Escherichia coli Double-Strand Uracil-DNA Glycosylase: Involvement in Uracil-Mediated DNA Base Excision Repair and Stimulation of Activity by Endonuclease IV†

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ABSTRACT: *Escherichia coli* double-strand uracil-DNA glycosylase (Dug) was purified to apparent homogeneity as both a native and recombinant protein. The molecular weight of recombinant Dug was 18 670, as determined by matrix-assisted laser desorption−ionization mass spectrometry. Dug was active on duplex oligonucleotides (34-mers) that contained site-specific U·G, U·A, ethenoC·G, and ethenoC·A targets; however, activity was not detected on DNA containing a T·G mispair or single-stranded DNA containing either a site-specific uracil or ethenoC residue. One of the distinctive characteristics of Dug was that the purified enzyme excised a near stoichiometric amount of uracil from U·G-containing oligonucleotide substrate. Electrophoretic mobility shift assays revealed that the lack of turnover was the result of strong binding by Dug to the reaction product apyrimidinic-site (AP) DNA. Addition of *E. coli* endonuclease IV stimulated Dug activity by enhancing the rate and extent of uracil excision by promoting dissociation of Dug from the AP·G-containing 34-mer. Catalytically active endonuclease IV was apparently required to mediate Dug turnover, since the addition of 5 mM EDTA mitigated the effect. Further support for this interpretation came from the observations that Dug preferentially bound 34-mer containing an AP·G target, while binding was not observed on a substrate incised 5′ to the AP-site. We also investigated whether Dug could initiate a uracil-mediated base excision repair pathway in *E. coli* NR8052 cell extracts using M13mp2op14 DNA (form I) containing a site-specific U·G mispair. Analysis of reaction products revealed a time dependent appearance of repaired form I DNA; addition of purified Dug to the cell extract stimulated the rate of repair.

Escherichia coli uracil-mediated DNA base excision repair (BER)[1] is initiated by uracil-DNA glycosylase which plays an important role in mutation avoidance (*1*−*4*). *E. coli* uracil-DNA glycosylase (Ung) is a monofunctional enzyme encoded by the *ung* gene (*5*) which produces a single polypeptide with a molecular weight of 25 558, as determined by MALDI mass spectrometry (*6*). The enzyme exhibits a ∼2-fold preference for uracil residues located in single-stranded DNA compared to double-stranded DNA containing U·G mispairs and is more active (2.4-fold) on U·G- rather than U·A-containing DNA (*7*). Catalysis occurs when the uracil-deoxyribose N1−C1′ glycosylic bond is destabilized and subjected to an activated water nucleophilic attack that results in the release of free uracil and the production of apyrimidinic-site DNA (*8*). Following hydrolysis, Ung dissociates from the AP-site and may implement a processive search mechanism to locate additional uracil nucleotides (*7*, *9*). With a turnover rate of ∼800 min^{-1}, measured on U·A-containing DNA (*10*) Ung is considered kinetically efficient compared to other monofunctional DNA glycosylases (*11*−*13*). Ung is inhibited noncompetitively by uracil ($K_i = 0.12$ mM), competitively by AP-sites ($K_i = 1\ \mu$M), and irreversibly by binding to the bacteriophage PBS1 and PBS2 uracil-DNA glycosylase inhibitor (Ugi) protein (*10*, *14*). Significant amino acid sequence homology exists between *E. coli* Ung and uracil-DNA glycosylases from other biological sources, such as human, which shares ∼56% identity with Ung (*15*, *16*).

Wiebauer and Jiricny (*17*) initially detected a novel BER repair process in HeLa cell extracts that excised aberrant thymine bases from T·G mispairs. Subsequent experiments revealed that the thymine-excision activity involved a DNA glycosylase which was designated mismatch-specific thymine-DNA glycosylase, TDG (*18*). This enzyme was purified from HeLa cell extracts, and identified as a 55 000 molecular weight polypeptide, based on active analysis following SDS−polyacrylamide gel electrophoresis (*19*). The substrate speci-

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[1] Abbreviations: BER, base excision repair; Ung, *E. coli* uracil-DNA glycosylase; UDG, human and herpes simplex virus uracil-DNA glycosylase; Ugi, PBS1 and 2 uracil-DNA glycosylase inhibitor protein; dsUDG and Dug, *E. coli* double-strand-specific uracil-DNA glycosylase; TDG, human mismatch-specific thymine-DNA glycosylase; MUG, *E. coli* mismatch-specific uracil-DNA glycosylase; ϵC, 3,N^4-ethenocytosine; ϵCDG, *E. coli* ϵC-DNA glycosylase; Endo IV, *E. coli* endonuclease IV; BSA, bovine serum albumin; AP, apurinic/apyrimidinic; *AP, incision on the 5′-side of AP-site; MALDI, matrix-assisted laser desorption−ionization; ORF, open reading frame; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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ficity of HeLa TDG was determined as U·G > T·G ≫ T·C ≫ T·T (*19*); however, efficient excision of 3,N^4-ethenocytosine residues from ethenoC·G-containing oligonucleotides was recently demonstrated (*20*, *21*). Unlike HeLa uracil-DNA glycosylase, TDG was reportedly inactive on U·A base pairs or uracil-containing single-stranded DNA and was not inhibited by either uracil or Ugi (*22*). PCR-mediated cloning of human TDG cDNA revealed that the gene (hTDG) contained an open reading frame encoding a 410 amino acid polypeptide with a deduced molecular weight of ~46 000 (*23*). A databank search disclosed a murine homologue with 88% identity; however, no amino acid sequence motifs common to other types of DNA glycosylases were identified, including uracil-DNA glycosylase (*23*). In a database search for proteins with a methyl-CpG-binding domain (MBD), Hendrich et al. (*24*) identified mouse and human full-length cDNAs encoding a protein that was designated MBD4. Subsequently, MBD4 was shown to bind preferentially to m^5CpG-TpG mismatches and possess DNA glycosylase activity against U·G and T·G mispairs. However, no significant amino acid sequence conservation was reported for MBD4 and TDG (*24*).

In a study of hTDG N- and C-terminal deletion mutants, Gallinari and Jiriciny (*25*) identified a 248 amino acid core region of hTDG capable of processing U·G but not T·G mispairs. This core hTDG region revealed limited homology with bacterial open reading frames found in *E. coli* and *Serratia marcescens* (*25*). The *E. coli* ORF (169 amino acids)[2] shared ~30% identity with the core hTDG sequence (*11*, *25*). When the *E. coli* recombinant protein was expressed in either reticulocyte lysates or *E. coli* cells, the extracts efficiently processed U·G but not T·G mispairs or U·A base pairs (*25*). Furthermore, excision of uracil residues from single-stranded DNA was not observed nor was the DNA glycosylase activity inhibited by Ugi (*25*). Like hTDG but unlike Ung, this *E. coli* enzyme was shown to efficiently remove ethenoC residues from duplex DNA (*21*). Thus, these results indicated that the protein encoded by ORF-169 was distinct from Ung and corresponded to a second class of *E. coli* uracil-DNA glycosylases. This activity was initially designated as double-strand-specific uracil-DNA glycosylase (dsUDG) (*25*), but later was referred to as mismatch-specific uracil-DNA glycosylase (MUG) (*11*) and ethenoC-DNA glycosylase (ethenoCDG) (*21*) by other investigators. Based on characterization presented in this manuscript, and in compliance with traditional *E. coli* nomenclature, we propose that dsUDG (double-strand-specific uracil-DNA glycosylase) be referred to as Dug.

E. coli Dug and Ung lack amino acid sequence homology (<10% identity); however, these proteins share common tertiary structural features as described by X-ray crystallography (*11*, *25*). Both enzymes utilize a nucleotide-flipping action to capture the target base in the active site, but use distinctively different mechanisms for substrate recognition (*11*). Dug purportedly inserts a "polypeptide wedge" into the DNA double helix at a U·G mispair to facilitate nucleotide-flipping by a "push" mechanism (*11*). The inserted polypeptide chain (NPSGLSR) is apparently stabilized by intercalation of Arg146 with the complementary strand base stack and by three hydrogen bonds formed by Gly143 and Ser145 that are absolutely specific for the widowed guanine (*11*). In contract, Ung mediates nucleotide-flipping by the "pinch-push−pull" mechanism that inserts a Leu191 into the DNA minor groove base stack but overall establishes few contacts with the complementary DNA strand (*16*, *26*). While X-ray crystallographic studies of Dug protein structure have offered significant insight into structure/function relationships, the biochemical characteristics of this enzyme, which differs substantially in size and amino acid sequence from hTDG, remains to be elucidated.

In the present study we have purified to apparent homogeneity a Ugi-insensitive double-strand-specific uracil-DNA glycosylase from *E. coli* (*ung*) cells that was identified as Dug. The *dug* gene was cloned, and the gene product was overproduced, purified, and characterized with respect to substrate specificity, product binding, and mechanism of action. We also demonstrated that *E. coli* endonuclease IV stimulates Dug by instigating catalytic turnover. Furthermore, we provide the first evidence that Dug initiates a complete BER reaction using M13mp2 DNA (form I) containing a site-specific U·G mispair.

MATERIALS AND METHODS

Materials. E. coli strains NR8051 [Δ(*pro-lac*) *thi ara*] and NR8052 [Δ(*pro-lac*) *thi ara trpE9777 ung-1*] were provided by T. A. Kunkel (NIEHS, National Institutes of Health) and have been previously described (*27*, *28*). *E. coli* JM109 [*recA1* e14(McrA$^-$) Δ(*pro-lac*) *thi gyrA96* (*Nal^r*) *endoA1 hsdR17* (r_k^- m_k^+) *relA1 supE44*/F′ *traD36 lacI^Q* Δ(*lacZ*)-*M15 proA$^+$B$^+$*] was obtained from New England BioLabs. *E. coli* uracil-DNA glycosylase (fraction V) and Ugi (fraction IV) were purified as described by Sanderson and Mosbaugh (*29*).

E. coli endonuclease IV (fraction V) was provided by B. Demple (Harvard University). Restriction endonucleases (*Eco*RI and *Hin*dIII), T4 polynucleotide kinase, and Vent DNA polymerase were purchased from New England BioLabs, and T4 DNA ligase from Gibco-BRL. Proteinase K and creatine phosphokinase (type I, rabbit) were from Sigma, and ribonuclease A was from Worthington Biochemicals.

M13mp2op14 (U·G) DNA (>95% form I) containing a site-specific U·G mispair[3] at nucleotide position 79 of the *lacZ*α gene was prepared as described by Sanderson and Mosbaugh (*30*). Plasmid pKK223−3 was obtained from Pharmacia Biotech Inc. Oligonucleotides AGCTTGGCTGCAGGTXGACGGATCCCCGGGAATT, where X at nucleotide position 16 corresponds to U (U-34-mer), C (C-34-mer), or T (T-34-mer), and two complementary oligonucleotides (34-mer) containing G or A opposite to X (G-34-mer and A-34-mer, respectively) were purchased from Midland Certified Reagent Company. The oligonucleotide containing 3,N^4-ethenocytosine (ϵC-34-mer) at the nucleotide position X was prepared by Genset. Oligonucleotides CCCAGTCACGTUATTGTAAAACG (U-23-mer), used in the construction of M13mp2op14 (U·G) DNA, and primers CCAAGCTTTTATCGCCCACGCACTACCAGCGCC (R-33-mer) and CAGAATTCATGGTTGAGGATATTTTG-

[2] ORF-169 is located between nucleotide positions 3,212,608 and 3,213,115 on the *E. coli* chromosome.

[3] Base pairs and mispairs are described by listing the base in the repaired strand first and then the base in the template strand.

GCTCCAG (L-33-mer), used in cloning and DNA sequencing experiments, were obtained from Oligos Etc. Oligonucleotides were deblocked, deprotected, purified by denaturing polyacrylamide gel electrophoresis, and 5′-end phosphorylated when appropriate using 6000 Ci/mmol [γ-^{32}P]ATP (DuPont-NEN) in place of ATP as previously described (*7*, *31*). Duplex oligonucleotides were prepared by annealing ^{32}P-labeled U-, T-, or ϵC-34-mer with the oligonucleotide G-34-mer or A-34-mer (*7*). The duplex oligonucleotide [^{32}P]AP/G-34-mer was created by reacting [^{32}P]U/G-34-mer with excess *E. coli* uracil-DNA glycosylase to produce a site-specific AP-site; the reaction was quenched by Ugi treatment, as described by Shroyer et al. (*8*). To prepare duplex DNA (34-mer) that contained an incision on the 5′-side of the defined AP-site ([^{32}P]*AP/G-34-mer)[4], a reaction mixture (50 μL) containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 pmol of [^{32}P]AP/G-34-mer, and 0.5 units of *E.coli* endonuclease IV (Endo IV) was incubated at 37 °C for 4 h. One unit of *E. coli* endonuclease IV releases 1 pmol of product/min as described by Levin et al. (*32*). Following incision of >98% of the AP-sites, endonuclease IV was heat-inactivated at 70 °C for 10 min.

Methods. Isolation and Cloning of the dug Gene. A DNA fragment was obtained from *E. coli* genomic DNA that contained an ORF (507-bp) encoding the *dug* gene by using the procedure described by Gallinari and Jiricny (*25*) with some modifications. Briefly, a single colony of *E. coli* NR8052 (*ung1*) was isolated, diluted into 100 μL of distilled water and boiled for 10 min to lyse the cells. After centrifugation for 10 min at 13 000 rpm in a Microspin 24S (Sorvall) centrifuge at 4 °C, the supernatant fraction was recovered and a sample (10 μL) was added to a primer annealing reaction mixture (100 μL) containing 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 100 pmol each of R- and L-33-mer oligonucleotide primer. The mixture was heated at 90 °C for 5 min, adjusted to 200 μM each of dATP, dTTP, dCTP, dGTP, and 2 units of Vent DNA polymerase was added to the reaction mixture (109 μL). DNA amplification was then performed for 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. The DNA fragment (523 bp) was isolated using 1.5% agarose gel electrophoresis and purified by the QIAquick gel extraction protocol (Qiagen). Following simultaneous restriction endonuclease digestion with *Eco*RI and *Hin*dIII, the DNA fragment (517-bp) was isolated, as before, and then inserted into the corresponding restriction endonuclease cleavage sites of pKK223−3 using T4 DNA ligase (*33*). The resulting construct (pKK-Dug) was isolated and the nucleotide sequence of the entire *dug* gene was determined for both DNA strands which verified the predicted ORF (*25*). DNA sequencing was conducted using an Applied Biosystems model 373A by the Center for Gene Research and Biotechnology (Oregon State University).

Purification of E. coli Double-Strand Uracil-DNA Glycosylase. Native double-strand uracil-DNA glycosylase was purified using a purification scheme similar to that initially described by Lindahl et al. (*10*) and modified by Bennett and Mosbaugh (*34*) for purifying *E. coli* Ung. *E. coli* NR8052 cells were grown at 37 °C in 40 L of YT medium (0.5% yeast extract, 0.8% tryptone, and 0.5% NaCl). After reaching a density of ~6.5 × 10^8 cells/mL (1 OD_{600} = 8 × 10^8 cells/mL), the cells were harvested by centrifugation at 3500*g* for 15 min in a GSA (Sorvall) rotor, and frozen at −80 °C. Cell pellets (100 g) were thawed on ice and resuspended in 1 L of sonification buffer composed of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 mM dithiothreitol. The cells were then disrupted by sonification and the lysate was centrifuged at 20000*g* for 20 min at 4 °C in a SS34 (Sorvall) rotor, as previously described (*34*). Following the addition of 2 × 10^5 units of Ugi (fraction IV), the cell-free extract was mixed with an equal volume of 1.6% (w/v) streptomycin sulfate in sonification buffer. One unit of Ugi inactivates 1 unit of Ung using reaction conditions as described by Bennett and Mosbaugh (*6*). After gentle stirring for 30 min at 4 °C, the precipitate was removed by centrifugation at 20000*g* for 20 min at 4 °C, and the supernatant fraction was designated fraction I. Pulverized ammonium sulfate was slowly added to fraction I to achieve a final concentration of 50% (saturation). Precipitated protein was removed by centrifugation at 20000*g* for 20 min at 4 °C. The recovered supernatant fraction was then adjusted to 80% (saturation) with ammonium sulfate and the precipitate collected as described above. The pellet was resuspended in 35 mL of UEB buffer [10 mM Hepes-KOH (pH 7.4), 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 M NaCl and 5% (w/v) glycerol] and dialyzed extensively against the same buffer; this material constituted fraction II. Fraction II (68 mL) was loaded onto a Sephadex G-75 column (6 cm^2 × 88 cm) equilibrated in UEB buffer, and eluted with the same buffer. Fraction (5 mL) were collected and assayed for double-strand uracil-DNA glycosylase activity. Active fractions were pooled, concentrated (~5-fold) using a Centriprep-10 concentrator (Amicon), dialyzed against HAB buffer [10 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol, 200 mM KCl], and designated fraction III. Fraction III (20 mL) was applied to a hydroxyapatite column (4.9 cm^2 × 8 cm) equilibrated with HAB buffer. The column was eluted with the same buffer and fractions (5.2 mL) were collected. To avoid a contaminating exonuclease activity, only fractions from the leading half of the activity peak were pooled. These were concentrated (~6-fold) as before, dialyzed against DAB buffer [30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 5% (w/v) glycerol] and corresponded to fraction IV. Fraction IV (7.5 mL) was loaded onto a single-stranded DNA agarose column (1.8 cm^2 × 8 cm) equilibrated in DAB buffer. The column was washed with 50 mL of equilibration buffer, and a 100 mL linear gradient of 0 to 700 mM NaCl in DAB buffer was applied. Fractions (3 mL) were collected and assayed for double-strand uracil-DNA glycosylase activity. Those fractions containing activity that bound to the resin and eluted in a symmetrical peak at ~220 mM NaCl were pooled and concentrated (~8-fold) as described above. After dialysis against DAB buffer, the preparation of native Dug (nDug) was designated fraction V, and stored at −80 °C.

Overproduction and Purification of Recombinant Dug. *E. coli* JM109 containing pKK-Dug was grown at 37 °C in 9 L of YT medium supplemented with 0.01% ampicillin. After reaching a cell density of ~6.5 × 10^8 cell/mL, 100 mL of 100 mM isopropyl-β-D-thiogalactopyranoside was added to induce *dug* expression and incubation was continued for 4 h at 37 °C. Cells were harvested by centrifugation and lysed

[4] *AP denotes an apyrimidinic-site cleaved on the 5′-side to produce 3′-hydroxyl nucleotide and deoxyribose 5-phosphate termini.

by sonification as described above. Ugi (3×10^6 units) was added to the cell-free extract (400 mL) to inactivate endogenous *E. coli* Ung activity. Recombinant Dug was purified as described above for the native enzyme with the following modification: (i) the pellet precipitated by 50−80% ammonium sulfate was resuspended in 20 mL of UEB buffer; (ii) fraction III was applied to a hydroxyapatite column (19.6 $cm^2 \times 3.6$ cm); (iii) fraction IV was loaded onto a single-stranded DNA agarose column (4.9 $cm^2 \times 16$ cm); and (iv) fraction V was purified using a DEAE-Sephadex A50 column (4.9 $cm^2 \times 6$ cm). Active fractions from the DEAE-Sephadex column were pooled, concentrated (∼2-fold) as described above, dialyzed against DAB buffer, and designated recombinant Dug (rDug) fraction VI.

Enzyme Assays. Standard uracil-DNA glycosylase reaction mixtures (10 μL) used for detecting *E. coli* Ung and Dug contained 25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.01 mM $ZnCl_2$, 0.1 mg/mL acetylated BSA, 0.1 pmol of [^{32}P]U/G-34-mer, and various amount of enzyme (2 μL) as indicated in the figure legends. Where appropriate, enzyme samples were diluted with Dug buffer. Incubation was conducted at 30 °C for 30 min and the reactions were then terminated by heat treatment at 70 °C for 3 min. Apyrimidinic sites generated by uracil removal from [^{32}P]U/G-34-mer were quantitatively cleaved following the addition of 0.1 units of *E. coli* endonuclease IV, incubation at 37 °C for 30 min, and reaction termination at 70 °C for 3 min. An equal volume of denaturing sample buffer was added to the reaction mixtures, which were then heated at 95 °C for 3 min. Samples (10 μL) were analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as previously described by Bennett et al. (*7*). Gels were dried under vacuum, autoradiography was performed using X-OMAT AR film (Eastman Kodak Co.), and quantification of ^{32}P radioactivity was performed using a PhosphorImager (Molecular Dynamics) and ImageQuant software. During the purification of *E. coli* Dug, enzyme activity was measured as described above except (i) reaction mixtures (100 μL) contained 20 μL of each fraction; (ii) 1000 units of Ugi was added to each reaction; (iii) 4 pmol of [^{32}P]U/G-34-mer was used as substrates; and (iv) incubation occurred at 30 °C for 16 h. Reactions were terminated with an equal volume of stop solution (2% SDS, 50 mM EDTA). Each sample was adjusted to 0.3 mg/mL yeast tRNA and 2 M ammonium acetate, and extracted with phenol:chloroform (1:1). DNA was then ethanol precipitated, and resuspended in 20 μL of distilled water. Samples (5 μL) were treated with *E. coli* endonuclease IV, denaturing polyacrylamide gel electrophoresis was conducted, and [^{32}P]DNA bands were detected as described above.

Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide slab gel electrophoresis was conducted as described by Bennett et al. (*6*) with some modifications. Briefly, samples were mixed with an equal volume of denaturation buffer containing 50 mM Tris-HCl (pH 6.8), 429 mM 2-mercaptoethanol, 30% (w/v) glycerol, 0.04% bromphenol blue, and 1% SDS. After heating at 100 °C for 3 min, samples were applied to a discontinuous SDS−polyacrylamide gel (3% stacking gel, 12.5% resolving gel) and electrophoresis was performed at room temperature and 100 V (stacking gel) and 200 V (resolving gel), until the tracking dye migrated ∼9 cm. Gels were fixed in 10% acetic acid and 50% methanol, and protein bands were detected by staining with 0.05% Coomassie Brilliant Blue G-250 (*6*), or by silver staining with RAPID-Ag−STAIN (ICN Radiochemicals).

Activity gel electrophoresis was similarly conducted except that the denaturation buffer contained 108 mM Tris-HCl (pH 6.8), 2.4 mM EDTA, 244 mM 2-mercaptoethanol, 12.5% (w/v) glycerol, 1.6% SDS, and 0.06% bromphenol blue. In addition, 2 mM EDTA was added to both the 3% stacking gel and 12.5% resolving gel, 50 μg/mL fibrinogen was included in the resolving gel, and electrophoresis was performed at 4 °C (*31*). Following electrophoresis, the gel was immersed in 3 gel volumes of SDS-extraction buffer [10 mM Tris-HCl (pH 7.5), 5 mM mercaptoethanol, and 25% (v/v) 2-propanol] and agitated for 30 min at room temperature. After repeating the SDS-extraction step, the gel was sliced horizontally into ∼3.5 mm sections that were crushed and incubated overnight (16 h) at 4 °C with vigorous shaking in 500 μL of elution buffer [25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.01 mM $ZnCl_2$, and 0.1 mg/mL acetylated BSA]. Samples (40 μL) were assayed for uracil-DNA glycosylase activity using [^{32}P]U/G-34-mer as substrate.

Denaturing DNA sequencing gels ($30 \times 40 \times 0.08$ cm) containing 12% acrylamide, 0.41% *N*,*N*′-methylene-bis-acrylamide, 8.3 M urea, and TBE buffer (90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA) were used to analyze uracil-DNA glycosylase reaction products as previously described (*7*). [^{32}P]DNA samples were mixed with an equal volume of denaturing sample buffer, heated at 95 °C for 3 min, and electrophoresis performed at 1200 V until the tracking dye migrate 18 cm. Autoradiography was conducted using X-OMAT AR film, and quantitation of [^{32}P]DNA bands was performed using PhosphorImager (Molecular Dynamics) and ImageQuant Software.

Band Mobility Shift Assays. Standard DNA binding reaction mixtures (10 μL) contained 25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.01 mM $ZnCl_2$, 0.1 mM acetylated BSA, 10 nM of various double-stranded [^{32}P]DNA probes, and rDug as indicated. Competitor oligonucleotide (400 nM C/G-34-mer) was mixed with duplex [^{32}P]34-mer probes on ice prior to adding rDug as indicated. DNA binding reactions were carried out at 30 °C for 30 min, samples (5 μL) mixed with 1.5 μL of 50% sucrose, and loaded onto a nondenaturing 6% polyacrylamide gel ($17 \times 14 \times 0.08$ cm). Electrophoresis was conducted at room temperature and 140 V with TAE buffer [40 mM Tris-acetate and 1 mM EDTA (pH 8.0)] until the bromphenol blue dye, which was loaded in adjacent lanes, migrated ∼6 cm (*35*). The gel was then dried and [^{32}P]DNA bands were detected by autoradiography. The relative amounts of free and mobility-shifted ^{32}P-labeled oligonucleotide were measured using a PhosphorImager and ImageQuant software.

Base Excision DNA Repair Reactions. Uracil-mediated base excision repair reactions were conducted with cell-free extracts of *E. coli* NR8052 using a procedure similar to that developed by Sanderson and Mosbaugh (*30*). *E. coli* grown at 37 °C in YT medium to mid-log phase were harvested by centrifugation, the cell pellet (2.5 g wet weight) was resuspended in 40 mL of sonification buffer, and cells were lysed with a Dismembrator (Fisher, model 300) as previously described (*34*). After centrifugation at 20000*g* for 20 min at

4 °C, 14 g of powdered ammonium sulfate was slowly added to the supernatant fraction (40 mL), and the protein precipitate formed after a 10 min incubation at 0 °C was recovered by centrifugation. The pellet was resuspended in 10 mL of R buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol and 10% (w/v) glycerol], dialyzed against the same buffer, and the cell-free extract protein concentration (~12 mg/mL) was determined.

Standard BER reaction mixtures contained 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 dATP, dTTP, dGTP, and dCTP, 5 mM phosphocreatine di-Tris salt, 200 units/ml of phosphocreatine kinase, 10 000 units/mL of Ugi, 10 μg/mL of M13mp2op14 (U·G) heteroduplex DNA (form I) and 1 mg/mL of *E. coli* NR8052 cell-free extract. In some cases, rDug was added to cell-free extracts prior to incubation at 30 °C for various amounts of time. Samples (100 μL) were removed, the reaction was terminated on ice, adjusted to 20 mM EDTA and then heated at 70 °C for 3 min. RNase A was then added to 80 μg/mL and incubated at 37 °C for 10 min. Following the addition of SDS to 0.5%, proteinase K was added to 190 μg/mL, and each sample was incubated for 30 min at 37 °C. The samples were subsequently extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, washed with 75% ethanol, and resuspended in 20 μL of TE buffer.

Repair of the M13mp2op14 DNA recovered from the BER reactions described above was analyzed as follows. DNA samples (5 μL) were incubated with 100 units of *E. coli* Ung for 30 min at 37 °C. One unit of Ung activity is defined as the amount of enzyme that release 1 nmol of uracil/h under standard conditions, as previously described (*6*). The reaction was terminated by addition of 1000 units of Ugi, then 1 unit of *E. coli* endonuclease IV was added and incubation continued for 30 min at 37 °C. After heating at 70 °C for 3 min, each sample (8 μL) was mixed with 2 μL of agarose gel sample buffer that contained 0.5% SDS, 50 mM EDTA, 25% (w/v) glycerol and 0.05% bromphenol blue. Samples (10 μL) were then loaded on to a 0.8% agarose gel (12 × 13 × 0.5 cm) containing TAE buffer [40 mM Tris-acetate and 1 mM EDTA (pH 8.0)] and 0.2 μg/mL ethidium bromide. Electrophoresis was performed in TAE buffer at 100 V until the tracking dye migrated ~70% of the gel length. Ethidium-bromide-stained DNA bands were visualized by transillumination (302 nm), and the relative percentage of form I and II DNA was determined using a concentration series of M13mp2op14 DNA standards (form I and II) run on the same gel. Ethidium bromide staining intensity was measured using an Image Store 7500 (Ultra Violet Products) gel documentation system and ImageQuant software. After correcting for the ~0.7-fold reduced staining intensity of form I DNA relative to form II DNA, the percentage of form I DNA in each sample was determined.

Protein Measurement. The protein concentration of *E. coli* crude extracts and partially purified Dug preparations was determined by the Bradford reaction (*36*) using the Bio-Rad Protein Assay. *E. coli* Ung (fraction V), Dug (fraction V and VI),[5] and Ugi (fraction IV) protein concentrations were determined by absorbance spectroscopy using molar extinction coefficients $\epsilon_{280\ nm} = 4.2 \times 10^4$, 2.4×10^4, and 1.2×10^4 L/mol cm, respectively[6] (*6*).

Mass Spectrometry. MALDI mass spectrometry was conducted using a custom-built time-of-flight mass spectrometer equipped with a two-stage delayed extraction source by the Mass Spectrometry Facilities and Service Core Unit (Environmental Health Science Center, Oregon State University). Approximately 1 μL of purified rDug (fraction VI, 0.16 mg/mL) was mixed with 3 μL of 4-hydroxy-α-cyanocinnamic acid in 0.1% trifluoroacetic acid, 33% acetonitrile. A droplet (~0.5 μL) of this analyte/matrix solution was deposited on a precrystallized matrix sample probe and allowed to air-dry. Mass spectra were produced by irradiation of the sample with 30 individual laser pulses and the summed signals were calibrated using ions from an external calibrant.

RESULTS

Detection of Ugi-Insensitive Uracil-DNA Glycosylase Activity in E. coli Cell-Free Extracts. To initiate this investigation of enzyme activities in *E. coli* cell extracts that catalyze the removal of uracil from DNA, 5′-end ^{32}P-labeled synthetic duplex oligonucleotides were prepared that contained either a site-specific U·A base pair or U·G mispair. The 34-mer DNA substrates (U/A-34-mer and U/G-34-mer) were reacted with various amounts of *E. coli* NR8051 (*ung*$^+$) or NR8052 (*ung*$^-$) cell extracts in the absence or presence of the uracil-DNA glycosylase-specific inhibitor protein (Ugi) as shown in Figure 1. AP-sites generated during the incubation were cleaved by treatment with exogenous *E. coli* endonuclease IV, which incised the deoxyribose-phosphate ester bond on the 5′-side of the AP-site, resulting in a [^{32}P]15-mer product. Analysis of reaction products by denaturing polyacrylamide gel electrophoresis revealed that both U/A-34-mer and U/G-34-mer were efficiently processed in the NR8051 cell extract (Figure 1A). When Ugi was included in these reaction mixtures, uracil-excision activity on the U/A-34-mer was significantly inhibited; however, a reduced level of activity was detected on the U/G-34-mer (Figure 1B). In the reaction mixtures containing the NR8052 cell extract, uracil-excision activity was observed solely on the U/G-34-mer substrate (Figure 1C). Furthermore, the activity was not inhibited by Ugi (Figure 1D). The relative amount of uracil excision detected for each DNA substrate, U/A-34-mer and U/G-34-mer, was determined and plotted in Figure 1, panels E and F, respectively.

E. coli NR8051 cell extract was active on both substrates, but more active (~1.6-fold) on the mispaired U/G-34-mer relative to the paired U/A-34-mer duplex. That the uracil-excision activity of the NR8051 cell extract was significantly inhibited by Ugi indicated that the enzyme responsible for the majority of uracil processing on both substrates was most likely Ung. The extent of Ugi-insensitive uracil-excision on U/G-34-mer observed in the NR8051 cell extract was comparable to that observed on U/G-34-mer in the NR8052 cell extract. Since this activity was not inhibited by Ugi and preferred the mispaired U/G-34-mer, we concluded it could not be synonymous with Ung.

Purification of the Ugi-Insensitive Uracil-DNA Glycosylase Activity. The Ugi-insensitive uracil-DNA glycosylase

[5] Fractions V and VI correspond to the final purification step for nDug and rDug, respectively.

[6] Molar extinction coefficient ϵ_{280nm} for Dug was calculated by the DNASTAR Protean computer program version 3.11.

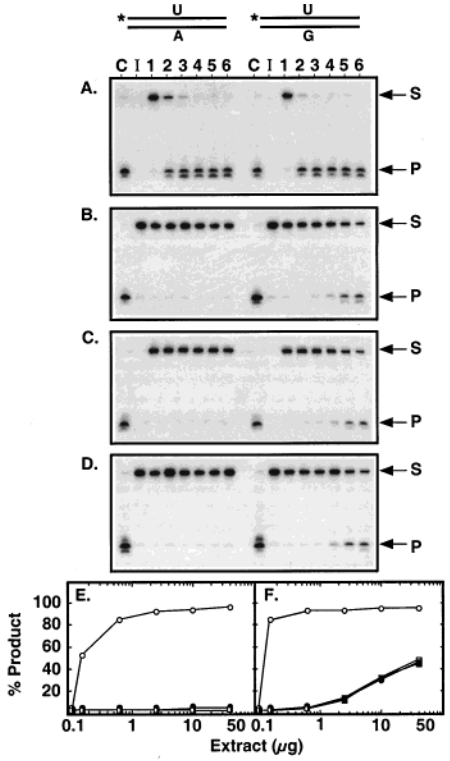

FIGURE 1: Detection of Ugi-insensitive uracil-DNA glycosylase activity in *E. coli* cell-free extracts. Two 5′-end ^{32}P-labeled duplex oligonucleotides ([^{32}P]U/A-34-mer and [^{32}P]U/G-34-mer) containing a site-specific uracil residue located at position 16 of the radioactively labeled strand were prepared as described under Materials and Methods. Reaction mixtures (100 μL) containing 25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.1 mM $ZnCl_2$, 0.1 mg/mL acetylated BSA, 0.1 pmol of [^{32}P]U/A- or [^{32}P]U/G-34-mer as indicated, and either 0, 0.16, 0.63, 2.5, 10, or 40 μg of extract protein (lanes 1−6, respectively) from *E. coli* NR8051 (*ung*$^+$) cells (A and B) or NR8052 (*ung*$^-$) cells (C and D) were incubated with (B and D) or without (A and C) Ugi (1000 units) at 30 °C for 2 h. A control reaction (lane C) was also prepared that lacked Ugi but contained 400 units of *E. coli* Ung. A similar reaction (lane I) was conducted following the addition of 1000 units of Ugi (B and D). After incubation, each reaction was terminated with stop solution and the DNA was isolated, AP-sites hydrolyzed, and reaction products resolved by denaturing polyacrylamide as described under Materials and Methods. *Arrows* indicate the location on the autoradiogram of unreacted [^{32}P]34-mer substrate (S) and [^{32}P]15-mer product (P). The amount of [^{32}P]DNA detected in each band was determined using a PhosphorImager and the percentage of product formed was calculated by dividing the amount of [^{32}P]-15-mer by that of [^{32}P]15-mer plus [^{32}P]34-mer and multiplying by 100. The percentage of product generated in reactions containing [^{32}P]U/A-34-mer (E) or [^{32}P]U/G-34-mer (F) substrate and various cell-free extracts is shown as *E. coli* NR8052 extract plus Ugi (□), NR8051 minus Ugi (○), NR8052 plus Ugi (■), and NR8052 minus Ugi (●).

activity was purified from *E. coli* strain NR8052 (*ung*$^-$) after Ugi was added to the cell extract to inactivate any residual Ung activity (see Supporting Information, Figure 1). Active fractions from the final purification step were pooled and analyzed by SDS−polyacrylamide gel electrophoresis, and a single protein band was visualized by silver staining (Figure 2A, lane I). Activity gel analysis was carried out to determine whether the protein band corresponded with the location of Ugi-insensitive uracil-DNA glycosylase activity (Figure 2B). Using the [^{32}P]U/G-34-mer cleavage assay, enzymatic activity was detected which comigrated with the silver-stained band (Figure 2). The apparent molecular weight of the

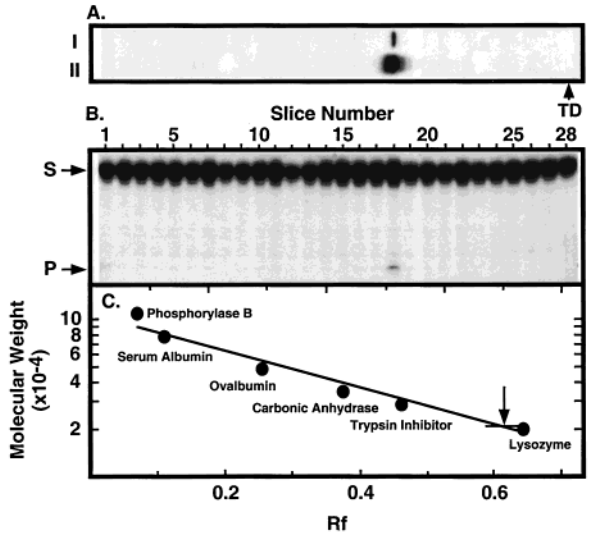

FIGURE 2: SDS−polyacrylamide gel electrophoresis of native and recombinant Dug preparations. (A) Two sets of samples (40 μL each lane, 4 lanes total) of nDug fraction V (2.2 μg) and rDug fraction VI (6.3 μg) were loaded onto a 12.5% SDS-polyacrylamide gel along with molecular weight standards, and electrophoresis was conducted as described under the Materials and Methods. Following electrophoresis, the gel was cut in half vertically between the sample sets and one-half of the gel containing nDug (lane I) and rDug (lane II) was silver-stained. The direction of migration was from left to right and the location of tracking dye (TD) is indicated by an *arrow*. (B) After electrophoresis, the other half of the gel was vertically cut between the sample lanes, SDS extracted, and each lane horizontally sliced into ∼3.5 mm segments. Protein was eluted and renatured from the gel slices as described in the Materials and Methods. Samples (40 μL) were assayed for uracil-DNA glycosylase activity, reaction products analyzed on a denaturing 12% polyacrylamide/8.3 M urea gel, and the autoradiogram corresponding to nDug (fraction V) is shown. The location of the [^{32}P]U/G-34-mer substrate (S) and [^{32}P]15-mer product (P) are indicated by *arrows*. (C) A standard curve (log M_r versus R_f) was generated based on the relative mobility of prestained protein molecular weight markers (Bio-Rad) for phosphorylase b (M_r 111 000), BSA (M_r 77 000), ovalbumin (M_r 48 200), carbonic anhydrase (M_r 33 800), trypsin inhibitor (M_r 28,600), and lysozyme (M_r 20 500). The apparent molecular weight of nDug was determined based on the Rf for the midpoint of the gel slice (fraction 18, *horizontal line*) as indicated by the *vertical arrow*.

polypeptide was calculated to be ∼21 000 from both the location of the silver-stained band and position of activity gel slice 18 (Figure 2C). Thus, the Ugi-insensitive enzyme was purified to apparent homogeneity and consisted of a single polypeptide, referred to as Dug.

Cloning, Overexpression, and Purification of Recombinant Dug. On the basis of the report of Gallinari and Jiricny (*25*), we decided to clone the *E. coli* gene encoded by an open reading frame of 169 amino acids which was associated with double-strand-specific uracil-DNA glycosylase activity. The nucleotide sequence of this ORF was amplified using *E. coli* NR8052 chromosomal DNA as template and cloned into the overexpression vector pKK223−3; the resultant construct was designated pKK-Dug. Attempts to transform *E. coli* NR8052 (*ung*$^-$) with pKK-Dug were unsuccessful; however, transformation of *E. coli* JM109, an *ung*$^+$ strain, was successful for overproduction of recombinant protein. To separate double-strand uracil-DNA glycosylase activity from the endogenous Ung activity of the host bacterial cells, Ugi was added to the cell extracts before performing purification. Recombinant Dug was purified using the procedure described

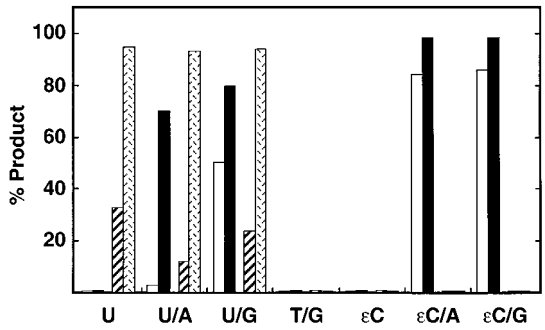

FIGURE 3: DNA substrate specificity of Dug and Ung. Seven sets of standard uracil-DNA glycosylase reaction mixtures containing either single- or double-stranded [^{32}P]34-mer oligonucleotide substrates with U-, U·A-, U·G-, T·G-, ϵC-, ϵC·A-, or ϵC·G-target residues were prepared as described under Materials and Methods. After adding 4 and 800 nM rDug (white and black bars, respectively) or 2 pM and 8 nM Ung (striped and stippled bars, respectively), each reaction (10 μL) was incubated for 30 min at 30 °C. Following the incubation, 5 μL of each reaction was quenched with 2.5 μL of buffer containing 0.3 M NaOH and 30 mM EDTA. Samples were heated for 30 min at 90 °C to cleave AP-sites and analyzed on a denaturing 12% polyacrylamide/8.3 M urea gel as described in Figure 1. The percentage of product ([^{32}P]-15-mer) formed was determined using a PhosphorImager.

for the native enzyme with some modifications. DEAE-Sephadex chromatography was included as the final step, where the activity was detected in the flow-through fractions. SDS−polyacrylamide gel electrophoresis analysis of the active fractions revealed a single protein with an apparent molecular weight of ~22 000, as visualized by silver staining. Thus, the purification procedure resulted in the isolation of an apparently homogeneous protein preparation (see Supporting Information, Figure 2).

SDS−Polyacrylamide Gel Analysis of Native and Recombinant Double-Strand Uracil-DNA Glycosylase. To explore the possibility that the native and recombinant protein might be the same enzyme, two sets of samples from each preparation were resolved by SDS−polyacrylamide gel electrophoresis and protein bands were visualized by silver-staining as shown in Figure 2A. Side by side comparison of their electrophoretic mobility showed that the two protein bands comigrated. Activity gel analysis established that the protein band corresponded to the Ugi-insensitive uracil-DNA glycosylase activity in both the nDug (Figure 2) and rDug (data not shown) preparations. From these observations, we inferred that the recombinant protein and native Dug were indistinguishable. Therefore, the recombinant double-strand uracil-DNA glycosylase preparation was referred to as recombinant Dug (rDug).

Molecular Weight Determination of Dug by MALDI Mass Spectrometry. Although the polypeptide molecular weight estimates (~21 000) of both the native and recombinant Dug proteins were close to the predicted molecular weight of the polypeptide (18 672) encoded by the ORF-169, mass determination by MALDI mass spectrometry was conducted to more accurately determine the molecular weight and verify the identity of Dug. The summed spectra produced one major singly charged peak of molecular weight 18 670 (see Supporting Information, Figure 3). Since the experimentally determined molecular weight of Dug was in excellent agreement with that predicted (±0.01%), it appeared that the protein did not undergo posttranslational modification.

Comparison of Dug and Ung Substrate Specificity. To compare the relative substrate specificity of *E. coli* Ung and Dug, 5′-end ^{32}P-labeled 34-mer oligonucleotides containing site-specific U, U·A, U·G, T·G, ϵC, ϵC·A, or ϵC·G residues were prepared. Each substrate was incubated with Ung or Dug to catalyze base excision, and the resulting AP-sites were cleaved by hot-alkali treatment rather than enzymatic cleavage by *E. coli* endonuclease IV, as single-stranded DNA is known to be a poor substrate for this enzyme (*7*, *37*). Analysis of reaction products by denaturing polyacrylamide gel electrophoresis revealed that Dug was more active on the ϵC-containing duplex oligonucleotide than on the mispaired U/G-34-mer, and that the excision of ϵC did not show strict preference for the opposite strand base (Figure 3). In contrast, Dug was considerably more active on the mispaired U/G-34-mer than the paired U/A-34-mer oligonucleotide. However, at a higher concentration of Dug (800 nM) the excision of uracil from the U/A-34-mer was significantly increased, whereas Dug activity on single-stranded U- and ϵC-34-mer, as well as duplex T/G-34-mer, was not detected. In contrast, Ung was more active on single-stranded U-34-mer, and about 2-fold more active on the mispaired U/G-34-mer relative to the paired U/A-34-mer duplex. Furthermore, Ung-mediated base-excision was not detected on substrates containing ϵC·G or T·G mispairs. When taken together, these results clearly illustrate a difference in the substrate specificity of Dug and Ung activities.

Effect of Uracil and Ugi on Dug and Ung Activity. Inhibition studies were conducted to examine the relative effect of free uracil and Ugi on Dug and Ung activity. Standard uracil-DNA glycosylase assays were performed in the presence of various amounts of free uracil or Ugi, and the percent of activity relative to the control was determined (Figure 4). Whereas, Ung activity determined on [^{32}P]U/G-34-mer was inhibited ~50% by 1 mM free uracil (Figure 4A), Dug activity was not significantly affected by as much as 2 mM free uracil. In addition, Ung was inhibited by Ugi, whereas, the activity of Dug was not affected by a 4-fold molar excess of Ugi (Figure 4B). These results provide additional evidence that the uracil-excision activity of the Dug preparation was an intrinsic property of the purified protein, and not the consequence of *E. coli* Ung contamination.

Effect of E. coli Endonuclease IV on Dug Activity. To examine the effect of *E. coli* endonuclease IV on the activity of Dug, the rate of uracil excision was determined for the [^{32}P]U/G-34-mer substrate in the absence or presence of endonuclease IV (see Supporting Information, Figure 4). Dug activity in standard assays using 2.5 nM Dug exhibited an initial fast reaction rate (0−1 min), but then the reaction dramatically slowed and essentially stopped after 1 h. Upon examination, the concentration of uracil removed (1.7 nM) after a 1 h incubation was determined to be less than the concentration of the enzyme added to the reaction. Therefore, the enzyme did not appear to turn over. In contrast, the addition of 10 nM endonuclease IV enhanced the reaction rate of Dug during and after the initial burst phase of the reaction. Endonuclease IV alone did not exhibit detectable uracil-DNA glycosylase or incision activity (data not shown). The maximum extent of uracil excision from the U/G-34-mer was increased ~5.5-fold relative to the Dug reaction conducted in the absence of endonuclease IV.

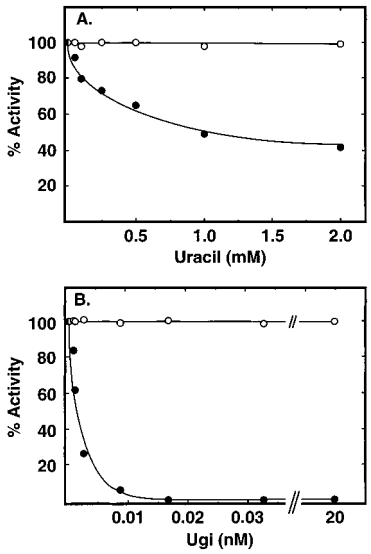

FIGURE 4: Effect of uracil and Ugi on Dug and Ung activity. Two sets of standard uracil-DNA glycosylase reaction mixtures were prepared containing 10 nM [^{32}P]U/G-34-mer and either 5 nM rDug (○) or 5 pM Ung (●) as described under Materials and Methods. To each set of reactions various amounts of (A) uracil (0, 0.05, 0.1, 0.25, 0.5, 1, and 2 mM) or (B) Ugi (0, 0.25, 0.5, 2, 8, 16, 32, and 20 000 pM) were added as indicated above. After incubation for 30 min at 30 °C, reactions were terminated by heating at 70 °C for 3 min, then treated with 0.1 units of *E. coli* endonuclease IV. Reaction products were analyzed on denaturing 12% polyacrylamide/8.3 M urea gels and [^{32}P]DNA bands were quantitatively measured using a PhosphorImager as described in Figure 1. Enzyme activity (%) was determined relative to the control reaction which lacked uracil or Ugi addition: 100% activity corresponded to 61 and 77 fmol of [^{32}P]U/G-34-mer converted to product for Dug and Ung, respectively.

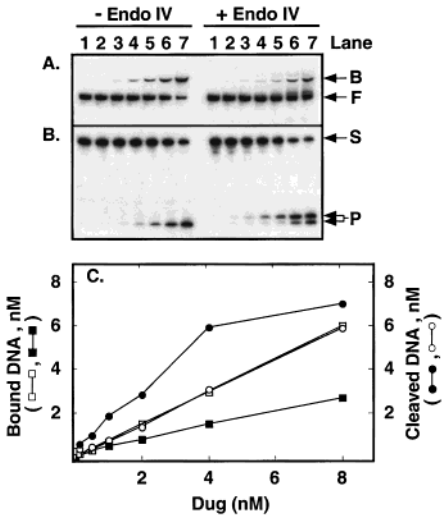

FIGURE 5: Effect of *E. coli* endonuclease IV on DNA binding and catalytic activity of Dug. (A) Electrophoretic mobility shift assay. Two sets of standard uracil-DNA glycosylase reaction mixtures containing 0, 0.25, 0.5, 1, 2, 4, and 8 nM rDug (lanes 1−7, respectively), and 10 nM [^{32}P]U/G-34-mer were prepared in the presence (+) or absence (−) of 10 nM *E. coli* endonuclease IV. After incubation at 30 °C for 30 min, one-half of each reaction mixture (5 μL) was mixed with 1.5 μL of 50% sucrose, and samples (5 μL) were analyzed by nondenaturing polyacrylamide (6%) gel electrophoresis as described under Materials and Methods. Autoradiography was performed and the location of the free (F) and bound (B) [^{32}P]DNA probe are indicated by *arrows*. (B) To the remainder of each reaction mixture (5 μL), 2.5 μL of stop solution containing 0.3 M NaOH and 30 mM EDTA was added, and each sample was heated at 90 °C for 30 min to cleave the generated AP-sites. Samples were then analyzed on a denaturing 12% polyacrylamide/8.3 M urea gel as described in Figure 1. Autoradiography was performed and the arrows indicate the location of the [^{32}P]34-mer DNA substrate (S) and reaction products (P) produced by NaOH treatment alone (−Endo IV) or by a combination of *E. coli* endonuclease IV and NaOH treatment (+Endo IV). (C) The ^{32}P radioactivity was measured for each band shown in panels A and B using a PhosphorImager. The amount of [^{32}P]34-mer detected (A) in the bound form was calculated for each reaction mixture conducted in the presence (■) or absence (□) of endonuclease IV. The amount of cleaved [^{32}P]U/G-34-mer (B) was determined for the reactions performed with (●) and without (○) endonuclease IV, and plotted as a function of the concentration of rDug in each reaction mixture.

Effect of E. coli Endonuclease IV on Dug DNA Binding and Catalysis. Electrophoretic mobility shift assays were conducted to determine whether Dug forms a stable protein−DNA complex. Standard Dug−DNA binding reactions containing 10 nM [^{32}P]U/G-34-mer and various amounts of Dug were incubated in the absence or presence of 10 nM endonuclease IV. Following the binding reaction, half of the sample was analyzed by nondenaturing polyacrylamide gel electrophoresis (Figure 5A). In the absence of endonuclease IV, the [^{32}P]U/G-34-mer probe was mobility shifted in a concentration-dependent manner. When endonuclease IV was included in the binding reaction mixture, the extent of Dug−DNA complex formation at equivalent Dug concentrations became less intense. These results demonstrated that endonuclease IV affected the stability of the Dug−DNA interaction.

The second aliquot of the binding reaction mixture was treated with hot-alkali and subjected to denaturing polyacrylamide gel electrophoresis to determine whether uracil-excision had occurred during the binding reaction (Figure 5B). Product analysis of binding reactions carried out in the absence of endonuclease IV showed a single [^{32}P]15-mer band that increases in intensity with increasing Dug concentration. Analysis of the reactions containing endonuclease IV revealed the presence of a second ^{32}P-labeled product band just above the [^{32}P]15-mer generated by hot-alkali treatment; this relatively slow-migrating band was the product of cleavage by endonuclease IV. The slow-migrating band occurred earlier in the concentration series than the lower band and represents enzyme turnover; the lower band presumably resulted from Dug-bound AP-site DNA that was inaccessible to endonuclease IV and was subsequently cleaved by the hot-alkali treatment. This interpretation was consistent with an initial β-elimination cleavage reaction catalyzed by hot alkali on the 3′-side of the AP-site, followed by loss of the ring-opened deoxyribose (4-hydroxy-2-pentenal) by a second β-elimination reaction (δ elimination) to produce a [^{32}P]15-mer with a 3′-terminal phosphate, and cleavage 5′ to the AP-site by endonuclease IV, which generates [^{32}P]15-mer with a 3′-terminal hydroxyl (*19*, *38*). Quantification of the DNA binding results revealed that, in the absence of endonuclease IV, the amount of Dug-bound DNA formed was equal to the amount of AP-site-containing DNA (product DNA) (Figure 5C). Moreover, the amount of oligonucleotide cleaved was approximately equal to the amount of enzyme in the reaction, suggesting that Dug bound tightly to the DNA following uracil excision. In the presence of endonuclease IV, the amount of bound DNA was significantly reduced, but the rate of uracil excision was enhanced (Figure 5C). These results imply that endonuclease

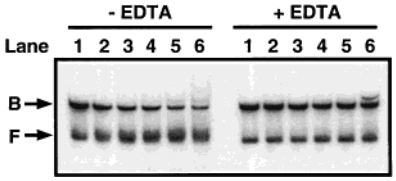

FIGURE 6: Effect of EDTA on *E. coli* endonuclease IV and Dug binding to DNA. DNA binding reaction mixtures (10 μL) containing 20 nM [^{32}P]U/G-34-mer, 20 nM rDug, and various amounts of *E. coli* endonuclease IV (0, 2, 5, 10, 20, and 40 nM: lanes 1−6, respectively) were prepared with (+) and without (−) 5 mM EDTA. After incubation at 30 °C for 30 min, samples (5 μL) were analyzed by nondenaturing 6% polyacrylamide gel electrophoresis as described under Materials and Methods. Autoradiography was performed and the location of the free (F) and bound (B) forms of [^{32}P]DNA are indicated by *arrows*. The amount of the [^{32}P]DNA probe detected in each band was determined using a PhosphorImager as described under Materials and Methods.

IV stimulated the dissociation of Dug from product DNA enabling the enzyme to turnover and participate in further catalytic events.

Effect of EDTA on Endonuclease IV-Mediated Dissociation of the Dug/AP-Site−DNA Complex. To determine if the catalytic activity of endonuclease IV was responsible for stimulating Dug activity, electrophoretic mobility shift assays were conducted to examine the endonuclease IV-mediated dissociation of Dug from AP-site-DNA in the absence or presence of 5 mM EDTA. Previously, Levin et al. (*39*) demonstrated that EDTA was a potent inhibitor of endonuclease IV activity. Binding reactions containing 20 nM [^{32}P]U/G-34-mer and an equal concentration of Dug resulted in the mobility shift of more than 90% of the [^{32}P]U/G-34-mer DNA probe, both in the presence and absence of EDTA (Figure 6, lane 1, left and right). This indicated that the DNA-binding of Dug was insensitive to EDTA. In the absence of EDTA (Figure 6, left), increasing amounts of endonuclease IV in the DNA binding reaction resulted in a linear decline in the amount of bound ^{32}P-labeled probe, confirming the results obtained in Figure 5. However, in the presence of EDTA (Figure 6, right), the stimulatory effect of endonuclease IV on the dissociation of Dug from its product DNA was significantly blocked. This result implied that incision of the AP-site by endonuclease IV was required to promote dissociation of Dug from its product DNA.

Ability of Dug To Bind Duplex DNA Containing an Incised AP-Site. Since endonuclease IV allowed the release of Dug from its product DNA, we postulated that Dug would not efficiently bind DNA containing an incision on the 5′-side of an AP-site. To test this proposition, incised AP-site duplex DNA (*AP/G-34-mer) was prepared. Three ^{32}P-labeled DNA duplexes (U/G-, AP/G-, and *AP/G-34-mer) were incubated with various amounts of Dug and electrophoretic mobility shift assays were performed. Analysis of the binding reactions showed that Dug bound preferentially to AP/G-34-mer relative to U/G-34-mer, and that Dug was not observed to bind to the *AP/G-34-mer containing an incision on the 5′-side of the AP-site (Figure 7). These findings supported the hypothesis that the endonuclease IV-mediated stimulation of Dug activity was the result of Dug dissociation from incised AP-sites.

Uracil-Mediated Base Excision Repair Initiated by Dug in E. coli Cell Extracts. To ascertain whether Dug may participate in uracil-mediated base excision repair in *E. coli*,

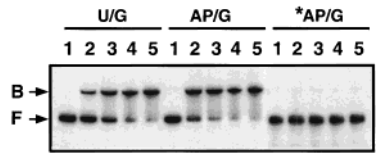

FIGURE 7: Ability of Dug to bind duplex DNA containing an incised AP-site. DNA binding reaction mixtures (10 μL) were prepared containing 0, 2.5, 5, 10, and 20 nM rDug (lanes 1−5, respectively), 10 nM of [^{32}P]U/G-, [^{32}P]AP/G-, or [^{32}P]*AP/G-34-mer (pretreated with *E. coli* endonuclease IV) as indicated, and 400 nM C/G-34-mer competitor oligonucleotide. After incubation at 30 °C for 30 min, samples (5 μL) were analyzed using nondenaturing 6% polyacrylamide gel electrophoresis as described under Materials and Methods. The location of the free (F) and bound (B) oligonucleotide probes are indicated by *arrows* on the autoradiogram.

we conducted BER assays using M13mp2op14 DNA (form I) containing a single site-specific U·G mispair. To eliminate Ung-initiated base excision repair, BER assays were performed using *E. coli* NR8052 (*ung*$^-$) cell extract in the presence of excess Ugi. Form I DNA was incubated with cell-free extracts for various times, DNA isolated, and then treated with excess of exogenous Ung and endonuclease IV in order to convert unrepaired molecules to form II DNA. Analysis of reaction products by agarose gel electrophoresis revealed the time dependent appearance of repaired form I DNA (Figure 8A, lanes 1−7, left). To investigate whether Dug was indeed involved in the repair reaction, we supplemented the BER reaction with purified rDug. Under this condition, the amount of Ung/Endo IV-resistant form I DNA formed was significantly increased (Figure 8A, lanes 1−7, right). Quantification of the reaction products revealed that the rate of uracil-DNA repair was enhanced and the extent of repaired form I DNA was increased by ∼2.5-fold after a 60 min reaction (Figure 8B). These results demonstrated that Dug participated in the *E. coli* base excision repair reaction, by carrying out uracil-excision in the first step of the pathway.

DISCUSSION

We have purified to apparent homogeneity a Ugi-insensitive double-strand specific uracil-DNA glycosylase from *E. coli ung-1* cell extracts. Several observations led to the conclusion that Dug was distinct from the *E. coli* Ung: (1) Dug activity was identified and purified in the presence of Ugi, a potent and irreversible inhibitor of Ung (*14*, *34*, *40*), (2) the molecular weight of Dug, as estimated by size exclusion chromatography (∼18 000) and SDS−polyacrylamide gel electrophoresis (∼21 000) was lower than that obtained for Ung (∼25 000), (3) Dug was inactive on the single-stranded uracil-containing substrates preferred by Ung (*7*, *41*), and (4) the turnover number of Dug, compared to Ung, was extremely low (*10*, *14*). Since many properties of nDug were similar to the *E. coli* ORF-169 gene product first referred to as dsUDG (*25*), we cloned the gene and purified the overproduced recombinant enzyme, and found it to be indistinguishable from Dug. Further, we showed by mass spectrometry analysis that the molecular weight of purified Dug (18 670) was in excellent agreement with the deduced molecular weight of the amino acid sequence, 18 672. In this study we have demonstrated that Dug acts on U·A-containing DNA, and has a relatively broad substrate specificity that was restricted to duplex DNA substrate;

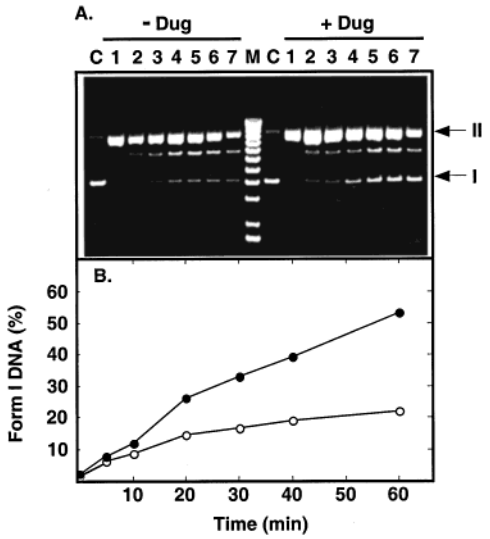

FIGURE 8: Uracil-mediated base excision DNA repair in *E. coli* (*ung*$^-$) cell-free extracts. (A) Two base excision repair reaction mixtures (800 μL) containing 10 μg/mL of M13mp2op14 (U·G) DNA and 1 mg/mL of *E. coli* NR8052 cell extract protein were prepared either with (+) or without (−) 80 pmol of exogenous rDug (fraction VI). Reactions were incubated at 30 °C, samples (100 μL) removed after 0, 5, 10, 20, 30, 40, and 60 min (lanes 1−7, respectively), 25 μL of 0.1 M EDTA was added, and the samples heated at 70 °C for 3 min to terminate the BER reaction. DNA was isolated, treated with *E. coli* uracil-DNA glycosylase and endonuclease IV, and then analyzed by 0.8% agarose gel electrophoresis as described under Materials and Methods. Mock-treated M13mp2op14 (U·G) DNA (1 μg) without cell-free extract and Ung/Endo IV treatment, and a sample containing 1 μg of a 1-kb DNA ladder (Gibco-BRL) were used as reference standards (lanes C and M, respectively). The *arrows* indicate the location of form I and II DNA bands detected by ethidium bromide stain. (B) DNA bands detected by ethidium bromide staining (A) were quantitatively measured using a transilluminator and digital camera imaging system as described in Materials and Methods. The amount of form I and II DNA was measured relative to corresponding DNA standards (6.3−100 ng). The percentage of form I DNA detected in samples with (●) and without (○) rDug addition was calculated by dividing the amount of form I (ng) by that of form I plus form II DNA and multiplying by 100.

therefore, the designations MUG (mismatch-specific uracil-DNA glycosylase) and ϵCDG (ethenocytosine-DNA glycosylase) appeared to be somewhat limiting. Hence, in accordance with the three-letter system of bacterial nomenclature, we advocate that dsUDG be referred to as Dug, double-strand specific uracil-DNA glycosylase.

One of the distinctive characteristics of Dug was that the purified enzyme appeared incapable of excising more than a stoichiometric amount of uracil residues from DNA. Formally, the observed lack of turnover is open to several interpretations: (1) Dug may function as a "suicide enzyme" that is inactivated after a single cleavage reaction, such as O^6-alkylguanine methyltransferase (*42*) or type I restriction endonucleases (*43*, *44*); (2) Dug may bind to the AP-site containing DNA reaction product with sufficient affinity that it is subsequently restricted from engaging new substrate; and (3) a cofactor(s) essential for efficient Dug function may be absent in the preparation or reaction mix. DNA-binding experiments involving band shift assays demonstrated that the low turnover of Dug was the result of strong binding to the reaction product AP·G-DNA. Furthermore, cleavage analysis of Dug−DNA binding reactions indicated that the vast majority of U·G-containing DNA in complex with Dug was converted into AP·G-containing DNA. We reasoned that since AP sites in *E. coli*, generated either spontaneously or through the action of DNA glycosylases, are subjected to a variety of AP endonuclease activity, the catalytic activity of Dug might be modulated by an AP-endonuclease. This was indeed the case as Dug activity was significantly stimulated (~5.5-fold) by the addition of an excess (4-fold) of endonuclease IV.

Stimulation of Dug activity was apparently promoted by enzyme dissociation from AP·G-containing DNA, since we observed less Dug−DNA complex in the presence versus the absence of endonuclease IV. Endonuclease IV is a class II AP-endonuclease that cleaves 5′ to an AP-site and whose activity is inhibited by EDTA (*39*). Interestingly, in the reactions including both Dug and endonuclease IV, a new reaction product was observed (Figure 5), one that presumably contained a 3′-terminal hydroxyl rather than the 3′-terminal phosphate created by alkali cleavage of Dug-generated AP-sites (*19*). Further, the increase in Dug activity stimulated by endonuclease IV corresponded to an increase in the amount of 3′-terminal hydroxyl product observed. Catalytically active endonuclease IV was required for stimulation, since Dug/Endo IV reactions conducted in the presence of 5 mM EDTA showed no detectable stimulation. Therefore, it appeared that Dug did not bind product DNA nicked by endonuclease IV; this was demonstrated directly in Figure 7. These results suggested that it was the interaction of endonuclease IV with DNA, rather than a Dug:Endo IV protein interaction, that promoted displacement of Dug from AP·G-containing DNA. To our knowledge, this study is the first to report on the interaction of a uracil-DNA glycosylase with a 5′-incised AP-site.

Waters et al. (*13*) investigated the effect of human AP-endonuclease 1 (HAP1) on the catalytic efficiency of human thymine-DNA glycosylase. Like Dug, purified hTDG was characterized by a very low turnover rate (~0.4 min^{-1}) and a high affinity (half-life ~10 h) for its reaction product, AP·G DNA (*11*, *45*). The presence of a 10-fold excess of endonuclease IV in hTDG reactions with T·G-containing DNA was not observed to affect the rate of thymine excision. In contrast, the addition of HAP1 was found to stimulate thymine removal in a concentration-dependent manner, although the stimulatory effect of an equimolar amount of HAP1 was modest, approximately 0.5-fold in a 5 h reaction (*13*). Interestingly, HAP1-mediated stimulation of hTDG activity was much greater on U·C-containing DNA (*13*). This finding is consonant with the observation that dissociation of hTDG, in the presence of 2 mM Mg^{2+}, is more rapid from DNA containing an AP-site opposite a C or ^{SMe}G nucleotide than from AP·G-DNA (*13*). Following the cloning of the hTDG cDNA and the purification of the recombinant protein, N- and C-terminal deletion analysis revealed that hTDG contained a 249 amino acid catalytic "core" capable of processing U·G but not T·G mispairs (*25*). It would be of interest to ascertain whether the hTDG catalytic core enzyme is stimulated by an AP-endonuclease, or whether the N-terminal portion of hTDG, which bears no sequence similarity to the N-terminus of Dug, is required. Determination of whether the stimulatory effect of HAP1 on hTDG catalytic efficiency is the result of a specific HAP1:hTDG protein interaction, a HAP1:DNA interaction, or some other mechanism must await further experimentation.

In an effort to clarify the role of Dug in DNA repair, Lutsenko and Bhagwat (*46*) constructed *E. coli* strains mutant in either or both *ung* and *dug*. Using a kanamycin reversion assay specific for a C to T mutation at the second C in the Dcm recognition sequence CCAGG located in the *kan* gene, no significant change in the frequency of kanamycin reversion was detected for the *E. coli ung-1 dug* double mutant relative to the isogenic *dug*$^+$ strain (*46*). In contrast, the reversion frequency of the *E. coli ung* mutant strain was elevated approximately 8−10-fold compared to wild-type (*ung*$^+$) strain (*46*). Whether the DNA sequence context of the kanamycin reversion target might modulate any potential anti-mutator effect of wild-type *dug* in this genetic assay is not clear. In this regard, we have presented evidence in this study that extracts of *E. coli ung-1* cells are proficient in carrying out complete uracil-mediated BER of a U·G mispair, and that the addition of purified Dug protein accelerates this process. These observations formally support the argument that Dug can participate in DNA repair, and that further elucidation of the role of Dug in BER is required.

Using a rifampicin-resistance forward mutation assay, Lutsenko and Bhagwat (*46*) did observe a small *dug*-dependent effect (∼2.7-fold) on mutation frequency. Interestingly, efficient excision of ϵC from ϵC·G mispairs was reported for *E. coli* extracts of *dug*$^+$, but not *dug*$^-$, strains (*46*). In the present study, we observed efficient excision of ϵC from ϵC·A as well as ϵC·G, mispairs. Taken together, these data further strengthen the hypothesis of Lutsenko and Bhagwat (*46*) and Saparbaev and Laval (*21*), that *dug* may encode the principal ϵC excision activity in *E. coli* cells.

In interpreting the double-strand uracil-DNA glycosylase X-ray crystal structure, Barrett et al. (*11*) suggested that the protein, lacking the specialized binding pocket of uracil-DNA glycosylase, achieved substantial specificity from interactions with the complementary strand. According to this interpretation, the substrate preference of Dug for U·G and T·G is due to an association with the guanine opposite U that is mediated by three hydrogen bonds absolutely specific for guanine (*11*). Thus, the carbonyl of Gly 143 forms strong hydrogen bonding interactions with the exocyclic 2-amino and endocyclic N1 groups of guanine, and the carbonyl of Ser 145 interacts similarly with the guanine exocyclic 2-amino group. These amino acids reside on a motif (NPSGLSR) that forms a "wedge" that penetrates the base stack of the DNA from the minor groove (*11*). Although this hypothesis is useful to explain the preference of Dug for U·G- over U·A-containing DNA, it is not entirely consistent with the efficient catalytic activity observed on ϵC·A in this study, nor with the very low activity against T·G-containing DNA observed by Saparbaev and Laval (*21*). In this regard, it is notable that, following the processing of a ϵC·A mispair, Dug was not observed to bind to the resultant AP·A-DNA in a electrophoretic mobility shift assay (data not shown). It is possible that the interaction of the enzyme with the guanine opposite the mispaired uracil or ethenocytosine may occur after nucleotide flipping. In the Dug crystal structure, the amino acids implicated in the interaction with the opposite guanine are Gly-143 and Ser-145, which are found in the "wedge" motif (NPSGLSR) (*11*). However, while these amino acids are not conserved in the related hTDG motif (MPSSSAR), nevertheless hTDG shows strong preference for U·G- and T·G-containing DNA over U·A (and T·A) base pairs. These amino acid differences may explain in part the differential activity of hTDG and Dug toward U·G- and ϵC·G-containing substrates reported by Saparbaev and Laval (*21*). As we have demonstrated, Dug loses affinity for the "widowed" guanine once the AP-site is 5′-incised by endonuclease IV. The structural basis for this loss of interaction and whether a similar result would be obtained for a 3′-incised AP-site are not known at this time.

We have demonstrated the involvement of Dug in initiating uracil-mediated base excision repair in *E. coli* using an M13mp2 form I DNA containing site-specific U·G mispair. An examination of the BER reaction in *E. coli* deficient in Ung activity revealed that ∼20% of the mispaired uracil was repaired after 60 min of incubation. To ensure that we were examining Ung-independent uracil base excision repair, an excess of Ugi was included in the reaction mixture to inactivate any residual Ung activity. The repair of mispaired uracil appeared to be mediated by Dug because the extent of repair was enhanced to ∼55% when the BER reaction was supplemented with purified Dug. This observation provides the first line of evidence that uracil-excision repair in *E. coli* can be instigated by the *dug* gene product in vivo. These results stand in contrast to those recently reported by Sandigursky et al. (*47*). Previously, repair of a site-specific U·G mispair in a closed circular plasmid was not detected in extracts of an *E. coli* strain deficient in Ung activity. On this basis, it was concluded that Ung was absolutely required for repair of U·G-containing DNA (*47*). In this study, we show that extracts of *E. coli ung-1* contain a Ugi-resistant uracil-excision activity attributable to the *dug* gene product and capable of initiating repair of a U·G mispair. In an earlier report, this laboratory found that in extracts of a human glioblastoma cell line, a small but detectable amount (∼7%) of uracil-excision repair appeared to be insensitive to Ugi, suggesting that a back-up repair pathway existed, and that hTDG activity may be involved in the repair of a U·T mispair (*30*).

Many DNA glycosylases have overlapping substrate specificities and may provide back-up functions for each other (*48*). It has been suggested that Dug may act as a back-up or alternative to Ung in the repair of U·G mispairs arising through spontaneous hydrolytic deamination of cytosine (*25*). In the present study, we have shown that Dug can initiate uracil-mediated base excision DNA repair in *E. coli*. However, the *dug* gene product does not appear to play a strong role in preventing G·C to A·T transition mutations. Since Dug has been shown to exhibit a strong preference for ϵC residues in double-stranded DNA (*21*), one might wonder whether an *E. coli* strain defective in *dug* would exhibit an elevated mutation rate for G·C to T·A transversions. An understanding of the biological role of *E. coli* Dug and that of its human homolog, TDG, must await further experimentation.

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SUPPORTING INFORMATION AVAILABLE

Figure 1 presents column purification profiles for Sephadex G-75, hydroxyapatite, and single-stranded DNA agarose chromatography of native double-strand uracil-DNA glycosylase isolated from *E. coli* NR8052 cells. SDS−polyacrylamide gel electrophoresis analysis of recombinant Dug during the purification procedure is shown in Figure 2. Molecular weight determination of rDug by matrix-assisted laser desorption−ionization mass spectrometry is provided in Figure 3. The effect of *E. coli* endonuclease IV on the rate and extent of the Dug mediated uracil excision reaction is demonstrated in Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Mol, C. D., Parikh, S. S., Putnam, C. D., Lo, T. P., and Tainer, J. A. (1999) *Annu. Rev. Biophys. Biomol. Struct. 28*, 101−128.
2. Duncan, B. K., and Weiss, B. (1982) *J. Bacteriol. 151*, 750−755.
3. Duncan, B. K., and Miller, J. H. (1980) *Nature 287*, 560−561.
4. Wang, Z., and Mosbaugh, D. W. (1988) *J. Bacteriol. 170*, 1082−1091.
5. Varshney, U., Hutcheon, T., and van de Sande, J. H. (1988) *J. Biol. Chem. 263*, 7776−7784.
6. Bennett, S. E., Jensen, O. N., Barofsky, D. F., and Mosbaugh, D. W. (1994) *J. Biol. Chem. 269*, 21870−21879.
7. Bennett, S. E., Sanderson, R. J., and Mosbaugh, D. W. (1995) *Biochemistry 34*, 6109−6119.
8. Shroyer, M. J. N., Bennett, S. E., Putnam, C. D., Tainer, J. A., and Mosbaugh, D. W. (1999) *Biochemistry 38*, 4834−4845.
9. Higley, M., and Lloyd, R. S. (1993) *Mutation Res. 294*, 109−116.
10. Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., and Sperens, B. (1977) *J. Biol. Chem. 252*, 3286−3294.
11. Barrett, T. E., Savva, R., Panayotou, G., Barlow, T., Brown, T., Jiricny, J., and Pearl, L. H. (1998) *Cell 92*, 117−129.
12. Roy, R., Brooks, C., and Mitra, S. (1994) *Biochemistry 33*, 15131−15140.
13. Waters, T. R., Gallinari, P., Jiricny, J., and Swann, P. F. (1999) *J. Biol. Chem. 274*, 67−74.
14. Bennett, S. E., Schimerlik, M. I., and Mosbaugh, D. W. (1993) *J. Biol. Chem. 268*, 26879−26885.
15. Olsen, L. C., Aasland, R., Wittwer, C. U., Krokan, H. E., and Helland, D. E. (1989) *EMBO J. 8*, 3121−3125.
16. Putnam, C. D., Shroyer, M. J., Lundquist, A. J., Mol, C. D., Arvai, A. S., Mosbaugh, D. W., and Tainer, J. A. (1999) *J. Mol. Biol. 287*, 331−346.
17. Wiebauer, K., and Jiricny, J. (1989) *Nature 339*, 234−236.
18. Wiebauer, K., and Jiricny, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5842−5845.
19. Neddermann, P., and Jiricny, J. (1993) *J. Biol. Chem. 268*, 21218−21224.
20. Hang, B., Medina, M., Fraenkel-Conrat, H., and Singer, B. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13561−13566.
21. Saparbaev, M., and Laval, J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8508−8513.
22. Neddermann, P., and Jiricny, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1642−1646.
23. Neddermann, P., Gallinari, P., Lettieri, T., Schmid, D., Truong, O., Hsuan, J. J., Wiebauer, K., and Jiricny, J. (1996) *J. Biol. Chem. 271*, 12767−12774.
24. Hendrich, B., Hardeland, U., Ng, H. H., Jiricny, J., and Bird, A. (1999) *Nature 401*, 301−304.
25. Gallinari, P., and Jiricny, J. (1996) *Nature 383*, 735−738.
26. Slupphaug, G., Mol, C. D., Kavli, B., Arvai, A. S., Krokan, H. E., and Tainer, J. A. (1996) *Nature 384*, 87−92.
27. Kunkel, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494−1498.
28. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
29. Sanderson, R. J., and Mosbaugh, D. W. (1996) *J. Biol. Chem. 271*, 29170−29181.
30. Sanderson, R. J., and Mosbaugh, D. W. (1998) *J. Biol. Chem. 273*, 24822−24831.
31. Longley, M. J., and Mosbaugh, D. W. (1993) *Methods Enzymol. 218*, 587−609.
32. Levin, J. D., Johnson, A. W., and Demple, B. (1988) *J. Biol. Chem. 263*, 8066−8071.
33. Wang, Z., Smith, D. G., and Mosbaugh, D. W. (1991) *Gene 99*, 31−37.
34. Bennett, S. E., and Mosbaugh, D. W. (1992) *J. Biol. Chem. 267*, 22512−22521.
35. Jiricny, J., Su, S.-S., Wood, S. G., and Modrich, P. (1988) *Nucleic Acids Res. 16*, 7843−7853.
36. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
37. Lindahl, T. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 135−192.
38. Bailly, V., and Verly, W. G. (1989) *Biochem. J. 259*, 761−768.
39. Levin, J. D., Shapiro, R., and Demple, B. (1991) *J. Biol. Chem. 266*, 22893−22898.
40. Lundquist, A. J., Beger, R. D., Bennett, S. E., Bolton, P. H., and Mosbaugh, D. W. (1997) *J. Biol. Chem. 272*, 21408−21419.
41. Lindahl, T. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3649−3653.
42. Lindahl, T., Demple, B., and Robins, P. (1982) *EMBO J. 1*, 1359−1363.
43. Eskin, B., and Linn, S. (1972) *J. Biol. Chem. 247*, 6183−6191.
44. Janscak, P., MacWilliams, M. P., Sandmeier, U., Nagaraja, V., and Bickle, T. A. (1999) *EMBO J. 18*, 2638−2647.
45. Waters, T. R., and Swann, P. F. (1998) *J. Biol. Chem. 273*, 20007−20014.
46. Lutsenko, E., and Bhagwat, A. S. (1999) *J. Biol. Chem. 274*, 31034−31038.
47. Sandigursky, M., Freyer, G. A., and Franklin, W. A. (1998) *Nucleic Acids Res. 26*, 1282−1287.
48. Krokan, H. E., Standal, R., and Slupphaug, G. (1997) *Biochem. J. 325*, 1−16.

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