2′-Deoxyribonolactone lesion produces G→A transitions in Escherichia coli

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ABSTRACT

2′-Deoxyribonolactone (dL) is a C1′-oxidized abasic site damage generated by a radical attack on DNA. Numerous genotoxic agents have been shown to produce dL including UV and γ-irradiation, ene-dye antibiotics etc. At present the biological consequences of dL present in DNA have been poorly documented, mainly due to the lack of method for introducing the lesion in oligonucleotides. We have recently designed a synthesis of dL which allowed investigation of the mutagenicity of dL in Escherichia coli by using a genetic reversion assay. The lesion was site-specifically incorporated in a double-stranded bacteriophage vector M13G*1, which detects single-base-pair substitutions at position 141 of the lacZα gene by a change in plaque color. In E.coli JM105 the dL-induced reversion frequency was 4.7 × 10⁻⁵, similar to that of the classic abasic site 2′-deoxyribose (dR). Here we report that a dL residue in a duplex DNA codes mainly for thymidine. The processing of dL in vivo was investigated by measuring lesion-induced mutation frequencies in DNA repair deficient E.coli strains. We showed a 32-fold increase in dL-induced reversion rate in AP endonuclease deficient (xth nfo) mutant compared with wild-type strain, indicating that the Xth and Nfo AP endonucleases participate in dL repair in vivo.

INTRODUCTION

2′-Deoxyribonolactone (dL) is one of the numerous oxidation products formed in DNA. The lesion is produced following radical hydrogen atom abstraction at the 1′ position of 2′-deoxyribose (1). This C1′-oxidized apurinic/apyrimidinic (AP) site has been reported to be produced through a variety of radical processes including the action of anticancer drugs [e.g. the neocarzinostatin chromophore which contains the enediyne residue (2–5), heterocyclic N-oxides of the tirapazamine family (6,7), cationic manganese porphyrins (8,9), copper phenanthroline complex Cu(OP)₂, a chemical nuclease (10–12), or γ-irradiation (13,14) and UV exposure (15–17). One of the main features of this abasic lesion resides in its alkali-labile sensitivity: the lactone leads to DNA strand scission by β and δ elimination processes (18–20) with formation of 5-methylene furanone as an ultimate product (8,10,21–23). For a long time this instability has been a limitation on knowledge of the chemistry and biological consequences of the lesion. To address the major questions concerning the specificity of its repair and its mutagenic potency, the disposal of short synthetic DNAs containing a single dL lesion is of prime importance. If very little is known about dL repair, it has been mainly due to the paucity of synthetic methods. In this context, our laboratory developed an efficient and general synthesis of oligodeoxyribonucleotides containing a dL lesion at a predetermined position in the sequence. This method is based on the use of a 7-nitroindole nucleotide (dNi) as a photo-active precursor of dL (20,24). The modified nucleoside dNi has a maximum absorption at 360 nm and illumination above 320 nm triggers a radical process where an intramolecular 1′-hydrogen abstraction is performed by the nitroindole moiety in its excited state, evolving quantitatively towards dL formation. It should be stressed that irradiation of DNA above 320 nm does not generate photodamage. Such a quantitative triggered method allows the production of oligonucleotide quantities for structural determination (25) and in vitro and in vivo biological studies. Other methods based on the photo-triggered formation of the dL lesion in short oligonucleotides have also been reported (11,15,16,26–31). Despite their lower selectivity and poorer yields, these methods enabled investigations on dL reactivity towards itself and certain DNA repair enzymes (19,32–34). Recently, a new approach based on the photo-activation of a nitroveratryl cyanohydrine motif to generate dL has also been described (35). Our starting hypothesis in this study is based on the structural analogy between dL and 2′-deoxyribose, a regular abasic site, which suggests that the AP site-specific repair enzymes might be involved in dL repair. The AP site is an intermediate of the base excision repair (BER) pathway in which DNA glycosylases excise mispaired and/or modified bases (36,37). The AP site lacks a base and is potentially cytotoxic and/or mutagenic (38,39). This non-informative lesion can lead to nucleotide misincorporation during the replication and transcription processes (40–43). During
processing of the abasic lesions by the BER pathway, the initial step involves AP endonucleases, which incise DNA at the 5' position adjacent to the baseless site. Two major enzymes were identified in Escherichia coli: exonuclease III (Xth), which represents >80% of the total AP endonuclease activity in wild type (WT) (44), and endonuclease IV (Nfo), which accounts for 5–10% of the total AP-endonuclease activity (45,46). If the baseless lesion is refractory to excision repair, it may be encountered by DNA polymerases. Numerous in vitro and in vivo studies of baseless lesions in E.coli came up with the establishment of an ‘A rule’: when a DNA polymerase is confronted with a non-instructional lesion, it tends to insert the dAMP nucleotide opposite the lesion rather than any of the three other nucleotides (47,48). Nevertheless, this ‘A rule’ is not always strictly obeyed. For example, in mammalian cells, a random incorporation of nucleotide opposite 2'-deoxyribobase has been found (49), whereas a dAMP was preferentially incorporated in human cells (50). In a previous study we have shown that the ‘A rule’ was obeyed for in vitro dL translesional synthesis by Klenow fragment exo’ (51). Greenberg and colleagues (32) found that DNA substrates containing a dL lesion are cleaved by human Ape1 and can react with DNA substrates containing a dL lesion are cleaved by human Nth (33), Fpg and human Nei-like protein 1 (hNEIL1) (34) to form DNA–protein covalent cross-links.

In the present study, we extended dL repair to a prokaryotic in vivo system using a bacteriophage-based genetic reversion assay in E.coli. Mutation frequencies and repair pathways for dL and its precursor 7-nitroindole (Ni) were determined and compared with those for 2'-deoxyribose (dR).

### MATERIALS AND METHODS

**Microbiological methods and E.coli strains**

All bacterial genetic procedures were performed as described (52,53); these included manipulation with single-stranded filamentous bacteriophage vectors. Rich medium was LB broth, supplemented when required with 100 μg/ml ampicillin or 50 μg/ml kanamycin or 20 μg/ml chloramphenicol or 10 μg/ml tetracycline. M9 minimal medium was supplemented with thiamine. Electrocompetent E.coli strains were prepared as described (54). Fresh SOC medium was used as the incubation buffer following electroporation (53).

The E.coli strains and the plasmid used in this study are listed in Table 1. AB1157 derivatives containing the Tn10 insertions at specific sites in the genome were constructed by PI transductions of the tetracycline resistance marker from the collection of strains for genetic mapping (55). The E.coli strains BH70F', BH110F' and BH160F' were obtained by direct electrottransfer of plasmid pCJ105 (Cmr') from CJ236 to BH70, BH110 and BH160 strains as described (56). The transformants were selected on media containing two antibiotics: either kanamycin (10 μg/ml) + chloramphenicol (10 μg/ml) or tetracycline (5 μg/ml) + chloramphenicol (10 μg/ml). Phenotypes of all strains were verified on antibiotic selective media and by their ability to propagate the M13 bacteriophage. In addition, the phenotype of the BH110F' strain was verified by plating on LB media containing methyl methanesulfonate at various concentrations (2–20 mM). Double-stranded M13G*1 DNA was a gift from Dr Lawrence A. Loeb (University of Washington, Seattle, WA) (57). Individual dark blue plaques were subcloned on top agar 1% containing isopropyl-β-d-thiogalactopyranoside, 5-bromo 4-chloro 3-indolyl β-d-galactoside (X-gal) and E.coli JM105 cells. An isolated dark blue plaque was grown in 3 ml LB for 12 h and single-stranded DNA was purified as described (53). The purity of the single-stranded DNA was verified by agarose gel electrophoresis.

**Enzymes**

Purification of the E.coli Nfo (endonuclease IV) and UNG (uracil-DNA glycosylase) were performed as described (46,58). Molecular biology products, restriction endonucleases and T4 DNA ligase were purchased from Roche (46,58). Molecular biology products, restriction endonucleases and T4 DNA ligase were purchased from Roche Diagnostics (Meylan, France). T4 polynucleotide kinase was purchased from New England BioLabs (Ozyme, Saint-Quentin en Yvelines, France).

**Oligonucleotides**

Nitroindole nucleoside phosphoramidite was synthesized according to the method described (24). The structures of 2'-deoxyribonitroindole abasic site precursor, 2'-deoxyribonolactone and 2'-deoxyribose are illustrated in Figure 1. Phosphorylated 41mer oligonucleotide dPO3-CTATTNCCGCCAGTGCGXAAATGCGGGATGTGGCTGCAAGGCCG) complementary to positions 117–157 of the lacZ sequence, where X is 2'-deoxycytidine (dC) or 2'-deoxyguanosine (dG) or 2'-deoxyribonitroindole (dNI) or 2'-deoxyuridine (dU), were synthesized by the standard phosphoramidite method with a DNA synthesizer (Perceptive Biosystem 8900 DNA).

### Table 1. Bacterial strains

| Strain | Genotype† | Origin | Source/reference |
|--------|-----------|--------|-----------------
| JM105  | [F', traD36, proA* B', lacIqΔ(lacZM15) endA, thi, rpsL (Str'), endA1, sbcB15 sbcC201 hsdR4 (rC-, mK-), Δ(lac-proAB)] | Laboratory stock | |
| BH110F' | nfo::Kan' [Δ(xth-pncA)90 X::Tn10]pCJ105 (Cmr') | AB1157 | Present study (BW528) |
| BH160F' | nth::Kat' [fps::Kat' X::Tn10]pCJ105 (Cmr') | AB1157 | Present study (BW372) |
| BH70F' | X::Tn10]pCJ105 (Cmr') | AB1157 | Present study |
| BH1100 | recA::Cmr' | JM105 | Laboratory stock |
| BH430 | uvrA' | JM105 | Laboratory stock |
| CJ236 | dna1 ung1 thi1 relA1[pCJ105 (Cmr')] | Laboratory stock | |

*The insertions X::Tn10 are the co-transducible tetracycline resistance markers with fpsg or xth.
Oligonucleotides were separated by denaturing 20% polyacrylamide gel electrophoresis (PAGE) and purified by elution from C18 column with a water–acetonitrile mixture (7:3) as described (53).

Construction of site-specifically modified double-stranded M13G*1

Construction of double-stranded M13G*1 vectors with site-specifically incorporated lesion precursors (dU or dNi) were performed as described (59). Briefly, 10 pmol of the synthetic 5′ phosphorylated 41mer oligonucleotide was hybridized to 0.2 µg single-stranded circular M13G*1 at a 25:1 primer-to-template ratio in 20 mM Tris–HCl, 10 mM MgCl2, 50 mM NaCl pH 7.5 buffer in a final volume of 10 µl. The solution was heated at 90°C for 3 min and then allowed to cool to 30°C before storage in an ice-water bath. The synthesis of the second strand was carried out by adding to the annealing reaction 3 U of T4 DNA polymerase, 3 U of T4 DNA ligase and 3 µl of 10X synthesis buffer containing 5 mM of each dNTP, 10 mM ATP, 100 mM Tris–HCl pH 7.5 and 20 mM dithiothreitol. The synthesis reaction was incubated in an ice bath for 5 min, then at 25°C for 5 min and finally at 37°C for 90 min. The reaction was stopped by adding 90 µl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) at 4°C. The reaction products were analyzed on a 0.8% agarose gel containing 3 µg/ml ethidium bromide in TAE (40 mM Tris–acetate and 1 mM EDTA). The double-stranded DNA was desalted using a Sephadex G50 column (volume 1 ml). Aliquots (1–2 µl) of the desalted phage DNA were used directly for transformation. The blue revertant plaques were screened on media containing X-gal (40 mg/ml) and the nucleotide sequences of the phage DNA from the blue plaques were determined. A sample of M13G*1 containing a single dR residue was prepared by treating 20 ng of double-stranded circular M13G*1 containing a 2′-deoxyuridine at position 141 with 1 nM of the UNG protein at 37°C for 60 min. The M13G*1 containing dL residue was prepared by illuminating the vector containing the nitroindole residue (dNi) at 365 nm for 14 h at 4°C. The illumination device used in this study was obtained from Fisher Bioblock Scientifique (Fisher Scientific SAS, Elancourt Cedex, France) and consisted of an illumination tube and a filter that selected wavelengths ranging from 310 to 400 nm centered at 365 nm (the maximum absorption for dNi is 360 nm) with an energy of 7 mW/cm². The illumination tube is guaranteed to be free from lower wavelengths (particularly 254 nm); under such conditions the main absorbing compound is the Ni residue, and DNA damage which requires more energetic radiations (260 nm for creating thymine dimers and <200 nm for generating reactive oxygen species) are excluded. The conversions dU→dR and dNi→dL was controlled by annealing the 32P-labeled modified primers with their complementary strand and treating the short duplexes with either 0.1 M NaOH at 37°C for 30 min or 100 mM Nfo at 37°C for 10 min. The alkali labile lesions and abasic site residues were then evidenced by denaturing PAGE. A sample of 10 ng of the lesion containing double-stranded M13G*1 phage was used to electroporate a 55 µl suspension of E.coli electrocompetent cells as described (54). Transformation mixtures were incubated in 900 µl of SOC medium at 37°C for 1 h followed by overnight incubation in 10 ml LB at 37°C. The double-stranded phage DNA was isolated using a Nucleospin-plasmid column (Macherey Nagel S.ar.l., Hoerdt, France). The presence and purity of M13G*1 DNA was controlled by agarose gel electrophoresis (0.8%, TAE buffer).

Lesion-induced reversion rate assay

Various strains of electrocompetent E.coli were transformed with double-stranded M13G*1 containing dR or dL or dNi followed by overnight incubation at 37°C with shaking. The centrifuged cultures containing about 1011 phage particles per millilitre were used for plating on media containing X-gal and E.coli JM105. After overnight incubation at 37°C, the dark blue plaques representing revertants were scored. Mutation frequency was calculated as the ratio of dark blue plaques to the total number of plaques. Three independent synthesis reactions were performed and five to seven independent transformations were carried out to determine mutation frequencies. The nucleotide sequence of the phage DNA from revertants was determined by dideoxy DNA sequencing using a Big-Dye terminator V3.1 cycle sequencing kit (ABI Applied Biosystems, France) according to the manufacturer’s instructions. Sequence reading was performed on a 3100 ABI Sequencer.

Altered restriction site mutagenesis

A 5′ phosphorylated primer d(CACAAACGAATGGATCTCATTAAAAGCCGTA) containing a mutated BamHI restriction site (C replaced by T in bold type) and the primer containing dNi residue were co-hybridized with single-stranded M13G*1 phage. Primer elongation, vector ligation, precursor conversion, cell transformation, growth and phage DNA recovery were performed as described above. A resulting 5 µg of the double-stranded M13G*1 phage DNA progeny was incubated overnight at 37°C with 40 U of BamHI in the reaction buffer supplied by the manufacturer and purified by ethanol precipitation. The BamHI-digested phage DNA was used to determine the mutation frequency.

RESULTS

Site-specific incorporation of dNi, dL and dR into M13G*1

We have designed a strategy allowing the introduction of dL in E.coli cells to study its mutagenic effect and its repair in vivo. The assay combines the use of a lesion precursor and the previously described bacteriophage-based genetic reversion assay. A bacteriophage vector M13G*1 detects single-base substitutions, namely X→C, T and A (where X is either a G or

Figure 1. Structure of the abasic lesions dL (2′-deoxyribonolactone) and dR (2′-deoxyribose) and of dNi (nitroindole precursor).
a modified residue) at position 141 of the lacZα gene by change from white-to-blue plaque color (57). To generate site-specifically modified circular duplex DNA containing G-C, C-C U-C and Ni-C base pairs at position 141, the 41 mer oligonucleotides complementary to the 117–157 region of the single-stranded M13G*1, containing dG-dC, dG-dC, dNi and dNi, respectively, were used as primers. About 90% of the 41 mer primers were fully extended in the presence of T4 DNA polymerase, T4 DNA ligase and the dNTPs (data not shown). Alkaline and Nfo treatments were used to ascertain conversion of the dU and dNi precursors into dR and dNi after UNG treatment and illumination, respectively. As shown in Figure 2, ≥95% of the dU- and dNi-containing oligonucleotide duplexes were resistant to the treatment, indicating the absence of abasic sites (Fig. 2, lanes 1, 6 and 7). Partial degradation of the dNi-oligonucleotide by excess Nfo is due to non-specific 3'-5' exonuclease activity (Fig. 2, lane 6) (60). As expected, UNG-treated and UV-irradiated duplex oligonucleotides were cleaved by alkaline and Nfo (Fig. 2, lanes 3, 4, 9 and 10), indicating efficient formation of the abasic site residues. However, 8–10% of the non-converted dNi precursor remains under the illumination conditions used (Fig. 2, lanes 9 and 10), and ~4–6% of dL undergoes spontaneous degradation by β and δ elimination (lane 8). The illumination and UNG treatment did not degrade the phage DNA and did not affect transformation efficiency (data not shown).

Analysis of the lesion-induced mutation frequencies in WT and mutant E. coli

A sufficient number of transformants (10^5–10^8 per microgram of DNA) to measure mutation frequency were obtained with the double-stranded phage M13G*1 containing dC-dC, dG-dC, dR-dC, dNi-dC and dL-dC nucleotide pairs. As expected, M13G*1 containing dC-dC mismatch induced blue plaques with high frequency (4 × 10^{-1}). The photochemical treatment may produce small amounts of undesirable DNA damage which in turn could contribute to mutagenesis. To address this possibility we irradiated the double-stranded M13G*1 vector containing a dG-dC base pair at position 141 under the same conditions as for the vector containing the dNi residue at 365 nm for 14 h at 4°C. The irradiated M13G*1 containing dG-dC produced only colorless plaques in E.coli JM105 and no blue plaques were found among 60 000 plaques screened. These results strongly indicate that the photochemical treatment and in vitro primer synthesis did not induce mutations at a detectable level under the experimental conditions used. As shown in Tables 2–4, the presence of dR, dNi and dL lesions in M13G*1 induced reversion to blue plaques with frequencies ranging from 2.2 × 10^{-5} to 14 × 10^{-5} in WT E.coli strains AB1157 and/or JM105 proficient in DNA repair.

In the present study, we used altered site mutagenesis (61) to select the dL-containing strand progeny. Two oligonucleotides, one containing dNi and the other containing a mutated BamHI restriction site, were used as primers to generate double-stranded M13G*1. The resulting M13G*1 with mutated BamHI site and dNi was UV-irradiated and used for the first round of transformation to E.coli BH70F'. The phage DNA from the transformants was then digested by BamHI and used for the second round of transformation, and the reversion frequencies in phage progeny from the first and second rounds of transformation were compared. Only a 2-fold increase in mutation frequency was found for the BamHI-mutated progeny [(1.0 ± 0.2) × 10^{-4}] compared with the non-digested progeny [(5.5 ± 5.6) × 10^{-5}].

To investigate the contribution of different DNA repair pathways to the elimination of dL, the modified M13G*1 was transformed into WT (AB1157 and/or JM105), AP endonuclease deficient (BH110F'), DNA glycosylase deficient (BH160F'), nucleotide excision repair (NER) deficient (BH430F') and recombination deficient (BH1100) E.coli strains. As shown in Table 2, >30-fold higher mutation frequency was observed in BH110F' strain compared with isogenic BH70F' WT strain. Although a 5-fold higher mean mutation frequency was observed in BH160F' and BH1100 strains compared with the respective WT strains, the differences were not statistically significant (p > 0.05). No detectable increase in reversion rate was observed in the BH430 strain compared with the isogenic WT JM105 strain.

The mutation frequencies induced by dR and dNi lesions in WT and mutant E.coli strains were also measured and compared with that of dL. As shown in Table 3, the dR-induced mutation frequencies in BH110F', BH160F' and BH1100F' were only 3–5-fold higher than those in WT strains, suggesting that dR is less mutagenic than dL in these repair deficient strains. Similarly to dL, no increase in dR-induced reversion rates was detected in the NER-deficient strain.

The synthetic precursor, the dNi nucleotide, is not a natural modification and it has been specially designed to generate dL in DNA under UV irradiation. As shown in Table 4, significant variation in dNi-induced mutation frequencies was detected between non-isogenic BH70F' and JM105 WT strains (p < 0.01), whereas no significant increase in mutation frequency was observed in BER-deficient mutants compared with the isogenic WT strain. In contrast, a >10-fold increase in reversion rate was observed in the homologous recombination-deficient strain. Surprisingly, there was a very low yield
Table 2. Mutation frequencies from in vivo replication of site-specifically incorporated dL residue into M13G*1 phage DNA

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Ratio of no. of blue plaques to no. of screened plaques</th>
<th>Mutation frequency</th>
<th>Averagea</th>
<th>Ratio of mutant to WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH70F' (WT)</td>
<td>Ex.1 4/32 800</td>
<td>1.21 × 10⁻⁴</td>
<td>(5.5 ± 5.6) × 10⁻⁵</td>
<td>1</td>
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<td></td>
<td>Ex.2 1/31 200</td>
<td>3.2 × 10⁻⁵</td>
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<tr>
<td></td>
<td>Ex.3 1/70 200</td>
<td>1.42 × 10⁻⁵</td>
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<tr>
<td>BH110F' (xth nfo)</td>
<td>Ex.1 12/3240</td>
<td>3.7 × 10⁻³</td>
<td>(1.8 ± 1.1) × 10⁻³</td>
<td>32</td>
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<td></td>
<td>Ex.2 48/39 000</td>
<td>1.2 × 10⁻³</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Ex.3 10/5760</td>
<td>1.7 × 10⁻³</td>
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</tr>
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<td></td>
<td>Ex.4 6/6000</td>
<td>1.2 × 10⁻³</td>
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<td></td>
<td>Ex.5 1/1000</td>
<td>1 × 10⁻³</td>
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<td>BH1160F' (nth fpg)</td>
<td>Ex.1 3/46 400</td>
<td>6.4 × 10⁻⁵</td>
<td>(2.9 ± 3.1) × 10⁻⁴</td>
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<td></td>
<td>Ex.3 10/58 400</td>
<td>1.7 × 10⁻⁴</td>
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<td>JM105 (WT)</td>
<td>Ex.1 1/13 550</td>
<td>7.3 × 10⁻⁵</td>
<td>(5.7 ± 1.4) × 10⁻⁵</td>
<td>1</td>
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<td></td>
<td>Ex.2 1/30 000</td>
<td>3.3 × 10⁻⁵</td>
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<tr>
<td></td>
<td>Ex.3 2/31 000</td>
<td>6.4 × 10⁻⁵</td>
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<tr>
<td></td>
<td>Ex.4 1/16 300</td>
<td>6 × 10⁻⁵</td>
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<tr>
<td></td>
<td>Ex.5 2/30 200</td>
<td>6.6 × 10⁻⁵</td>
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<td></td>
<td>Ex.6 1/21 600</td>
<td>4.6 × 10⁻⁵</td>
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<td>BH1430 (uvrA)</td>
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<td>(3.3 ± 1.8) × 10⁻⁵</td>
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<td></td>
<td>Ex.2 2/141 000</td>
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<td>Ex.3 1/29 600</td>
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<td>BH11100 (recA)</td>
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<td></td>
<td>Ex.3 1/10 000</td>
<td>1 × 10⁻⁴</td>
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</tr>
</tbody>
</table>

aFrequency numbers are the average ± standard deviation of three to five independent experiments.

Table 3. Mutation frequencies from in vivo replication of site-specifically incorporated dR residue into M13G*1 phage DNA

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
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<th>Mutation frequency</th>
<th>Averagea</th>
<th>Ratio of mutant to WT</th>
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<tbody>
<tr>
<td>BH70F' (WT)</td>
<td>Ex.1 4/57 200</td>
<td>7 × 10⁻⁵</td>
<td>(6.4 ± 3) × 10⁻⁵</td>
<td>1</td>
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<tr>
<td></td>
<td>Ex.2 1/33 200</td>
<td>3 × 10⁻⁵</td>
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<tr>
<td></td>
<td>Ex.3 1/10 900</td>
<td>9 × 10⁻⁵</td>
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<td>BH110F' (xth nfo)</td>
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<td>1.28 × 10⁻⁴</td>
<td>(2.1 ± 0.8) × 10⁻⁴</td>
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<td></td>
<td>Ex.2 4/15 600</td>
<td>2.56 × 10⁻⁴</td>
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<tr>
<td></td>
<td>Ex.3 26/102 000</td>
<td>2.55 × 10⁻⁴</td>
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<tr>
<td>BH160F' (nth fpg)</td>
<td>Ex.1 10/40 800</td>
<td>2.45 × 10⁻⁴</td>
<td>(3.2 ± 1.0) × 10⁻⁴</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Ex.2 3/10 800</td>
<td>2.8 × 10⁻⁴</td>
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<td>Ex.3 1/2300</td>
<td>4.34 × 10⁻⁴</td>
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<td>JM105 (WT)</td>
<td>Ex.1 2/30 000</td>
<td>6.66 × 10⁻⁵</td>
<td>(4.7 ± 2.6) × 10⁻⁵</td>
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<td>Ex.2 17/252 800</td>
<td>6.7 × 10⁻⁵</td>
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<td>Ex.3 2/48 000</td>
<td>4.2 × 10⁻⁵</td>
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<td>Ex.4 5/410 000</td>
<td>1.2 × 10⁻⁵</td>
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<td>BH430 (uvrA)</td>
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<td>3 × 10⁻⁵</td>
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<td>Ex.3 1/32 800</td>
<td>3 × 10⁻⁵</td>
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<td>BH11100 (recA)</td>
<td>Ex.1 7/25 000</td>
<td>2.8 × 10⁻⁴</td>
<td>(2.0 ± 0.7) × 10⁻⁴</td>
<td>4.3</td>
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<td>Ex.4 4/36 400</td>
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<td>Ex.5 6/26400</td>
<td>2.3 × 10⁻⁴</td>
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aFrequency numbers are the average ± standard deviation of three to five independent experiments.
of transformants for dNi-containing phage DNA in the NER-deficient BH430 strain. The number of plaques did not allow determination of the reversion frequency for this mutant.

Analysis of the mutation spectra induced by dL, dNi and dR

The phage DNAs from revertants were sequenced in order to determine the nature and occurrence of mutations resulting from in vivo replication of modified vectors. As shown in Table 5, all mutations were detected at the target site and no other sequence changes were detected within a 200 nt region containing the target site. Since the M13G*1-based reversion assay was designed to detect all base substitutions, except X®G base substitution and frameshifts, the majority of dL, dR and dNi-induced mutations were X®A,C,T mutations. As expected, in 79% of the cases dAMP was introduced opposite dR, confirming the previous observations (62,63). In contrast, the spectrum of mutations induced by dL and dNi did not obey the ‘A rule’. For dL, TMP was the most frequently incorporated nucleotide (55%) followed by dAMP (18%) and dGMP (12%). A significant number (11%) of two-nucleotide inversions GX®AG (where X is dL) were also observed. On the other hand, both TMP (40%) and dAMP (42%) were preferentially introduced opposite dNi. An inversion GX®TG (3%) and insertion of an extra C accompanied with a T deletion (8%) were also detected for dNi. The difference in mutation spectra obtained for dL and dNi further confirms the photoconversion of dNi to dL within a phage vector.

DISCUSSION

Free-radical attack and oxidation produce a tremendous multiplicity of DNA modifications in vivo. Consequently, the toxicity and the mutagenic effect of a given lesion are
difficult to assess. A variety of efficient and reliable methods have been developed for the construction of site-directed mutations in extra-chromosomal vector DNA using synthetic oligonucleotides with defined lesions in both prokaryotic and eukaryotic cells (64). To incorporate the very unstable dL lesion, which is 20 times less stable than dR at 37°C (20), we chose a strategy, described by Cheng et al. (57) for an unstable exocyclic guanine adduct, based on the site-directed incorporation of a lesion into the double-stranded M13mp2-derived bacteriophage vector M13G*1. This approach allows the incorporation of dNi and dU (the stable precursors of dL and dR, respectively) following conversion to the baseless lesions and immediate transformation into cells.

Currently, very little is known about repair mechanisms of dL in E.coli. Previous studies have shown that dL is a substrate for some AP endonucleases (32, and our unpublished results) and DNA glycosylases/AP lyases (33). Conversely, the repair of dR in E.coli is well studied. Various overlapping repair pathways including AP endonucleases Xth and Nfo (65), DNA glycosylases/AP lyases Fpg and Nth (66,67), the UvrABC complex, which is central in the NER pathway (68) and the RecA protein, which is central in recombination and translesion DNA synthesis (69), are involved in dR repair.

In the present study, we used the M13G*1 reversion assay to explore the importance of AP endonucleases, DNA glycosylases, the NER pathway and homologous recombination in the handling of dL sites in E.coli. In addition, the repair of dR and dNi was also investigated.

Under the illumination conditions used, ≥90% of dNi was converted into dL. Importantly, the UV irradiation of non-modified M13G1 did not induce mutations. The mutation frequencies for the site-specifically incorporated dL nucleoside in WT E.coli strains were low [(5.5–5.7) × 10^{-5}] compared with mismatched dC (4 × 10^{-4}). The presence of dL in the (−) strand may lead to replication bias towards non-damaged (+) DNA strand in vivo and therefore mask the mutagenic effect of the lesion. However, using altered site mutagenesis we have shown that replication bias towards non-damaged (+) strand was not significant. Interestingly, the mutation frequencies for dR [(3.0-6.4) × 10^{-5}] and dNi [(14.0-2.2) × 10^{-5}] in WT E.coli strains were also low, suggesting that these modified residues are not highly mutagenic. However, low mutagenesis could also be due to efficient repair of dR, dL and dNi. Indeed, we showed that in the xth nfo strain the dL-induced mutation frequency increased ≥30-fold compared with WT. This result strongly suggests that in E.coli the AP endonucleases play a major role in the removal of dL. In contrast, either slight or no increase in dL-induced reversion frequency were observed in recA, fpg nth and uvrA strains as compared with WT strain suggesting that the oxidized AP sites are not substrates for AP lyases, homologous recombination and NER in vivo.

AP endonuclease deficient E.coli strain has a weak spontaneous mutator phenotype suggesting that dR residues are not highly mutagenic in vivo (65). In agreement with this observation, we found only a 3-fold increase of dR-induced reversion in E.coli xth nfo mutant. This is at variance with the 30-fold increase observed with dL, implying that the oxidized AP site might be more mutagenic than dR if not repaired. In vitro the Fpg and Nth proteins can cleave dR via β-elimination with high efficiency (66,67) and may participate in repair of AP sites. However, it was shown that fpg and nth, in contrast with xth and xth nfo mutants, are not sensitive to alkylation DNA damage, suggesting that DNA glycosylases/AP lyases play a minor role in repair of AP sites in vivo (70,71). Interestingly, we found a significant 5-fold increase of dR-induced reversion frequency in E.coli fpg nth mutant, suggesting that the DNA glycosylases/AP lyases suppress mutations induced by dR in vivo. It was also shown that in E.coli, AP site-directed mutagenesis requires activation of the SOS response system (72). This damage-inducible pathway involves the RecA-dependent induction of the error-prone DNA polymerases that lack exonuclease proofreading ability. Such polymerases are more capable of bypassing an AP site in vitro than polymerases of high fidelity (73). However, it has recently been shown that RecA also participates in error-free recombinational repair which predominates over mutagenic translesion replication (74). We found a 4-fold increase of dR-induced reversion frequency in E.coli recA mutant, suggesting that in non-induced cells RecA participates in error-free repair of dR residues.

Since dNi is a non-natural nucleoside, it may not be recognized by excision repair in E.coli. Indeed, no significant increase in dNi-induced mutation frequencies was observed in BER-deficient mutants compared with isogenic corresponding WT E.coli strains. Because of the very low yield of transformants in BH430 strain, nothing can be said about the role of NER in dNi-induced mutagenesis. However, we speculate that replication of dNi-containing phage may depend on NER function. Similarly to dR, a 10-fold increase in dNi-induced reversion rate was found in E.coli recA mutant, suggesting that RecA may participate either in non-mutagenic DNA translesion synthesis (TLS) or in the removal of dNi.

The mutagentic consequences of replicating M13 DNA containing dL, dR or dNi nucleotides were examined. Despite structural similarity between dL and dR, the ‘A rule’ (47,48) was not respected for either dL or dNi. We detected preferential incorporation of TMP opposite to dL, whereas TMP and dAMP were incorporated with the same insertion frequency opposite to dNi. Furthermore, in addition to base substitution, dL and dNi induced complex mutations which were combinations of deletion and insertion. Importantly, the presence of residual dNi in irradiated phage DNA cannot be considered to have a significant contribution to the dL-induced mutation spectrum. As shown in Table 5, the ‘complex mutations’ for these two lesions show a completely different distribution. They represent 13% of the total for dNi in 76 sequenced clones versus 4% for dL in 84 sequenced clones. Both dL and dNi preferentially induce X→A base substitution and complex mutations, implying that translesional synthesis machinery does not discriminate these structurally different lesions efficiently. On the other hand, AP endonucleases recognize dL but not dNi. A structural study by high-field NMR spectroscopy is in progress to determine the geometry of dNi pairing with natural bases.

Previously, we demonstrated a neat preferential incorporation of dAMP opposite dL and dNi in vitro: by Klenow fragment DNA polymerase I devoid of 3’ exonucleolytic activity (KF exo') (51). In contrast, in vivo we have found preferential incorporation of TMP opposite to dL and dNi. We propose that discrepancies between in vivo and in vitro mutagenesis might be due to several factors: First, it was
shown that the chemical structure of a lesion is not the only determinant of the spectra and efficiency of mutagenesis (75–77). Indeed, the neighboring sequences used for in vitro studies (5'-AXG-3') and in the present work (5'-GXA-3') were different. Importantly, in vitro replication performed on short pieces of DNA may give rise in some cases to sequence-dependent results due to a realignment process in the primer–matrix duplex. Secondly, the nature of DNA polymerases involved in TLS may influence mutational spectra. It has been postulated that in eukaryotes TLS is a ‘two-polymerase affair’ involving an ‘insertor polymerase’ and an ‘extender polymerase’ (78). In E. coli, polymerase V could reestablish a stalled polymerase III holoenzyme at the lesion site and extend primer 3’ ends, suggesting that prokaryotic TLS might be also catalyzed by two polymerases (73). Thirdly, the interactions between lesion and cellular proteins might affect TLS. Recently it has been demonstrated that dL may cross-link with E. coli Nth and human polymerase β, indicating that dL reactivity towards cellular proteins may have also important consequences on repair and mutagenesis (33,79).

In conclusion, in the present study, we have demonstrated that dL is as mutagenic as the AP site in non-induced E. coli. Despite their structural analogy, repair pathways for dL and dR are not similar. In vivo the dL lesion is processed by the AP endonucleases Xth and Nfo which are involved in abasic lesion repair. Unlike dR, the dL residue is a rather poor substrate for Fpg. Nth and recombinational repair. The absence of an increase of mutagenesis in uvrA mutant strongly suggests that dL is not a substrate for NER pathway. Finally, the most common mutagenic response to the presence of dL is the incorporation of TMP. These results show that a small difference in the DNA lesion structure can have tremendous effect on repair and mutagenesis.

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REFERENCES


73. Tang, M., Pham, P., Shen, X., Taylor, J.S., O'Donnell, M., Woodgate, R. and
Goodman, M.F. (2000) Roles of E.coli DNA polymerases IV and V in
recombinational repair predominates over mutagenic translesion
75. Litinski, V., Chenna, A., Sagi, J. and Singer, B. (1997) Sequence context is
an important determinant in the mutagenic potential of 1,N6-
ethenodeoxyadenosine (epsilonA): formation of epsilonA basepairs and
elongation in defined templates. Carcinogenesis, 18, 1609–1615.
understanding of the role of DNA adduct conformation in defining
mutagenic mechanism based on studies of the major adduct (formed at
N(2)-dG) of the potent environmental carcinogen, benzo[a]pyrene.
77. Shimizu, H., Yagi, R., Kimura, Y., Makino, K., Terato, H., Ohyama, Y. and
Ide, H. (1997) Replication bypass and mutagenic effect of alpha-
deoxyadenosine site-specifically incorporated into single-stranded
79. DeMott, M.S., Beyret, E., Wong, D., Bales, B.C., Hwang, J.T.,
DNA polymerase beta by the oxidative DNA lesion