Long-patch Base Excision DNA Repair of 2-Deoxyribonolactone Prevents the Formation of DNA-Protein Cross-links with DNA Polymerase β<sup>+</sup><sup>3</sup>

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Oxidized abasic sites are a major form of DNA damage induced by free radical attack and deoxyribose oxidation. 2-Deoxyribonolactone (dL) is a C1’-oxidized abasic site implicated in DNA strand breakage, mutagenesis, and formation of covalent DNA-protein cross-links (DPCs) with repair enzymes such as DNA polymerase β (polβ). We show here that mammalian cell-free extracts incubated with Ape1-incised dL substrates under non-repair conditions give rise to DPCs, with a major species dependent on the presence of polβ. DPC formation was much less under repair than non-defective conditions, with extracts of either polβ-proficient or -deficient cells. Partial base excision DNA repair (BER) reconstituted with purified enzymes demonstrated that Flap endonuclease 1 (FEN1) efficiently excises a displaced oligonucleotide containing a 5’-terminal dL residue, as would be produced during long-patch (multinucleotide) BER. Simultaneous monitoring of dL repair and dL-mediated DPC formation demonstrated that removal of the dL residue through the combined action of strand-displacement DNA synthesis by polβ and excision by FEN1 markedly diminished DPC formation with the polymerase. Analysis of the patch size distribution associated with DNA repair synthesis in cell-free extracts showed that the processing of dL residues is associated with the synthesis of ≥2 nucleotides, compared with predominantly single nucleotide replacement for regular abasic sites. Our observations reveal a cellular repair process for dL lesions that avoids formation of DPCs that would threaten the integrity of DNA and perhaps cell viability.

Cellular DNA is under constant assault by DNA-damaging agents of both endogenous and exogenous sources (1, 2). Loss of DNA bases generates abasic (AP) sites, perhaps the most common DNA lesions, which can be mutagenic and cytotoxic if not repaired appropriately (3, 4). AP sites are formed by spontaneous hydrolysis of the N-glycosyl bonds or through the removal of damaged or mismatched bases by various DNA glycosylases (5, 6). In either case, the resulting AP sites are repaired by the base excision DNA repair (BER) pathway. In mammalian cells, the major AP endonuclease, Ape1 (also called Apex, HAP1, or Ref-1), incises the 5’-phosphodiester bond of the AP site to generate a BER intermediate that contains a single-strand break bracketed by 3’-hydroxyl and 5’-deoxyribose-5-phosphate (5’-dRP) termini.

Subsequent steps may follow either of two distinct BER sub-pathways that replace either a single nucleotide (short-patch BER) or multiple nucleotides (long-patch BER). In short-patch BER, most 5’-dRP excision is attributable to DNA polymerase β (polβ), specifically the dRP lyase activity of its amino-terminal 8-kDa domain (7, 8). The DNA polymerase activity of polβ is also the major enzyme for DNA synthesis during short-patch BER, as demonstrated in vitro by using purified enzymes and cell extracts from wild-type and polβ-null mouse embryonic fibroblasts (MEFs) (9, 10). The long-patch BER pathway involves strand displacement repair synthesis of at least two nucleotides and the excision of the 5’-dRP residue as part of a flap oligonucleotide released by the FEN1 nuclease (11–13).

polβ may initiate strand displacement DNA synthesis, but involvement in long-patch BER of other DNA polymerases, such as polδ and polε, has been suggested (14–16). A reconstituted enzyme system was developed for long-patch BER of a reduced AP site utilizing purified Ape1, polβ, polδ, proliferating cell nuclear antigen (PCNA), FEN1, and DNA ligase I, where polδ substituted for polβ when PCNA was present in the reaction (12). PCNA-dependent long-patch BER has also been demonstrated in extracts of polβ-deficient MEFs, but it was shown to be dependent on using a circular DNA substrate (9, 17). An additional variation of BER has been suggested; because some bifunctional DNA glycosylases associated with AP lyase activity can carry out incision of AP sites by β-elimination, resulting in 3’-blocked ends that must be removed by enzymes such as Ape1 and polynucleotide kinase prior to repair DNA synthesis (18, 19).

Oxidative damage to DNA, mediated by free-radicals and reactive oxygen species, produces structurally distinct AP sites that are handled differently by BER enzymes (18). Such lesions include 2-deoxyribonolactone (dL), a C1’-oxidized AP site, which has been reported to be introduced into DNA by numerous genotoxic agents including long-wave UV and ionizing radiation, organometallic oxidants, copper-phe- nanthrone chemical nuclease, and the chromophore of the antitumor agent neocarzinostatin (20–22). Little was known about repair of dL until the recent development of synthetic oligonucleotides that yield a site-specific dL through a photosensitive nucleotide analog (23–26). Initial investigation of the reaction of dL with Escherichia coli endonuclease III, a DNA glycosylase with an associated AP lyase activity, revealed formation of a stable DNA-protein cross-link (DPC) between the dL lesion and the catalytic lysine of the enzyme (27). However, the AP endonuclease activity of E. coli exonuclease III (Exo III) and endonuclease IV (Endo IV) process dL lesions efficiently (28), and such 5’-incision would prevent AP lyase enzymes from acting on dL. Consistent
with these observations, the dL-induced mutation frequency was highly elevated in E. coli-deficient in both major AP endonucleases (29). The major human AP endonuclease Ape1 also incises dL residues rather efficiently, leaving a 5'-terminal oxidized AP residue (30, 31). However, an Ape1-incised dL residue forms stable DPC with polβ, the next BER enzyme, dependent on its catalytic lysine (residue 72) for its dRP lyase activity (31). This DPC linkage is evidently a stable amide bond between the lysine-72 ε-nitrogen and the dL C1-carbonyl (18, 31).

Although in vitro studies with purified E. coli proteins and mammalian polβ have provided useful information about chemical mechanisms involved in dL-mediated DPC (31, 32), the biological significance of such DPC formation and the cellular repair of dL lesions remains unclear. In the present study, we utilized mammalian cell-free extracts to determine whether different cellular proteins are prone to dL-mediated DPC formation. To explore possible repair of dL lesions, and its correlation with DPC formation, the complete repair of closed circular DNA substrate with a defined dL residue was analyzed for evidence of both sub-pathways of BER. We found that polβ is indeed the major cellular protein associated with formation of dL-specific DPC, but that DPC formation can be largely prevented by rapid removal of dL through mechanisms of long-patch BER.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides containing a dL precursor residue (1'-t-butylationuriyldolate; tBU) (31) were provided by Dr. M. Greenberg (Johns Hopkins University, MD). Other oligonucleotides were synthesized and high-performance liquid chromatography purified by Operon Technologies. pGEM-3Zf(+) plasmid and helper phage (R408) were obtained from Promega. Radionucleotides were from PerkinElmer Life Sciences. HeLa and polβ-proficient (MB16tsA, clone 1B5) or -deficient (MB19tsA, clone 2B2) SV-40 immortalized MEF cell lines were obtained from American Type Culture Collection. T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, Klenow fragment DNA polymerase, E. coli Exo III, and all restriction enzymes were from New England Biolabs. Proteinase K was from Sigma/Aldrich. Recombinant human Ape1 and E. coli Endo IV were purified as described previously (33). Human polβ was kindly provided by Drs. R. Prasad and S. H. Wilson (NIEHS, National Institutes of Health). Goat anti-Polβ polyclonal antibody was from Santa Cruz Biotechnology. Recombinant human FEN1 was expressed and purified to apparent homogeneity as previously described (34), by utilizing an expression vector pET-FCH that was provided by Dr. R. Bambara (University of Rochester). E. coli uracil-DNA glycosylase (Ung) was provided by Dr. D. W. Mosbaugh (Oregon State University). The p21 peptide GRKRRQTSMTDFYHS-KRRLIFS that binds PCNA (35) was synthesized by Sigma-Genosys.

Preparation of DNA Substrates—Oligonucleotides used in this study are listed in TABLE ONE. Duplex 31-mer DNA substrates were prepared by hybridization of tBU-30 or U-30 to T1. The substrates utilized for FEN1 cleavage were constructed by hybridization of upstream primer (Up-19) and downstream primer (U-17 or tBU-17) with T2 template DNA. Each substrate was 3’-end labeled by incorporation of α-32PdCTP using the exonuclease-free Klenow fragment of DNA polymerase I. Closed circular pGEM-3Zf(+) plasmid DNA containing a site-specific lesion was constructed as previously described (36), except that different primers were employed. Briefly, single-stranded pGEM plasmid (+) DNA was produced with the aid of helper phage (R408) and purified by the CTAB DNA precipitation method (36). Each upstream primer (Up-T, Up-U, and Up-tBU) was 5’-phosphorylated and annealed to single-stranded pGEM DNA. During the preparation of 32P-labeled pGEM DNA substrates that were employed in patch size distribution analysis, each upstream primer was 5’-end-labeled using T4 polynucleotide kinase and a molar excess of α-32PdATP. To construct 32P-labeled pGEM DNA substrates used for DPC detection, 5’-32P-labeled downstream primer (Down), in addition to the upstream primer Up-tBU, was also hybridized to single-stranded pGEM DNA. Each primed template was subjected to a primer extension reaction followed by ligation with T4 DNA ligase. Covalently closed circular duplex DNA for either unlabeled or 32P-labeled pGEM (T/A), (U/A), and (tBU/A) were isolated by cesium chloride gradient centrifugation as described previously (36). Typically, isolated plasmid DNA was found to contain >98% form I molecules as determined by 1% agarose gel.

To generate a site-specific dL residue in DNA substrates, 2–5 pmol of DNA duplex containing a tBU residue was subjected to the photolysis reaction as previously described (31). The efficiency of the photo-conversion was typically >90% when monitored by dL-specific DNA cleavage using hot-alkali treatment and subsequent analysis of DNA by denaturing polyacrylamide gel electrophoresis. Where indicated, DNA containing a dL lesion was subjected to the treatment with catalytic amounts of either Endo IV or Ape1 to generate a site-specific dL-phosphate (5’-dL) residue. Oligonucleotide and plasmid DNA containing a uracil residue was treated with a catalytic amount (1–10 units) of

| TABLE ONE  
DNA oligonucleotides used for DNA substrate constructions |
|---------------------------------|---------------------------------|---------------------------------|
| Oligonucleotides DNA substrates | DNA sequences                   |
| Primers                        |                                 |
| U-30                           | 5’-GTCACGTGCTGCAUACGACGTGCTGAGCCCT-3’ |
| tBU-30                         | 5’-GTCACGTGCTGCApBUACGACGTGCTGAGCCCT-3’ |
| Up-19                          | 5’-GTCACGTGCTGCAATTCGCTG-3’     |
| U-17                           | 5’-UACGACGTGCTGAGCCCT-3’       |
| tBU-17                         | 5’-tBUACGACGTGCTGAGCCCT-3’     |
| Templates                      |                                 |
| T1                             | 5’-GAGGCTACGACGTCGTGAGCAGCAGTCAGAC-3’ |
| T2                             | 5’-GAGGCTACGACGACGCAATGCGACAGTCAGAC-3’ |
| Plasmid DNA substrates         |                                 |
| Up-T                           | 5’-ATCCCTCTAGAGTCGACCTGAGG-3’  |
| Up-U                           | 5’-ATCCCTCTAGAGUCCGACCTGAGG-3’  |
| Up-tBU                         | 5’-ATCCCTCTAGAGtBUACGACCTGAGG-3’ |
| Down                           | 5’-CATGCAAGCTGAGTATCTAT-3’     |

* The underlined nucleotides U and tBU denote uridine and 1’-t-butylationuriyldolate residues, the precursor for dL, respectively.
uracil-DNA glycosylase to prepare DNA substrates containing a site-specific AP site.

**Preparation of Cell-free Extracts**—HeLa and MEF cell-free extracts were prepared from confluent cells as previously described (17), and dialyzed extensively against 20 mM Hepes-KOH (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenethylsulfonyl fluoride, 10% (v/v) glycerol, and protease inhibitor mixture (Sigma-Aldrich). Protein concentrations of the cell-free extracts were determined using a Bio-Rad Protein Assay reagent. The levels of polβ in cell-free extracts were monitored by Western blot analysis as described previously (37).

**Analysis of Cross-linking Reactions**—Standard cross-linking reactions contained 50 mM Hepes-KOH (pH 7.5), 20 mM NaCl, 0.5 mM dithiothreitol, 2 mM EDTA, 5% (v/v) glycerol, 0.1 mg/ml bovine serum albumin, 10 mM 32P-labeled 31-mer DNA substrate, and protein concentrations as indicated in the figure legends. Following incubation at 30 °C for the specified times, reactions were terminated by the addition of SDS-PAGE loading buffer (31) and heating at 100 °C for 5 min. DPCs and free DNA were resolved by 8% SDS-PAGE, and 32P radioactivity associated with DNA-protein cross-links was quantified using a PhosphorImager and the ImageQuant program (Amersham Biosciences).

**In Vitro DNA Repair Assay with Purified Enzymes**—A partial reconstitution of long-patch BER was performed in reactions containing 50 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 8 mM MgCl2, 5% (v/v) glycerol, 0.1 mg/ml bovine serum albumin, 10 mM 3′-end 32P-labeled 31-mer DNA substrate, and various BER enzymes as indicated. Where indicated, 50 μM each of dATP, dCTP, and dGTP were included to permit only limited repair related strand displacement DNA synthesis. For the FEN1 assay, reactions also contained the indicated concentrations of FEN1 enzyme and duplex DNA substrates bearing unannealed 5′-flap structures. After incubation at 30 °C for the specified times, reactions were terminated by addition of formamide loading buffer (31) and heating at 100 °C for 3 min. 32P-DNA products were resolved by 15% polyacrylamide, 7 M urea gel electrophoresis.

**Analysis of BER Reaction Products**—BER reactions were performed at 30 °C using a standard reaction buffer containing 100 mM Hepes-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM each of dNTPs, 5 mM phosphocreatine, 200 units/ml phosphocreatine kinase, 5–10 μg/ml of the appropriate pGEM DNA substrate, and cell-free extract as indicated in the figure legends. To monitor DPC formation with unrepaird dL residues, a 32P-labeled pGEM (dL/A) substrate was employed in the reaction. Following incubation for the specified times, reaction products were digested with 10 units of BamHI and HindIII for 1 h at 37 °C, and then analyzed by 8% SDS-PAGE. To distinguish between either the short- or long-patch BER pathways, a modified restriction analysis method was conducted in the presence of EDTA to suppress nuclease activity; AP endonucleases and dRP lyases are usually active under these conditions and might form DPC (27, 31). To allow incision of dL residues under these conditions, we added catalytic amounts of the EDTA-resistant enzyme Endo IV and either 30 nM polβ (lanes 1, 3, and 5) or 10 μg of HeLa cell-free extract, without (lanes 2, 4, and 6) or with (lane 7) prior heat treatment for 10 min at 70 °C. The reactions were then further incubated with 0.1 unit of proteasine K (lanes 5 and 6) or mock treated (lanes 1–4 and 7) for 20 min at 50 °C. The products were separated by SDS-PAGE and detected using a PhosphorImager. The band positions of the M, markers are indicated at the left.

**RESULTS**

**dL-mediated DPC Formation in Cell-free Extracts**—We showed previously that Apel efficiently incises DNA at dL lesions to yield 5′-terminal dL-5-phosphate (5′-dL) residues (30, 31). During attempted excision of a 5′-dL residue, polβ becomes covalently cross-linked to the oxidized lesion (31). To understand the biological significance of such DPC formation, we have now conducted *in vitro* assays with cell-free extracts and determined major cellular proteins involved in dL-mediated DPCs. A 32P-labeled oligonucleotide DNA substrate bearing a site-specific dL residue (formed from the precursor by UVA exposure) was substituted for either the 18U precursor lesion (lanes 1 and 2) or a photochemically (UVA) generated dL residue (lanes 3–7) was co-incubated for 2 h at 30 °C in 25-μl reactions with 4 nM Endo IV and either 30 nM polβ (lanes 1, 3, and 5) or 10 μg of HeLa cell-free extract, without (lanes 2, 4, and 6) or with (lane 7) prior heat treatment for 10 min at 37 °C. The reactions were then further incubated with 0.1 unit of proteasine K (lanes 5 and 6) or mock treated (lanes 1–4 and 7) for 20 min at 50 °C. The products were separated by SDS-PAGE and detected using a PhosphorImager. The band positions of the M, markers are indicated at the left.
Long-patch BER of dL Prevents Cross-link Formation

FIGURE 2. Major DPC formed with dL in MEF cell-free extracts dependent on polβ. A, standard cross-linking reaction mixtures (20 μl) containing 10 μl 32P-labeled 31-mer duplex DNA containing a 5′-dL residue were incubated at 30 °C with 200 nM polβ for 120 min (lane 1), with 10 μg of POLB+/+ MEF cell-free extract for 0, 2, 10, 60, and 120 min (lanes 2–6), or with 10 μg of POLB−/− MEF cell-free extract for 0 and 120 min (lanes 7 and 8). Samples were subjected to SDS-PAGE and detected using a PhosphorImager. The resulting DPC bands are indicated by arrows. B, Western blot analysis of 10 μg of POLB+/+ or POLB−/− MEF cell-free extract (lanes 1 and 2, respectively) are shown, along with 0, 0.13, 0.5, 2, and 8 pmol of purified polβ (lanes 3–7).

FIGURE 3. Reduction of polβ-dL DPC by pre-treatment of dL-DNA with cell-free extract. A, shown is a partial segment of the pGEM (dl/A) DNA substrate with a site-specific dL residue bracketed by unique BamHI and HindIII restriction sites. The location of the 32P-dCMP residue is indicated by an asterisk. B, preincubation mixtures (10 μl) containing 10 μg 32P-labeled Ape1-incised pGEM (dl/A) DNA and 5 μg of wild-type MEF cell-free extract were incubated at 30 °C for 0, 10, 30, 60, and 90 min (lanes 1–5) in standard BER reaction buffer as described under “Experimental Procedures.” The reactions were terminated by the addition of EDTA to a final concentration of 5 mM, supplemented with 200 nM polβ, and further incubated for 2 h at 30 °C. Following digestion with BamHI/HindIII, the samples were resolved by SDS-PAGE and analyzed using a PhosphorImager. C, quantification of polβ-dL DPC as shown in panel B. The values were standardized against the maximum DPC formed (8, lane 1). The mean of two experiments is shown.

Reduced dl-mediated DPC Formation under DNA Repair Conditions—Although the removal of 5′′-dRP residues by polβ is a key step in accomplishing short-patch BER (17, 40), the formation of DPC in reactions with 5′-dL is problematic. In principle, long-patch BER (17, 40) would avoid this problem. We therefore examined reactions of dl with cell-free extracts under the conditions that would allow long-patch BER.

To protect the substrate from nonspecific degradation and to promote the assembly of long-patch BER proteins (38, 41), a circular plasmid DNA substrate was constructed containing a site-specific 5′-dL residue (Fig. 3). This DNA substrate was first incubated with MEF cell-free extracts under the DNA repair conditions in the presence of dNTP, MgCl2, and an ATP regenerating system (17). After various incubation times, the DNA was subjected to the cross-linking reaction with an excess amount of purified polβ to detect the level of 5′-dL residues that remained available to form DPC. The analysis showed that DPC with polβ decreased as a function of the preincubation time (Fig. 3B; see also supplemental Fig. S2). Quantification showed that DPC formation was reduced to 50% after a 10-min preincubation, to 25% after 20 min, and effectively eliminated after a 60-min preincubation with an MEF cell-free extract (Fig. 3C).

The elimination of dL residues cross-linkable to polβ suggested that dL modification, removal, or competition with other proteins to form DPC was taking place in the extracts. Examination of reactions of the dL plasmid substrate with increasing amounts of either POLB+/+ or POLB−/− extracts revealed that no detectable DPC were being formed (Fig. 4A, lanes 2–5 and 12–15, respectively; see also supplemental Fig. 4).
Long-patch BER of dL Prevents Cross-link Formation

FIGURE 5. In vitro reconstituted long-patch BER mediates repair of dL and inhibits formation of dL-mediated DPC. A, a duplex 3'-32P-labeled 31-mer DNA substrate (10 nm) containing a site-specific dL (top of panel A) was incubated for 60 min at 30 °C with different combinations of 1 nm Ape1, 10 nm polβ, and 1 nm FEN1 as indicated. The reactions were conducted in the absence (lanes 1–5) or the presence (lanes 6–10) of a dNTP mix excluding dTTP. The asterisk indicates the position of the radiolabel, X denotes the dL residue, and the underlined nucleotide sequence represents the DNA segment that would be displaced by the incorporation of 7 nucleotides with dCMP, dAMP, and dGMP. After the incubation, one-half of each reaction mixture was analyzed by SDS-PAGE, and the 32P-labeled DNA bands were visualized using a PhosphorImager. B, quantification of DPC formation. The values were standardized against the maximum DPC formed (lanes 6 and 16) and are plotted as a function of cell extract protein. Open bars, POLB 1/2; closed bars, POLB 1/2. The mean of two experiments is shown.

Inhibition of dL-mediated DPC by In Vitro Reconstitution of Long-patch BER—To determine whether the processing of dL residue can occur by long-patch BER to prevent DPC formation, we examined the profiles of both DNA processing and DPC formation in the reactions reconstituted with various repair components involved in long-patch BER (Fig. 5). Untreated 3'-32P-labeled dL DNA substrate displayed heterogeneous mobility on a sequencing gel, probably due to cleavage at the heat-labile dL site during gel electrophoresis (Fig. 5A, lane 1). Treatment of the DNA substrate with Ape1 converted the majority of the DNA substrate to the 18-mer DNA cleavage product, consistent with incision at the 5' site of the dL residue by Ape1 (30, 31). Additional treatments with polβ and FEN1, or both, did not mediate further processing of DNA in the absence of dNTPs (Fig. 5A, lanes 3–5). However, the inclusion of dATP, dCTP, and dGTP to permit limited DNA repair synthesis (only 7 nucleotides, specified by the substrate DNA sequence) in a reaction containing Ape1, polβ, and FEN1 produced a distinct DNA product of 11 nucleotides (Fig. 5A, lane 10). The generation of the 11-mer is consistent with strand displacement DNA synthesis of 7 nucleotides by the polymerase, followed by removal of the displaced DNA flap by FEN1. Parallel analysis of the same reaction mixtures by SDS-PAGE displayed the profile of DPC formation, which was dependent on both Ape1 and polβ (Fig. 5B, lanes 4 and 5). The generation of DPC was markedly reduced when the reaction allowed the combined action of repair synthesis by polβ and flap excision by FEN1 (Fig. 5, A and B, lane 10). polβ-mediated repair synthesis alone did not block dL-mediated DPC formation (Fig. 5B, lane 9), which indicates the importance of FEN1-mediated removal of the dL-containing fragment in preventing cross-linking.

To test directly whether FEN1 can indeed excise a 5'-dL flap, the endonuclease activity of FEN1 was assayed with a pre-assembled flap DNA structure. Processing of flaps by FEN1 occurred about equally efficiently for 5'-dL, 5'-dRP, or the dL-precursor residue (Fig. 6), consistent with published observations that FEN1 tolerates a variety of flap modifications (42). Overall, these results demonstrated that repair of dL by long-patch BER averts the formation of dL-mediated DPC.

Altered Patch Size Distribution Associated with dL-mediated DNA Repair—To examine how dL lesions affect the mode of DNA repair synthesis, we analyzed the repair patch size distribution for dL lesions compared with regular AP sites. Briefly, the approach (36) relies on the incorporation of 2-deoxyribonucleoside-α-phosphorothioates during DNA synthesis, which renders the repaired DNA region resistant to subsequent digestion by E. coli Exo III. As illustrated in Fig. 7A, the pGEM-derived plasmid DNA substrates utilized in this experiment contained the target dL or AP site on the (−) strand 17 nucleotides 5' and 12 nucleotides 3', respectively, to unique HindIII and BamHI sites. A 32P-dAMP label was introduced 5' to the target, and the repair patch size distribution was evaluated by determining the length of 32P-DNA

FIGURE 4. Suppression of DPC formation in MEF extracts under DNA repair conditions. A, standard BER reaction mixtures (20 μl) containing 10 nm 32P-labeled pGEM (dL/A) DNA (see Fig. 3) and 0.3, 1.2, 5, and 20 μg of cell-free extract of POLB 7/8 (lanes 1–5 and 6–10) or POLB 7/8 (lanes 11–15 and 16–20) were prepared in the absence (lanes 1–5 and 11–15) or the presence (lanes 6–10 and 16–20) of 200 nm of added purified polβ. After incubation for 2 h at 30 °C, each reaction product was subjected to BamHI/HindIII treatments with polβ (Fig. 4, A1, lanes 6 and 16) and are plotted as a function of cell extract protein. Open bars, POLB 7/8; closed bars, POLB 7/8. The mean of two experiments is shown.

S3). Minor bands with slower mobility were also observed, but these occurred with both POLB 7/8 or POLB 7/8 extracts (Fig. 4A), and so were not related to the dL-mediated DPC with polβ. However, supplementation of either extract with a large amount of purified polβ showed the expected DPC with the polymerase (Fig. 4A, lanes 6 and 16 versus 7 and 17), the formation of which diminished with an increasing proportion of added extract (Fig. 4A, lanes 6–10 and 16–20; quantification in Fig. 4B). Considering that the level of DPC with polβ was reduced without a compensating increase in DPC with other proteins (Figs. 3C and 4A), the observed decrease in polβ-DPC formation most likely resulted from repair (or modification) of dL residues, rather than a simple competition between polβ and other proteins to form DPC. Collectively, the results imply the existence of dL-repair mechanisms that prevent the cross-linking of polβ with dL.

DNA ladder was prepared by BamHI/HindIII digestion of the undigested DNA substrate to the 18-mer DNA cleavage product, consistent with published observations that FEN1 tolerates a variety of flap modifications (42). Overall, these results demonstrated that repair of dL by long-patch BER averts the formation of dL-mediated DPC.

Altered Patch Size Distribution Associated with dL-mediated DNA Repair—To examine how dL lesions affect the mode of DNA repair synthesis, we analyzed the repair patch size distribution for dL lesions compared with regular AP sites. Briefly, the approach (36) relies on the incorporation of 2-deoxyribonucleoside-α-phosphorothioates during DNA synthesis, which renders the repaired DNA region resistant to subsequent digestion by E. coli Exo III. As illustrated in Fig. 7A, the pGEM-derived plasmid DNA substrates utilized in this experiment contained the target dL or AP site on the (−) strand 17 nucleotides 5' and 12 nucleotides 3', respectively, to unique HindIII and BamHI sites. A 32P-dAMP label was introduced 5' to the target, and the repair patch size distribution was evaluated by determining the length of 32P-DNA ladder.
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FIGURE 6. Excision of 5'-dL flaps by FEN1. Duplex DNA substrates (10 ng) preassembled with a 5' flap containing a 5'-terminal AP site (lanes 1–3), the tBU precursor lesion (lanes 4–6), or a dL residue (lanes 7–9) were incubated with 0.1, 0.5, and 1 nmol FEN1 for 30 min at 30 °C. The reaction products were analyzed on a 15% polyacrylamide/urea DNA sequencing gel and visualized using a PhosphorImager. Schematic representations of the substrates are depicted for each set of lanes; the asterisks indicate the positions of the 32P-label in each band.

FIGURE 7. DNA repair patch size distributions for AP and dL substrates. A, A segment of pGEM DNA with site-specific target residues and BamHI and HindIII restriction sites. X denotes an AP site, the precursor tBU lesion, or a dL site. The location of the 32P-dAMP label is indicated by an asterisk. Repair DNA synthesis initiating at the target site occurs in the direction of the arrow. BER reaction mixtures (100 μl) containing 20 μM each of dNTPs in place of dNTPs were incubated for 30 min at 30 °C. The mean ± S.D. for three experiments are shown.

For AP repair, the mean ± S.D. for three experiments are shown. For repair of dL reactions scored as single-nucleotide BER may include incomplete repair intermediates in which a single nucleotide has been inserted but no further reaction has yet occurred. The effect of the p21 peptide on the 32P-radioactivity for each band was quantified using a PhosphorImager. The relative intensity for each band was determined by dividing the amount of 32P radioactivity detected in that band by the total 32P signal detected for all bands in the same lane and multiplying by 100%. The proportion of repair products with a single-nucleotide patch (black bars) and the sum of those with multinucleotide patches (white bars) are expressed as percent distributions. This quantitative analysis represents the relative amount of the single short-patch (black bars) versus long-patch (white bars) BER products, and may not reflect the visually perceived intensities of the image in panel B. The mean ± S.D. for three experiments are shown.

For dL repair, in the polβ-deficient extract (Fig. 7B, lanes 8 and 9; Fig. 7C). The products of the dL reactions scored as single-nucleotide BER may include incomplete repair intermediates in which a single nucleotide has been inserted but no further reaction has yet occurred. The effect of the p21 peptide in the PDB+/+ extract compared with the PDB−/− extract was consistent with the generation of these products by polβ. Activation of the dL precursor also generates a proportion of strand breaks with 3'-phosphate residues (30, 31), which could also generate some single-nucleotide repair product following the action of polynucleotide kinase (18, 19).

Complete Repair of dL Is Mediated Exclusively by Long-patch BER—To verify that the complete repair of dL residues indeed occurred through long-patch BER, we analyzed the repaired DNA products by restriction digestion approach adopted from a published procedure (38). As shown in Fig. 8A, pGEM plasmid DNA substrates that con-
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DNA sequencing gel. As an internal standard (proportion of 3 in this way (30, 31). Incubation of the dL substrate under long-patch BER, respectively. The DNA products would require repair DNA synthesis of two nucleotides (Fig. 8). Under these conditions, only the completely repaired DNA product digestion, with the unligated products yielding a 13-mer fragment. For AP sites, discrete 32P-DNA restriction fragments were observed (Fig. 8A). The ligated products of such single-nucleotide BER are expected to generate a 32P-labeled 30-mer DNA fragment from BamHI and HindIII digestion, with the unligated products yielding a 13-mer fragment. Under these conditions, only the completely repaired DNA product would contain the recognition sequence for AccI to generate a 13-mer 32P-DNA fragment by co-digestion with BamHI. For long-patch BER, the reactions were conducted in the presence of 32P-dCTP and unlabeled dTTP. Under these conditions, incorporation of 32P-dCMP would require DNA repair synthesis of two nucleotides (Fig. 8A). Digestion of the repaired DNA with BamHI/HindIII or HincII/BamHI was expected to produce 30-mer or 14-mer 32P-DNA fragments, respectively, indicating complete repair by the long-patch BER pathway.

For AP sites, discrete 32P-DNA restriction fragments were observed under both short- and long-patch conditions (Fig. 8B, lanes 9 – 12, respectively). DNA repair synthesis was specific for damaged DNA, as reactions with control pGEM (T/A) lanes 1 – 4 did not produce any detectable 32P-DNA restriction fragments (Fig. 8B, lanes 1 – 4). For dL residues, in contrast, under the short-patch BER conditions complete repair was not observed (Fig. 8B, lane 5), and only a small amount of 32P-dTTP incorporation into dL-DNA was registered as the 32P-13-mer (Fig. 8B, lane 5 and 6). The small amount of ligated product observed with the dL substrate likely corresponds to repair of the minor proportion of 3'-phosphate residues present in dL substrates prepared in this way (30, 31). Incubation of the dL substrate under long-patch BER conditions, however, produced the 32P-DNA products expected for digestion by the corresponding restriction enzymes (Fig. 8B, lanes 7 and 8; compare with lanes 11 and 12 for AP site repair), which indicated complete repair. We note that the amount of DNA repair synthesis associated with complete long-patch BER for dL appeared to equal to that for the long-patch component of AP repair (Fig. 8B, compare lanes 7 and 11). This observation suggests that long-patch BER of dL may occur as efficiently as it does for an AP site, or the same factor is limiting for both. The repair of a site-specific dL lesion has not been reported previously, but our results clearly demonstrated that the long-patch BER pathway is capable of handling dL lesions, which are resistant to processing by short-patch BER mechanisms.

DISCUSSION

Cellular DNA exposed to free radical attack and oxidation due to metabolic by-products or agents such as ionizing radiation contains various DNA modifications, including several types of oxidized abasic sites (18, 46). Assessing the biological consequences of individual lesions, such as dL residues, has been difficult because of the complex nature of free radical DNA damage (18) and, until recently, a lack of methods to generate the lesions site-specifically. Our recent work had already shown that dL residues have the potential to generate DPC with polβ (31), which makes the analysis of this lesion especially important. In this study we have used DNA substrates containing a site-specific dL residue to examine repair by either purified BER enzymes or cell-free extracts, and we have demonstrated the complete repair of dL residues by long-patch BER.

The abundance of the highly active Ape1 protein in most cell types (47, 48) indicates that dL residues in DNA would likely be rapidly incised to yield a 5'-dL moiety at the nick (30). It is this cleaved species that forms DPC with polβ (31), which raised the question of whether other cellular proteins might also undergo such a cross-linking reaction. Indeed, incubation of Ape1-treated dL-DNA with cell extracts yielded several distinct DPC products, with the major species evidently due to polβ (Fig. 3). Because polβ is the major 5'-dRP excision activity in mamalian cells (7, 8), these results indicate that efficient DPC formation with 5'-dL lesions depends on the dRP lyase activity of polβ (31). More recently pol has been ascribed with a dRP lyase activity (49), but another study suggested that pol could be trapped as a covalent Schiff base intermediate at 5'-dRP residues (50). Although our own studies indicate that recombinant pol can form DPC with 5'-dL residues, there was no significant dL-mediated DPC in the cell-free extracts corresponding to the molecular mass of ~80 kDa (Fig. 1) expected for pol (51).

Several other dL-mediated DPCs, of lower intensity than the polβ product, were observed to involve cross-linking to relatively small polypeptides, which was not prevented by a heat pretreatment of the extracts (Fig. 1). These other DPC may be due to abundant DNA-binding proteins, such as histones, participating in non-enzymatic reactions with the lactone.

A recent study showed that the oxazine, a major nitric oxide-induced guanine base damage, forms stable DPC with various DNA glycosylases (human OGG1 and E. coli Fpg, AlkA, and EndoVIII) at a much higher rate than found for histone and HMG proteins (52). Oxazine and dL are base and sugar lesions, respectively, but both involve DPC formation via a lactone structure that is prone to react with nucleophilic molecules. The two major oxazine-mediated DPC species detected in human cell extracts (52) involve as-yet-unknown proteins other than hOGG1, the enzyme that plays a major role in the recognition and removal of the oxidized guanine base (53, 54). For DPC formation between 5'-dL DNA and polβ, the cross-linking efficiency (Fig. 2) may be affected not only by

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4 M. DeMott and B. Demple, unpublished data.
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the intrinsic reactivity of the active-site lysine-72 and the strong affinity of the enzyme for 5'-dRP residues (55), but also by Ape1-mediated recruitment during BER (56). On the other hand, we found that the polβ dRP lyase lags behind its polymerase activity (57), which could offset the potential for DPC formation during BER.

Long-path BER seems to provide an effective mechanism to avoid DPC formation during the excision of dL residues. A partial long-patch BER reaction reconstituted with Ape1, polβ, and FEN1 allowed the excision of 5'-dL on a displaced oligonucleotide flap (Fig. 5), with the product further processed by ligation (Figs. 7 and 8). However, once DPC were formed, either FEN1 or cell-free extract appeared unable to process the DPC on a displaced DNA flap (31) (see supplemental Figs. S4 and S5).

Although short-path BER appears to be the predominant mode for the repair of AP sites or base lesions converted to AP sites by monofunctional DNA glycosylases, several in vitro and in vivo studies also suggest a significant contribution of long-patch BER (17, 40, 58, 59). Our data for AP site repair are consistent with these observations (Fig. 7). Repair of the dL lesion, in contrast, was accompanied exclusively by multinucleotide repair synthesis (Fig. 7). As a consequence, in cell-free extracts proficient for long-patch BER, the formation of dL-mediated DPC was dramatically diminished (Fig. 4). The cross-linking reaction between polβ and dL is not a fast one (31), and evidently the long-path BER steps occur rapidly enough to prevent most of the possible DPC formation.

It has not been established what factors are involved in the selection between the short- or long-path BER mode. For DNA base lesions, the nature of the participating DNA glycosylase is important (59). DNA glycosylases with associated AP lyase activity lead mainly to short-patch BER, whereas monofunctional DNA glycosylases lead to a mixture of the short- and long-patch pathways.

The exclusive use of the long-patch pathway for dL, a natural product, echoes a similar finding for the synthetic tetrahydrofuran analog often used as a stable substitute for AP sites. However, under conditions of oxidative stress that might accompany the formation of relatively large numbers of dL residues, BER would also be engaged with handling many other base and deoxyribose lesions. Under these circumstances, it is unclear how the coordination that seems to operate generally in BER can be maintained. It seems possible that, in such circumstances, Ape1-incised dL residues may remain in the DNA for longer periods, increasing the opportunity for DPC formation withpolβ and polκ (enzymatically), and with other cellular proteins (non-enzymatically). The study of dL will provide opportunities for analyzing these issues and the switching mechanisms that govern the short- versus long-path BER distribution under varying circumstances of damage load and repair enzyme availability. Developing methods to determine the extent to which dL-mediated DPC are formed in vivo is an important goal to enable such an analysis.
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