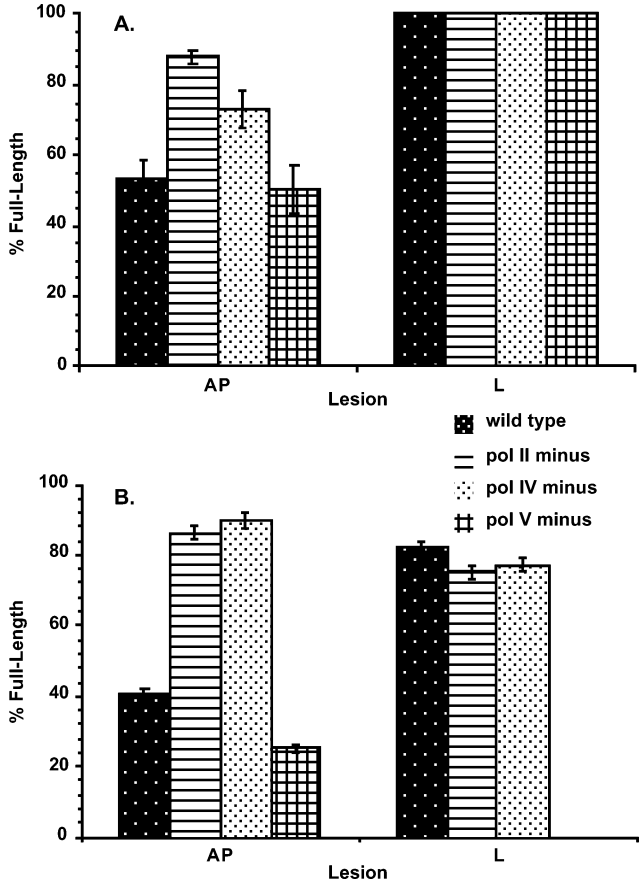


FIGURE 3: Percent full-length replication product formed from AP and L lesions in wild-type and various bypass polymerase-deficient forms of *E. coli* as a function of the 5′-adjacent nucleotide: dT (A); dC (B). (% full length is defined in terms of full-length and −1 frameshift products only.)

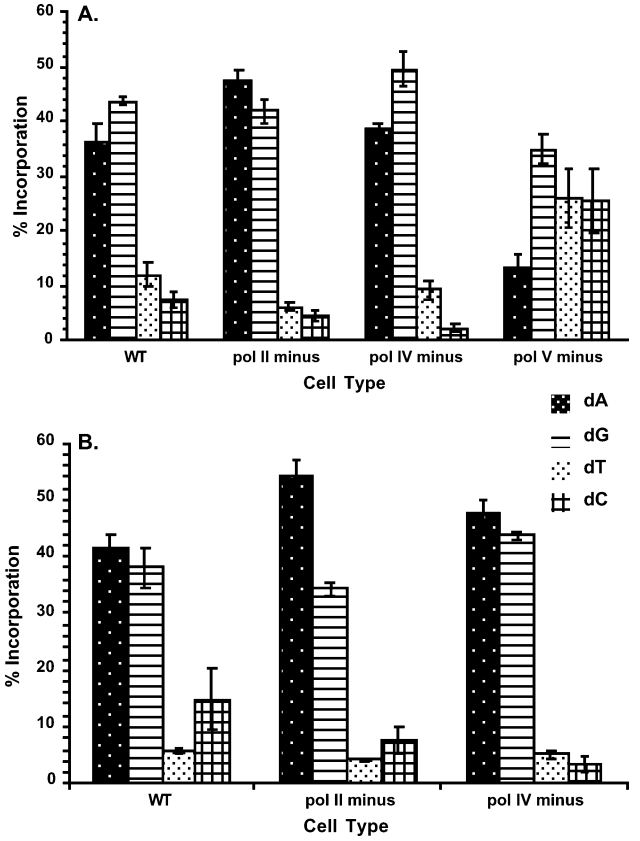


FIGURE 4: Nucleotide incorporation opposite 2-deoxyribonolactone (L) in the full-length replication product formed in wild-type and various bypass polymerase-deficient forms of *E. coli* as a function of the 5′-adjacent nucleotide: dT (A); dC (B).

polymerase deletions. The fraction of full-length product was affected little if at all when L was replicated in pol II- or pol IV-deficient cells; whereas removing pol II or pol IV significantly increased the percent full-length product formed from replication of AP sites. The effect of pol V on 2-deoxyribonolactone replication was dramatic and highly dependent upon the identity of the 5′-adjacent nucleotide (Figure 3). Removing pol V from the cell had no effect on the amount of full-length product when the 5′-adjacent nucleotide was thymidine. However, single nucleotide deletions were the sole product when the plasmid containing 5′-dCL was replicated. In contrast, more modest reductions in the fraction of full-length replication product generated from AP-containing plasmids (5′-dT and 5′-dC) were observed in pol V-deficient *E. coli*.

Translesion Synthesis in Full-Length Products. Differences in the replication of 2-deoxyribonolactone (L) (Figure 4) and an AP site (Figure 5) were readily apparent. The high level of dG incorporation opposite L in 5′-d(TL) and 5′-d(CL) plasmids is most noteworthy. In wild-type *E. coli* the ratio of dG:dA incorporation opposite 2-deoxyribonolactone depended upon the local sequence (Table 3) and varied from 0.9 to almost 2.2. In contrast, replication of the AP lesion in wild-type *E. coli* followed the A-rule regardless of whether dT or dC was present on the 5′-side of the lesion (Figure 5) (*23*, *24*). The behavior of the plasmids containing AP is consistent with previous studies on this lesion or its model (F) (*35*).

The effects of deleting pol II or pol IV on translesion synthesis of lactone-containing plasmids were minor and in accordance with the observed effects on survival and fraction of full-length product. Little change in translesion synthesis was observed upon replication of 5′-d(TL)- and 5′-d(CL)-containing plasmids in *E. coli* lacking either of these SOS polymerases (Figure 4). However, the full-length product formed from the 5′-d(TL) plasmid [full-length product is not formed from the 5′-d(CL) plasmid] in pol V-deficient cells had a significant increase in dC and dT incorporation opposite the lesion, which came mostly at the expense of dA (Figure 4A).

Deleting pol II or pol IV also had modest effects on translesion synthesis opposite AP. REAP analysis of replication of AP-containing plasmid in pol II- or pol IV-deficient cells revealed small increases in translesion incorporation of dA (Figure 5). The increase in percent dA incorporation appeared to come at the expense of pyrimidine incorporation opposite AP. Although deleting pol V insignificantly altered the amount of full-length product formed from replication of plasmids containing an AP site adjacent to thymidine (Figure 3), nucleotide incorporation selectivity opposite the lesion in the presence of a 5′-dT was significantly different than in wild-type *E. coli* (Figure 5A). The progeny produced from the AP site containing a 5′-dT formed in transfected pol V minus cells contained a high level of dC incorporation compared to wild-type *E. coli*. Deoxycytidine incorporation opposite the AP site occurred at the expense of dA and dT. Translesional incorporation of dC in plasmid containing a dC 5′-adjacent to the AP site was higher in pol V-deficient

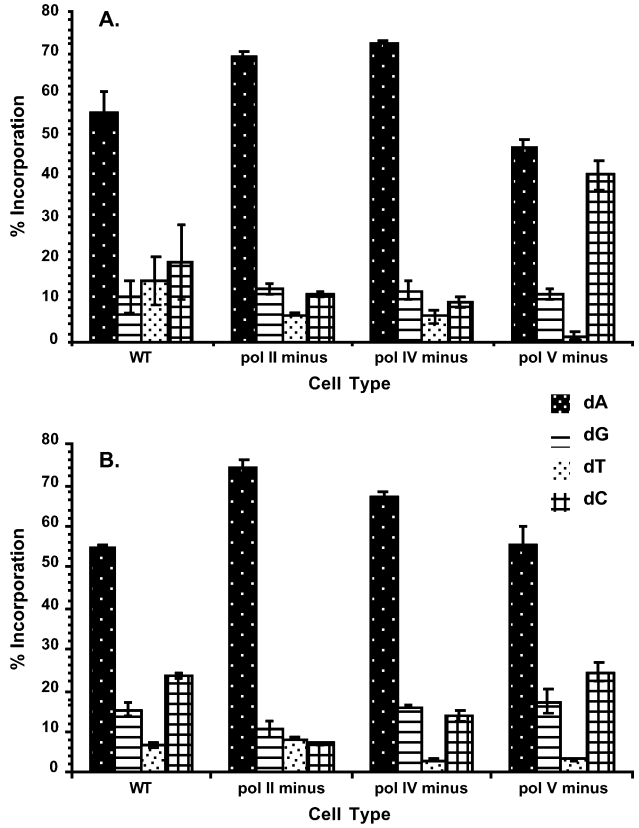


FIGURE 5: Nucleotide incorporation opposite an abasic site (AP) in the full-length replication product formed in wild-type and various bypass polymerase-deficient forms of *E. coli* as a function of the 5′-adjacent nucleotide: dT (A); dC (B).

cells than in the progeny produced in pol II minus or pol IV minus transfected cells. However, it was not statistically different from those produced in wild-type *E. coli*.

DISCUSSION

2-Deoxyribonolactone is formed in DNA by a variety of oxidizing agents. Recent reports suggest that the lesion is produced in higher yields than previously thought when DNA is exposed to γ-radiolysis (*3*, *12*, *13*). In vitro experiments demonstrated that L is unique among abasic lesions in that it forms DNA−protein cross-links with enzymes involved in DNA repair (endonuclease III, DNA polymerase β) (*25*, *26*). The lesion also inhibits DNA replication in vitro by Klenow (*21*). In this regard the lesion is similar to a typical abasic site (AP) (*22*). However, it is worth noting that Klenow exo$^-$ exhibited only a 2-fold preference for dA versus dG incorporation opposite L. In the past, knowledge concerning the effects of L in vivo was inferred from experiments in which the lesion is generated randomly by a DNA damaging agent, such as NCS (*15*, *16*). In this study we utilized established single-stranded shuttle vector methods and the ability to independently synthesize oligonucleotides containing L to determine the effects of the lesion in *E. coli* unambiguously (*19*, *20*, *29*, *33*, *34*). These experiments indicate that the effects of 2-deoxyribonolactone in single-stranded vectors are distinct from those of an AP site.

Abasic sites exhibit high levels of cytotoxicity, which is typically determined by counting colonies that grow after plating the transfection mixture on a lawn of bacteria (*35*, *36*). Colonies are screened utilizing β-galactosidase expression. Plasmids containing the full-length sequence produce blue colonies. In our experiments accurate lethality determination required that we account for a −1 frameshift product, which is not detectable by counting blue colonies. We also accounted for an 18-nucleotide deletion product, which provides a false positive when counting colonies and inflates the true survival rate. This larger deletion is unrelated to the abasic lesions. Control experiments revealed that it is a consequence of incompletely ligated plasmid, which is processed upon electroporation into the *E. coli*. Processing of incompletely ligated shuttle vectors in vivo has been observed previously (*37*). Moreover, a recent report using the REAP method to characterize comparably lethal lesions also accounted for this artifact, albeit in a different manner (*38*). The percent survival was determined by first comparing the number of colonies produced from cells transfected with plasmid containing an abasic site to those in which cells were transfected with a plasmid containing thymidine instead of a lesion. This number was then scaled accordingly after determining the relative amounts of full-length, 1-nucleotide deletion, and 18-nucleotide deletion. The relative amounts of these deletions were determined via PAGE of PCR-amplified plasmid. The AP site and 2-deoxyribonolactone (L) exhibited between ~2% and 6% survival in wild-type *E. coli*. The survival rates observed here are comparable to those in which yeast cells were transfected with oligonucleotides containing AP (*39*, *40*).

The trend toward lower survival of repair-deficient cells transfected with L- or AP-containing plasmid was consistent with previous studies of the latter lesion and its analogue (F) in yeast or *E. coli* (*39*, *40*). However, this trend is not universally observed in all species studied. A recent study in mammalian cells indicated that bypass polymerases were not necessary for high bypass efficiency of F (*41*). Removing pol V consistently had the most deleterious effect on cell survival (Figure 1). The interaction of pol V with F has been characterized in vitro and in vivo (*40*, *42*). However, this is the first report describing the involvement of pol V in replicating DNA containing 2-deoxyribonolactone.

Bypass polymerases also influenced the distribution of full-length and single-nucleotide deletions. The amount of deletion products formed from AP in wild-type *E. coli* was dependent upon flanking sequence (Figure 3). Furthermore, most previous studies in *E. coli* were carried out using the abasic site analogue (F). Hence, direct comparison to previous reports of relative amounts of deletion and full-length replication products is difficult. Ide and Livneh observed 89% and 79% full-length product from plasmids containing the 5′-d(GFG) sequence transfected into wild-type *E. coli* (*36*, *40*). The plasmids used in this study contained the same 3′-adjacent nucleotide and resulted in lower percentages of full-length product when the 5′-nucleotide was dC but comparable amounts of single-nucleotide deletion when a thymidine was present at this position.

The amounts of deletion products obtained in wild-type cells from replication of the AP site were greater than those observed in our studies on 2-deoxyribonolactone. Furthermore, they were significantly higher than an earlier in vivo study on AP lesions in which in one instance the 5′- and 3′-adjacent nuclei were identical (dT and dG, respectively) (*35*). The effects of deleting individual polymerases were

Table 3: Effect of Local Sequence on Translesional Synthesis during Replication of 2-Deoxyribonolactone (L) Plasmid in Wild-Type *E. coli*

	% translesional incorporation				
local sequence	dA	dG	dT	dC	dG:dA
C-T-L-G	36.5 ± 3.1	43.8 ± 0.7	12.1 ± 2.2	7.6 ± 1.6	1.2 ± 0.1
C-C-L-G	41.7 ± 2.0	38.0 ± 3.7	5.7 ± 0.6	14.8 ± 5.5	0.9 ± 0.1
C-C-L-C	27.0 ± 1.7	58.8 ± 1.3	8.2 ± 1.4	5.9 ± 2.5	2.2 ± 0.2
C-C-L-A	29.0 ± 1.0	59.7 ± 0.7	8.3 ± 0.6	3.0 ± 0.3	2.1 ± 0.1
C-C-L-T	37.1 ± 0.4	39.7 ± 1.5	15.9 ± 0.3	7.3 ± 1.6	1.1 ± 0.1
A-G-L-G (NCS)	38.6 ± 4.0	54.8 ± 4.0	2.6 ± 0.1	4.0 ± 0.1	1.4 ± 0.2

different for the two lesions. Deleting either pol II or pol IV significantly increased the fraction of full-length AP replication product to more than 70% from the approximately 40−50% level in wild-type cells. In contrast, replication of AP in pol V minus cells resulted in at most a modest decrease in the percentage of full-length product, depending upon the identity of the 5′-adjacent nucleotide. Direct comparisons to previous in vitro or in vivo experiments are not possible, as we are aware only of experiments addressing the effects of pol II, pol IV, or pol V on replication of DNA containing the AP model site (F).

When the effects of alternative polymerases on lethality and percent full-length product are considered in conjunction with one another, they support the view that pol V, pol II, and pol IV compete for AP lesions (*43*). The data suggest that AP sites are bypassed more efficiently by pol V and that this enzyme is more likely to produce full-length product. In comparison, pol II and pol IV are less capable of rescuing a damaged template containing AP or F and are more likely to produce product containing a frameshift.

Replication of 2-deoxyribonolactone was unlike that of an AP lesion. Higher percentages of full-length product were generally formed. For instance, no deletions were formed from plasmid containing a 5′-dT, and less than 20% deletions are generated during replication of the 5′-d(CL) sequence. Furthermore, unlike replication of the AP sites, deleting pol II or pol IV had little or no effect on the distribution of full-length and deletion products. These data also correlate with the lack of an effect of pol II or pol IV deletion on survival of *E. coli* transfected with 2-deoxyribonolactone-containing plasmid (Figure 1). In contrast, the effect of deleting pol V from the cells on L replication was dramatic and strongly affected by sequence context. When the 5′-adjacent nucleotide was thymidine, no deletion products were observed. In contrast, replication of the 5′-d(CL) plasmid in cells lacking pol V produced 100% single-nucleotide deletion products. We believe that the distribution of full-length and deletion products is strongly influenced by the 5′-adjacent nucleotide and is related to the nucleotide incorporated opposite the lactone abasic site.

The REAP method allows one to measure millions of translesion synthesis events in a single experiment, rapidly providing a statistically meaningful sampling of data (*33*, *34*). The product distribution obtained from translesion synthesis of a template containing L in wild-type *E. coli* was consistently comprised of greater than 75% dA and dG in six different local sequence contexts (Table 3, Figure 4). Deoxyguanosine incorporation was preferred over dA in five of the six replicated plasmids. Although significant amounts of dA are incorporated opposite 2-deoxyribonolactone, it would be inappropriate to suggest that replication of this lesion follows the A-rule. Given that incorporation frequencies opposite a lesion can vary from system to system, it was important to carry out experiments on AP-containing plasmids simultaneously. Analysis of these data (Figure 5) provided the expected preferential incorporation of dA. The adherence to the A-rule (*23*) was consistent with literature precedent for other in vivo studies on AP (or F) lesions in *E. coli* (*35*, *36*, *38*, *40*). Moreover, they clearly show that the in vivo replication of a single-stranded vector containing 2-deoxyribonolactone in *E. coli* is distinct from a typical abasic site.

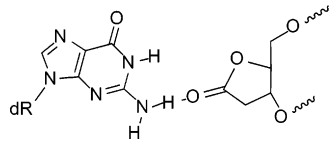


FIGURE 6: Possible hydrogen bonding between 2-deoxyribonolactone and 2′-deoxyguanosine.

The explanation for why 2-deoxyribonolactone induces higher levels of dG incorporation opposite itself compared to the AP lesion is uncertain. Structural studies on duplexes containing various abasic lesions, including L, do not reveal significant differences that would predict this (*44*−*48*). However, a fundamental difference between L and AP lesions is that the former presents a hydrogen bond acceptor (C1 carbonyl) when the lesion is intrahelical, whereas an AP site presents a hydrogen bond donor to a polymerase. We suggest that the elevated level of dG incorporation opposite 2-deoxyribonolactone is attributable to hydrogen bonding between the N2-amino group and the lactone carbonyl (Figure 6). Admittedly, this hypothesis is compromised by the fact that the tetrahydrofuran analogue (F) has no functional group capable of hydrogen bond participation at all, and it exhibits mutational frequencies comparable to that of an AP lesion (*33*, *34*, *41*). Further experiments are needed to elucidate the structural basis of this effect.

Although we found that the preference of nucleotide incorporation opposite L is quite different than opposite AP, deleting pol II or pol IV from cells had a small effect on translesion synthesis of both lesions. Translesion incorporation of dA generally, but not universally, increased at the expense of the pyrimidines. In contrast, deleting pol V had a large effect on 2-deoxyribonolactone translesion synthesis (Figure 4). Translesional incorporation of thymidine or dC increased 2−3-fold in the plasmid where full-length product was formed (5′-dT). This occurred mostly at the expense of dA incorporation, which decreased almost 3-fold. The apparent preference for pol V-mediated dA incorporation opposite 2-deoxyribonolactone is consistent with preferential incorporation of this nucleotide opposite F in vitro (*42*). Deoxyguanosine incorporation opposite L in full-length product decreased more modestly when any of the SOS

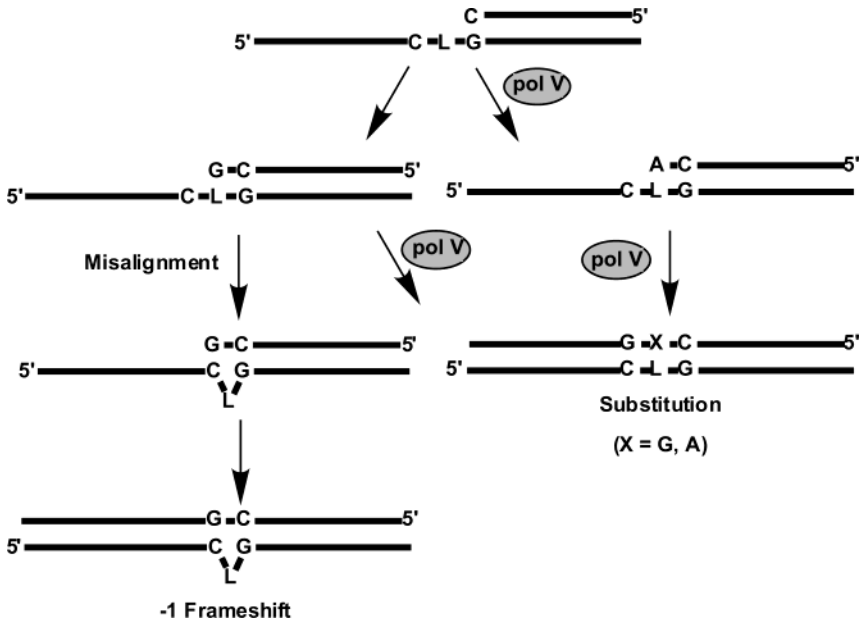


FIGURE 7: Misalignment mechanism for the formation of the −1 frameshift product during the replication of 2-deoxyribonolactone (L) within 5′-d(CLG).

polymerases were deleted and suggests that either pol I or pol III is mainly responsible for its incorporation opposite the lesion. The SOS polymerase(s) is (are) required for extension past the lesion in order to avoid −1 frameshift formation. Alternatively, one could propose that pol II and/or pol IV takes up the mantle for dG incorporation opposite the lactone in the absence of pol V. With no evidence supporting this explanation at this time, Ockham's razor would suggest the former as the more likely explanation.

Deleting pol V had a similar effect on translesion synthesis of AP in plasmid containing a 5′-adjacent thymidine but not dC (Figure 5). Translesion incorporation of dC opposite AP in the template containing 5′-dT increased significantly at the expense of dA. Local sequence [5′-d(TXG), X = AP] does not provide an explanation for increased dC incorporation in pol V minus cells, nor are we aware of any experiments with isolated polymerases (e.g., pol II and pol IV) which show increased dC incorporation opposite an AP site (*49*). However, unexpected results in shuttle vector experiments are not unheard of. For instance, thymidine was incorporated 39% of the time opposite F in a 5′-d(GFG) sequence in wild-type *E. coli* (*36*). One possible explanation that is consistent with literature precedent is that nucleotide incorporation opposite L is inhibited, resulting in slippage and insertion of a second dC opposite the 3′-template dG (*50*). Upon realignment this results in a dC opposite the lesion.

A misalignment mechanism can explain the formation of −1 frameshift products during the replication of 2-deoxyribonolactone (Figure 7). The identity of the nucleotide(s) incorporated opposite the lesion in the single-nucleotide deletion product is not readily determined in the REAP experiment, but the misalignment mechanism is consistent with observations. The first observation is that −1 frameshift occurs when the 5′-adjacent nucleotide is dC but not dT or dG (Table 2). In addition, there is a high level of dG incorporation opposite L in the full-length product. We propose that the misalignment mechanism is driven in by the greater strength of a dG·dC base pair compared to dA·dT, resulting in deletion product from the 5′-d(CL) plasmids. The identity of the nucleotide bonded to the 3′-phosphate of L plays a small role (Table 2). A combination of base stacking and the strength of the 3′-adjacent base pair may influence the amount of deletion product. On the 3′-side the weaker hydrogen bonding and poorer base stacking of the dT·dA base pair present in the plasmid containing the 5′-d(CLT) sequence gives rise to the largest amount of −1 frameshift product. Previously, a correlation was observed between thermal stability of duplexes containing F and their digestion by endo IV (*51*). The less stable duplexes gave rise to lower rates of hydrolysis. In our situation, the less thermally stable duplex may be expected to be most prone to misalignment. This explanation is speculative, and we also cannot rule out a kinetic role in which primer extension past the lesion is faster when a 5′-dT is present in the template. The data also indicate that pol V plays a role in extension by preventing misalignment. Although the level of dG incorporation opposite L in the 5′-d(TLG) plasmid is only modestly affected by deleting this polymerase, deletions are the sole product formed from the 5′-d(CLG) plasmid in pol V minus cells.

CONCLUSIONS

The effects of the commonly formed oxidized abasic lesion, 2-deoxyribonolactone (L), have been examined for the first time by combining a shuttle vector method (REAP) with the ability to independently synthesize oligonucleotides containing L. The effects of L in *E. coli* are distinct from those of a typical AP site. The most notable difference is that large amounts of dG are incorporated opposite the lactone, whereas replication of the AP lesion follows the A-rule. A structural explanation for these differences is unavailable at this time, but the difference in hydrogen bonding groups at the C1 position in 2-deoxyribonolactone (acceptor) and AP (donor) lesions is an attractive starting point for future investigation. In addition, significant levels of −1 frameshift products are observed in wild-type *E. coli* when in 5′-d(CLX) sequences, where X is a native deoxy-

ribonucleotide. The propensity for translesional dG incorporation and the strength of the dG·dC base pair are believed to be the driving force behind formation of single-nucleotide deletion products.

These experiments also substantiate previous proposals concerning the mutagenicity of NCS. Previously, the dC of 5′-d(AGC) sequences was shown to account for the majority of dG·dC to dA·dT transitions in the cI gene of λ phage exposed to NCS (*15*, *16*). In addition, a very small number of deletions were detected in the phage studies. It was subsequently realized that the nucleotide damaged by NCS was a hot spot for 2-deoxyribonolactone formation (*28*). We showed that no deletions are formed from replication of L in this sequence and that the lesion is a source for dC → dT transitions (Tables 2 and 3). Fortuitously, the propensity for L to induce dG incorporation translesionally reduces the mutagenic effects of the lesions produced by the neocarzinostatin chromophore in 5′-d(AGC) sequences. Other agents that produce the lactone, such as γ-radiolysis, do not exhibit such sequence preference, and formation of 2-deoxyribonolactone will likely be more mutagenic in these instances. Our experiments also show that the error-prone polymerase pol V contributes to translesional incorporation of dA during replication of a template containing 2-deoxyribonolactone.

Finally, AP sites are often referred to as noninstructive lesions. Although it is certainly true that AP lesions cannot form Watson−Crick- type base pairs with other nucleotides, the experiments presented above indicate that these molecules interact with polymerase enzymes in different ways. In the overall context of their effects on *E. coli*, it appears that abasic lesions are instructive. It is reasonable to conclude that structural variations between 2-deoxyribonolactone (L) and abasic lesions (AP) are the source of these differences.

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SUPPORTING INFORMATION AVAILABLE

Description of the method and equations used for determining lethality. This material is available free of charge via the Internet at http://pubs.acs.org.

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