Escherichia coli Uracil- and Ethenocytosine-Initiated Base Excision DNA Repair: Rate-Limiting Step and Patch Size Distribution†

Jung-Suk Sung‡ and Dale W. Mosbaugh*,‡,§,||

Departments of Environmental and Molecular Toxicology, Biochemistry and Biophysics, and the Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331-7301

Received November 5, 2002; Revised Manuscript Received January 29, 2003

ABSTRACT: The rate, extent, and DNA synthesis patch size of base excision repair (BER) were measured using *Escherichia coli* GM31 cell-free extracts and a pGEM (form I) DNA substrate containing a site-specific uracil or ethenocytosine target. The rate of complete BER was stimulated (∼3-fold) by adding exogenous *E. coli* DNA ligase to the cell-free extract, whereas addition of *E. coli* Ung, Nfo, Fpg, or Pol I did not stimulate BER. Hence, DNA ligation was identified as the rate-limiting step in the *E. coli* BER pathway. The addition of exogenous DNA polymerase I caused modest inhibition of BER, which was overcome by concomitant addition of DNA ligase. Repair patch size determinations were performed to assess the distribution of DNA synthesis associated with both uracil- and ethenocytosine-initiated BER. During the early phase (0−5 min) of the BER reaction, the large majority of repair events resulted from short patch (1-nucleotide) DNA synthesis. However, during the late phase (>10 min) both short and long (2−20 nucleotide) patches were observed, with long patch BER progressively dominating the repair process. In addition, the patch size distribution was influenced by the ratio of DNA polymerase I to DNA ligase activity in the reaction. A novel mode of BER was identified that involved DNA synthesis tracts of >205 nucleotides in length and termed very-long patch BER. This BER process was dependent upon DNA polymerase I since very-long patch BER was inhibited by DNA polymerase I antibody and addition of excess DNA polymerase I reversed this inhibition.

Base excision DNA repair serves as a strategic cellular defense system to protect against the accumulation of DNA damage in prokaryotic and eukaryotic cells (*1*). The uracil-mediated BER[1] pathway functions as part of this system to remove uracil residues that accumulate as U·A base pairs in DNA following the incorporation of dUMP in place of dTMP during DNA synthesis (*1*, *2*). This BER pathway also removes uracil produced by the deamination of cytosine in DNA, which results in the formation of premutagenic U·G mispairs (*1*, *3*−*5*). The uracil-mediated DNA repair process plays an important role in maintaining genetic stability since inactivation of uracil-mediated BER produces genotoxic, mutagenic, and lethal consequences (*1*, *6*, *7*).

Uracil-mediated BER is initiated by uracil-DNA glycosylase activity that cleaves the *N*-glycosylic bond linking the uracil base to the deoxyribose phosphate backbone of DNA (*8*). In *Escherichia coli*, two genetically distinct monofunctional forms of uracil-DNA glycosylase have been purified to apparent homogeneity and characterized (*9*, *10*). While these two enzymes share a common catalytic activity, they exhibit very different substrate specificity. *E. coli* uracil-DNA glycosylase (Ung) acts preferentially on uracil residues located in single-stranded DNA and also recognizes uracil in duplex DNA (i.e., U·G and U·A target sites) with only moderately reduced efficiency (*8*, *11*). In contrast, double-strand uracil-DNA glycosylase (Dug, also termed Mug) removes uracil from DNA containing U·G or U·T mispairs but inefficiently recognizes U·A base pairs and lacks detectable activity on single-stranded uracil-DNA (*9*, *10*, *12*). In addition, Dug efficiently excises 3,N^4-ethenocytosine residues from double-stranded DNA but not from single-stranded DNA (*9*, *10*). These two enzymes can be distinguished in cell-free extracts based on differential sensitivity to the *Bacillus subtilis* bacteriophage PBS-1 and -2 uracil-DNA glycosylase inhibitor (Ugi) protein (*13*). Ugi efficiently inhibits Ung activity by forming an essentially irreversible Ung·Ugi complex; however, Dug activity is not inhibited by Ugi (*13*, *14*).

Both Ung and Dug initiate uracil-mediated BER by catalyzing the release of free uracil and creating an apyrimidinic site in DNA (*1*, *8*, *10*). Completion of the BER pathway requires three additional steps: (i) AP-site incision

† This work was supported by National Institutes of Health Grants GM32823 and ES00210 and the Nucleic Acids and Protein Facilities and Services Core of the Environmental Health Sciences Center. This is Technical Report 11939 from the Oregon Agricultural Experiment Station.

* To whom correspondence should be addressed. Tel: (541) 737-1797. Fax: (541) 737-0497. E-mail: mosbaugd@ucs.orst.edu.

‡ Department of Environmental and Molecular Toxicology.

§ Department of Biochemistry and Biophysics.

|| Environmental Health Sciences Center.

[1] Abbreviations: BER, base excision repair; AP, apurinic/apyrimidinic; ϵC, 3,N^4-ethenocytosine; dRP, deoxyribose 5′-phosphate; dRPase, 5′-deoxyribosephosphodiesterase; Fpg, formamidopyrimidine-DNA glycosylase; PCNA, proliferating cell nuclear antigen; moi, multiplicity of infection; form I, covalently closed circular DNA; form II, form I DNA containing one or more phosphodiester bond discontinuities; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; dNMP[αS], 2′-deoxyribonucleoside α-thiolmonophosphate; bp, base pair(s); nt, nucleotide(s).

10.1021/bi027115v CCC: $25.00
Published on Web 03/27/2003

by an AP endonuclease; (ii) incorporation of one or more nucleotides during DNA repair synthesis; and (iii) ligation of the phosphodiester backbone by DNA ligase. In *E. coli*, exonuclease III (Xth) and endonuclease IV (Nfo) have been implicated in the AP-site cleavage reaction (*15*, *16*). Both enzymes promote incision of the phosphodiester bond on the 5′-side of the AP site, leaving a 3′-hydroxyl containing nucleotide and deoxyribose phosphate (dRP) residue at the 5′ terminus (*17*, *18*); however, approximately 90% of the AP endonuclease activity detected in *E. coli* has been attributed to Xth (*18*). Removal of the 5′-terminal dRP residue may involve the action of a hydrolytic deoxyribophosphodiesterase (dRPase) or an AP-lyase activity (*19*, *20*). Initial reports indicated that exonuclease I (SbcB) and RecJ exhibited dRPase activity capable of participating in BER (*21*, *22*). Recently, these observations were challenged by Piersen et al. (*20*) who were unable to detect dRPase activity in SbcB and RecJ preparations but observed robust dRPase activity associated with 2,6-dihydroxy-5*N*-formamidopyrimidine DNA glycosylase (Fpg). Fpg utilizes β-elimination catalysis to remove dRP residues from incised AP sites located on 5′-termini and has been described as the major EDTA-resistant enzyme responsible for dRP release in *E. coli* cell-free extracts (*23*). The 5′-terminal dRP residue may also be removed by the 5′ to 3′ exonuclease activity of *E. coli* DNA polymerase I (Pol I), which has been shown to excise 5′-dRP as part of a di-nucleotide product (*24*). Following dRP removal, gap-filling DNA synthesis by DNA polymerase I is required to replace the excised nucleotide(s) (*12*). If the 5′-dRP residue is not removed prior to DNA synthesis, then DNA polymerase I will displace the dRP-containing strand via a strand displacement reaction (*25*). In this case, the displaced strand is eventually cleaved at the branch point by the structure-specific 5′ nuclease associated with DNA polymerase I (*26*, *27*). The extent of strand displacement may vary at different repair sites since the DNA polymerase and 5′ to 3′ exonuclease activities of DNA polymerase I are not precisely coordinated (*28*). The BER process is completed by *E. coli* DNA ligase that covalently joins juxtaposed 3′-hydroxyl and 5′-phosphoryl termini (*29*).

Several studies have investigated the DNA synthesis patch size associated with complete BER using cell-free extracts of *E. coli* (*13*, *29*−*32*), *Saccharomyces cerevisiae* (*33*), *Xenopus laevis* oocytes (*34*), bovine testis (*35*), mouse embryonic fibroblast (*36*), Chinese hamster ovary (*37*), and various human cells including adenocarcinoma (*32*, *38*), glioblastoma (*38*), cervical carcinoma (*39*, *40*), and lymphoid (*41*). From these studies, two distinct BER pathways have been identified: one involving single nucleotide gap-filling DNA synthesis (short patch BER) and the other involving incorporation of two to 20 nucleotides (long patch BER). Using a short oligonucleotide (30-mer) DNA substrate containing either a U·G or a U·A target site and *E. coli* cell extracts, Dianov et al. (*30*) initially reported that DNA synthesis associated with uracil-initiated BER was limited in large part to replacement of a single nucleotide. A similar observation was made using an in vitro reconstituted enzyme system composed of purified *E. coli* Ung, Nfo, RecJ, Pol I, and DNA ligase utilizing a 30-mer oligonucleotide substrate containing a U·G mispair (*21*, *29*). However, in the absence of RecJ protein, a larger repair patch was detected that constituted between two and 18 nucleotides (*29*). Dianov and Lindahl (*29*) also observed that a low concentration of DNA polymerase I facilitated short patch BER in the in vitro BER reaction. Thus, the repair patch size could be influenced by the relative concentration of BER enzymes. In contrast to the observations of Dianov and Lindahl, Sandigursky et al. (*31*) reported that the repair of uracil-containing plasmid DNA by *E. coli* cell-free extracts invariably resulted in long patch BER; specifically, DNA synthesis tracts of >11 nt and <19 nt in length. Recently, Sung et al. (*13*) reported that uracil-initiated BER conducted with an M13mp2 form I uracil-DNA substrate in *E. coli* cell extracts consisted of predominantly (~90%) long patch BER that ranged from two to 20 nucleotides. The investigations by Sung et al. (*13*) and Sandigursky et al. (*31*) utilized a closed circular DNA; thus, use of a small oligonucleotide substrate appears to bias BER toward short patch repair. This bias is thought to occur because short oligonucleotides may not provide a platform sufficient for the interaction of DNA polymerase and other BER proteins to carryout long patch BER (*13*, *31*, *42*).

Although uracil-initiated BER has been studied extensively, the factor(s) that mediate the choice between short and long patch BER in *E. coli* remain to be elucidated. In addition, the rate-limiting step in the *E. coli* uracil-mediated BER pathway has not been identified. In a previous report, we examined the kinetics of uracil-mediated BER in *E. coli* cell extracts using a closed circular duplex DNA substrate containing a site-specific U residue (*13*, *32*). In the present study, a similar approach was utilized to assess the influence of *E. coli* uracil-DNA glycosylase, endonuclease IV, deoxyribophosphodiesterase, DNA polymerase I, and DNA ligase supplementation of *E. coli* GM31 cell-free extracts on the BER efficiency and patch size. Both uracil- and ethenocytosine-initiated BER have been examined, and the rate-limiting step associated with each has been identified. We also demonstrate that alteration of the balance between DNA polymerase I and DNA ligase modulates the repair patch size distribution. The results provide the first evidence that very-long repair patch BER (>205 nucleotides) occurs and that this repair process is mediated by DNA polymerase I.

MATERIALS AND METHODS

Materials. Oligodeoxynucleotides ATCCTCTAGAGTX-GACCTGCAGG, C-23-mer (X = C), U-23-mer (X = U), and ϵC-23-mer (X = ϵC) were synthesized and purified by Midland Certified Reagent Company; 5′-end phosphorylated oligodeoxynucleotides were prepared as described previously (*10*). pGEM-3Zf(+) plasmid DNA and helper phage (R408) were procured from Promega. *E. coli* strain GM31 was obtained from *E. coli* Genetic Stock Center (Yale University), and the isogenic strains BH156 (*ung*), BH157 (*dug*), and BH158 (*ung dug*) were provided by A. S. Bhagwat (Wayne State University). *E. coli* JM109 was obtained from New England Biolabs. *E. coli* Ung, Dug, T4 DNA polymerase, and bacteriophage PBS-2 Ugi proteins were purified as described by Sung et al. (*13*). Nfo was provided by B. Demple (Harvard University), and Fpg was obtained from S. Mitra (University of Texas Medical Branch, Galveston). *E. coli* exonuclease III (Xth) and restriction endonuclease *Bsr*I, *Hind*III, and *Bam*HI were purchased from New England

Biolabs. Antiserum specific for *E. coli* DNA polymerase I raised by immunization of rabbits was a gift from L. A. Loeb (University of Washington).

Preparation of Base Excision Repair DNA Substrates. Closed circular pGEM-3Zf(+) plasmid DNA containing a site-specific uracil or ethenocytosine residue was constructed essentially as described by Sung et al. (*13*). Briefly, *E. coli* JM109 cells harboring the pGEM-3Zf(+) phagemid DNA were infected with helper phage (R408) at an moi of 100 and propagated for 14 h at 37 °C. Single-stranded pGEM-3Zf(+) DNA was purified using the CTAB DNA precipitation method (*43*). To construct the DNA substrates used in the repair patch size analysis, 5′-end ^{32}P-labeled C-23-mer, U-23-mer, and ϵC-23-mer were prepared and annealed to the single-stranded pGEM-3Zf(+) DNA to construct homoduplex pGEM (C·G) or heteroduplex (U·G) and (ϵC·G) DNA, respectively, as previously described (*10*). Each primed template was subjected to primer extension and ligation reactions, and the resulting covalently closed circular duplex DNA reaction product was isolated by ethidium bromide cesium chloride gradient centrifugation, as described previously (*13*). Centrifugation was performed using a VTi80 rotor (Beckman) at 50 000 rpm for 14 h at 20 °C. Form I DNA was isolated, extracted, concentrated, and dialyzed against TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) as previously described (*13*). The isolated DNA was found to contain >98% form I molecules as determined by 1% agarose gel electrophoresis.

Preparation of E. coli Cell-Free Extract. BER proficient *E. coli* cell-free extracts were prepared as described by Sung et al. (*13*), with minor modifications. Briefly, *E. coli* GM31 and BH158 cells were grown at 37 °C in 100 mL of TYN medium (1% tryptone, 1% yeast extract, and 0.5% NaCl) to mid-log phase, harvested, resuspended in 4 mL of sonification buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 mM dithiothreitol), and lysed on ice by sonification. Cell debris was removed by centrifugation at 20 000*g* for 20 min at 4 °C. The supernatant fraction was collected, and protein was precipitated following the addition of powdered ammonium sulfate (0.35 g per mL of cell extract). The precipitated protein was recovered by centrifugation, resuspended in 1 mL of R-buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and 10% (w/v) glycerol), and dialyzed against the same buffer. Protein concentrations were determined using the Bio-Rad Protein assay, described by Bradford (*44*).

Base Excision DNA Repair Reaction. Standard reaction mixtures (100 μL) containing 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM each of dATP, dTTP, dGTP, and dCTP, 5 mM phosphocreatine di-Tris salt, 200 units/mL phosphocreatine kinase, 2 mg/mL *E. coli* cell-free extract protein, and 20 μg/mL of the appropriate pGEM (form I) DNA substrate were prepared on ice. When appropriate, exogenous purified proteins were added to the reaction mixtures 3 min prior to the addition of DNA substrate. After incubation at 30 °C for various times, the reactions were terminated by the addition of 25 μL of 0.1 M EDTA and then heated at 70 °C for 3 min. RNase A was added to 80 μg/mL, and reaction mixtures were incubated at 37 °C for 10 min. Each reaction was then adjusted to 0.5% SDS and 190 μg/mL proteinase K, incubated for 30 min at 37 °C, and the pGEM DNA was isolated and resuspended in 25 μL of TE buffer as described by Sung et al. (*13*).

Analysis of Base Excision DNA Repair Reaction Product. DNA samples (2 μL), isolated as described above, were subjected to 1% agarose gel electrophoresis (*13*, *32*). The DNA concentration of each sample was quantitatively measured relative to a series of pGEM DNA standards (10−80 ng) that were analyzed on the same gel, as described by Sanderson et al. (*32*). The fluorescence intensity at 300 nm of the ethidium-bromide stained DNA was measured using a gel documentation system (Ultra Violet Products Ltd.), and the DNA concentration was determined using the ImageQuant computer program (Molecular Dynamics, Inc.). After determining the DNA concentration, each DNA sample (80 ng) was treated with Ung (50 units) and Nfo (0.5 units) for 30 min at 37 °C when uracil-initiated BER was examined or with Dug (0.1 pmol) and Nfo (0.5 units) for 30 min at 37 °C when ethenocytosine-initiated BER was analyzed. Following incubation, the reactions were terminated by heating at 70 °C for 3 min, and form I DNA that was resistant to the combined Ung/Nfo or Dug/Nfo treatment (i.e., repaired) was resolved from form II DNA by 1% agarose gel electrophoresis (*32*, *38*). The amount of form I DNA was determined by comparing the fluorescent intensity of the ethidium-bromide stained reaction products to that of co-electrophoresed form I and II DNA standards (10−80 ng). The percentage of form I DNA was calculated after correcting for the reduced (~0.7-fold) staining intensity of the form I DNA relative to form II DNA, as described previously (*13*).

Analysis of Base Excision Repair DNA Synthesis. Standard BER reaction mixtures were prepared as described above, except that 10 μCi/mL of [α-^{32}P]dCTP (6000 Ci/mmol) was included in each reaction. Following incubation at 30 °C for 0−90 min, DNA products were isolated, and each DNA sample (100 ng) was then incubated with excess *Bsr*I restriction endonuclease at 65 °C for 1 h. Restriction fragments were resolved by 5% nondenaturing polyacrylamide gel electrophoresis (*13*). After drying the gel, DNA bands were visualized with a PhosphorImager, and the intensity of various [^{32}P]DNA bands was measured using the ImageQuant computer program (Molecular Dynamics).

Determination of Repair Patch Size. Standard BER reaction mixtures were prepared except that a 2′-deoxyribonucleoside α-thiotriphosphate was added in place of each of the four 2-deoxyribonucleoside triphosphates, and ^{32}P-labeled pGEM DNA (form I) was used as the BER substrate. The pGEM DNA substrate contained a ^{32}P-labeled dAMP residue located 12 nt upstream of the target uracil or ethenocytosine and two nucleotide downstream of *Bam*HI restriction site on the (−) strand DNA. Following the BER reactions, DNA products were isolated as described above. Each DNA sample (100 ng) was treated with 5 units of *Hind*III for 30 min at 37 °C. The reaction was terminated at 70 °C for 5 min, and samples were incubated in the presence or absence of *E. coli* exonuclease III (100 units) for 1 h at 37 °C. Exonuclease III was then inactivated by heating at 70 °C for 5 min, and the [^{32}P]DNA was then cleaved with 5 units of *Bam*HI for 30 min at 37 °C. The resulting [^{32}P]-DNA products were resolved by 12% polyacrylamide, 8.3 M urea gel electrophoresis, visualized, and quantified using PhosphorImager as described previously (*13*). The relative amount of ^{32}P label detected in each band was determined

by dividing the amount of ^{32}P radioactivity detected per band by the total ^{32}P signal detected for all bands in the same lane and multiplying by 100.

DNA Polymerase Activity Assay. *E. coli* DNA polymerase I activity was measured in reaction mixtures (100 μL) containing 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM each of dATP, dTTP, dGTP, and dCTP, 5 mM phosphocreatine di-Tris salt, 200 units/mL phosphocreatine kinase, 10 μg of activated calf thymus DNA, 24 pmol of [^{3}H]dTTP (120 cpm/pmol), and 1 pmol of *E. coli* DNA polymerase I. Various amounts of antiserum specific for *E. coli* DNA polymerase I were incubated on ice with the cell extract and DNA polymerase I samples prior to the addition of DNA substrate. Reaction mixtures were incubated at 30 °C for 60 min and terminated on ice by the addition of 200 μL of 1 mg/mL BSA in 0.1 M sodium phosphate prior to precipitation with 1 mL of 10% (saturation) trichloroacetic acid. Precipitates were collected on #30 glass fiber filters (Schleicher and Schuell), washed with 18 mL of 1 N HCl in 0.1 M sodium pyrophosphate, dehydrated with 95% ethanol, and dried under a heat lamp, as described by Mosbaugh (*45*). The amount of precipitated acid-insoluble [^{3}H]DNA was measured with a liquid scintillation spectrometer using Formula-989 (Packard) fluor.

RESULTS

Effect of Exogenous DNA Ligase and Fpg Protein on Uracil-Initiated BER in E. coli Cell Extracts. To investigate the rate-limiting step associated with *E. coli* uracil-initiated BER, exogenous DNA repair enzymes were added to *E. coli* GM31 cell extracts, and the kinetics of BER were determined using an assay that has been previously described by Sung and Mosbaugh (*10*). Briefly, this assay utilized a pGEM DNA (form I) substrate containing a site-specific U·G mispair that served as the target to initiate BER. The assay was designed to detect the complete repair process following uracil excision, AP-site incision, DNA repair synthesis, and ligation. After performing standard BER reactions, the pGEM DNA was isolated from the reaction mixture and treated with excess *E. coli* Ung and Nfo to cleave any residual uracil-containing DNA molecules. This treatment converted the unreacted form I DNA to form II DNA molecules to eliminate any unrepaired DNA substrate. Completely repaired form I DNA was insensitive to the Ung/Nfo treatment and was easily resolved from form II DNA by agarose gel electrophoresis. Thus, the extent of complete reaction could be determined from the amount of Ung/Nfo insensitive (repaired) form I DNA detected relative to that of form II DNA.

We initially examined the ability of exogenous dRPase and DNA ligase activities to influence the rate and extent of the uracil-mediated BER reaction. *E. coli* Fpg protein and DNA ligase were independently added to standard BER reaction mixtures and incubated for various times, as indicated in Figure 1. Following the reactions, DNA products were isolated, treated with excess *E. coli* Ung and Nfo, and analyzed by agarose gel electrophoresis (Figure 1A). This process effectively eliminated unreacted and incompletely repaired DNA molecules from the pool of repaired DNA (form I) molecules, as demonstrated by the control reactions (Figure 1A, lanes S and C). The amount of repaired and unrepaired DNA was determined for each set of BER reactions performed without addition, or with addition of either DNA ligase or Fpg protein (Figure 1A, lanes 1−6, 7−12, and 13−18, respectively). After quantitatively determining the amount of form I and II DNA detected by ethidium bromide staining, the percentage of repaired form I DNA was plotted versus reaction time (Figure 1B). The results revealed that addition of DNA ligase caused an increase in the rate (~3-fold) and extent of repair in early time points (0−30 min) as compared to the control reaction performed without addition. On the other hand, the addition of Fpg protein did not alter the rate or extent of BER relative to the control reaction (Figure 1B). The inability of Fpg to affect the reaction rate was not due to enzyme inactivity

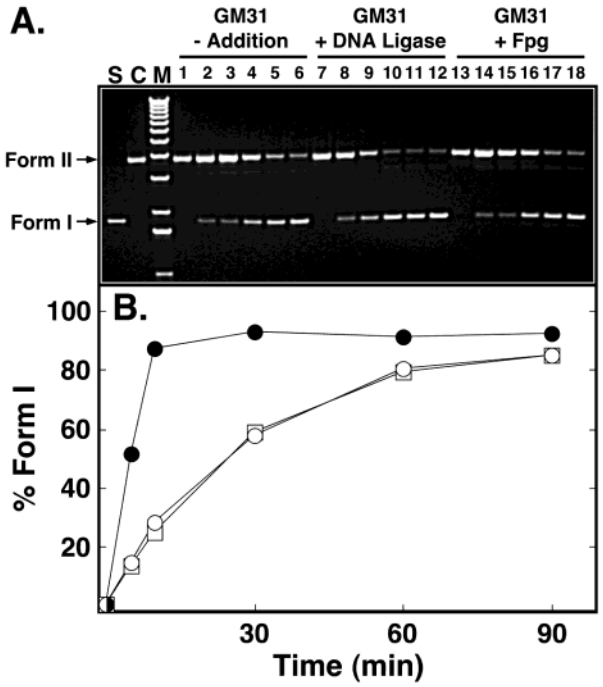

FIGURE 1: Effect of DNA ligase and Fpg protein supplementation on the rate and extent of uracil-initiated base excision repair in *E. coli* GM31 cell extracts. (A) Three sets of standard BER reaction mixtures (100 μL/assay) containing 20 μg/mL of pGEM (U·G) DNA and 2 mg/mL of *E. coli* GM31 cell extract protein were prepared as described under Materials and Methods except that *E. coli* DNA ligase (0.15 μM) or Fpg (0.2 μM) was added to some reaction mixtures as indicated above. Incubation was carried out for 0, 5, 10, 30, 60, and 90 min at 30 °C (lanes 1−6, 7−12, and 13−18, respectively). After terminating the reactions, pGEM DNA was isolated, subjected to Ung/Nfo treatment, and analyzed by 1% agarose gel electrophoresis as described under Materials and Methods. As a control, pGEM (U·G) DNA was mock-reacted and treated with excess Ung and Nfo (lane C). Untreated pGEM (U·G) DNA (80 ng) and a sample containing 1 μg of a 1-kilobase pair DNA ladder (Life Technologies, Inc.) were utilized as reference standards (lanes S and M, respectively). The arrows indicate the location of form I and II DNA bands detected by ethidium bromide staining. (B) The fluorescent intensity of ethidium bromide stained DNA bands was quantified using a gel documentation system (Ultra Violet Products Ltd.) and the ImageQuant computer program (Molecular Dynamics Inc.) as previously described by Sung et al. (*13*). The percentage of repaired form I DNA was calculated by dividing the amount of form I DNA by the sum of form I and II DNA and multiplying by 100. The extent of repair detected in each sample was plotted as a function of incubation time for the following sets of reactions: *E. coli* GM31 cell extract without additional protein (○), supplemented with *E. coli* DNA ligase (●) and Fpg (□).

because the protein preparation was shown to efficiently act as an AP-lyase on the same form I DNA containing an AP-site (data not shown). The observation that DNA ligase but not Fpg stimulated the rate of BER provided initial evidence that the ligation step was rate-limiting in the *E. coli* uracil-initiated BER pathway.

Specificity of Uracil-Mediated DNA Repair Synthesis. To assess the distribution of DNA repair synthesis associated with uracil-mediated BER involving the pGEM DNA substrate, standard BER reactions were performed in the presence of [α-^{32}P]dCTP and pGEM DNA containing a site-specific U·G mispair. After various reaction times, ^{32}P-labeled DNA products were isolated and digested with restriction endonuclease *Bsr*I, and the [^{32}P]DNA fragments were resolved by nondenaturing polyacrylamide gel electrophoresis (Figure 2). While there are 12 *Bsr*I recognition sites in the pGEM DNA sequence, *Bsr*I digestion produces only nine DNA fragments that are larger than 100 bp (Figure 2A). Among these DNA fragments, the 272-bp fragment contained the target uracil residue located 205 nucleotides upstream from the *Bsr*I restriction site between the 272- and the 603-bp fragments. Following electrophoresis, PhosphorImage analysis of the ^{32}P-labeled DNA fragments revealed that [^{32}P]dCMP incorporation was preferentially detected in the 272-bp fragment during early time points (0−30 min) and that the amount of incorporation into this fragment increased in a time-dependent manner (Figure 2B, lanes 7−12). Interestingly, significant [^{32}P]dCMP incorporation also was observed in the 603-bp fragment. However, the [^{32}P]-dCMP incorporation into the 603-bp fragment was delayed relative to the 272-bp fragment since detectable ^{32}P-labeling initially appeared 30 min after initiating the reaction (Figure 2B, lanes 10−12). Since the 603-bp fragment is located immediately downstream from the 272-bp fragment in the direction of the uracil-initiated DNA synthesis (Figure 2A), the results suggested that the [^{32}P]dCMP incorporation associated with the 603-bp fragment may have evolved from BER DNA synthesis initiated at the uracil residue.

Control reactions were conducted using pGEM DNA (homoduplex) containing a C·G base pair at the target site to determine the extent of [^{32}P]dCMP incorporation that was initiated from the uracil target site. Under this condition, all DNA fragments derived from the homoduplex substrate (including 272- and 603-bp fragments) accumulated low levels of incorporation that were attributed to background (Figure 2B,C, lanes 1−6). When compared to the C·G DNA following the 60 min reaction (Figure 2C, lane 5), the relative amount of [^{32}P]dCMP incorporation observed for the U·G DNA substrate (Figure 2C, lane 11) was increased by 20-fold in the 272-bp DNA fragment and 8-fold in the 603-bp DNA fragment. The results suggested that the uracil residue stimulated DNA synthesis as a consequence of uracil-mediated BER. Thus, it was not surprising that the incorporation of [^{32}P]dCMP into these two DNA fragments was remarkably reduced when the reaction was performed in the presence of Ugi (Figure 2B,C, lane 14) or when *E. coli* BH158 (*ung*$^-$ *dug*$^-$) cell extract was used in place of *E. coli* GM31 (Figure 2B,C, lane 15). When taken together, these results indicated that the majority of DNA synthesis associated with the U·G DNA substrate was uracil-initiated and instigated by uracil-DNA glycosylase activity. Therefore, uracil-initiated BER DNA synthesis that resulted from [^{32}P]-dCMP incorporation into the 603-bp DNA fragment apparently involved repair patches of at least 205 nucleotides since the uracil residue was located upstream (205 nt) of the *Bsr*I site that produced this DNA fragment. Similar results indicating uracil-mediated BER specific [^{32}P]dCMP incorporation into the 603-bp DNA fragment were also obtained when *E. coli* BH156 (*ung*) or BH157 (*dug*) cell extracts were substituted for *E. coli* GM31 (*46*). We hereafter refer to this type of repair that produces long DNA synthesis tracts as very-long patch BER.

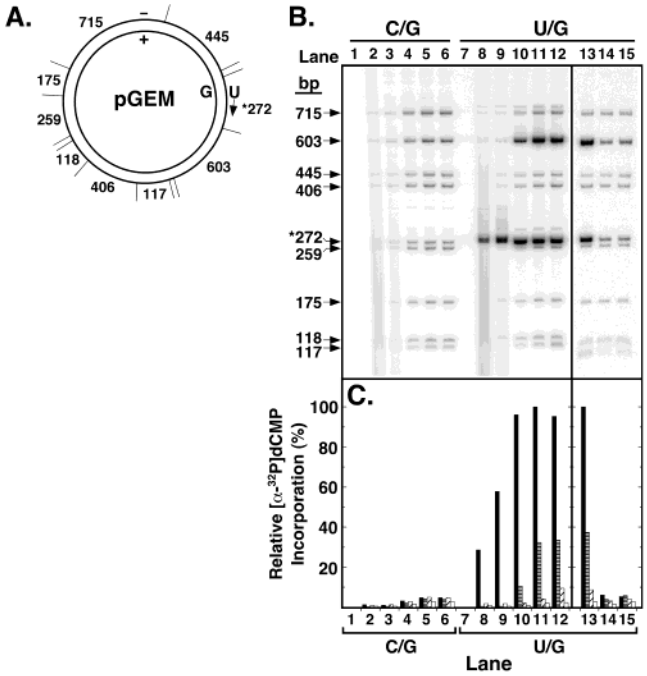

FIGURE 2: Specificity of uracil-mediated BER DNA synthesis in *E. coli* GM31 cell extracts. (A) *Bsr*I restriction map of pGEM DNA indicating restriction sites (hash marks) and the size (bp) of DNA restriction fragments is shown. The location of the uracil (U) residue in the (−) strand of pGEM DNA and the direction of DNA repair synthesis (arrow) during BER are indicated. (B) Two sets of standard BER reaction mixtures (100 μL/assay) containing 2 μg/mL of pGEM (C·G) or (U·G) DNA, 2 mg/mL of *E. coli* GM31 cell extract protein, and 10 μCi/mL of [^{32}P]dCTP were prepared. The reaction mixtures for lanes 13−15 were similarly constructed with pGEM (U·G) DNA except that 1 μL of Ugi dilution buffer (lane 13), or 1 μL of Ugi (1000 units) was included (lane 14), or *E. coli* BH158 (*ung*, *dug*) cell extract replaced the GM31 cell extract (lane 15). BER reactions were incubated for 0, 5, 10, 30, 60, and 90 min at 30 °C (lanes 1−6 and 7−12, respectively) and 90 min at 30 °C (lanes 13−15). Following each reaction, DNA products were isolated, and each DNA sample (100 ng) was treated with 2.5 units of *Bsr*I for 1 h at 65 °C, as described under Materials and Methods. The resulting DNA restriction fragments were then resolved by 5% nondenaturing polyacrylamide gel electrophoresis and visualized using a PhosphorImager. The location of the DNA fragment (*272) that contained the site-specific uracil is indicated by an arrow, as are the locations of eight other fragments. (C) The amount of ^{32}P radioactivity of each DNA band was determined using the ImageQuant program. After subtracting the background radioactivity, the [^{32}P]dCMP incorporation was normalized for each DNA fragment by dividing the amount of ^{32}P radioactivity detected by the number of cytosine residues located in each corresponding DNA fragment. The highest normalized value was then designated 100%, and the percent of the [^{32}P]dCMP incorporation in other DNA fragments was calculated relative to that value. The data for DNA fragments of 272-bp (black bar), 603-bp (horizontally striped bar), 117-bp (diagonally striped bar), and 445-bp (white bar) were plotted for each reaction shown in panel B.

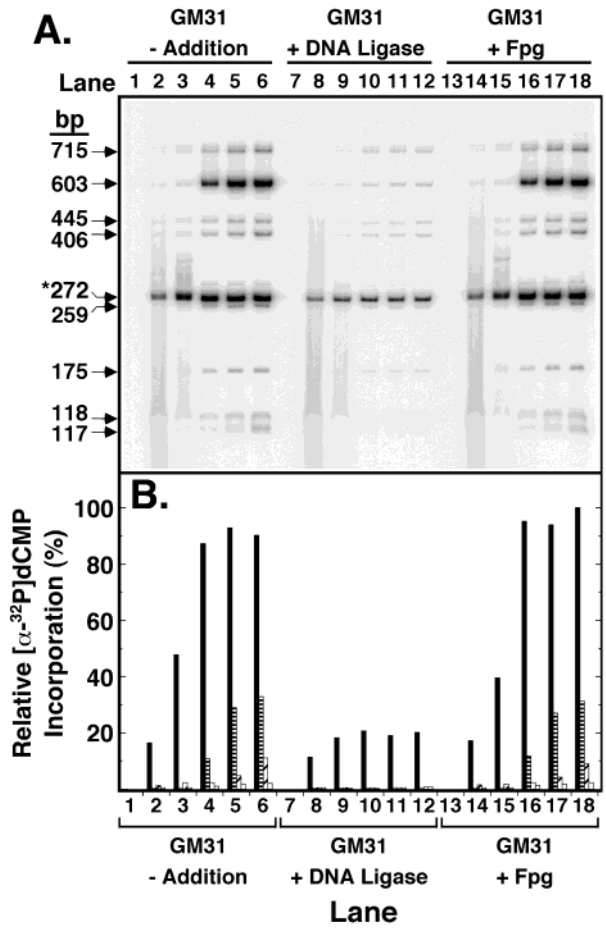

FIGURE 3: Effect of DNA ligase and Fpg protein on the specificity of uracil-mediated BER DNA synthesis. (A) Three sets of standard BER reaction mixtures were prepared as described in Figure 1 except that 10 μCi/mL of [^{32}P]dCTP was added with or without the addition of exogenous *E. coli* DNA ligase (0.15 μM) and Fpg (0.2 μM) as indicated above. Following incubation for 0, 5, 10, 30, 60, and 90 min at 30 °C (lanes 1−6, 7−12, and 13−18, respectively), DNA samples (100 ng) were isolated, subjected to *Bsr*I digestion, and resolved by 5% nondenaturing polyacrylamide gel electrophoresis as described under Materials and Methods. [^{32}P]-DNA fragments (arrows) were identified using a PhosphorImager, and the 272-bp DNA fragment that contained the uracil residue was located (*272). (B) The percentage of relative [^{32}P]dCMP incorporation into the DNA fragments of *272-bp (black bar), 603-bp (horizontally striped bar), 117-bp (diagonally striped bar), and 445-bp (white bar) was determined as described in Figure 2 and plotted for each lane shown in panel A.

Uracil-Initiated BER Patch Size Distribution Is Influenced by Exogenous DNA Ligase. On the basis of the results presented in Figures 1 and 2, we postulated that very-long patch repair resulted from the rate-limiting step in the uracil-initiated BER process, DNA ligation. To test this hypothesis, we examined whether the addition of exogenous DNA ligase to the BER reaction might reduce the extent and distribution of DNA repair synthesis. As before, uracil-initiated BER was conducted using *E. coli* GM31 cell extracts with and without supplementation of *E. coli* DNA ligase or Fpg. Following each BER reaction, *Bsr*I restriction analysis was performed on the repaired [^{32}P]DNA, and the amount of [^{32}P]dCMP incorporation was analyzed for each of the *Bsr*I-DNA fragments (Figure 3). When compared to the control reaction, performed without supplementation, the results revealed that the addition of DNA ligase greatly reduced the overall amount of [^{32}P]dCMP incorporation into the U·G DNA substrate (Figure 3A). In the presence of exogenous DNA ligase, [^{32}P]dCMP incorporation into the 272-bp fragment reached a plateau after 10 min, whereas in the absence of exogenous DNA ligase, maximal DNA repair synthesis was observed at 30 min (Figure 3B). In addition, the extent of DNA synthesis was decreased in the DNA ligase supplemented reaction. However, the reduced level of [^{32}P]dCMP incorporation was not attributed to an inefficient repair process since addition of DNA ligase was previously shown to stimulate the rate of complete BER (Figure 1). Rather, the results were consistent with the premise that exogenous DNA ligase restricted the overall extent of DNA repair synthesis thereby reducing the repair patch size and enhancing the efficiency of complete BER. In support of this interpretation, it was observed that the incorporation of [^{32}P]-dCMP into the 603-bp fragment was reduced to the background level when DNA ligase was added to the reaction (Figure 3B). Thus, DNA ligase supplementation promoted three interrelated responses: (i) restriction of DNA synthesis associated with the 272-bp fragment, (ii) inhibition of very-long patch BER, and (iii) stimulation of the rate of complete BER. On the other hand, Fpg supplementation did not affect any of these parameters (Figure 3).

To directly determine whether adding DNA ligase or Fpg altered the size of the DNA synthesis tracts associated with uracil-initiated BER, we evaluated the BER patch size distribution using the method described previously by Sung et al. (*13*). The approach relies on the incorporation of 2′-deoxyribonucleoside α-thiolmonophosphates during uracil-initiated BER to render the repaired DNA strand resistant to in vitro digestion with *E. coli* exonuclease III. In this experiment, the pGEM (U·G) DNA substrate contained the uracil target located on the (−)-strand, 17 nucleotides upstream (5′-side) of the unique *Hind*III site, and 13 nucleotides downstream (3′-side) of the unique *Bam*HI site (Figure 4A). To evaluate the repair patch size distribution, the length of the [^{32}P]DNA fragments produced after sequential treatment of the BER DNA products with *Hind*III, exonuclease III, and *Bam*HI was determined by denaturing polyacrylamide gel electrophoresis. Under these conditions, exonuclease III was expected to digest the ^{32}P-labeled strand (repaired strand) from the cleaved *Hind*III site in the 3′ to 5′ direction until encountering the first phosphorothioate linkage at the 3′-boundary of the repair patch. Patch size analysis was conducted following time course reactions of uracil-initiated BER using *E. coli* GM31 cell extracts in the presence and absence of exogenous DNA ligase or Fpg (Figure 4B). As expected, exonuclease III digestion of the [^{32}P]DNA isolated from both the mock and the 0 min reactions did not produce a detectable repair patch since 2′-deoxyribonucleoside α-thiolmonophosphate incorporation did not occur in the absence of cell extract or without incubation (Figure 4B, U and 0 min). On the other hand, when BER reaction products were examined following incubation for 5−90 min, a set of discrete [^{32}P]DNA fragments were obtained that defined the size distribution of the repair patches (Figure 4B). Following quantitative analysis, the repair patch size distribution was plotted for the 5 and the 60 min reactions (Figure 4C, D). One-nucleotide repair patches appeared to be the most prevalent type of repair in the 5 min reaction conducted without supplementation (Figure 4B, white bars), whereas the repair patch distribution

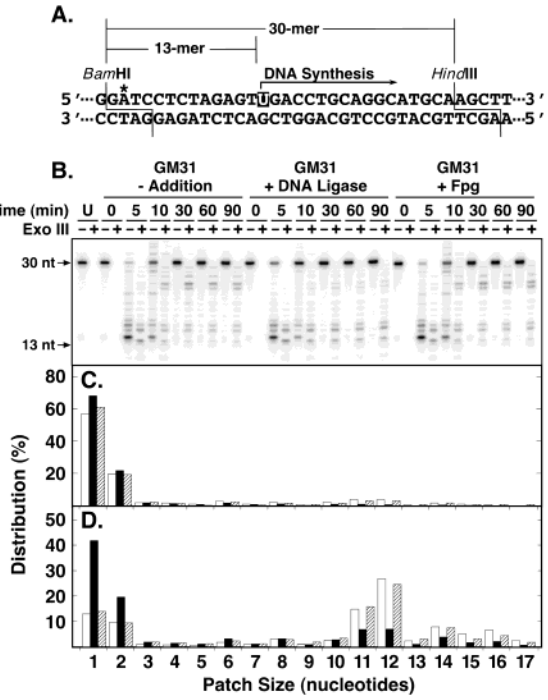

FIGURE 4: Effect of DNA ligase and Fpg protein on the uracil-initiated BER patch size distribution. (A) Partial nucleotide sequence of the pGEM (U·G) DNA substrate used to conduct uracil-initiated BER DNA synthesis patch size analysis is shown that contains the *Bam*HI and *Hind*III restriction endonuclease sites. A site-specific [^{32}P]dAMP residue (*) was introduced between the uracil target and the *Bam*HI site to facilitate measuring the repair patch size as described under Materials and Methods. BER DNA synthesis was initiated at the uracil target (U), and 2′-deoxyribonucleoside α-thiolmonophosphate incorporation occurred in the direction of the arrow. Following DNA synthesis, sequential treatment with *Hind*III, exonuclease III, and *Bam*HI produced [^{32}P]DNA fragments of 14−30 nucleotides that corresponded to BER patch sizes of 1−17 nucleotides, respectively. (B) Three sets of standard BER reaction mixtures (100 μL/assay) containing 20 μg/mL of pGEM (U·G) [^{32}P]-DNA, 20 μM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP-[αS], and 2 mg/mL of *E. coli* GM31 cell-free extract were incubated in the presence and absence of exogenous *E. coli* DNA ligase (0.15 μM) or Fpg (0.2 μM) for 0, 5, 10, 30, 60, and 90 min at 30 °C as indicated above. As a control, pGEM (U·G) [^{32}P]DNA (2 μg) was mock-reacted in the absence of cell extract proteins (U). Following the BER reaction, DNA products were isolated, and each DNA sample (100 ng) was digested with 5 units of *Hind*III and then treated with 100 units of exonuclease III (+) or mock treated (−) as indicated above. After these treatments, the DNA was cleaved with 5 units of *Bam*HI, and the ^{32}P-labeled DNA fragments were resolved by 12% polyacrylamide, 8.3 M urea gel electrophoresis as described under Materials and Methods. The location of [^{32}P]-DNA size markers are indicated by arrows. Reference markers were generated by digesting of pGEM (U·G) [^{32}P]DNA (100 ng) with excess *Hind*III and *Bam*HI (30 nt) and by subsequently treating the ^{32}P-labeled oligonucleotide (30-mer) with excess Ung and Nfo (13 nt). The relative amount of ^{32}P radioactivity detected in each DNA band corresponding to repair patch sizes of 1−17 nucleotides was determined for the 5 min (C) and 60 min (D) reactions in panel A, using the method described by Sung et al. (*13*). The frequency of each BER patch was expressed as the percent distribution of the observed repair patches. The results for reactions digested with exonuclease III are shown as follows: GM31 cell extract without addition (white bar), with DNA ligase (black bar), and with Fpg protein (striped bar).

associated with the 60 min reaction revealed that the large majority of BER occurred via a long patch mechanism and that short patch (1-nucleotide) repair accounted for only ∼12% of the BER events (Figure 4D, white bars). However, the overall patch size distribution was biphasic with the majority of the patch sizes corresponding to either 1−2 nucleotides or 11−12 nucleotides in length. The addition of DNA ligase promoted a significant reduction in the proportion of long patch BER and increasing the percentage of 1-nucleotide repair patches by ∼4-fold (Figure 4D, black bars). While supplementation with DNA ligase profoundly reduced the repair patch size distribution, addition of Fpg did not apparently alter the profile relative to the control in either the 5 or the 60 min reaction (Figure 4C, D, striped bars).

Influence of DNA Ligase and Fpg on Dug-Mediated BER of Ethenocytosine Residues. To examine the effect of *E. coli* DNA ligase and Fpg on the efficiency of Dug-mediated BER in *E. coli* GM31 cell extract, we repeated the kinetic analysis using a pGEM form I DNA substrate that contained a site-specific ethenocytosine residue in place of the uracil target. As before, the BER reaction time course was examined, and the isolated DNA products were subjected to a combined treatment with Dug and Nfo to convert unrepaired form I DNA to form II DNA. The extent of ethenocytosine-initiated BER was determined following agarose gel electrophoresis. The results indicated that the addition of DNA ligase stimulated the initial rate of repair (∼3-fold) when compared to the control reaction (no addition), whereas the rate of BER was virtually unchanged for the reaction conducted in the presence of exogenous Fpg (data not shown). Further characterization of the DNA products by *Bsr*I restriction analysis indicated that very-long patch DNA repair synthesis also occurred during ethenocytosine-initiated BER in the *E. coli* GM31 cell extract and that very-long patch repair was abolished by the addition of DNA ligase but not Fpg protein (*46*).

Repair patch size experiments were performed to further examine the influence of DNA ligase and Fpg supplementation on the Dug-mediated ethenocytosine-initiated BER patch size distribution. During the early stage of the BER reactions (5 min), neither the addition of DNA ligase nor Fpg appeared to affect the repair patch size distribution that resulted in mostly 1-nucleotide repair tracks (data not shown). As the reaction progressed, the vast majority of repair occurred via the long patch pathway as illustrated by the 60 min reaction conducted without supplementation. Under this condition, when >75% of the substrate was completely repaired, most of the repair patches involved incorporation of 11 or 12 nucleotides. During the latter stage of the reaction, the addition of DNA ligase but not Fpg increased the amount of short patch repair by ∼4-fold. These findings were very similar to those obtained for the uracil-initiated BER reactions.

Ability of Various Enzymes to Influence the Efficiency of the Complete BER Reaction. To examine the effect of various BER enzymes on the efficiency of uracil-initiated BER, increasing amounts of *E. coli* Ung, Nfo, and Pol I were individually added to the *E. coli* GM31 cell extract, and standard BER reactions were conducted to determine the extent of complete repair. While the addition of Ung and Nfo did not influence the efficiency of uracil-initiated BER, supplementation with DNA polymerase I caused a modest reduction in the extent of BER (Figure 5A). Interestingly, the inhibitory effect observed by adding DNA polymerase I was reversed by concomitant addition of exogenous *E. coli*

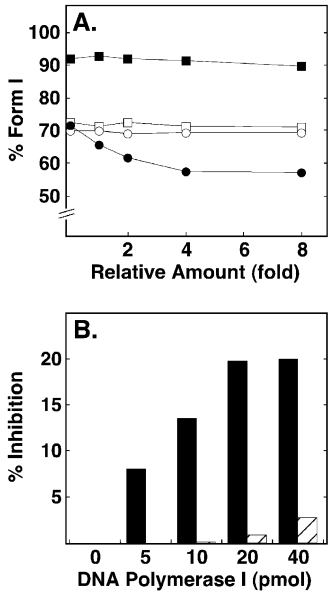

FIGURE 5: Influence of *E. coli* Ung, Nfo, PolA, and Lig supplementation on uracil-initiated BER. (A) Four sets of standard BER reaction mixtures (100 μL/assay) containing 20 μg/mL of pGEM (U·G) DNA and 2 mg/mL of *E. coli* GM31 cell extract protein were prepared except that various amounts of exogenous *E. coli* BER enzymes were added as follows: 0, 25, 50, 100, and 200 units/assay of Ung (○); 0, 1, 2, 4, and 8 units/assay of Nfo (□); 0, 5, 10, 20, and 40 units/assay of DNA polymerase I (●); 0, 5, 10, 20, and 40 units/assay of DNA polymerase I plus 20 units/assay of DNA ligase (*n*). After incubation for 60 min at 30 °C, the pGEM DNA was recovered, subjected to Ung/Nfo treatment, and analyzed by 1% agarose gel electrophoresis as described under Materials and Methods. The fluorescent intensity of ethidium bromide-stained DNA bands (form I and II) was determined as described in Figure 1, and the percentage of repaired form I DNA in each sample was plotted as a function of the relative amount of each enzyme added to the reaction. (B) The inhibition of uracil-initiated BER activity was determined based on the amount of decrease in the repaired form I DNA observed following exogenous enzyme supplementation. To determine the percentage of inhibition, the amount of repaired form I DNA detected in each enzyme-supplemented reaction was divided by that detected in the reaction without supplementation and then multiplied by 100. The data were plotted as a function of the amount of DNA polymerase I added in the presence (striped bar) or absence (black bar) of exogenous DNA ligase.

DNA ligase (Figure 5B). However, the level of attenuation was dependent on the molar ratio of exogenous DNA polymerase I to DNA ligase. Essentially no inhibition of BER was observed when excess DNA ligase was added relative to the amount of exogenous DNA polymerase I (Figure 5B, striped bar). Inhibition of BER was only detected when the ratio of DNA polymerase I to DNA ligase was equal to or greater than one. Thus, the ability of DNA polymerase I to inhibit the process of complete BER depended on the balance between DNA polymerase I and DNA ligase and was not solely influenced by the DNA polymerase I concentration.

The reaction kinetics of complete uracil-initiated BER were examined to determine the influence of DNA polymerase I supplementation in the presence and absence of exogenous DNA ligase (Figure 6). The results confirmed our previous observation since the addition of DNA polymerase I alone reduced the amount of repaired form I DNA when compared to the control reaction (Figure 6B). After the 60 min reaction, the amount of repaired form I DNA detected was ~30% less when the reaction was conducted in the

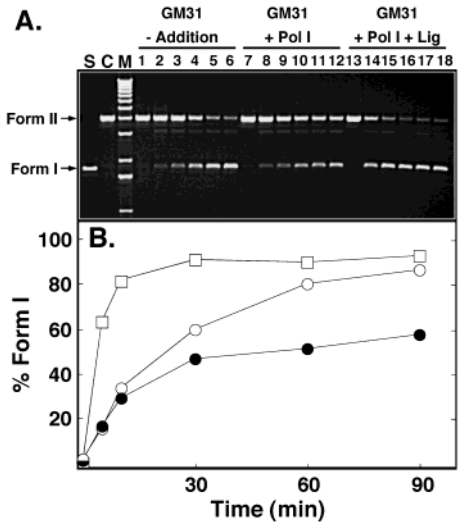

FIGURE 6: Inhibitory affect of DNA polymerase I on uracil-initiated BER was attenuated by DNA ligase. (A) Three sets of standard BER reaction mixtures (100 μL/assay) containing 20 μg/mL of pGEM (U·G) DNA and 2 mg/mL of *E. coli* GM31 cell extract protein were incubated for 0, 5, 10, 30, 60, and 90 min at 30 °C (lanes 1−6, 7−12, and 13−18, respectively) in the presence and absence of exogenous *E. coli* DNA polymerase I (0.2 μM) or DNA ligase (0.15 μM) as indicated. Following the repair reactions, pGEM DNA was recovered, subjected to Ung/Nfo treatment, and analyzed by 1% agarose gel electrophoresis as described under Materials and Methods. Untreated pGEM (U·G) DNA (lane S), mock-reacted DNA treated with Ung and Nfo (lane C), and a 1-kilobase pair DNA ladder (lane M) were utilized as reference standards. The arrows indicate the location of the form I and II DNA bands detected by ethidium bromide staining. (B) The percentage of repaired form I DNA in each sample was calculated as described in Figure 1 and plotted as a function of incubation time for the following reactions: *E. coli* GM31 cell extract without addition (○), with addition of *E. coli* DNA polymerase I (●) and with both DNA polymerase I and DNA ligase (□).

presence versus the absence of exogenous DNA polymerase I. As before, this inhibitory affect was counteracted when both exogenous DNA polymerase I and DNA ligase were added to the reaction (Figure 6B). Moreover, the addition of both DNA polymerase I and DNA ligase caused an increase in the initial rate and maximum extent of repair when compared to the control, conducted without protein supplementation. Collectively, these results imply that DNA polymerase I and DNA ligase exhibit antagonistic roles toward facilitating BER in the *E. coli* GM31 cell extract.

Stimulation of Long Patch BER by DNA Polymerase I Is Alleviated by Supplementation with DNA Ligase. To further investigate the relationship between DNA polymerase I and DNA ligase on uracil-initiated BER, repair patch experiments were conducted using *E. coli* cell extracts supplemented with DNA polymerase I in the presence and absence of exogenous DNA ligase. Time course reactions were performed, and DNA repair products were analyzed as described in Figure 7A. Inspection of the BER patch size distribution obtained for the control (Figure 7B) and DNA polymerase I supplemented reaction (Figure 7C) revealed that the addition of DNA polymerase I stimulated the formation of longer repair patches and reduced the occurrence of short patch (1-nucleotide) repair by ~3-fold. However, the DNA polymerase I-mediated shift in patch size distribution was partially reversed when exogenous DNA ligase was introduced along with DNA polymerase I in the BER reaction

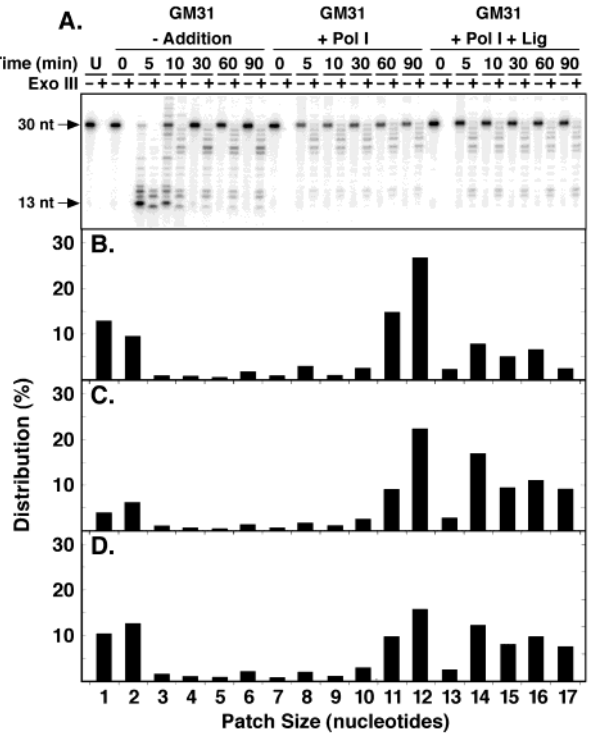

FIGURE 7: Effect of DNA polymerase I supplementation with or without DNA ligase on the BER patch size distribution. (A) Three sets of standard BER reaction mixtures (100 μL/assay) were prepared as described in Figure 4 with or without addition of exogenous *E. coli* DNA polymerase I (0.2 μM) and DNA ligase (0.15 μM) as indicated. BER reactions were conducted for 0, 5, 10, 30, 60, and 90 min at 30 °C, and product DNA recovered from each reaction was subjected to the BER patch size analysis as described under the Materials and Methods. The relative amount of ^{32}P radioactivity detected in each band (14−30 nt) was determined for the 60 min reactions in panel A, as described in Figure 4. The distribution of repair patch size for reactions digested with exonuclease III are shown as follows: *E. coli* GM31 cell extract without addition (B), with DNA polymerase I (C), and with DNA polymerase I plus DNA ligase (D).

(Figure 7D). This observation was most clearly illustrated by examining the percent distribution of 1−2 nucleotide repair patches produced in the three reactions (Figure 7B−D). Collectively, these results illustrated that increasing the DNA polymerase I concentration drove the BER reaction to produce longer repair patches and that DNA ligase mitigated this response.

Repair DNA Synthesis in Very-Long Patch BER Is Affected by the Ratio of DNA Polymerase I and DNA Ligase. *Bsr*I restriction analysis was conducted to investigate whether the addition of DNA polymerase I influenced the extent of DNA repair synthesis associated with very-long patch BER. As before, [^{32}P]dCMP incorporation into pGEM DNA containing the U·G mispair was monitored following nondenaturing gel electrophoresis of *Bsr*I digested DNA repair products as described in Figure 8. An examination of *Bsr*I DNA fragments revealed that supplementation of the BER reaction with DNA polymerase I dramatically increased the rate of [^{32}P]dCMP incorporation into the 603-bp fragment and several other fragments, as compared to the control reaction conducted without supplementation (Figure 8A). The relative incorporation of [^{32}P]dCMP was plotted for the 259-, 272-, 445-, and 603-bp DNA fragments obtained from the control (Figure 8B) and DNA polymerase I supplemented reaction

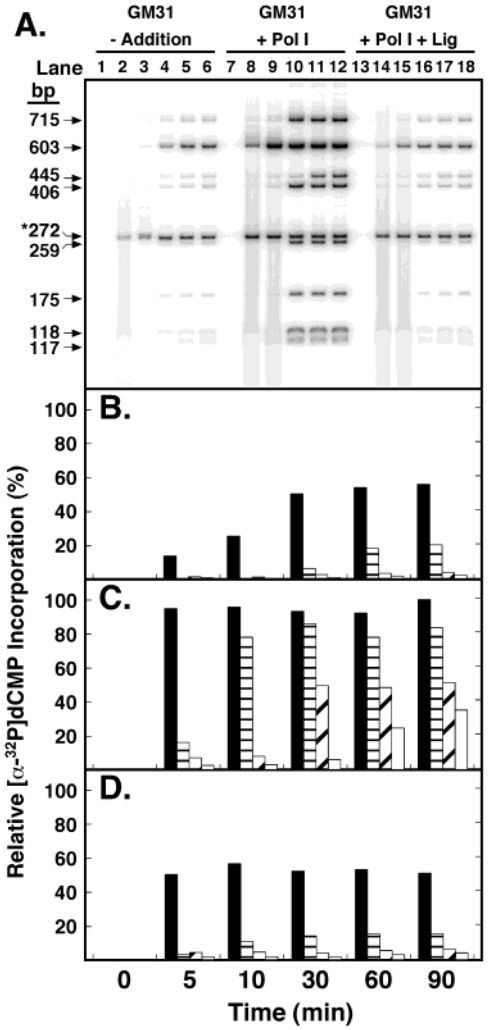

FIGURE 8: Influence of DNA polymerase I in the presence and absence of DNA ligase supplementation on the specificity of uracil-initiated BER DNA synthesis. (A) Three sets of standard BER reaction mixtures (100 μL/assay) containing 20 μg/mg of pGEM (U·G) DNA, 2 mg/mL of *E. coli* GM31 cell extract protein, and 10 μCi/mL of [^{32}P]dCTP were prepared with or without the addition of exogenous *E. coli* DNA polymerase I (0.15 μM) and DNA ligase (0.2 μM) as indicated. Repair reactions were incubated for 0, 5, 10, 30, 60, and 90 min at 30 °C (lanes 1−6, 7−12, and 13−18, respectively), DNA products were isolated, and DNA samples (100 ng) were subjected to *Bsr*I digestion and resolved by 5% nondenaturing polyacrylamide gel electrophoresis as described under Materials and Methods. The locations of ^{32}P-labeled restriction DNA fragments were visualized by PhosphorImager and are indicated by arrows. The [^{32}P]DNA fragment containing the uracil residue is indicated (*272). The percentage of relative [^{32}P]dCMP incorporation into the DNA fragments of *272-bp (black bar), 603-bp (horizontally striped bar), 259-bp (diagonally striped bar), and 445-bp (white bar) was calculated using the method as described in Figure 2 and plotted for each of the 60 min reactions (lanes 5, 11, and 17) shown in panel A. The data were plotted as follows: *E. coli* GM31 cell extract without addition (B), with DNA polymerase I (C), and with DNA polymerase I plus DNA ligase (D).

(Figure 8C). These results revealed that exogenous DNA polymerase I significantly enhanced DNA synthesis during BER that started with the 272-bp DNA fragment and progressed sequentially around the pGEM DNA molecule. In contrast, the addition of DNA polymerase I and DNA ligase suppressed the DNA polymerase I-mediated stimulation of DNA repair synthesis (Figure 8A, D). Under this condition, greater accumulation of [^{32}P]dCMP incorporation

into the 272-bp fragment was observed earlier in the reaction (5 and 10 min) than at corresponding times in the control reaction (Figure 8B, D, respectively).

To investigate whether DNA polymerase I was the major DNA polymerase responsible for DNA repair synthesis associated with very-long patch BER, DNA polymerase I antiserum was added to the *E. coli* GM31 cell extract as described in Figure 9. Before conducting the uracil-initiated BER reactions, DNA polymerase assays were performed to ascertain that the DNA polymerase I antibody neutralized the DNA polymerase activity (Figure 9A). As expected, the results showed that the DNA polymerase I antiserum caused a dose-dependent decrease in DNA polymerase I activity. Standard uracil-initiated BER reactions were then conducted under various conditions as described in Figure 9B. Following incubation for 60 min, [^{32}P]DNA products were isolated and subjected to the *Bsr*I restriction analysis to determine the distribution of [^{32}P]dCMP incorporation during DNA repair synthesis. The control reaction was incubated with the *E. coli* GM31 cell extract but without addition of either DNA polymerase I or antiserum (Figure 9B, lane 1). Addition of preimmune serum had no apparent influence on [^{32}P]dCMP incorporation into the *Bsr*I DNA fragments (Figure 9B, lane 2). In contrast, the addition of DNA polymerase I antiserum resulted in a diminution of [^{32}P]dCMP incorporation into the 603-bp fragment (Figure 9B, lanes 3 and 4). However, the addition of exogenous DNA polymerase I restored [^{32}P]-dCMP incorporation into both the 603-bp fragment (Figure 9B, lane 5) and other DNA fragments (Figure 9B, lanes 6 and 7), depending on the amount of DNA polymerase I added to the BER reaction. The amount of [^{32}P]dCMP incorporation into the following fragments (272-, 603-, and 259-bp) was quantitatively analyzed and plotted for each reaction (Figure 9C). From these results we determined that the addition of DNA polymerase I antiserum (2 μL) reduced the amount incorporation into the 272- and 603-bp fragment by 1.4- and 3-fold, respectively (Figure 9C, lanes 3 and 4). In contrast, addition of purified DNA polymerase I in the presence of neutralizing antiserum increased in the level of [^{32}P]dCMP incorporation as follows; 1.1-, 1.4-, and 1.8-fold in the 272-bp fragment; 1.6-, 2.7-, and 3.5-fold in the 603-bp fragment; 1.3-, 2.8-, and 7.6-fold in the 259-bp fragment by 1, 2, and 5 units of purified DNA polymerase I, respectively (Figure 9C, lanes 5−7).

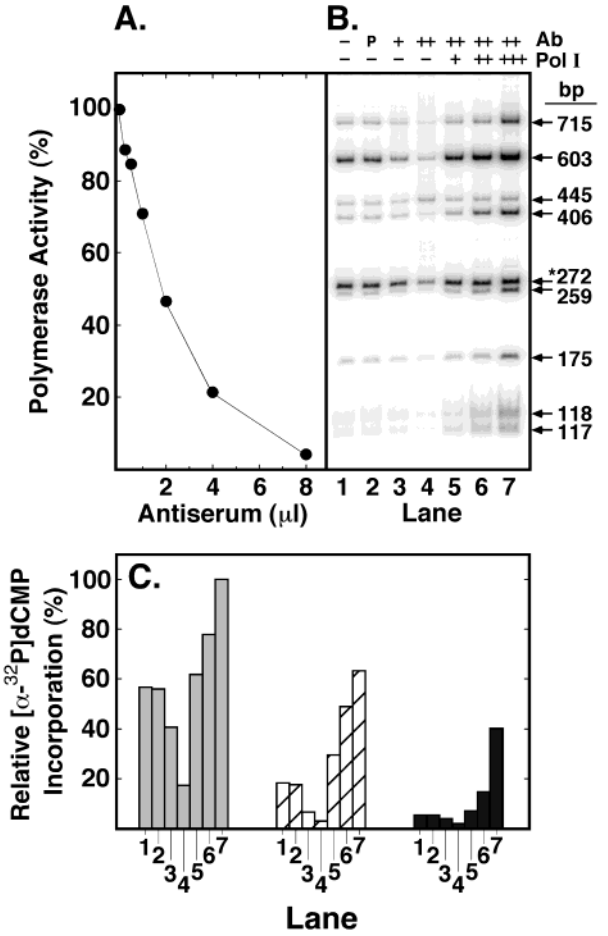

FIGURE 9: Influence of PolA neutralizing antibody and DNA polymerase I supplementation on uracil-initiated BER DNA synthesis. (A) DNA polymerase activity was determined under the standard BER reaction condition using reaction mixtures (100 μL) containing 1 unit of DNA polymerase I, 10 μg of activated calf thymus DNA, and [^{3}H]dTTP (120 cpm/pmol). Reaction mixtures were supplemented with 0, 0.25, 0.5, 1, 2, 4, and 8 μL of antiserum raised against *E. coli* DNA polymerase I prior to the addition of DNA substrate. After incubation for 60 min at 30 °C, reaction mixtures were subjected to DNA polymerase activity assays as described under Materials and Methods. The percentage of DNA polymerase activity detected in samples containing antiserum was determined by dividing the amount of [^{3}H]dTMP incorporated in the presence of antiserum by the amount of [^{3}H]dTMP incorporated in the absence of antiserum and then multiplied by 100. Incorporation of 210 pmol of [^{3}H]dTMP represented 100% DNA polymerase activity. The mean values from two independent experiments were plotted. (B) Standard BER reactions (100 μL/assay) containing 20 μg/mL of pGEM (U·G) DNA and 2 mg/mL of *E. coli* GM31 cell extract protein were carried out with the following: no addition (lane 1); 2 μL of rabbit preimmune (P) serum (lane 2); 2 (+) and 4 μL (++) of antiserum (lanes 3 and 4, respectively); and 1 (+), 2 (++), and 4 (+++) units of DNA polymerase I in the presence of 2 μL of antiserum (lanes 5−7, respectively). After incubation for 60 min at 30 °C, DNA products were isolated, and DNA samples (100 ng) were subjected to *Bsr*I digestion and resolved by 5% nondenaturing polyacrylamide gel electrophoresis as described under Materials and Methods. The locations of ^{32}P-labeled restriction DNA fragments were visualized by PhosphorImager and are indicated by arrows. (C) The percentage of relative [^{32}P]dCMP incorporation into the DNA fragments of *272-bp (gray bar), 603-bp (striped bar), and 259-bp (black bar) was determined as described in Figure 2 and plotted for each lane corresponding to panel B.

DISCUSSION

We have examined the DNA repair synthesis patch size distribution associated with both uracil- and ethenocytosine-initiated BER in *E. coli* cell-free extracts. Unlike several previous investigations that utilized small duplex oligonucleotide substrates containing a damaged base to characterize the repair patches associated with BER (*29*, *30*, *47*), a covalently closed circular pGEM DNA substrate with a defined (U·G or ϵC·G) target was employed in this study. The use of this relatively large size pGEM DNA molecule allowed repair patch size determination over an extended region, as compared to studies conducted with oligonucleotide substrates. Under the conditions examined, both uracil- and ethenocytosine-mediated BER events were observed that produced short, long, and very-long DNA repair synthesis patches. To our knowledge, this report is the first investigation into the patch size of ethenocytosine-mediated *E. coli* BER and presents the first evidence for very-long patch DNA repair synthesis (>205 nucleotides) associated with both uracil- and ethenocytosine-initiated BER. Furthermore, very-

long patch DNA synthesis was uniformly detected in BER reactions conducted with *E. coli* GM31, BH156, and BH157 cell-free extracts. Previous studies that utilized pSFFura (*31*) and M13 (form I) DNA (*13*) to monitor uracil-initiated BER apparently did not detect very-long patch BER because of a limitation in experimental design. While both studies used duplex circular DNA substrates and restriction endonuclease analysis conducted to detect DNA repair synthesis, only a 21-nucleotide region downstream of the uracil target site was monitored for DNA synthesis. Since these experiments were not designed to detect very-long DNA synthesis tracts, perhaps it is not surprising that the very-long patch BER eluded detection.

Several observations supported the interpretation that the very-long DNA synthesis tracts detected using pGEM (U·G) DNA resulted from uracil-initiated BER. First, the very-long DNA synthesis tracts were associated with form I DNA molecules that were resistant to cleavage by a combined treatment with Ung and Nfo, indicating that complete uracil-initiated BER occurred on these molecules. Second, repair DNA synthesis within the 603-bp fragment was dependent on the presence of the uracil residue located in the upstream (272-bp) DNA fragment. Third, specific incorporation of [^{32}P]dCMP into the 603-bp fragment was significantly reduced when Ugi, a potent and irreversible inhibitor of Ung (*48*), was added to the cell-free extract. Fourth, very-long DNA synthesis tracts were abolished in repair reactions conducted with *E. coli* BH158 (*ung dug*) cell-free extracts, in which both *ung* and *dug* were inactivated. This observation is consistent with pervious reports that *E. coli* BH158 cells are defected in uracil-initiated BER (*13*, *49*). Collectively, these observations strongly suggested that the very large repair patches resulted from uracil-initiated BER.

The results presented here indicate that phosphodiester bond formation by DNA ligase is the slowest step in the *E. coli* BER pathway. A previous report by Srivastava et al. (*47*) using a reconstituted human enzyme system composed of uracil-DNA glycosylase (10 nM UNGΔ84), AP-endonuclease (10 nM HAP), DNA polymerase β (10 nM POL-β), and DNA ligase I (100 nM LIG 1) identified the dRP lyase activity associated with DNA polymerase β as the rate-determining step of this in vitro BER reaction. Our results involving *E. coli* uracil-mediated BER stand alone and do not contradict the findings involving the human reconstituted BER system since different approaches and biological systems were investigated. The rate-limiting step in *E. coli* BER most likely occurs either because of a limited amount of DNA ligase activity or limited accessibility of DNA ligase to the nicked DNA molecule during the repair process. We propose that the ligation efficiency during BER is influenced by four intertwined biochemical reactions that include DNA chain elongation, strand displacement, displaced strand excision, and ligation. Lundquist and Olivera (*50*) previously reported that *E. coli* DNA polymerase I transiently generates displaced single-stranded overhangs during nick translational DNA synthesis reactions, as would be expected to occur during long patch BER. On the basis of in vitro experimental results, it appears that the DNA polymerase and 3′-5′ exonuclease activities of DNA polymerase I compete for the displaced single-stranded DNA (*50*). Furthermore, DNA polymerase activity reportedly acts in favor of the 5′-3′ exonuclease with a probability of 55% at each nucleotide incorporated (*50*). As a consequence of strand-displacement DNA synthesis, single-stranded DNA overhangs are produced that serve as intermediates to long patch BER. Cleavage of these single-stranded DNA overhangs is a prerequisite for producing a ligatable nick and completing the BER process (*27*, *29*). Thus, a delay in the action of 5′-3′ exonuclease because of extended DNA synthesis would be expected to impede the ligation step and reduce the rate of BER. The exact amount of strand displacement and the in vivo factors that facilitate the production of single-stranded DNA overhangs during the BER reaction has not yet been determined. However, the competing action of DNA polymerase I and DNA ligase would be expected to play a significant role in regulating the rate and extent of BER since these enzymes mediate chain elongation and chain termination, respectively. Consistent with this interpretation, our results show that the ratio of DNA polymerase I to DNA ligase activity significantly influenced both the ligation efficiency and the BER patch size. Specifically, the addition of DNA ligase to the *E. coli* cell-free extract resulted in a drastic diminution of longer repair patches and increased 1-nucleotide DNA repair synthesis. In contrast, supplementation of the cell-free extracts with DNA polymerase I enhanced patch size, whereas a simultaneous addition of DNA ligase negated this affect. These observations reinforce the concept that the balance between DNA polymerase I and ligase plays a key factor in determining the rate and mode of BER.

The rate of Dug-mediated repair of ethenocytosine residues also appeared to be depend on the ligation step during the BER process. Unlike Ung-mediated BER, after excision of uracil or ethenocytosine, Dug binds tightly to its AP-site containing DNA product; however, cleavage of the AP-site by AP endonuclease (Nfo) or AP lyase (Fpg) stimulates catalytic turnover of Dug (*10*, *46*). We observed that the rate of ethenocytosine-initiated BER did not change following the addition of Fpg, while significant stimulation occurred in the presence of excess DNA ligase. Since Nfo and Xth have been reported to comprise >90% of the AP-endonuclease activity detected in *E. coli*, AP-sites generated by Dug would be expected to be frequently processed by a class II AP-endonuclease activity (*18*). One-nucleotide gaps would result from the removal of 5′-terminal dRP residues by either a deoxyribophosphodiesterase or AP-lyase activity (*20*). Short patch (1-nucleotide) BER would follow after gap-filling DNA synthesis and DNA ligation reactions. The 5′-3′ exonuclease action of DNA polymerase I does not efficiently remove 5′-terminal dRP residues (*25*). However, a previous study demonstrated that the 5′-dRP residue can be slowly released as part of a short oligonucleotide (two nucleotides) upon incubation with DNA polymerase I (*24*). This may explain, in part, the biphasic distribution of BER that we detected, as well as the origin of the short patches that corresponded to two nucleotides in length. During long patch BER, a strand displacement reaction most likely displaced the DNA strand containing the 5′-dRP moiety, and subsequent cleavage of the generated DNA flap was carried out by the 5′-3′ exonuclease activity of DNA polymerase I, which acts efficiently on this DNA substrate (*25*, *27*, *28*).

It is generally accepted that DNA repair synthesis associated with BER is performed by *E. coli* DNA polymerase I

(*51*). The results presented in this report extend this observation and suggest that DNA polymerase I also functions during very-long patch BER DNA synthesis. After correcting for the dCMP content of the *Bsr*I DNA fragments produced following BER, very-long patch repair was estimated to comprise ~2% of BER events. In this regard, a parallel may exist between the function of DNA polymerase I in BER and its role in nucleotide excision repair (NER). Previous studies have demonstrated that DNA polymerase I conducts DNA repair synthesis during the gap-filling reaction of NER (*52*−*54*). While the majority of DNA repair synthesis during *E. coli* NER consisted of ~12 nucleotides, a small fraction (1−10%) of repair synthesis resulted in tracts of >1500 nucleotides and was referred to as long patch excision repair (*55*, *56*). A requirement for DNA polymerase I in long patch NER was demonstrated utilizing *E. coli* strains defective in DNA polymerase II or III (*55*, *57*). Furthermore, it was recognized that the frequency of long patch NER increased in *E. coli pol*A mutants defective in 5′-3′ exonuclease activity (*55*). By analogy, the 5′-3′ exonuclease function of DNA polymerase I might be important in determining the frequency of very-long patch BER in *E. coli*.

Very-long patch BER was not detectable when *E. coli* cell-free extracts were supplemented with DNA ligase. One possible explanation for this observation was that additional DNA ligase promotes phosphodiester bond formation during strand displacement DNA synthesis thereby reducing long DNA repair synthesis tracts. Indeed, the length of DNA repair synthesis associated with very-long patch BER could be manipulated by altering the balance between DNA polymerase I and DNA ligase in the *E. coli* cell extract. While this interpretation seems reasonable, an alternative explanation that DNA ligase modulates short and long patch repair by protein−protein interactions with various BER proteins cannot be excluded. For example, DNA ligase might interact with DNA polymerase I or other auxiliary factors that influence the patch size. In the mammalian BER pathway, the ligation step is coordinated through complicated protein−protein interactions such as those between XRCC1 and DNA ligase III (*58*), DNA polymerase β and DNA ligase I (*59*, *60*), and PCNA and DNA ligase I (*61*, *62*). Several studies have suggested that the sliding clamp replication protein, PCNA, acts as a scaffold that promotes assembly of BER proteins at an incised AP-site and stimulates the activity of DNA ligase I and flap endonuclease 1 during long patch BER in mammalian cells (*63*−*66*). A similar model for *E. coli* BER might be considered in light of the recent reported that the *E. coli* β clamp protein, a homologue of PCNA, interacts with DNA ligase and DNA polymerase I (*67*). Furthermore, the association of the β clamp protein with DNA polymerase I was shown to increase the processivity of DNA synthesis (*67*). Whether the DNA polymerase I/β-clamp protein interaction or other protein−protein interactions influence BER patch size and mediate very-long patch DNA repair synthesis has not been determined. Additional investigation is required to define the molecular mechanisms of very-long patch repair DNA synthesis and elucidate its biological significance.

ACKNOWLEDGMENT

We wish to thank the Center for Gene Research and Biotechnology for access to the PhosphorImager and to Dr. Samuel Bennett for insightful discussions and critical reading of the manuscript.

REFERENCES

1. Mosbaugh, D. W., and Bennett, S. E. (1994) *Prog. Nucleic Acid Res. Mol. Biol. 48*, 315−370.
2. Focher, F., Mazzarello, P., Verri, A., Hubscher, U., and Spadari, S. (1990) *Mutation Res. 237*, 65−73.
3. Duncan, B. K., and Miller, J. H. (1980) *Nature 287*, 560−561.
4. Frederico, L. A., Kunkel, T. A., and Shaw, B. R. (1990) *Biochemistry 29*, 2532−2537.
5. Lindahl, T. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3649−3653.
6. Ingraham, H. A., Dickey, L., and Goulian, M. (1986) *Biochemistry 25*, 3223−3230.
7. Radany, E. H., Dornfeld, K. J., Sanderson, R. J., Savage, M. K., Majumdar, A., Seidman, M. M., and Mosbaugh, D. W. (2000) *Mutation Res. 461*, 41−58.
8. Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., and Sperens, B. (1977) *J. Biol. Chem. 252*, 3286−3294.
9. Saparbaev, M., and Laval, J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8508−8513.
10. Sung, J.-S., and Mosbaugh, D. W. (2000) *Biochemistry 39*, 10224−10235.
11. Bennett, S. E., Sanderson, R. J., and Mosbaugh, D. W. (1995) *Biochemistry 34*, 6109−6119.
12. Barrett, T. E., Savva, R., Panayotou, G., Barlow, T., Brown, T., Jiricny, J., and Pearl, L. H. (1998) *Cell 92*, 117−129.
13. Sung, J.-S., Bennett, S. E., and Mosbaugh, D. W. (2001) *J. Biol. Chem. 276*, 2276−2285.
14. Bennett, S. E., and Mosbaugh, D. W. (1992) *J. Biol. Chem. 267*, 22512−22521.
15. Gossard, F., and Verly, W. G. (1978) *Eur. J. Biochem. 82*, 321−332.
16. Ljungquist, S. (1977) *J. Biol. Chem. 252*, 2808−2814.
17. Warner, H. R., Demple, B. F., Deutsch, W. A., Kane, C. M., and Linn, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4602−4606.
18. Doetsch, P. W., and Cunningham, R. P. (1990) *Mutation Res. 236*, 173−201.
19. Franklin, W. A., and Lindahl, T. (1988) *EMBO J. 7*, 3617−3622.
20. Piersen, C. E., McCullough, A. K., and Lloyd, R. S. (2000) *Mutation Res. 459*, 43−53.
21. Dianov, G., Sedgwick, B., Daly, G., Olsson, M., Lovett, S., and Lindahl, T. (1994) *Nucleic Acids Res. 22*, 993−998.
22. Sandigursky, M., and Franklin, W. A. (1992) *Nucleic Acids Res. 20*, 4699−4703.
23. Graves, R. J., Felzenszwalb, I., Laval, J., and O'Connor, T. R. (1992) *J. Biol. Chem. 267*, 14429−14435.
24. Price, A. (1992) *FEBS Lett. 300*, 101−104.
25. Mosbaugh, D. W., and Linn, S. (1982) *J. Biol. Chem. 257*, 575−583.
26. Lyamichev, V., Brow, M. A., and Dahlberg, J. E. (1993) *Science 260*, 778−783.
27. Xu, Y., Potapova, O., Leschziner, A. E., Grindley, N. D., and Joyce, C. M. (2001) *J. Biol. Chem. 276*, 30167−30177.
28. Xu, Y., Grindley, N. D., and Joyce, C. M. (2000) *J. Biol. Chem. 275*, 20949−20955.
29. Dianov, G., and Lindahl, T. (1994) *Curr. Biol. 4*, 1069−1076.
30. Dianov, G., Price, A., and Lindahl, T. (1992) *Mol. Cell. Biol. 12*, 1605−1612.
31. Sandigursky, M., Freyer, G. A., and Franklin, W. A. (1998) *Nucleic Acids Res. 26*, 1282−1287.
32. Sanderson, R. J., Bennett, S. E., Sung, J.-S., and Mosbaugh, D. W. (2001) *Prog. Nucleic Acid Res. Mol. Biol. 68*, 165−188.
33. Wang, Z., Wu, X., and Friedberg, E. C. (1993) *Mol. Cell. Biol. 13*, 1051−1058.
34. Matsumoto, Y., and Bogenhagen, D. F. (1991) *Mol. Cell. Biol. 11*, 4441−4447.
35. Singhal, R. K., Prasad, R., and Wilson, S. W. (1995) *J. Biol. Chem. 270*, 949−957.
36. Bennett, S. E., Sung, J.-S., and Mosbaugh, D. W. (2001) *J. Biol. Chem. 276*, 42588−42600.
37. Frosina, G., Fortini, P., Rossi, O., Carrozzino, F., Raspaglio, G., Cox, L. S., Lane, D. P., Abbondandolo, A., and Dogliotti, E. (1996) *J. Biol. Chem. 271*, 9573−9578.
38. Sanderson, R. J., and Mosbaugh, D. W. (1998) *J. Biol. Chem. 273*, 24822−24831.

39. Fortini, P., Pascucci, B., Parlanti, E., Sobol, R. W., Wilson, S. H., and Dogliotti, E. (1998) *Biochemistry 37*, 3575−3580.
40. Fortini, P., Parlanti, E., Sidorkina, O. M., Laval, J., and Dogliotti, E. (1999) *J. Biol. Chem. 274*, 15230−15236.
41. Dianov, G., Bischoff, C., Piotrowski, J., and Bohr, V. A. (1998) *J. Biol. Chem. 273*, 33811−33816.
42. Biade, S., Sobol, R. W., Wilson, S. H., and Matsumoto, Y. (1998) *J. Biol. Chem. 273*, 898−902.
43. Del Sal, G., Manfioletti, G., and Schneider, C. (1989) *Biotechniques 7*, 514−520.
44. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
45. Mosbaugh, D. W. (1988) *Nucleic Acid Res. 16*, 5645−5659.
46. Sung, J.-S. Characterization of *Escherichia coli* double-strand uracil-DNA glycosylase and analysis of uracil-initiated base excision DNA repair, Ph.D. Thesis, Oregon State University, 2002.
47. Srivastava, D. K., Berg, B. J. V., Prasad, R., Molina, J. T., Beard, W. A., Tomkinson, A. E., and Wilson, S. H. (1998) *J. Biol. Chem. 273*, 21203−21209.
48. Bennett, S. E., Schimerlik, M. I., and Mosbaugh, D. W. (1993) *J. Biol. Chem. 268*, 26879−26885.
49. Lutsenko, E., and Bhagwat, A. S. (1999) *J. Biol. Chem. 274*, 31034−31038.
50. Lundquist, R. C., and Olivera, B. M. (1982) *Cell 31*, 53−60.
51. Lindahl, T. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 135−192.
52. Caron, P. R., Kushner, S. R., and Grossman, L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4925−4929.
53. Hanawalt, P. C., Cooper, P. K., Ganesan, A. K., and Smith, C. A. (1979) *Annu. Rev. Biochem. 48*, 783−836.
54. Husain, I., Van Houten, B., Thomas, D. C., Abdel-Monem, M., and Sancar, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6774−6778.
55. Cooper, P. K. (1982) *Mol. Gen. Genet. 185*, 189−197.
56. Kuemmerle, N., Ley, R., and Masker, W. (1981) *J. Bacteriol. 147*, 333−339.
57. Cooper, P. K., and Hanawalt, P. C. (1972) *J. Mol. Biol. 67*, 1−10.
58. Nash, R. A., Caldecott, K. W., Barnes, D. E., and Lindahl, T. (1997) *Biochemistry 36*, 5207−5211.
59. Dimitriadis, E. K., Prasad, R., Vaske, M. K., Chen, L., Tomkinson, A. E., Lewis, M. S., and Wilson, S. H. (1998) *J. Biol. Chem. 273*, 20540−20550.
60. Prasad, R., Singhal, R. K., Srivastava, D. K., Molina, J. T., Tomkinson, A. E., and Wilson, S. H. (1996) *J. Biol. Chem. 271*, 16000−16007.
61. Montecucco, A., Rossi, R., Levin, D. S., Gary, R., Park, M. S., Motycka, T. A., Ciarrocchi, G., Villa, A., Biamonti, G., and Tomkinson, A. E. (1998) *EMBO J. 17*, 3786−3795.
62. Levin, D. S., Bai, W., Yao, N., O'Donnell, M., and Tomkinson, A. E. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12863−12868.
63. Matsumoto, Y. (2001) *Prog. Nucleic Acid Res. Mol. Biol. 68*, 129−138.
64. Jonsson, Z. O., Hindges, R., and Hubscher, U. (1998) *EMBO J. 17*, 2412−2425.
65. Tom, S., Henricksen, L. A., and Bambara, R. A. (2000) *J. Biol. Chem. 275*, 10498−10505.
66. Tom, S., Henricksen, L. A., Park, M. S., and Bambara, R. A. (2001) *J. Biol. Chem. 276*, 24817−24825.
67. Lopez de Saro, F. J., and O'Donnell, M. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 8376−8380.

BI027115V