DNA Polymerase β and Flap Endonuclease 1 Enzymatic Specificities Sustain DNA Synthesis for Long Patch Base Excision Repair*

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DNA polymerase β (pol β) and flap endonuclease 1 (FEN1) are key players in pol β-mediated long-patch base excision repair (LP-BER). It was proposed that this type of LP-BER is accomplished through FEN1 removal of a 2- to 11-nucleotide flap created by pol β strand displacement DNA synthesis. To understand how these enzymes might cooperate during LP-BER, we characterized purified human pol β DNA synthesis by utilizing various BER intermediates, including single-nucleotide-gapped DNA, nicked DNA, and nicked DNA with various lengths of flaps all with a 5'-terminal tetrahydrofuran (THF) residue. We observed that nicked DNA and nicked-THF flap DNA were poor substrates for pol β-mediated DNA synthesis; yet, DNA synthesis was strongly stimulated by purified human FEN1. FEN1 did not improve pol β substrate binding. FEN1 cleavage activity was required for the stimulation, suggesting that FEN1 removed a barrier to pol β DNA synthesis. In addition, FEN1 cleavage on both nicked and nicked-THF flap DNA resulted in a one-nucleotide gapped DNA molecule that was an ideal substrate for pol β. This study demonstrates that pol β cooperates with FEN1 to remove DNA damage via a “Hit and Run” mechanism, involving alternating short gap production by FEN1 and gap filling by pol β, rather than through coordinated formation and removal of a strand-displaced flap.

The mammalian genome suffers from endogenous and exogenous insults that modify DNA. These modifications can be a small single-base lesion, bulky DNA adduct, base dimer, or other type of alteration. Among them, the single-base lesion is the most common form of DNA damage observed in mammalian cells, because it arises from both exogenous DNA-damaging agents (1) as well as from endogenous biological processes resulting in base alklylation (2, 3) and oxidation (4, 5). Additionally, endogenous cytosine deamination and base loss resulting from hydrolysis of the glycosidic bond are important sources of DNA damage (6–10). It has been estimated that a single-base lesion caused by hydrolytic depurination alone occurs at a frequency of 2 × 10⁻¹⁰ per human cell per day (3). Thus, DNA base modifications account for a large proportion of the total cellular DNA damage. To preserve genomic stability, mammalian cells have evolved a robust defense mechanism to repair these damaged bases.

The repair of small single-base lesions is accomplished through base excision repair (BER) (11). BER is often initiated by a DNA glycosylase that cleaves the N-glycosydic bond of a damaged base, leaving an apurinic/apyrimidinic site, also referred to as an abasic site or AP site (3, 12). Subsequently, AP endonuclease (APE) cleaves the sugar-phosphate backbone at the 5’-side of the AP site resulting in 3’-hydroxyl and 5’-deoxyribose phosphate (dRP) groups at the margins of a one-nucleotide gap in DNA (13, 14). DNA polymerase β (pol β) inserts a nucleotide into the gap leaving nicked DNA with a 5’-dRP flap (15). At this point, the repair will be directed into different sub-pathways depending, at least in part, on whether or not the sugar residue in the flap (i.e. 5’-dRP) has been further modified (i.e. oxidized or reduced). With the unmodified dRP group, pol β removes the 5’-dRP group through its associated lyase activity (16), resulting in nicked DNA that will be sealed by DNA ligase. This sub-pathway is designated as short-patch or single-nucleotide base excision repair (SN-BER). However, if the sugar group is oxidized or reduced and not removed by the pol β dRP lyase activity (16–18), DNA ligase cannot seal the nick (15) and repair occurs through an alternate long patch base excision repair (LP-BER) sub-pathway, involving removal and replacement of several nucleotides (19–22). It has been proposed that, in LP-BER, pol β performs strand displacement synthesis generating a flap that is then removed by another enzyme (19, 22). Thus, pol β is considered a key player in both SN-BER and LP-BER (17, 23–25).

The LP-BER sub-pathway mechanism requires an additional enzyme to effectively remove the modified 5’-dRP-containing flap. This enzyme, flap endonuclease 1 (FEN1), has been characterized as a multifunctional endo/exonuclease that specifically recognizes and removes a DNA flap (26, 27). FEN1 was initially identified as an essential enzyme involved in Okazaki fragment processing (28). Later, FEN1 was implicated in maintaining genomic stability (29–32). Both in vivo and in vitro studies have demonstrated that FEN1 plays a critical role in LP-BER by cleaving a DNA flap structure with damaged DNA (19, 30, 33–36). LP-BER may also involve replicative DNA polymerases pol δ and pol ε (20, 37, 38) as well as accessory proteins such as proliferating cell nuclear antigen (PCNA) (18, 20, 39) and poly(ADP-ribose) polymerase-1 (PARP-1) (36, 40).

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1 The abbreviations used are: BER, base excision repair; APE, AP endonuclease; dRP, deoxyribose phosphate; pol, polymerase; SN-BER, single-nucleotide base excision repair; LP-BER, long patch base excision repair; FEN1, flap endonuclease 1; PCNA, proliferating cell nuclear antigen; PARP-1, poly(ADP-ribose) polymerase-1; THF, tetrahydrofuran; nt, nucleotide(s); XRCC1, x-ray cross-complementing protein 1; TEMED, N,N,N’,N’-tetramethylethylenediamine.
APE may also participate in LP-BER, because it can physically interact with pol β (41, 42) and stimulate FEN1 endonuclease activity (43, 44). In addition, a BER protein complex that contains uracil-DNA-glycosylase, APE, pol β, DNA ligase I, PARP-1, and FEN1 has been identified through both affinity column chromatography and photoaffinity labeling (45–47), suggesting an interaction and cooperation among these proteins in LP-BER. Thus, it is expected that protein-protein interactions and cooperation could be important features of LP-BER.

Depending on the involvement of PCNA, LP-BER has been further classified into a PCNA-dependent sub-pathway, where pol β is involved, and a PCNA-independent sub-pathway, where pol β is the only DNA polymerase that mediates LP-BER DNA synthesis (21, 22, 32, 48). However, a recent study demonstrated that PCNA can also physically interact with pol β (49), raising the possibility that PCNA may have a role even in pol β-dependent LP-BER. Most studies are consistent with the idea that pol β contributes a majority of the DNA synthesis during LP-BER and that FEN1 is required for processing (i.e. cleavage) the DNA flap intermediate (19, 32, 35, 50). Thus, pol β and FEN1 coordination may be an essential element of LP-BER.

The substrate specificities of pol β and FEN1 could allow these enzymes to perform reactions in a sequential order during LP-BER. In one scenario, pol β has been proposed to independently displace the downstream DNA strand and create a flap for FEN1 cleavage (19, 21). A recent study found that FEN1 stimulated pol β-mediated DNA synthesis on a LP-BER substrate, and conversely, pol β stimulated FEN1 cleavage on a LP-BER flap substrate (48). Interestingly, PARP-1 also stimulated pol β LP-BER DNA synthesis, but this stimulation required the presence of FEN1 (36), suggesting that the stimulation of pol β activity by PARP-1 was somehow mediated through FEN1. Overall, these results indicate that pol β and FEN1 interact functionally. However, the mechanism by which these two enzymes collaborate to achieve efficient LP-BER is not known. Mechanistically, FEN1 could stimulate the substrate binding step and/or catalysis of pol β. Alternatively, FEN1 may remove intermediates that pose a block to pol β-mediated LP-BER DNA synthesis. Thus, in this study, we dissected the functional coordination between pol β and FEN1 on LP-BER substrates in vitro. We initially characterized pol β DNA synthesis on various LP-BER intermediates and found them to be poor substrates for pol β DNA synthesis. Accordingly these LP-BER intermediates would represent barriers to accomplish BER. pol β relied on FEN1 cleavage to remove the barriers and proceed with LP-BER DNA synthesis. A “Hit and Run” mechanism is proposed to describe how these enzymes coordinate their enzymatic activities to accomplish LP-BER. Specifically, alternating pol β gap filling and FEN1 gap generation results in an efficient “gap translation.”

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by Integrated DNA Technologies (Corvalis, IA). Radionucleotides (γ-32P]ATP (7000 mCi/ mmol) (MP Biomedicals, Irvine, CA) and (α-32P]dATP (3000 mCi/mmole) and MicroSpin G-25 columns were purchased from Amersham Biosciences (GE Healthcare, Piscataway, NJ). Deoxynucleotide triphosphates were from Roche Diagnostics Corp. Optikinase and terminal deoxynucleotidyl transferase were from US Corp. (Cleveland, OH). All other reagents were from Sigma-Aldrich.

Protein Expression and Purification—Human pol β and FEN1 were expressed and purified as described previously (48). The enzyme concentrations were determined from absorbance at 280 nm (51). The extinction coefficients used for pol β and FEN1 as calculated by ProtParam were 21,170 M⁻¹ cm⁻¹ and 21,880 M⁻¹ cm⁻¹, respectively. Oligonucleotide Substrates—Oligonucleotides were designed to generate DNA substrates, including a single-nucleotide gap, a single-nucleotide gap with a 5′-tetrahydrofuroran (THF) on the downstream oligonucleotide, a nicked DNA, and various lengths of downstream single-stranded flaps each with a 5′-THF residue in the context of nicked DNA. These substrates mimic various proposed SN and LP-BER DNA intermediates. The oligonucleotide sequences are given in Table I. Each substrate was constructed by annealing an upstream primer (U) and a downstream oligonucleotide (D) to a 31-mer template (T). pol β DNA synthesis was measured by following extension of a 5′-labeled upstream primer. The upstream primer was 5′-radiolabeled with [γ-32P]ATP and Optikinase. The unincorporated [γ-32P]ATP was removed with a G-25 spin column. The substrates utilized for FEN1 cleavage or mobility shift assays were radiolabeled at either the 5′- or 3′-end of the downstream oligonucleotides with [γ-32P]ATP and Optikinase or with α-32P]dATP and terminal deoxynucleotidyl transferase. These radiolabeled oligonucleotides were then purified using 12 or 15% polyacrylamide, 7 M urea denaturing gel electrophoresis. A radiolabeled oligonucleotide (U or D) and a corresponding U or D oligonucleotide were annealed to a template at a molar ratio of 1:5.1:5.1, respectively, to generate gapped and nicked DNA or nicked DNA substrates with various length flaps (Table I).

Gel Mobility Shift Assay—The gel mobility shift assay was performed in the binding buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mg/ml bovine serum albumin, 0.1% Nonidet P-40, and 5% glycerol. pol β was preincubated with nicked-THF flap DNA or nicked DNA substrate in the presence or absence of FEN1 at 37 °C for 8 min. An aliquot of each reaction mixture (8 μl) was loaded onto a 1% agarose-1% polyacrylamide gel. The gel was prepared in 0.25% Tris borate-EDTA buffer by combining equal volumes of 2% acrylamide and 0.6% bisacrylamide with melted 2% agarose gel in a total volume of 80 ml. Ammonium persulfate (200 μl of 10%) and 20 μl of TEMED were then added into the gel mixture, which was then poured into a horizontal gel electrophoresis apparatus. The enzyme-DNA complex and unbound DNA were then separated by electrophoresis in 0.25× Tris borate-EDTA buffer at 100 V for 1 h in the cold room (∼4 °C). Radioactive signals were detected with a PhosphorImager (Amersham Biosciences-GE Health) and quantified with ImageQuant version 1.2 software. The concentration of enzyme-DNA complex was calculated between the equation, C = |E| × |I| / (|E| × |I| + Kd), where C is the concentration of enzyme-DNA complex, |E| is the intensity of DNA-protein complex, and |I| is the intensity of free DNA. Apparent Kd was obtained by fitting the data to a quadratic equation, ([pol β-DNA] = 0.5 × ([pol β + Kd + DNA]) − ([0.25 × ([pol β + Kd + DNA]) − ([pol β-DNA])])1/2, where [pol β-DNA] is the concentration of pol β-DNA complex, pol β is the concentration of pol β, Kd is the apparent dissociation constant, and DNA is the concentration of DNA.

Enzymatic Assays—pol β DNA synthesis was determined by measuring nucleotide insertion into a DNA substrate. The 5′-end of the upstream primer of these substrates was radiolabeled with [γ-32P]ATP, as described above. In all cases, the 5′-end of the downstream oligonucleotide was phosphorylated. The reaction was performed in buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.2 mM EDTA, and 0.1 mM mg/ml bovine serum albumin. pol β, as indicated, was incubated with 50 nm DNA substrate in the presence of 5 mM MgCl2 and 5 μM dCTP. The reaction mixture was assembled on ice, transferred to 37 °C, and incubated for 10 min. The FEN1 cleavage assay was performed under the same conditions except that the downstream oligonucleotides of the substrates were radiolabeled at the 3′-end. The substrates were separated from the products by 15 or 18% polyacrylamide, 7 M urea denaturing gels, and products were detected by a PhosphorImager and quantified by ImageQuant as described above.

Measurement of kobs of pol β and FEN1—The apparent rate (kobs) of pol β nucleotide insertion with nicked-THF flap DNA and nicked DNA substrates in the absence and presence of FEN1 was measured by incubating 1 nM of each enzyme with 200 nM DNA in the same buffer as the one for pol β activity assay. The reaction was initiated with 5 mM MgCl2 and incubated at 37 °C. Aliquots (10 μl) were removed from the reaction mixture at various time intervals (0.5–5 min). The apparent rate of FEN1 cleavage on nicked-THF flap and nicked DNA substrates in the absence and presence of pol β was also measured. The substrates for measuring FEN1 cleavage were radiolabeled at the 5′-end of their downstream oligonucleotides. The slopes of the linear time courses were used to calculate the respective turnover numbers where kobs = Vobs[E]total.

RESULTS

FEN1 Stimulates Initiation of LP-BER—Previous studies had shown that FEN1 stimulated LP-BER DNA synthesis activity of pol β (36, 48), thereby facilitating LP-BER. To explore
the mechanism of this effect, we initially examined pol β/H9252 DNA synthesis on a nicked-THF flap DNA substrate in the presence of FEN1. This substrate mimics the initial LP-BER transient intermediate, because the single-nucleotide gap has already been filled and the lyase activity of pol β/H9252 will not remove the 5′-THF residue (16–18). DNA synthesis was measured over an 8-min time interval (Fig. 1). pol β/H9252 was incubated with the substrate in the presence of 5 μM dNTPs with or without FEN1. In the absence of FEN1, only a small amount (≈5%) of the nicked-THF flap substrate (50 nM) was extended by one nucleotide (lanes 1–8) and B. Addition of FEN1 enhanced the amount of pol β DNA synthetic products by ≈5-fold (lanes 9–16), and this was predominantly in the form of a single-nucleotide insertion synthesis. LP-BER DNA Synthesis of pol β Is Poor on Nicked DNA Substrates—To further understand the influence of FEN1 on pol β LP-BER DNA synthesis, we examined pol β nucleotide insertion on substrates that resemble various LP-BER intermediates. These included a nicked DNA, a nicked-THF flap, a nicked-THF-1nt flap, a nicked-THF-3nt flap, a nicked-THF-5nt flap, and a nicked-THF-15nt flap (Table I and Fig. 2). pol β-mediated DNA synthesis was lowest on the nicked DNA and nicked-THF flap DNA substrates, as compared with the other substrates (Fig. 2). DNA synthesis on longer nicked-THF flaps was lower than that with single-nucleotide gapped and 1nt-gap-THF DNA substrates that mimic SN-BER intermediates (Fig. 2). This was most obvious at lower enzyme concentrations (0.1 and 0.5 nM) where substrate depletion had little influence on the rate of product formation (Fig. 2, compare lanes 10 and 11 with 2 and 3, and lanes 14 and 15 with 6 and 7, respectively). Use of a higher concentration of pol β with the various lengths of nicked-THF flap substrates converted most of the labeled substrates into extended products indicating that all these substrates were fully annealed (Fig. 2, and data not shown). Gel mobility shift assays showed that the binding of pol β onto the nicked and various nicked-THF flap substrates was significantly decreased compared with gapped DNA (data not shown). This indicated that the nicked structures may not support pol β binding, thereby, compromising pol β nucleotide insertion. Thus, the LP-BER intermediates representing nicked DNA appear to form a barrier to pol β DNA synthesis. Overall, our results demonstrate that pol β DNA synthesis on nicked-THF flap and nicked DNA is poor; therefore, the development of a nicked flap intermediate during LP-BER would be a rate-limiting step of LP-BER DNA synthesis.

**Fig. 1.** FEN1 stimulates initiation of LP-BER. A, a nicked-THF flap DNA substrate that mimics an initiating intermediate of LP-BER was employed to examine the influence of FEN1 on pol β-mediated initiation of LP-BER. The substrate was made by annealing a downstream oligonucleotide, D_{THF flap}, and an upstream primer, U, to a template primer, T (Table I). The substrate was radiolabeled at the 5′-end of the upstream primer (asterisk) and illustrated above the gel. The 5′-end of the downstream oligonucleotide was phosphorylated. pol β (0.5 nM) was incubated with 50 nM substrate, 5 μM dNTPs, and 5 mM MgCl₂ at 37 °C in the absence (lanes 1–8) or presence (lanes 9–16) of 25 nM FEN1. The reaction was allowed to proceed for various periods (1, 2, 3, 4, 5, 6, 7, or 8 min) and product formation was quantified as described under “Experimental Procedures.” B, quantitative analysis on pol β DNA synthesis in the absence (▲) and presence of FEN1 (■) from panel A.
will delay progression of LP-BER. FEN1 could alleviate this block by removing a nucleotide at the nicks, stimulating pol β DNA synthesis, or by stabilizing the interaction between pol β and these DNA substrates allowing an effective pol β-mediated strand displacement synthesis or, alternatively, by destabilizing (i.e. melting) the downstream strand ahead of pol β DNA synthesis. To examine these possibilities, we initially measured DNA synthesis at various concentrations of pol β in the absence of enzymes.
and presence of FEN1 (5 nM) using a nicked-THF flap or a nicked DNA substrate (Fig. 3A). In the absence of FEN1, pol β DNA synthesis on both substrates was poor unless a high enzyme concentration was utilized. At lower concentrations of pol β (0.1–0.5 nM, lanes 2–4), very little product was produced in the absence of FEN1. FEN1 stimulated pol β DNA synthesis with both substrates (Fig. 3A, lanes 9–11), and the stimulation was most pronounced when substrate depletion did not interfere with the rate of product formation (i.e. at lower pol β concentrations).

Next, we examined the requirement for the cleavage activity of FEN1 in the stimulation of pol β by making use of a nuclease-deficient FEN1 mutant, FEN1<sup>D181A</sup> (52). This mutant has the same substrate binding affinity as wild type enzyme, but has a severe cleavage defect (52), particularly on nicked-THF flap and nicked DNA (data not shown). Unlike wild-type FEN1, the mutant failed to significantly stimulate pol β with both substrates (Fig. 3B). Therefore, FEN1 cleavage activity was critical for stimulating pol β-mediated LP-BER DNA synthesis.

The ability of FEN1 to modulate the binding affinity of pol β on nicked-THF flap and nicked DNA was examined by a gel mobility shift assay (Fig. 4). The assay was performed in the absence of magnesium to ensure that no FEN1 cleavage would occur. Results in Fig. 4 demonstrated that FEN1 (5 nM) did not significantly alter the binding of pol β to nicked-THF flap (Fig. 4A) and nicked DNA (Fig. 4B). In addition, a higher concentration of FEN1 failed to alter the amount of pol βDNA complex (Fig. 4, lanes 14 and 15), suggesting a lack of FEN1 effect on pol β binding to these substrates. Taken together, these results allow us to conclude that FEN1 stimulates pol β LP-BER DNA synthesis through 5′ cleavage of the nicked-THF flap or nicked DNA.

FEN1 Cleavage of Nicked-THF flap DNA and Nicked DNA Creates a One-nucleotide Gap—FEN1 cleavage on a conventional nicked flap DNA substrate usually generates three species of products: nicked DNA, single-nucleotide-gapped DNA, and cleaved flap (26, 27). These products result from FEN1 cleavage at the bifurcated junction between the flap and the downstream double-stranded DNA and FEN1 endonucleolytic cleavage of the first nucleotide in the downstream duplex DNA. FEN1 cannot make a cleavage precisely at the junction between the THF group and double-stranded DNA (35); instead, FEN1 endonucleolytically cleaves the first nucleotide of the downstream double-stranded DNA. This results in removal of a single nucleotide with a 5′-THF group, thereby generating a 1-nt gap. The results in Fig. 5A demonstrate that, in the presence of pol β, FEN1 effectively removed a nicked-THF flap primarily producing a 1-nt gap (Fig. 5A, lanes 6–9). Progressive FEN1 exonucleolytic cleavage products were also observed;
However, these were only minor species. As expected, pol β alone did not generate any cleavage product (Fig. 5A, lanes 2–5).

FEN1 cleavage of the nicked DNA substrate also resulted in a 1-nucleotide gap (Fig. 5B, lanes 6–9). The results in Fig. 5C showed that production of the 1-nucleotide gap was greater with increasing FEN1 concentrations in the presence of pol β (0.5 nM). A lower concentration of FEN1 (i.e., 5 nM) converted most of the nicked-THF flap substrates into 1-nucleotide gap, whereas 25 nM FEN1 converted almost all of the substrate into the 1-nucleotide gap product (Fig. 5C). Taken together, these results demonstrated that FEN1 cleavage on both nicked-THF flap and nicked substrates created a 1-nucleotide gap molecule, which is an ideal substrate for pol β. This implied that pol β substrate binding affinity would be improved, because it could preferentially bind to the 1-nucleotide gap, the FEN1 cleavage product. Therefore, we examined pol β substrate binding under conditions where FEN1 can effectively cleave the nicked-THF flap and the nicked DNA, thereby, generating a 1-nucleotide gap (i.e., 5 nM FEN1 and increasing concentrations of pol β). The results indicated that FEN1 cleavage significantly improved pol β DNA binding (Fig. 6). This was observed at the lower concentrations of pol β used (Fig. 6, compare lanes 2–7 with lanes 10–15).

In the experiments summarized in Table II, the relative binding of pol β to the “nicked-THF flap” DNA substrate and to a 1-nucleotide gap was determined as a function of various concentrations of FEN1. The results indicated that the apparent binding affinity of pol β increased at higher FEN1 concentrations. At 5 nM FEN1, when most of the nicked-THF flaps were converted into 1-nucleotide gaps, the relative binding affinity of pol β increased 6-fold, approaching that for a 1-nucleotide gap substrate. Thus, FEN1 cleavage appeared to remove the nicked-THF flap, thereby creating a 1-nucleotide gap molecule to which pol β could avidly bind. The results in Fig. 6 also illustrated that pol β was preferentially bound to the 1-nucleotide gap DNA even in the presence of a 10- or 20-fold excess of FEN1 (Fig. 6, lanes 14 and 15). This implies that FEN1 cannot effectively compete with pol β for binding to the 1-nucleotide gap and pol β preferentially bound to the FEN1 cleavage product. Similar results were obtained for pol β binding with a nicked DNA substrate (data not shown), suggesting that FEN1 employs the same mechanism to stimulate pol β DNA synthesis on the nicked DNA substrate.

If FEN1 creates a preferred 1-nucleotide gap substrate for pol β, FEN1 cleavage of the nicked THF flap and the nicked DNA substrates in situ should significantly increase the overall rate of pol β DNA synthesis. This could be evaluated by quantitatively measuring the rate of nucleotide insertion by pol β in the presence of FEN1. The rate of nucleotide insertion with FEN1 cleavage should approach that observed for an authentic single-nucleotide-gapped substrate. The apparent turnover number for nucleotide insertion by pol β with 5 μM dCTP was determined with a saturating concentration of a nicked-THF flap, a nicked DNA, and a single-nucleotide gap in the presence and absence of FEN1. This dCTP concentration represented a physiologically relevant concentration of dCTP in human cells (67). The observed rates (kcat) for pol β under these conditions are given in Table III. In the absence of FEN1, pol β DNA synthesis with nicked-THF flap and nicked DNA substrates was 6- and 12-fold lower than that for the 1-nucleotide gap substrate, respectively. In contrast, the rate of nucleotide insertion for all three substrates in the presence of FEN1 was similar to that for the 1-nucleotide gap substrate (−0.1 s−1). Thus, from the viewpoint of pol β catalysis, this confirmed that FEN1 removed the block in the nicked-THF flap and nicked substrates, creating a
nicked-THF flap and nicked DNA substrate.

**DISCUSSION**

A working model of LP-BER proposed that pol β initiates strand displacement synthesis (36, 48, 53) and proceeds to displace a 2- to 11-nt flap with DNA damage at the 5′-end (19). The damage-containing flap is then removed by FEN1 and thus provides a nicked substrate for DNA ligation. This model suggests that pol β catalyzes strand displacement DNA synthesis independent of FEN1. However, the fact that FEN1 stimulates pol β LP-BER DNA synthesis *in vitro* (48) raises the possibility that an LP-BER intermediate poses a barrier to pol β DNA synthesis leading to poor strand displacement activity. Our results demonstrated that FEN1 stimulated pol β DNA synthesis on substrates representing the intermediates formed in initiation and elongation phases of LP-BER (Fig. 1). We also examined pol β DNA synthesis on substrates that mimic LP-BER intermediates with a displaced flap. Much weaker DNA synthesis by pol β was found on all these substrates than on a 1-nt gap. Overall, the lowest activities were found on the LP-BER initial intermediate (nicked-THF flap), and on the LP-BER elongation intermediate, nicked DNA (Fig. 2). This indicated that pol β-mediated DNA strand displacement synthesis was blocked by LP-BER intermediates. The LP-BER initial intermediate and the nicked DNA intermediate appeared to be major barriers and would inhibit the pathway *in vivo*. This also implies that pol β alone does not effectively conduct strand displacement DNA synthesis during LP-BER.

We hypothesized that FEN1 cleavage could promote pol β DNA synthesis by enhancing pol β binding to LP-BER intermediates or stimulating pol β DNA synthesis by removing a barrier. We found that FEN1 cleavage was required for the stimulation of pol β DNA synthesis (Figs. 3 and 4). In addition, FEN1 protein alone could not affect the binding of pol β to the initial LP-BER intermediate or nicked DNA (Fig. 5). Thus, removal of the LP-BER barriers by FEN1 appeared to be essential for the pol β LP-BER DNA synthesis observed here. We concluded that FEN1 stimulated pol β LP-BER DNA synthesis by relieving a kinetic barrier to the reaction rather than by facilitating pol β-mediated strand displacement synthesis.

Because FEN1 cleavage on nicked-THF flap and nicked DNA primarily results in a single-nucleotide gap (Fig. 5), the binding affinity of pol β on the resulting DNA product *in situ* approached that of a 1-nt gap (Fig. 6 and Table II). The rate of pol β DNA synthesis in the reaction mixture with nicked and nicked-THF flap substrates increased 4- to 9-fold by FEN1 cleavage; again approaching the rate of pol β DNA synthesis observed on the 1-nt-gapped substrate (Table III). The altered substrate binding and catalytic properties of pol β are consistent with FEN1 removal of LP-BER barriers creating single-nucleotide gap substrates for pol β. Thus, FEN1 cleavage creates an ideal substrate (1-nt gap) for pol β DNA synthesis from a poor substrate (nicked-THF flap or nicked DNA). Alternating pol β 1-nt gap-filling DNA synthesis and FEN1 cleavage leads to an efficient LP-BER DNA synthesis. Therefore, we propose that, during LP-BER, FEN1 cleavage creates short gaps for pol β to perform efficient gap-filling reactions. This model predicts that the rate of FEN1 cleavage might be faster than that for pol β DNA synthesis on both nicked-THF flap and nicked DNA. Our results confirmed this prediction by showing that the turnover number for FEN1 cleavage on the intermediates was severalfold faster than that for pol β DNA synthesis (Table III). This demonstrates that FEN1 can cleave the LP-BER barriers fast enough so that the reaction is limited by pol β DNA synthesis. This further indicates that FEN1 cleavage and pol β gap-filling occurs in a specific sequential order to accomplish LP-BER. Because the values tabulated in Table III are rate constants, their magnitude represents velocities normal-
that substrate binding, catalysis, and dissociation of pol 
mediated by the substrate specificity of each enzyme. We propose 
FEN1 by a unique substrate-switching fashion that is deter-
quent pol 
providing pol 
affinity to the gap (data not shown), FEN1 rapidly dissociates 
-THF residue creating a 1-nt gap. Due to its lower binding 
loads onto the nicked-THF flap and excises one nucleotide with 
molecule, represents the first LP-BER intermediate. FEN1 
lyase activity. The resultant product, nicked-THF flap DNA 
will on catalytic activities (19, 22), we show that pol 
cannot efficiently create an authentic flap by strand displacement DNA synthesis. Indeed, FEN1 would be expected to cleave a nicked-THF flap structure before pol 
would be able to insert a nucleotide into the nick. Dianov et al. (21) identified a 5'-dRP-3'-nt flap released by mouse fibroblast cell extracts during pol 
-mediated LP-BER in vitro. This suggested that FEN1 cleaved a flap created by pol 
-mediated LP-BER strand displacement synthesis. Our results demonstrate that pol 
alone does not efficiently catalyze strand displacement DNA synthesis under physiological dNTP concentrations. It must rely on FEN1 to remove the LP-BER barrier intermediates creating the 1-nt gap. The short flap released during cell extract-mediated BER also raises the possibility that other proteins could participate in the Hit and Run mechanism increasing the size of the 
excised flap. In addition to the 1-nt gap, it is well known that 
pol 
is able to bind and efficiently fills gaps of 2–6 nucleotides 
in length through processive DNA synthesis (54). The principle 
patch size for LP-BER is often reported to be 2 nucleotides (55) 
consistent with a single FEN1 cleavage event after pol 
has mediated single-nucleotide gap filling.

Many BER and DNA replication proteins physically interact with pol 
and FEN1 in vivo. These proteins include PCNA (49, 56), APE (41, 43), Werner syndrome protein (57–59), PARP-1 (36, 46), DNA ligase I (45), x-ray cross-complementing protein 1 (XRC1) (60), and p53 (61). These observations suggest that 
these multiprotein interactions must be regulated during BER. For example, PCNA physically interacts with both FEN1 and pol 
(49, 56) and stimulates FEN1 endo- and exonuclease 
cleavage activity by 5- to 50-fold (56). APE and Werner protein 
stimulates both pol 
DNA synthesis and FEN1 cleavage (41, 43, 57–59). PARP-1 physically interacts with pol 
(40) but does not affect DNA polymerase activity (36). However, PARP-1 can stimulate FEN1 flap-cleavage suggesting that it could play an important role in LP-BER (36). Interestingly, PARP-1 stimulates pol 
LP-BER DNA synthesis only in the presence of 
, suggesting that PARP-1 indirectly stimulates pol 

### TABLE II

| Enzymatic assay | Accessory enzyme | BER DNA intermediate, k_{obs} |
|----------------|------------------|-------------------------------|
|                |                  | Nicked-THF flap | Nicked 1-nt gap |
| Pol β          | None             | 0.01             | 0.005 | 0.12 |
|                | FEN1             | 0.09             | 0.090 | 0.11 |
| FEN1           | None             | 0.04             | 0.02  | ND* |
|                | Pol β            | 0.04             | 0.03  | ND  |

*The respective DNA synthesis (pol β) or cleavage (FEN1) reactions were determined as outlined under "Experimental Procedures." 
*ND, not determined. The activity on a 1-nt gap DNA substrate is significantly lower than that on a nicked substrate.

### TABLE III

| Enzymatic assay | Accessory enzyme | BER DNA intermediate, k_{obs} |
|----------------|------------------|-------------------------------|
|                |                  | Nicked-THF flap | Nicked 1-nt gap |
| Pol β          | None             | 0.01             | 0.005 | 0.12 |
|                | FEN1             | 0.09             | 0.090 | 0.11 |
| FEN1           | None             | 0.04             | 0.02  | ND* |
|                | Pol β            | 0.04             | 0.03  | ND  |

*The relative DNA synthesis (pol β) or cleavage (FEN1) reactions were determined as outlined under "Experimental Procedures." 
*ND, not determined. The activity on a 1-nt gap DNA substrate is significantly lower than that on a nicked substrate.

**Fig. 6.** FEN1 cleavage of nicked-THF flap facilitates pol β DNA binding. Gel mobility shift assay was performed in a reaction mixture containing increasing concentrations of pol β (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 nm) at 5 nm FEN1. The reaction mixture was incubated at 37 °C for 8 min. Enzyme-DNA complexes and free DNA were resolved in a 1% agarose-1% polyacrylamide gel. Reaction mixture without enzyme was run in lane 1. The substrate was radioactively labeled at the 3'-end of the downstream oligonucleotide (asterisk) and schematically illustrated above the gel. Experimental details are described under "Experimental Procedures."
DNA synthesis through stimulating FEN1 activity (i.e. production of the 1-nt gap). This is consistent with the interpretation that PARP-1 may be essential for an efficient LP-BER in vivo (40, 62). XRCC1 physically interacts with pol β (60, 63). A recent study demonstrated that the pol β-XRCC1 interaction might enhance the efficiency of DNA Lig III during BER (64). p53 also directly interacts with pol β and stabilizes pol β binding onto an AP site (61). This interaction is essential for the p53-mediated stimulation of BER (61). However, it is not known whether this interaction can alter pol β enzymatic activities and the mechanism by which p53 stimulates SN- and LP-BER. Although our current knowledge about the role of the multiprotein interactions in LP-BER and SN-BER is limited, the pol β/FEN1-mediated LP-BER Hit and Run mechanism may be accomplished even more efficiently within a multiprotein complex. It should be interesting to examine this possibility in the future.

With the minimal mechanism developed here, we can now examine the influence of other BER proteins on the coordination and efficiency of LP-BER. This can be achieved by combining both in vitro enzymology and in vivo mutational analyses approaches. Taken together, these results should dissect the contribution of protein-protein interactions and/or kinetic coordination during BER.

Several alternative LP-BER sub-pathways have been identified in mammalian cellular systems according to utilization of alternate DNA polymerases. Co-existing LP-BER sub-pathways may provide functional redundancy, so that one sub-pathway will function if the other is compromised. This notion indicates that LP-BER is important for the cell in repairing DNA lesions that cannot be repaired by SN-BER. In the LP-BER sub-pathways that involve DNA replicative polymerases, pol δ and/or ε (19, 22, 65) or pol β and pol δ/ε (53), it has been proposed that the DNA damage is removed through cleavage of a 2- to 11-nt flap by FEN1 (19, 65). Tethered by PCNA, pol δ/ε can perform processive strand displacement synthesis, thereby effectively creating a longer flap for FEN1 cleavage. Thus, this sub-pathway is featured by PCNA stimulation of pol strand displacement synthesis (28) and FEN1 cleavage of a flap (56). Functional cooperation between pol δ/ε and FEN1 removes the DNA damage strand through “a nick translation” mechanism (66). In contrast, the pol β/FEN1-catalyzed LP-BER sub-pathway proposed in this study employs a “gap translation” to remove the damaged DNA strand. Because of the efficient gap-filling activity of pol β, the Hit and Run mechanism may be more efficient than PCNA-dependent LP-BER, because PCNA-dependent LP-BER involves additional steps such as loading of PCNA by replication factor C, loading of a DNA polymerase by PCNA, strand displacement DNA synthesis, and finally unloading of DNA polymerase making a way for subsequent FEN1 cleavage and DNA ligation. These steps would appear to be costly in terms of enzyme coordination and energy utilization. It should be of functional interest to compare the relative efficiencies of a pol β-mediated LP-BER with PCNA-dependent LP-BER sub-pathway.

In summary, by dissecting the functional interaction and coordination of pol β and FEN1 during a pol β-mediated LP-BER, we demonstrated that pol β cooperates with FEN1, in a

**Fig. 7. The Hit and Run mechanism of pol β/FEN1-mediated long patch base excision repair.** AP endonuclease makes an endonucleolytic cleavage at the 5’-end of an abasic site resulting in a 5’-dRP in a one-nucleotide-gapped DNA. If the dRP moiety is reduced or oxidized, pol β employs its polymerase activity to fill the gap creating a nicked-reduced/oxidized sugar flap, because its dRP lyase cannot remove the modified sugar residue. pol β rapidly dissociates from the nicked-THF flap product permitting FEN1 access, which cleaves the THF flap resulting in a 1-nt gap. pol β then fills the gap producing a nick. Subsequent FEN1 removal of one nucleotide from the 5’-end of the nick leads to another 1-nt gap for pol β to fill in. Alternating FEN1 cleavage and pol β DNA synthesis shown as enzyme binding, catalysis, and dissociation (Hit and Run), leads to a removal of the modified dRP moiety and results in replacement of 2–11 nucleotides (long patch) until DNA ligase seals the nick and terminates LP-BER.
specific sequential order determined by the substrate specificity of each enzyme. We propose and define a Hit and Run mechanism of pol β-mediated LP-BER that is accomplished through alternating catalytic cycles of pol β and FEN1 on different LP-BER intermediates. We suggest that the LP-BER pathway is initiated by FEN1 removal of a nicked-THF flap, followed by alternating pol β gap filling and FEN1 cleavage reactions on nicked DNA intermediates, thereby eventually forming a 2- to 11-nt repair synthesis patch.

Acknowledgments—We are grateful to Drs. J. K. Horton and S. Aoyagi for critical reading of the manuscript.

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J. Biol. Chem. 2005, 280:3665-3674.
doi: 10.1074/jbc.M412922200 originally published online November 22, 2004

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