AN ABSTRACT OF THE THESIS OF

Jung-Suk Sung for the degree of Doctor of Philosophy in Genetics presented
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Title: Characterization of Escherichia coli Double-strand Uracil-DNA
Glycosylase and Analysis of Uracil-initiated Base Excision DNA Repair.

Abstract approved: ____________________  Dale W. Mosbaugh

Escherichia coli double-strand uracil-DNA glycosylase (Dug) was purified
to apparent homogeneity from bacteria that were defective in uracil-DNA
glycosylase (Ung). After cloning the dug gene, recombinant Dug was
overexpressed, purified, and characterized with respect to activity, substrate
specificity, product DNA binding, and mechanism of action. Purified Dug
excised both uracil and ethenocytosine specifically from double-stranded
DNA substrates. One distinctive characteristic of Dug was that the purified
enzyme removed a near stoichiometric amount of uracil from DNA containing
U/G mispairs. The observed lack of turnover was attributed to tight binding
of Dug to the apyrimidinic-site (AP) contained in the DNA reaction product.
Catalytic activity was stimulated in the presence of E. coli endonuclease IV
that caused AP-site incision and dissociation of Dug. By using enzyme
complementation experiments, Dug was shown to initiate uracil-initiated base
excision repair (BER) in E. coli (ung) cell-free extracts. The relative rate of
repair of uracil- and ethenocytosine-containing DNA in isogenic E. coli cells
that were proficient or deficient in Ung and/or Dug was measured using a
novel competition assay. Complete ethenocytosine-initiated BER displayed an
absolute requirement for Dug and occurred at the same rate as uracil-initiated
BER in the presence of both Ung and Dug. However, the rate of Dug-
mediated ethenocytosine-DNA repair was 8-fold faster than that of uracil-DNA mediated by Dug. The distribution of BER patch sizes associated with both uracil- and ethenocytosine-containing DNA showed similar results. In both cases, DNA repair synthesis utilized predominantly a long patch BER mechanism involving the incorporation of 2-20 nucleotides. A previously unidentified "very long patch" mechanism of BER involving the incorporation of more than 200 nucleotides was identified and shown to be mediated by DNA polymerase I. The rate-limiting step associated with uracil-initiated BER was found to involve DNA ligase and the distribution of BER patch size was modulated by the ratio of DNA polymerase I and DNA ligase. The fidelity of DNA repair synthesis associated with complete uracil-DNA BER was measured using E. coli cell-free extracts that were proficient or deficient in Ung activity and determined to be $5.5 \times 10^4$ and $19.7 \times 10^4$, respectively.
Characterization of *Escherichia coli* Double-strand Uracil-DNA Glycosylase and Analysis of Uracil-initiated Base Excision DNA Repair

by

Jung-Suk Sung

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Jung-Suk Sung, Author
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Characterization of *Escherichia coli* Double-strand Uracil-DNA Glycosylase and Analysis of Uracil-initiated Base Excision DNA Repair

1. INTRODUCTION

1.1 Uracil Residues in DNA

1.1.1 Incorporation of dUMP During DNA Synthesis

Uracil residues can be incorporated into the genomic DNA of a variety of organisms, including *Escherichia coli* (1, 2), *Bacillus subtilis* (3, 4), virus (5, 6), and mammalian cells (7-9). Incorporation occurs because deoxyuridine triphosphate (dUTP) can be efficiently utilized as a precursor for DNA replication in place of deoxythymidine triphosphate (dTTP) by both prokaryotic and eukaryotic DNA polymerases (1, 10, 11). Therefore, the replacement of dUTP for dTTP produces a U/A rather than T/A basepair. The ability of DNA polymerases to incorporate dUMP into newly synthesized DNA suggests that dUTP and dTTP are not readily discriminated within the nucleotide binding pocket of the enzyme when associated with the primer/template. For example, the $K_m$ values of *E. coli* DNA polymerase I for dTTP and dUTP do not differ greatly at 4.1 and 5.6 $\mu$M, respectively (2). Similarly, *E. coli* DNA polymerase III exhibits $K_m$ values of 2.4 and 2.6 $\mu$M for dTTP and dUTP, respectively (2). Mammalian DNA polymerases $\alpha$, $\beta$, and $\gamma$ also display $K_m$ values of similar magnitude for dTTP and dUTP (10, 12). Thus, it is not the discrimination by DNA polymerases, but rather the intracellular pool size of dUTP relative to the dTTP pool size that directs the level of uracil incorporation.

In *E. coli*, dUTP biosynthesis is an obligatory intermediate in the *de novo* synthesis of dTTP; hence, incorporation of dUMP into the *E. coli* genome is unavoidable (3, 7, 13). Based on the estimate that intracellular levels of dUTP and dTTP in *E. coli* are 0.5 $\mu$M and 150 $\mu$M, respectively, approximately 1
uracil residue would be incorporated into DNA for every 300 thymine nucleotides polymerized (14).

The pathway of de novo dUTP synthesis in *E. coli* is carried out by the conversion of rUDP to dUDP by ribonucleotide reductase and phosphorylation of dUDP to dUTP (15). In addition, the action of dCTP deaminase directly converts dCTP to dUTP (16, 17). In *E. coli*, uracil incorporation into DNA is minimized by the enzymatic activities of deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) encoded by the *dut* gene (14, 18, 19). The enzyme dUTPase catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate, lowering the level of dUTP available to DNA polymerases (14). *E. coli* mutants defective in dUTPase exhibited a dramatically increased dUTP pool size and a high level of uracil incorporation into the DNA (20, 21).

The intracellular pool size of dUTP in mammalian cells was found to be significantly smaller than that of *E. coli* (7, 8). The absence of a mammalian counterpart to the *E. coli* dCTP deaminase and the presence of an abundant dUTPase appeared to account for the difference in the molar ratio of dUTP to dTTP between mammalian and bacterial cells (22, 23). The ratio of dUTP to dTTP was initially estimated in human lymphoid cells and indicated that less than 1 dUMP is incorporated into DNA for every $10^5$ dTMP polymerized (7, 8). However, measurements of the number of uracil residues per cellular genome have appeared to vary from 1,000 to 498,000 (9, 24). These differences may be explained by variations in the biological samples as well as the detection methods, which include gas chromatography-mass spectrometry, HPLC with UV detection, anion-exclusion chromatography, and single-cell gel electrophoresis (7, 9, 24-26).

1.1.2 Introduction of Uracil by Cytosine Deamination

Uracil is also introduced into the genome by spontaneous deamination of cytosine residues in DNA. As a consequence, a U/G mispair is produced
from the previous C/G base pairs (27). Two chemical mechanisms have been proposed for the deamination of cytosine in solution (27, 28). One involves the direct attack at the 4-position of the pyrimidine ring by a water molecule or a hydroxyl ion to release ammonia and requires protonation of the base at the N3-position. The other postulated pathway involves an addition-elimination reaction with the formation of an unstable dihydrocytosine as an intermediate. Further addition of a second water molecule results in the loss of ammonia group (deamination) to yield dihydouracil which is followed by the elimination of water to produce uracil. The spontaneous rate of cytosine deamination had been initially determined at extremes of temperature or pH and extrapolated to determine deamination rates under physiological conditions (28, 29). The rate of spontaneous cytosine deamination was estimated to be $7 \times 10^{13}$ and $1 \times 10^{10}$ sec$^{-1}$ for double- and single-stranded DNA, respectively, using a sensitive genetic assay under physiological conditions (37 °C, pH 7.4). (30). Since the rate of deamination is more than 100 times greater for single-stranded DNA, it appeared that the process of replication, recombination, and transcription that involve transient localized denaturation of DNA could accelerate cytosine deamination (31). In addition, for the mispairs of C/C and C/T, the cytosine deamination rates were experimentally determined to be $0.4 \times 10^{10}$ and $1.3 \times 10^{10}$ sec$^{-1}$, respectively (32). The similar rates of deamination with these mispairs to that observed with single-stranded DNA reinforced the notion of increased cytosine deamination rate for non Watson-Crick base pairs since pyrimidine-pyrimidine mispairs are known to distort the DNA helix (32).

The deamination of cytosine can also be enhanced by a number of chemical alterations or by certain intercalating agents. For example, sodium bisulfite has been shown to cause cytosine deamination with high concentrations (~2 M) under acidic conditions of pH ~5 (33). This reaction is processed by an acid-catalyzed addition-elimination reaction with the intermediate formation of 5,6-dihydouracil-6-sulfonate (33). Following
bisulfite elimination, a uracil residue is generated (33). Under physiological conditions of pH 7.4 and 37 °C, the rate of cytosine deamination by sodium bisulfite (10 mM) was measured to be $3.5 \times 10^{-10} \text{ sec}^{-1}$ as compared to $0.6 \times 10^{-10} \text{ sec}^{-1}$ for untreated DNA (34). Thus, the exposure to a low concentration of sodium bisulfite appeared to be mutagenic under physiological conditions. Unlike sodium bisulfite, nitrous acid reacts relatively nonspecifically with cytosine since it also results in the deamination of adenine and guanine residues in DNA (31). Nitrous acid promotes deamination of cytosine in double stranded DNA almost as efficiently as in single-stranded DNA (35), whereas sodium bisulfite attacks cytosine exclusively in single-stranded regions of DNA (33).

The formation of uracil through cytosine deamination can occur from direct photolysis of DNA upon ultraviolet (UV) irradiation at < 300 nm (36, 37). UV irradiation produces many cytosine photoproducts including monomeric form of cytosine hydrates and cytosine glycols (38-40), and dimeric species such as cyclobutane dimers (C<>T, T<>C, C<>C) and pyrimidine-pyrimidone (6-4) photoproducts (39). Elimination of a water molecule from uracil hydrates ultimately generates uracil (41). It was shown that UV-induced cytosine hydrates persist in irradiated poly(dG)/poly(dC) for extended periods of time, resulting in the formation of uracil hydrate and uracil at neutral pH (42). Deamination of cytosine residues in cyclobutane pyrimidine dimers was previously observed using poly(dC)/poly(dI) irradiated with UV light (280 nm) with a half-life of 2 hour at 37 °C (43). More recently, using a natural DNA substrate, the rate of deamination of cytosine in C<>C cyclobutane dimers was measured as $1.5 \times 10^6 \text{ sec}^{-1}$, corresponding to a half life of 5 days at 37°C (44). This result indicated that the formation of pyrimidine dimers in DNA led to an increase in cytosine deamination by six orders of magnitude from the spontaneous deamination rate.
1.1.3 Biological Consequences of Uracil Residues in DNA

The importance of avoiding uracil incorporation during DNA synthesis was initially obtained from in vivo studies of *E. coli* *dut* strains (1, 2, 21). This mutant was also identified as a *sof* mutant because the lack of dUTPase caused accumulation of short Okazaki-like DNA fragments (45). Since the *sof* mutants showed elevated levels of dUTP leading to an increased incorporation of dUMP into *E. coli* chromosomal DNA, it was proposed that uracil incorporation and its subsequent removal from DNA could lead to the production of short Okazaki fragments (1). The temperature-sensitive *dut-1* strain displayed lethality at the permissive temperature that appeared to be associated with the generalized degradation of newly synthesized uracil-containing DNA (2). Moreover, bacteriophage (T4, λ, and M13) containing uracil were unable to establish a productive infection in *dut* mutants of *E. coli* due to uracil-DNA degradation (46-48). Identification of the *E. coli* ung gene, encoding uracil-DNA glycosylase (Ung), and studies utilizing an ung-1 mutant strain led to the elucidation of the mechanism for uracil-DNA-induced degradation (46, 49). When T4 phage was grown in *E. coli* *dut ung* mutant cells, the progeny phage DNA contained as much as 30% of its thymine replaced by uracil residues (21, 47). These uracil-containing phage were restricted from propagation in wild-type *E. coli* cells due to phage DNA degradation, but productively infected and propagated in *E. coli* ung mutant cells (21). These results demonstrated that the lethality associated with high levels of uracil residues in DNA of wild-type cells is a result of DNA degradation initiated by uracil-DNA glycosylase.

While uracil and thymine are structurally similar, the methyl group of thymine projects into the major groove of duplex DNA and provides an important contact point in protein-DNA interaction. Thus, substitution of uracil for thymine may result in the disruption of protein-DNA interactions required for normal cell processes. The effect of uracil incorporation on
protein-DNA interactions was first reported in the binding of repressor to lac operator DNA. Substitution of uracil for thymine at position 13 of the lac operator resulted in a 10-fold decrease in lac repressor binding affinity on this element (50). A reduced protein-DNA affinity was also seen for cAMP-responsive element binding factor when uracil was substituted for thymine at position -4 in the cAMP response element (51). Interference of uracil-DNA was also observed in the interaction between the origin of replication region of herpes simplex virus type 1 (HSV-1) and the origin binding protein (51). Similarly, the incorporation of uracil residues into restriction endonuclease recognition sites have been shown to result in a reduced rates of cleavage by HpaI, HindII, and HindIII endonucleases (52).

Deamination of cytosine to uracil results in a U/G mispair, which if left unrepaired can lead to a G/C to A/T transition mutation. Several previous studies have implied that in both prokaryotic and eukaryotic cells the majority of spontaneous mutations result from cytosine deamination (48, 53-55). Analysis of spontaneous mutation spectrum in the E. coli lacI gene revealed that 93% of mutations occurred from G/C to A/T transitions (54). Previously, the E. coli ung mutant was shown to exhibit a mutator phenotype since the mutation frequency of G/C to A/T transition was increased ~30-fold in ung cells relative to isogenic ung proficient E. coli (48). Similarly, a 20-fold increase in spontaneous mutation rate was observed in an ung mutant strain of Saccharomyces cerevisiae (55). This mutator phenotype was abolished when the yeast cells were transformed with a multicopy plasmid carrying the gene encoding uracil-DNA glycosylase (55). Furthermore, determination of the spontaneous mutation spectrum in Chinese hamster ovary cellular DNA disclosed that the majority of mutations were base substitution, and 81% of these were G/C to A/T transition (53).
1.2 Uracil-DNA Glycosylase

Uracil-DNA glycosylase catalyzes the cleavage of the N-glycosylic bond linking the uracil base to the deoxyribose phosphate backbone of DNA, thereby initiating the uracil-excision DNA repair (49). The enzyme hydrolyzes uracil in both single and double-stranded DNA arising from either dUMP incorporation or cytosine deamination, resulting in free uracil and abasic site-containing DNA (49). Uracil-DNA glycosylase was first detected in _E. coli_, and has since been purified from a wide range of biological sources including bacteria, yeast, plants, and mammalian cells (49, 56-60). Thus, uracil-DNA glycosylase appears to be a ubiquitous enzyme, reflecting the importance of uracil removal from DNA. The biological significance of this enzyme is underscored by its high degree of evolutionary conservation, as indicated by the ~56 % amino acid sequence homology between proteins isolated from _E. coli_ and human (61).

1.2.1 _Escherichia coli_ Uracil-DNA Glycosylase

_E. coli_ uracil-DNA glycosylase (Ung) was the first DNA glycosylase identified and purified to apparent homogeneity (49). As analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the enzyme was determined to be monomeric with an apparent molecular weight of 24,500 (49). Activity of the enzyme appeared to have no requirement for divalent cations or other cofactors and occurs in the presence of EDTA (49). The $K_m$ value for dUMP residues in DNA was determined as 40 nM using PBS-1 DNA substrate and the enzyme turnover number was ~800 uracil residues released per minute (49). Subsequently, the ung gene was cloned under the control of both bacteriophage $\lambda_{ph}$ and the endogenous _E. coli_ promoter and the Ung protein was overexpressed in _E. coli_ at a level of 100-fold above that exhibited by wild type cells (62). The open reading frame of the ung gene encoded a 229-amino acid polypeptide with a deduced molecular weight of 25,664 (63).
Analysis of the amino acid sequence revealed that the N-terminal methionine residue was removed from the mature protein but without any other posttranslational modifications (63).

Uracil-DNA glycosylase specifically releases uracil from single- and double-stranded DNA, but not from dUMP, dUTP, and RNA (49, 64-66). Removal of uracil by Ung from single-stranded DNA appeared to be about twice as efficient as that from double-stranded DNA while U/G mispairs are preferred ~2-fold over U/A base pairs in oligonucleotide DNA (49, 67, 68). DNA containing 5-bromouracil, pyrimidine dimers, or deaminated purine residues do not appear to be substrates for Ung (49). However, the enzyme has been shown to be able to hydrolyze 5-fluorouracil and the cytosine-derived oxidative DNA damage products, such as 5-hydroxyuracil and 5,6-dihydroxyuracil (69-71). Following the excision step, Ung produces free uracil and an AP-site in DNA. Free uracil was shown to be a noncompetitive inhibitor of Ung with $K_i = 2 \times 10^{-3}$ M for single-stranded DNA and $K_i = 0.12 \times 10^{-3}$ M for double-stranded DNA (49). In addition, some uracil derivatives, such as 6-aminouracil, 5-azauracil, and 5-fluorouracil inhibit the enzyme activity at concentrations of 4 mM (49). Ung is also inhibited by the its other reaction product, the AP-site, at concentrations approximately 2-3 orders of magnitude lower than that of uracil (72).

Catalytic activity of Ung appeared to be influenced by the size of DNA substrate, since little uracil removal was detected from small oligonucleotides (tetra-, penta- and hexa-mers) (49). Further determination of the minimum substrate size and the preferred composition of the 3' and 5' ends for the Ung activity was conducted using various synthetic oligonucleotides (64). The results revealed that a 5'-terminal uracil residue can be removed from DNA only if the 5'-end was phosphorylated, whereas oligonucleotides containing uracil at the 3'-terminus or at the second position from the 3'-OH end were not substrates for Ung. However, uracil located in the second position from the 3'-terminus was hydrolyzed but depended upon the presence of a 3'-PO$_4$ group.
Thus, the oligonucleotide trimer containing uracil residue located at the phosphorylated 5'-terminus appeared to provide the minimum substrate requirement for Ung activity (64). In addition, the phosphodiester bond 3' to the uracil residue was found to be crucial for substrate binding by uracil-DNA glycosylase, suggesting important protein-DNA contact point(s) are required for efficient uracil removal (73).

The substrate specificity of Ung has been determined based on the sequence context surrounding the target uracil residue. For example, uracil removal from the consensus sequence 5'-(A/T)UA(A/T)-3' was ~17-fold greater than removal from the consensus sequence 5'-(G/C)U(T/G/C)-3' based on an analysis that involved 70 different nucleotide sequences (74). Generally, substrates containing uracil in GC-rich sequence or flanked 3' by a thymine were poor substrates while uracil removal was efficient in AT-rich sequences (74). Although Ung prefers single-stranded DNA substrates, excision of uracil from loop regions of hairpins was shown to be inefficient, probably because this conformation of the sugar-phosphate backbone is not favorable for efficient binding by the enzyme (75). In the presence of single-stranded DNA binding protein, the hairpin structures are melted and Ung can efficiently remove the uracil residue (76). These results suggest that both sequence context and DNA structure may affect the efficiency of uracil excision, thereby influencing the mutation frequency at specific sites in genomic DNA.

In an earlier study, E. coli Ung-defective mutants were isolated after N-methyl-N'-nitro-N-nitrosoguanidine-induced mutagenesis by mass screening of 3000 colonies for uracil-DNA glycosylase activity in vitro (46). Several mutants, from ung-1 to ung-8, were identified, which expressed various levels of residual uracil-DNA glycosylase activity (46). Among those, E. coli ung-1 appeared to grow normally and exhibited the lowest enzymatic activity of ~0.02% relative to the level of Ung activity in wild-type E. coli cells. It was later identified that this ung-1 DNA sequence contained a single guanine
deletion at the codon 43 which caused premature termination after translation of 16 amino acids (77). To avoid the low level of leaky expression of Ung observed in *E. coli ung-1* mutant, the insertion mutant *ung::Tn10* was constructed (78). No dramatic phenotypic differences between *ung-1* and *ung::Tn10* alleles were observed, suggesting that the *ung* gene is not essential under growth conditions (46, 78).

1.2.2 Mechanism of Action

Two possible enzymatic mechanisms of *E. coli* Ung for locating sequential uracil residues in DNA have been investigated previously (68, 79, 80). A processive mechanism occurs as a DNA-scanning process while the enzyme remains bound to the DNA and locates sequential uracil residues by facilitated diffusion (68, 79). In contrast, a distributive mechanism for locating the next uracil residues following catalysis requires dissociation of the enzyme from the DNA and random three-dimensional diffusion (81). It was first reported that uracil-DNA glycosylase acted with partial processivity by using covalently-closed circular plasmid DNA (79). The processivity of Ung was shown to decrease with increasing salt concentrations, a phenomenon observed for other processive DNA metabolizing enzymes (68, 82, 83). Further determination of the mechanism of action of Ung in searching for uracils was derived using a concatemeric polynucleotide substrate with uracil residues located at intervals of 25 nucleotides on one DNA strand (68). The results revealed that *E. coli* Ung locates successive uracil residues by a processive mechanism in the absence of NaCl. The nature of the base opposite to uracil sites did not appear to affect the search mechanism. In addition, it has been demonstrated that the processive mechanism exhibited by Ung was similar to that observed for restriction endonuclease *EcoRI* (68, 82, 84).

Mechanistic details of uracil-DNA glycosylase regarding to substrate binding and catalysis of uracil excision have arisen from the elucidation of the crystal structures and mutational studies. The tertiary protein structures have
been determined for human and herpes simplex virus type-1 (HSV-1) uracil-DNA glycosylases (UDG) (85-89), and *E. coli* Ung (90-92) by X-ray crystallography. The structure of the enzyme appeared to be highly conserved among these homologues, with a central, four-stranded parallel β-sheet surrounded by α-helices. On the basis of protein structural data, interactions between 13 amino acids and uracil-containing DNA were identified for the human uracil-DNA glycosylase (88). Subsequently, a comparison of the amino acid sequence alignment between the *E. coli* and human enzymes revealed that *E. coli* Ung possessed 12 (Gln-63, Asp-64, His-67, Phe-77, Ser-88, Asn-123, Ser-166, His-187, Ser-189, Leu-191, Ser-192, and Arg-195) conserved amino acid residues attributed to DNA interaction (88, 89). Among those, conserved Asp-64 and His-187 residues had been previously proposed to be essential residues for the activity of the enzyme by using photochemical cross-linking experiments involving Ung and oligonucleotide dT₂₀ (93). Site-directed mutations of the human gene was performed and the resulting mutant UDG enzymes were expressed, purified and the biochemical properties were determined (86). Six human UDG residues were identified as important, Gln-144, Asp-145, Ser-169, Asn-204, Ser-270, and His-268, which are conserved in *E. coli* Ung. Mutations to Asn-204 abolished the ability to bind poly(rU) whereas mutations to each of the five other residues retained poly(rU) binding (86). From these results, it was established that Gln-144, Asp-145, and His-268 are involved in the reaction mechanism of the N-glycosylic bond cleavage (86). Structural analysis of human UDG indicated that amino acids Gln-144, Asp-145, Tyr-147, and Asn-204 line one side of the uracil binding pocket, and His-268, Ser-169, and Ser-270 line the other side of the active site, while Phe-158 lies along the bottom of the pocket and stacks with the uracil pyrimidine ring (85, 86). Evidence supporting the common role of these conserved amino acids has emerged from the structural studies of *E. coli* Ung (90-92).
The role of conserved aspartic acid and histidine residues in recognition of the DNA substrate was examined in HSV-1 UDG using surface plasmon resonance. Catalytic residues Asp88 and His210 were both mutated to asparagine (D88N and H210N) and examined for the ability to influence binding to uracil- or AP-site-containing single- and double-stranded DNA (94). Interaction of the mutants with single stranded DNA was found to be stronger than double stranded DNA and binding to a U/G mispair was tighter than binding to a U/A mispair (94). In addition, there was negligible interaction between the mutants and single- or double-stranded DNA lacking uracil, or with DNA containing AP-sites, suggesting that the enzyme recognized the uracil in the DNA rather than the DNA itself. Since the affinity for DNA appeared to be similar between the two mutants, the residues Asp88 and His210 have been suggested to play a purely catalytic role in the HSV-1 UDG enzyme (94).

Previously, three different catalytic mechanisms had been proposed regarding to the role of conserved aspartic acid and histidine residues in uracil excision. Structural data obtained with HSV-1 UDG suggested Asp-147 as a general base, which activates a water molecule that attacks the C1' position, and His-210 as a general acid, which protonates O2 of uracil, thereby enhancing the destabilization of the N1-C1' bond (87). On the other hand, the imidazole group of His-268 in human UDG has been implicated in the direct nucleophilic attack on the N1-C1' glycosylic bond as well as second nucleophilic attack of water molecules providing H- and OH-group addition to the N1 and C1' atoms (86). It has been also proposed that a single nucleophilic attack by His-268 on a water molecule may abstract a proton and create an OH nucleophile that acts on the glycosylic bond (86). Recently, a role of the conserved histidine residue in the catalytic mechanism was further elucidated in *E. coli* Ung using H187D mutant protein (95). The properties of this mutant differed from Ung, since the mutant protein exhibited a 55,000-fold reduction in specific activity and a shift in pH optimum from pH 8.0 to
Additionally, the mutant was less susceptible to inhibition by uracil, 6-aminouracil, and 5-fluorouracil and displayed altered substrate preference on oligonucleotide substrates containing U/G > U > U/A target sites. On the basis of both biochemical and structural analysis, a refined catalytic mechanism was proposed: *E. coli* Ung Asp-64 residue activates a water molecule which attacks the C1' position following the approach of His187 to the uracil O2. This induced electron density withdrawal causes the N1-C1' bond to be polarized as a result of resonance stabilization of the uracil base through interactions with Gln-63, Asn-123, and His-187 (95).

Another important conserved feature of the enzyme is a loop above the active site containing a solvent-exposed leucine residue (e.g. Leu-191 in *E. coli*, Leu-272 in human) that has been proposed to facilitate nucleotide flipping (85). The biological importance of this leucine loop has been underscored by structures of the human and *E. coli* enzymes complexed with the irreversible uracil-DNA glycosylase protein inhibitor (Ugi) (85, 90). Structural analysis of human UDG bound to U/A and U/G containing DNA revealed that the leucine residue penetrates the DNA base stack from the DNA minor groove (89). The role of the leucine loop was implicated in a three distinctive structural features to describe the catalytic mechanism of uracil-DNA glycosylase as a "pinch-push-pull" model: (i) distortion of the DNA phosphodiester backbone in an initial UDG-DNA complex imposed by a "pinch" action of three serine residues, providing a mechanism for initial uracil detection, (ii) expulsion of the dUMP residue from the double-stranded DNA base stack by a "push" action mediated by the insertion of the side chain of a conserved leucine residue, and (iii) capturing the uracil by a "pull" action into a binding pocket of the enzyme, promoting concerted condensation of the surrounding catalytic residues to form a productive complex specific for N1-C1' glycosyl cleavage (88, 89, 96, 97). On the other hand, a recent study of the kinetic analysis using a hydrolyzable duplex DNA substrate and rapid chemical-quenched-flow methods revealed that the leucine insertion step
occur after nucleotide flipping but before excision (98). From these results, it was proposed that the inserted leucine loop acts as a doorstep to prevent the return of the flipped-out uracil residue, thereby facilitating the capture of the uracil in the active site of Ung (98). Thus, this study defines the catalytic action of Ung as "pull-push" as opposed to a "push-pull" mechanism.

1.3 Thymine-DNA Glycosylase

Spontaneous hydrolytic deamination of 5-methylcytosine forms thymine, thus generating a G/T mispair in DNA that needs to be repaired to avoid C to T transition mutagenesis. The repair of this mispaired thymine residue involves thymine-DNA glycosylase (TDG), which hydrolyzes the N-glycosyl bond between the sugar-phosphate backbone and a mispaired thymine. This activity was initially detected in simian cell extracts (99), and the corresponding human activity was subsequently demonstrated in nuclear extract of HeLa cells as a DNA glycosylase (100). Purification of TDG from HeLa cell extracts yielded a protein that migrated with an apparent molecular weight of 55,000 in denaturing polyacrylamide gel electrophoresis (101). Subsequently, two species of cDNAs encoding TDG were identified, differing in the lengths of the 3' untranslated region but not in their coding sequences (102). The full length cDNA encoded a protein of 410 amino acids with a calculated molecular mass of 46,000 daltons (102). Overexpression and purification of the recombinant enzyme allowed further biochemical characterization of TDG and led to the observation that cleavage occurs on double-stranded DNA, but not on single-stranded DNA, containing T/G as well as mispaired T/C, and T/T in the order T/G >> T/C > T/T (102). Several investigations for determining the effect of neighboring sequence context on TDG activity established the order of preferred nucleotides 3' to the mispaired thymine which decreases along the series CpG·TpG > TpG·TpA > GpG·TpC > ApG·TpT (103-105). Accordingly, it has been suggested that the enzyme has evolved to correct T/G mispairs arising as the result of
spontaneous hydrolytic deamination of 5-methylcytosine in CpG dinucleotides (104).

Further investigation of the substrate specificity of TDG using synthetic oligonucleotides revealed that the enzyme also catalyzed uracil removal efficiently from U/G, U/T, and U/C mispairs, but not from U/A base pairs or single-stranded DNA (102). This activity on uracil-containing substrates was distinct from UDG activity since the uracil excision by TDG was not inhibited by Ugi (102). The apparent $k_{cat}$ of uracil excision from U/G mispair appeared to be ~20-fold higher than that of thymine removal from T/G mispair (106). Additionally, thymine-DNA glycosylase had a higher binding affinity for double-stranded oligonucleotides containing a U/G mispair than for those containing T/G mispairs (102). Thus, aside from counteracting the mutagenic potential of 5-methylcytosine deamination events, the uracil-DNA glycosylase activity of TDG appeared to function to remove uracil residues that accumulate as a result of cytosine deamination, suggesting a back-up role for TDG in uracil-DNA repair to the more efficient UDG activity (103, 107).

Analysis of the substrate specificity of TDG revealed that the enzyme also catalyzed the removal of the cytosine adduct, $3$, $N^4$-ethenocytosine (106, 108). Determination of the $k_{cat}/K_m$ value of the human TDG on various substrates indicated that the specificity of the enzyme for ethenocytosine is ~5.5 times higher than for thymine, but ~4.5 times lower than for uracil (106). Additionally, ethenocytosine-DNA glycosylase activity of TDG was not observed using single-stranded DNA and the enzyme did not show particular preference for the base opposite to ethenocytosine in double-stranded DNA (106). Recently, it was observed that the enzyme copurified with a 5-methylcytosine DNA glycosylase activity from extracts of chicken embryos (109). The chicken enzyme was found to be a homolog of human TDG and shared ~80% identity in amino acid sequence (109). In contrast, human TDG did not display a significant 5-methylcytosine excision activity, while the
enzyme was found to be able to process 5-fluorouracil opposite mismatched guanine as well as adenine (110).

Substrate interaction of TDG has been examined using a synthetic oligonucleotide containing a uracil substrate analog, 2'-fluorouracil, that was bound but not cleaved by the enzyme (111). From the results of DNase I footprinting and methylation interference studies with 2'-fluorouracil containing DNA, bound TDG was found to protect a 14 nucleotide region on the target containing strand and a 19-nucleotide stretch on the opposite strand (111). The same study also revealed that TDG does not contact specifically the N7 position of the mispaired guanine; rather, a specific interaction was detected with the N7 position of the guanine immediately flanking the mispaired uracil on its 3'-side (111). This observation appeared to be consistent with the finding that U/G or T/G mismatches within CpG sequence are most efficiently processed by TDG (103-105). In addition, thymine mispaired opposite O-6-methylguanine or 2-amino-6-methylaminopurine was shown to be efficiently hydrolyzed, indicating that the 6- and 1-positions of the opposite guanine may not interact with the enzyme (104). Interestingly, one molecule of purified human TDG enzyme was found to be capable of removing only one thymine molecule from DNA containing a T/G mispair due to the tight binding of the enzyme to the AP-site that formed following hydrolysis of thymine (105). Recently, it was demonstrated that human AP endonuclease 1 (HAP1) stimulated turnover of the TDG acting on T/G mismatch-containing substrates but this modest increase in activity only occurred when HAP1 was added in a molar excess over TDG in the reaction (112). Since this stimulatory effect of HAP1 on TDG activity occurred in a HAP1 concentration dependent manner and was not detected with E. coli endonuclease IV, a protein interaction between TDG and HAP1 was proposed (112). However, neither direct evidence for this protein-protein interaction nor the mechanism to describe the role of HAP1 in stimulating TDG activity has been reported.
In a study of deletion of amino acids from the C-terminal and N-terminal ends of human TDG, a 248 amino acid core region of the enzyme was identified to be capable of processing U/G but not T/G mispairs (107). Although amino acid sequence analysis of the entire human TDG protein failed to show significant homology with other DNA repair proteins in both prokaryotes and eukaryotes, this core domain of TDG was found to share ~30% identity with an *E. coli* open reading frame encoding 169 amino acids (107). When the *E. coli* recombinant protein was expressed in either reticulocyte lysate or *E. coli* cells, the extracts efficiently processed U/G but not T/G mispairs (107). Furthermore, excision of uracil residues from single-stranded DNA was not observed and the detected DNA glycosylase activity was not found to be inhibited by Ugi (107). Thus, the enzyme was initially designated as double-strand-specific uracil-DNA glycosylase (dsUDG), and later also referred to as mismatch-specific uracil DNA glycosylase (Mug). In an attempt to understand the structural aspects of TDG, a co-crystal structure of the recombinant *E. coli* enzyme with AP-site-containing DNA and TDG alone have recently been determined (113). The results revealed significant structural similarities between Mug and the functionally related *E. coli* Ung despite very limited conservation at the amino acid sequence level (< 10% identity) (107). Additionally, important functional features of Ung, such as nucleotide-flipping mechanism, were also found in Mug whereas the mechanism for substrate recognition appeared to be distinctively different (113). Structural data were used to propose that Mug purportedly inserts a polypeptide "wedge" into the DNA double helix at a U/G mispair to facilitate nucleotide flipping by a "push" action (113). On the basis on this interpretation, the inserted polypeptide chain (NPSGLSR) is stabilized by intercalation of Arg-146 with the complementary strand base stack and by three hydrogen bonds formed by Gly-143 and Ser-145 that are absolutely specific for the "widowed" guanine. However, abstraction of a proton to activate the water molecule to a hydroxyl ion by a general-base mechanism as
observed in UDG cannot be provided by the amide side chain of Asn-18 in Mug. In addition, the catalytic histidine residue conserved in UDG, which protonates the O2 of the bound deoxyuridine to promote hydrolysis, was found to be replaced by an arginine in E. coli Mug and a methionine in human TDG (113). Thus, these enzymes cannot mediate general-base or general-acid catalyzed hydrolysis and instead must rely on weak nucleophilic attack by a coordinated water molecule (113). While the X-ray crystallographic studies of the enzyme provided some insight into structure/function relationships, neither the biochemical characteristics nor the biological significance of Mug (dsUDG) were established at the onset of this research project.

1.4 Uracil-DNA Glycosylase Inhibitor Protein

1.4.1 Bacteriophage PBS2 Uracil-DNA Glycosylase Inhibitor Protein

*Bacillus subtilis* bacteriophage PBS1 and the clear-plaque variant, PBS2, are unique since their double-stranded genomic DNA naturally contains uracil in place of thymine residues (114, 115). Accordingly, the overall composition of PBS2 DNA is enriched for A+U content (72%) (114). In order to achieve this uracil-rich genome, the bacteriophage has evolved strategies to increase the intracellular pool of dUTP during DNA replication and to inactivate uracil-DNA repair. Following bacteriophage PBS2 infection, the expression of several early gene products was found to increase the intracellular dUTP pool size while depleting the dTTP pool size. These include dTMP 5'-phosphatase (116), dCTP deaminase (117), dUMP kinase (118), and dUTPase inhibitor (119). In addition, the uracil-containing genome of PBS2 is preserved in the host uracil excision environment by induction of a phage-encoded uracil-DNA glycosylase inhibitor protein, Ugi (120).

Ugi was initially purified from PBS2-infected *Bacillus subtilis* and was found to be a heat-stable protein with a native molecular weight of ~18,500, as determined by gel filtration chromatography (121, 122). However, Ugi
appeared to migrate anomalously during sodium dodecyl sulfate polyacrylamide gel electrophoresis as a monomeric protein of ~3,500 daltons (123). This apparent molecular weight discrepancy was resolved upon the determination of the complete nucleotide sequence of the ugi gene, which revealed that the inhibitor protein encodes a polypeptide with a deduced molecular weight of 9,477 daltons with 84 amino acids (123, 124). The PBS2 ugi gene was cloned from the PBS2 genomic library and the sequence of the ugi gene combined with nucleotide deletion analysis revealed a promoter like region (-10 and -35 consensus sequences) similar to other Bacillus subtilis genes and a Shine-Dalgarno sequence (123). The amino acid composition of Ugi revealed the protein to be highly acidic (pI=4.2) with 12 glutamic and 6 aspartic acid residues (123). Expression of the ugi gene in E. coli cells resulted in physiological properties consistent with phenotypes of ung mutants including an increased spontaneous mutation frequency of ~10-fold (122). Subsequently, the ugi gene was successfully overproduced in E. coli and recombinant Ugi protein was purified to apparent homogeneity for further characterization (125). Using mass spectrometry, Ugi was determined to be ~9,475 daltons, in excellent agreement with the deduced amino acid sequence of the cloned ugi gene (124). The molecular weight determination of Ugi in the native form by sedimentation equilibrium centrifugation revealed that the protein exists as a homogeneous monomer in solution (124).

Several in vitro studies indicated that the inhibitory action of Ugi occurred with uracil-DNA glycosylases isolated from a variety of biological systems including Escherichia coli, Micrococcus luteus, Saccharomyces cerevisiae, herpes simplex virus types 1 and 2, rat liver (nuclear and mitochondrial), human placenta, and human KB cells (59, 72, 121-123, 126, 127). Other DNA glycosylases with uracil excision activity, identified as single-strand-selective monofunctional uracil-DNA glycosylase (SMUG-1), and methyl-CpG-binding domain (MBD4), have been shown to be resistant to inhibition by Ugi (102, 128, 129). In addition, DNA metabolizing enzymes such as 3-methyladenine-
DNA glycosylase, hypoxanthine-DNA glycosylase, DNA polymerase I, DNA polymerase α, β, and γ, did not display reduced activity in the presence of Ugi (123, 126). These results clearly indicate that inhibition by Ugi is specific for uracil-DNA glycosylase.

1.4.2 Mechanism of Inhibition

Ugi has been observed to form a complex with *E. coli* Ung that was shown to be essentially irreversible under physiological conditions (123, 124). The molecular weight of this Ung•Ugi complex was determined to be ~36,000 by gel filtration chromatography and analytical ultracentrifugation, indicating a 1:1 stoichiometry of Ung to Ugi protein (123, 124). When this complex was treated with 8 M urea followed by urea polyacrylamide gel electrophoresis and enzyme renaturation, the activities of both Ung and Ugi were detected from the dissociated complex (124). Additionally, dissociation of the complex by SDS-polyacrylamide gel electrophoresis revealed no alteration of the electrophoretic mobility or activity of the resolved proteins (124). Thus, these results suggested that the action of Ugi inhibition involves protein binding and not covalent protein modification by Ugi.

Several studies further demonstrated that Ugi binds at the DNA binding site of uracil-DNA glycosylase. The following observations stand in support of Ugi acting as DNA mimic; (i) the association of Ugi with Ung prevented enzyme binding of Ung to DNA (124), (ii) Ugi blocked the enzyme from forming an Ung•DNA complex (124), (iii) the addition of Ugi to UV cross-linking reactions prevented the formation of cross-linked Ung x dT₂₀ (93), (iv) X-ray crystallographic analysis of Ugi in complex with uracil-DNA glycosylase from human (85, 86) and HSV-1 (87) revealed that Ugi complexes with uracil-DNA glycosylase at the DNA binding domain. Furthermore, Ugi appeared to interact with uracil-DNA glycosylase by mimicking the phosphodiester backbone structure of DNA (85, 90). Recently the crystal structure of Ugi and its complex with *E. coli* Ung was resolved (90). The
structural analysis from this study indicated that most carboxylate side chains in Ugi superimposed with the DNA phosphate backbone, including the DNA strand that is not contacted by Ung (90).

The mechanism for interaction between Ung and Ugi has been investigated using stopped-flow kinetic analysis. The studies indicated that the association of Ugi with Ung involves a two-step kinetic mechanism (130). The first step is initiated by a reversible Ung/Ugi interaction to form a precomplex, distinguished by the dissociation constant $K_D=1.3 \ \mu M$. The second step results in the formation of the final irreversible Ung•Ugi complex characterized by the rate constant $k=195 \ \text{sec}^{-1}$. Thus, complex formation involves a preliminary "docking" step followed by a "locking" reaction through which the two proteins achieve optimal alignment and become very tightly bound (130). The slower transition to the locked configuration suggests that one or both proteins undergo a conformational change in structure (130). Based upon nuclear magnetic resonance comparison of free Ugi to the Ugi in complex with Ung, the structure of Ugi appeared to undergo some conformational change, possibly correlating with the kinetic analysis of a two step mechanism (131). Structural analysis of the Ugi complexed with *E. coli* Ung revealed that the locked complex involved not only the alignment of hydrogen bonding interactions along the interface between Ung and Ugi but also the local conformational change at Ugi Glu20 (90).

1.5 Ethenocytosine Residues in DNA

1.5.1 Formation of Ethenocytosine by Exposure to Vinyl Chloride

3,N$^4$-ethenocytosine ($\varepsilon$C) is a modified exocyclic DNA base and is categorized as an ethenobase adduct together with 1,N$^6$-ethenoadenine ($\varepsilon$A), N$^2$-3-ethenoguanine (3-$\varepsilon$G), and 1,N$^2$-ethenoguanine ($\varepsilon$G). Ethenobase derivatives were initially found to be formed from exposure of DNA to the industrial chemical, vinyl chloride (132, 133). Vinyl chloride is used in the
production of synthetic polymers, vinyl carbamate, and ethyl carbamate, a component of certain foods and alcoholic beverages. Subsequently, a number of structurally diverse chemicals were shown to react with nucleobases either directly or via metabolic intermediates to yield etheno adducts *in vitro* or *in vivo* (134, 135). These chemicals include urethane, halogenated aldehyde, bifunctional epoxide, α-haloketone, cyclophosphamide, *trans*-4-hydroxyl-2-nonenal, and mucochloric acid. These toxins have been investigated because vinyl chloride was shown to be a human carcinogen that was implicated in forming angiosarcoma of the liver which is a normally very rare tumor (136). Thereafter, vinyl chloride was found to be genotoxic in bacteria and a variety of other organisms (137, 138).

Covalent binding of vinyl chloride to cellular macromolecules is dependent upon its metabolic conversion, by microsomal monooxygenases, into chloroethylene oxide, which can rearrange spontaneously to chloroacetaldehyde (139). In humans and rats, it has been shown that oxidation of vinyl chloride is catalyzed by a cytochrome P450 2E1-mediated mechanism and this reaction occurs mainly in the liver (140-142). These bifunctional electrophile metabolites, chloroacetaldehyde and chloroethylene oxide, act as strong alkylating agents that form a hydroxyethan bridge between the exocyclic amino group and ring nitrogens of adenine, guanine, and cytosine bases in DNA, resulting in εA/T, εG/C, and εC/G residues, respectively (133, 143).

Although it was initially thought that the major reactive metabolite was chloroacetaldehyde, evidence was obtained *in vivo* and *in vitro* that its precursor, chloroethylene oxide, is more active and carcinogenic (144, 145). The formation of hydrated ethenocytosine has been demonstrated following *in vitro* reaction of chloroacetaldehyde or chloroethylene oxide with cytosine in ribo- and deoxyribopolynucleotides (133, 146). Under physiological condition this hydrate was slowly converted to ethenocytosine and the half-life of ethenocytosine in poly(rC) was measured to be 4.9 h at pH 7.25 at 50 °C (133).
The first in vivo detection of ethenobase adducts including ethenocytosine in DNA was made in liver hydrolysates from rats exposed to vinyl chloride (147). More recently, ethenobase adducts were quantitatively measured in the liver of rats exposed to 500 ppm vinyl chloride for 1, 2, 4, and 8 weeks (148). The results indicated that both ethenocytosine and ethenoadenine accumulated with steady-state levels of ~60 fmol/μmol and ~30 fmol/μmol, respectively, while the number of ethenoguanine appeared to be 10- to 100-fold greater.

1.5.2 Formation of Endogenous Ethenocytosine Residues in DNA

Ethenobase adducts can be also generated as a consequence of reactions between DNA and the products of lipid peroxidation (149, 150). After the development of the highly sensitive immunoaffinity/32P-postlabeling method that led to the measurement of background levels of ethenocytosine and ethenoadenine in tissues from unexposed (to vinyl chloride) rodents and humans, all tissues analyzed so far have shown endogenous levels of ethenocytosine and ethenoadenine (135, 151, 152). In an earlier study, the formation of ethenoguanosine was observed under physiological conditions, in the presence of lipid hydroperoxide, from in vitro reaction of deoxyguanosine with trans-4-hydroxy-2-nonenal, which is a major α,β-unsaturated aldehyde released during lipid peroxidation (153). It has been demonstrated that the reactive species involved in the formation of ethenobase adducts in DNA was the metabolite 2,3-epoxy-4-hydroxy-nonanal (154). Elevated levels of ethenocytosine and ethenoadenine were observed in liver DNA from the mutant rats that displayed copper accumulation and increased lipid peroxidation (155). Similarly, increased levels of ethenobase adducts have been reported in human, and in various tissues, in association with stimulated oxidative stress or inflammation that may stimulate overproduction of peroxyl radicals (156, 157). Since the peroxyl radicals are key participants in lipid peroxidation (158), these results provided additional
evidences for the in vivo generation of endogenous ethenobase adducts as products of lipid peroxidation.

1.5.3 Biological Consequences of Ethenocytosine Residues in DNA

Exocyclic base modifications, such as ethenocytosine, can lead to genetic mutations due to possible alteration of coding information at the lesion site. The mutagenic properties of the ethenocytosine were initially determined in E. coli by examining the fidelity of in vitro replication and transcription when modified synthetic polynucleotides containing the adduct were used as templates (159-162). The results revealed that ethenocytosine residues could direct significant misincorporation of thymine or adenine by E. coli DNA polymerase I and caused incorporation of uracil and adenine during transcription (161). Similarly, replication of the ethenocytosine-containing DNA template by the Klenow fragment of DNA polymerase I leads to C/G to T/A transitions and C/G to A/T transversions (163). This mutagenic specificity of ethenocytosine was subsequently confirmed in vivo (164). DNA replication by mammalian DNA polymerase α and δ past the ethenocytosine adducts also resulted in C/G to T/A transitions and C/G to A/T transversions while polymerase β generated mostly C/G to G/C transversions (165). These results were consistent with the findings on the mutations in tumors from human liver angiosarcomas associated with exposure to vinyl chloride, since the major class of mutations observed was C/G to T/A transitions at codon 13 of the c-Ki-ras-2 gene, which corresponded to the ethenocytosine mutagenic properties (166).

Ethenocytosine residues have been implicated in association with the damage-inducible response involving the UV modulation of mutagenesis (UVM). UVM was initially detected based on an increase in mutation fixation at a site-specific ethenocytosine lesion borne on transfected M13 viral single-stranded DNA in E. coli cells pretreated with a variety of DNA damaging agents, such as UV, alkylating agents, and H2O2 (167, 168). This response
appeared to be distinct from the SOS response in that the genetic requirements for UVM did not require functional recA, umuD, and umuC genes (169). In addition, UVM occurred in cells under conditions where the SOS functions were not induced (169). UVM induction has been shown to enhance insertion of thymine opposite both ethenocytosine and ethenoadenine, accounting for the mutagenic effect at ethenocytosine (C/G to A/T transversions) but for the antimutagenic outcome at ethenoadenine (170). The mechanism underlying the UVM response at ethenocytosine lesions has not been fully elucidated. However, in E. coli cells that were not induced for UVM, the mutation frequency at ethenocytosine appeared to be very low, indicating that the lesion is a weak mutagen (171). In contrast, ethenocytosine was shown to be highly mutagenic in mammalian simian kidney (COS) cells and produced C/G to A/T transversions and C/G to T/A transitions (171).

1.5.4 Repair of Ethenocytosine Residues

Several studies have provided the evidence that ethenobases including ethenocytosine can be repaired through the base excision repair DNA pathway. The first report on the excision of ethenobase adducts came from the observation of the release of free ethenoguanine and ethenoadenine bases from chloroacetaldehyde-treated DNA, after incubation with cell-free extracts obtained from a rat brain tumor cell line (172). Cell-free extracts from human cells have been shown to contain a protein that has a strong affinity for duplex oligonucleotide DNA containing the ethenoadenine residue (173). Subsequently, it has been demonstrated that human cell extracts could release all ethenobases including ethenocytosine from chloroacetaldehyde-treated DNA (174). In this experiment, both ethenocytosine and ethenoadenine were excised at similar rates, while two forms of ethenoguanine were released much more slowly under identical conditions (174).

The DNA glycosylase activity responsible for the excision of ethenoadenine in human cells was isolated and displayed the same activities
as purified alkylpurine-DNA glycosylase (ANPG) (175). Ethenoadenine-DNA glycosylase activity was also identified in *E. coli* and yeast in association with the AlkA and MAG proteins, respectively (176). However, ANPG appeared to be irrelevant to the repair of ethenocytosine in DNA since the partially purified ethenocytosine-DNA glycosylase activity from HeLa cells was found to be separate from ethenoadenine-DNA glycosylase activity (177). A knockout mouse lacking ANPG was then used as a genetic approach to verify this *in vitro* data and showed that only ethenocytosine but not ethenoadenine was released from duplex DNA by the mouse cell extracts, indicating that there was a different gene product for ethenocytosine repair (178).

Recently, the enzyme for ethenocytosine-DNA glycosylase activity was isolated from *E. coli* cell extracts (106). Amino acid sequence analysis of two peptides obtained from partial trypsin proteolysis of the enzyme revealed that this protein is identical to double-strand uracil-DNA glycosylase (dsUDG), which had been previously identified as a recombinant protein that correlated with the core domain of human thymine-DNA glycosylase (TDG) (107). Recombinant human TDG also exhibited an activity that excises ethenocytosine from duplex oligonucleotide DNA (106). In addition, kinetic analysis of TDG indicated that ethenocytosine was the more preferred substrate relative to uracil and thymine (106). In a separate study, further purification of ethenocytosine-DNA glycosylase activity from HeLa cells confirmed that it was the TDG (108). Both *E. coli* and human ethenocytosine DNA glycosylases appeared to remove the ethenocytosine base from all four mismatches with a comparable efficiency (106). Recently, *in vitro* DNA repair assays with HeLa cell extracts have been conducted using ANPG and ethenoadenine-containing DNA, which revealed that the repair was mediated via both short and long patch BER (179). However, the experimental evidence for either the complete repair of ethenocytosine or the involvement of ethenocytosine-DNA glycosylase activity in the BER process has not been described.
1.6 Base Excision DNA Repair in *Escherichia coli*

Covalent alterations of DNA bases, which may have promutagenic or cytotoxic effects, are the major consequences of endogenous DNA damage caused by deamination, hydrolysis, reactive oxygen species, and various metabolites (60, 180). To counteract these threats, cells have evolved defense mechanisms that repair damaged bases in DNA. In the simplest type of DNA repair, the damage to the base is directly removed by a one step mechanism, such as demethylation of O\(^6\)-methylguanine by *E. coli* Ada protein (181). However, most damaged bases in DNA are repaired by more complex excision repair systems that include nucleotide excision DNA repair (NER), mismatch DNA repair (MMR), and base excision DNA repair (BER). Among these, BER is distinct from other repair pathways because the first step involves damage recognition and removal of the base damage using a DNA glycosylase.

Uracil-DNA glycosylase was first discovered in *E. coli* and constituted a new class of DNA repair enzymes (49, 56). Following this discovery, fundamental information concerning the molecular mechanism of BER was derived from the *E. coli* system. From numerous efforts involving the identification, purification, and characterization of the enzymes responsible for BER, the multiple step process of the BER pathway was profiled. In addition, the *in vitro* reconstitution of uracil-initiated BER with purified *E. coli* enzymes led to the establishment of a general model for the BER pathway (182). This model pathway has been represented as a major BER mechanism in both prokaryotes and eukaryotes, which consists of five sequential reactions; (i) recognition and excision of the damaged base by a DNA glycosylase generating an AP-site, (ii) cleavage of the phosphodiester bond on the 5'-side of the resulting AP-site by AP endonuclease, (iii) removal of the abasic 5'-terminal deoxyribose phosphate residue by a deoxyribophosphodiesterase (dRPase) activity, (iv) filling in the resulting one
nucleotide gap through repair DNA synthesis by DNA polymerase, and (v) ligation of the nick by DNA ligase to restore the intact DNA molecule. The existence of one or more alternative BER pathway(s) has been demonstrated in mammalian system as well as in *E. coli* (182, 183). The alternative pathway(s) have been shown to differ in the size of the repair DNA synthesis patch (2 to ~10 nucleotides) (182, 183), thereby referred to as "long patch" BER, and generally considered as a minor pathway relative to the major "one-nucleotide" or "short patch" BER pathway.

1.6.1 Base Damage Recognition and Excision by DNA Glycosylases

DNA glycosylases recognize base damage and are the key enzymes for the initiation of BER. The common feature of these enzymes is that they hydrolyze the N1-C1' glycosylic bond between the target base and deoxyribose, thus releasing a free base and leaving an AP-site in DNA (49). In general, DNA glycosylases found in *E. coli* are relatively small monomeric proteins (<30,000 molecular weight) that do not require cofactors for their activity. Most DNA glycosylases have a more relaxed substrate specificity that allows removal of several structurally different damaged bases, while a few are highly specific for a particular form of base damage and exhibit a narrow substrate specificity. Some DNA glycosylases also have an associated AP lyase activity that cleaves the phosphodiester bond on the 3'-side of the AP-site. DNA glycosylases that contain an AP-site incision activity are termed "bifunctional" DNA glycosylases and are distinct from typical "monofunctional" DNA glycosylases that remove the damaged base producing a DNA product with an intact aldehydic AP site.

Monofunctional DNA glycosylases in *E. coli* include Ung (Section 1.2) and the major DNA glycosylases that remove alkylated bases. DNA glycosylase activities that excise alkylated bases in *E. coli* have been identified in associated with the *tag* and *alkA* gene products (184, 185). The Tag protein is fairly specific for 3-methyladenine (186), and was originally called 3-
methyladenine-DNA glycosylase, although the enzyme also removes 3-methylguanine with ~70-fold lower efficiency (187). In contrast, AlkA has a broad substrate specificity and removes both damaged purines and pyrimidines, such as 3-methyladenine, 3-methylguanine, 7-methylguanine, 7-methyladenine, O2-alkylcytosine, and O2-alkylthymine (185, 188, 189). Additionally, hypoxanthine, 5-formyluracil, 5-hydroxymethyluracil have also been shown to be removed by AlkA (190, 191). Comparison of the amino acid sequence deduced from the tag and alkA genes revealed that there is no significant homology between these two alkylbase-DNA glycosylases, suggesting that the precise mechanism of action of the enzymes are distinct (192). It has been shown that the resistance to alkylating agents (adaptive response) in E. coli is strongly and specifically induced by treating cells with small amounts of alkylating agents which induces the production of AlkA, AlkB, and Ada proteins, encoded by genes in the same operon, while Tag is constitutively expressed (60, 193).

In E. coli, DNA glycosylases involved in the repair of oxidized base damages are often found associated with bifunctional activities. These include two subgroup of E. coli DNA glycosylases found to remove oxidized bases: endonuclease III (Endo III) and related enzymes that remove oxidized pyrimidines and formamidopyrimidine-DNA glycosylase (Fpg) protein that processes oxidized purines. Endo III was initially identified as an endonucleolytic activity that degrades UV-irradiated DNA (194), but was subsequently found to be a DNA glycosylase with associated AP lyase activity (195). The DNA glycosylase activity of Endo III was found to have a broad substrate specificity and removes ring-saturated, ring-opened, and ring-fragmented pyrimidines. These include thymine glycol, 5,6-dihydrothymine, 5-hydroxy-6-hydrothymine, urea, uracil glycol, and 5-hydroxy-6-hydouracil (60, 180). The enzyme is encoded by the nth gene (196) and contains an iron-surfacer cluster (197). Kinetic analysis utilizing a form I DNA substrate containing targets of either thymine glycol or AP-site revealed the steps
involved in Endo III mediated catalysis; (i) protonation of the ring oxygen of
the deoxyribose residue, (ii) formation of a Schiff base between the
deoxyribose and the base, (iii) formation of a Schiff base between the enzyme
and the C1 aldehyde of the sugar, and (iv) release of the damaged base and β-
elimination of the 3'-phosphate (198). A Schiff base intermediate has been also
demonstrated for several structurally different bifunctional DNA glycosylases
including Fpg and T4 endonuclease V (199-201). Two other E. coli DNA
glycosylases, endonuclease VIII (Endo VIII) and endonuclease IX (Endo IX),
are functionally related to Endo III. Endo VIII, encoded by the nei gene, also
displays AP lyase activity as well as base excision activity against thymine
glycol and 5,6-dihydrothymine (202). Although Endo VIII and Endo III have
common substrates, there is no significant amino acid sequence similarity
between these two proteins (180). In addition, Endo VIII and Exo III are found
to be significantly different from each other in terms of excision rates (203).
Endo IX is less well characterized but has been found to act on urea and β-
ureidoisobutyric acid residues in DNA, but not on thymine glycol or
dihydrothymine residues (204). Based upon the overlapping substrate
specificity and the previous observation that nth mutants are not
hypersensitive to DNA damaging agents (H₂O₂ or γ-radiation) (205), Endo VIII
and Endo IX may serve as a back-up function for Endo III in removal of
oxidized pyrimidine bases in E. coli.

Oxidized purine bases such as 8-oxoguanine (8-oxoG), 2,6-diamino-4-
hydroxy-5-N-methylformamidopyrimidine (fapy) are excised by the Fpg
protein (also called MutM and Fapy-DNA glycosylase) in E. coli (206, 207).
The enzyme appeared to be the major DNA repair activity that recognizes 8-
oxoG in E. coli DNA and the inactivation of the fpg gene results in an increased
frequency of G to T transversion mutations (208), which was consistent with
the demonstration that 8-oxoG lesions lead primarily to G to T transversions
(209). In addition to the oxidized purine products, Fpg also can recognizes
some oxidative pyrimidine products including urea residues, 5-
hydroxycytosine, 5-hydroxyuracil, uracil glycol, and thymine glycol (70, 210).
The enzyme has been extensively purified following overexpression of cloned fpg gene as a 30,200 molecular weight protein (211). Analysis of the amino acid sequence of Fpg showed no significant similarity to other DNA glycosylases, but revealed a putative C-terminal zinc finger domain (211). The zinc finger domain appeared to be important for the normal function of Fpg since amino acid substitution at Cys-244 in this domain resulted in a loss of both DNA glycosylase and DNA binding activities (212). Mutagenic 8-oxoG residues in DNA are also removed by functional co-operation of two additional E. coli enzymes, MutT and MutY proteins. MutT is not a DNA glycosylase, but acts as a 8-oxo-dGTPase to eliminate 8-oxo-dGTP from the dNTP pool (213), while MutY excises adenine opposite 8-oxoG, which may be incorporated during replication because various DNA polymerases preferentially insert adenine opposite 8-oxoG in the template strand (214). The mutY gene has been cloned and encodes a 350 amino acid polypeptide of 39,123 daltons (215). The predicted amino acid sequences of MutY display homology (~66% similarity) to that of Endo III (215). The enzyme has been purified to near homogeneity and displays catalytic activity that also removes adenine from A/G and A/C mispair containing DNA; however, A/G was a considerably better substrate for the enzyme (214).

Recent structural studies with AlkA and Endo III suggested that there is a related mechanism for substrate recognition between monofunctional and bifunctional DNA glycosylases, although the catalytic mechanisms are somewhat different (201, 216, 217). In both AlkA (216) and Endo III (218), the helix-hairpin-helix motif and Pro/Gly-rich stretch with a conserved aspartic acid residue are found to comprise the active site of the enzymes. Additionally, for both DNA glycosylases the target base is apparently flipped out of the double-stranded DNA helix and accommodated in a substrate binding pocket (201, 216). In Endo III this pocket is rich in hydrophilic residues that interact with the flipped-out base directly via water-mediated
hydrogen bonds. Nucleophilic attack follows and involves a conserved aspartic acid in the active site of the enzyme (201). In AlkA, this substrate binding pocket is rich in hydrophobic residues and thus suitable to interact with a number of electron-deficient bases (216, 217). In most bifunctional DNA glycosylases, it has been suggested that the formation of a Schiff base forms as a covalent enzyme-substrate intermediate and following its transformation mediates strand cleavage, degradation of deoxyribose, and regeneration of the free enzyme (199-201). The formation of covalent intermediates between enzyme and substrate has not been observed for monofunctional DNA glycosylases such as E. coli AlkA and Ung.

1.6.2 Apurinic/apyrimidinic Site Cleavage by AP Endonucleases

1.6.2.1 Apurinic/apyrimidinic Sites

Apurinic/apyrimidinic (AP) sites are noncoding lesions that can be mutagenic, cytotoxic, and lethal to the cell (60). AP-sites in DNA are not only produced enzymatically by the action of various DNA glycosylases, and thus, appear as major intermediates in BER (180), but also formed by depurination or depyrimidination reactions that occur via nonenzymatic hydrolysis or by exposure to ionizing radiation (219, 220). The rate constant of spontaneous in vitro depurination has been determined to be $4 \times 10^{-9} \text{s}^{-1}$ by measurement of quantitative release of radiolabeled purine bases from double-stranded DNA at 70 °C and pH 7.4 (219). It was subsequently shown that spontaneous depyrimidination occurs at a rate ~100 times more slowly than depurination (221).

The cytotoxicity of AP-sites has been demonstrated in E. coli cells that were deficient in exonuclease III and contain a temperature-sensitive mutation in the dut gene encoding dUTPase (222). At the non-permissive temperature, these E. coli cells were inviable due to the accumulation of the cytotoxic AP-sites generated by Ung, which excised uracil residues that were incorporated
at high frequency (222). It has been suggested that the cytotoxicity of AP-sites may also be related to the chromosomal strand breaks at the AP-sites, which at high enough frequency result in lethality (223). The mutagenic potential of AP-sites was initially proposed from the observation that incorrect nucleotides were incorporated at higher than normal frequency during the in vitro copying of a poly(dAdT) substrate that was treated with acid to promote base release (224). Additionally, it has been shown that DNA and RNA polymerase in *E. coli* preferentially insert adenine opposite AP-sites (225-228). In *E. coli*, AP-site directed mutagenesis was found to be dependent on the activation of the SOS response (229). This damage inducible pathway involves the induction of several genes that promote a transient mutator phenotype by altering the fidelity of DNA replication, recombination, and repair (60). DNA polymerases that lack exonuclease proofreading ability, such as DNA polymerase IV and V, have been shown to be activated during the SOS response (230), and such error-prone polymerases were more capable of bypassing the AP-sites compared to DNA polymerase I and III (231, 232). Accordingly, it has been postulated that AP-sites are responsible for G/C to T/A transversion mutations associated with SOS activation (233).

AP-sites in DNA exist as an equilibrium between the open-sugar ring α, β unsaturated aldehyde, the open-sugar ring α, β unsaturated hydrate, and the α- and β-cyclic hemiacetals (234, 235). Open-sugar ring aldehyde constitutes about 1% of total AP-sites in duplex DNA, but are the most predominant chemical species in terms of reactivity (236). AP-sites can undergo several chemical reactions, which lead to cleavage of the phosphodiester bonds in the absence of catalytic action of enzymes (236). Chemical cleavage is mediated by β-elimination reactions catalyzed by nucleophiles that can occur either by the removal of a proton from the CH₂ group of the deoxyribose α to the carbonyl group at C-1 or by the formation of Schiff base between amine and C-1 carbonyl group of the ring-open aldehyde. Both of these events are followed by β-elimination resulting in a phosphodiester cleavage of DNA on
the 3'-side of the AP-site, which leaves an α, β unsaturated aldehyde at the 3'-terminus and a phosphate group at the 5'-terminus of the DNA incision (236). Under conditions of excess catalyst, the 3' α,β unsaturated aldehyde can undergo δ-elimination, which is actually a second β-elimination reaction, resulting in the release of the unsaturated baseless sugar moiety and yielding a single nucleotide gap flanked by 3' and 5' phosphate termini (60, 236). Nonenzymatic cleavage of AP-site containing DNA by reacting with polyamines, such as spermine and spermidine, or by heat and alkali treatment have been utilized in numerous studies for the purpose of AP-site detection (101, 236-238).

1.6.2.2 AP Endonucleases

AP sites are recognized and cleaved on either side of the sugar residue by AP endonucleases, resulting in a phosphodiester incision of the DNA backbone. According to their cleavage sites, AP endonucleases in E. coli can be divided into two groups, the class I AP endonucleases which cleave on the 3'-side of the AP-site and class II AP endonucleases which cleave 5' to the AP-site (239). Class I AP endonucleases catalyze AP-site cleavage by a δ-elimination reaction, but not by a hydrolytic mechanism (238, 240) and are thereby referred as AP lyases. These activities are associated with bifunctional DNA glycosylases, such as E. coli Endo III, Fpg, and MutY proteins (Section 1.6.1). It has been shown that AP lyase activity found in Endo III and its related DNA glycosylases as well as MutY mediate cleavage of the phosphodiester bond at AP-sites by β-elimination, thus generating 5'-phosphoryl and 3' unsaturated aldehyde termini in DNA (180). On the other hand, the AP lyase activity of Fpg protein resulted in 5'-phosphoryl and 3'-phosphoryl termini, since the reactions undergo successive β- and δ-elimination (241). Both of these types of 3' termini can not be efficiently utilized by DNA polymerase, and thus, need to be further processed to restore a functional DNA primer-template for BER (182). Since the majority of AP-
sites in *E. coli* appear to be incised by class II AP endonucleases, it has been suggested that the AP lyase function associated with some DNA glycosylases may not be biologically important or plays a secondary role in the incision of nascent AP-sites in DNA (60). Unlike AP lyase activity, class II AP endonucleases cleave phosphodiester bonds hydrolytically, resulting in a 3'-hydroxyl nucleotide and a 5'-deoxyribose phosphate termini and are true nucleotidyl hydrolases. Class II AP endonuclease activity was found to be associated with *E. coli* exonuclease III and endonuclease IV.

Exonuclease III (Exo III) is the major AP endonuclease in *E. coli*, which comprises ~90% of the total cellular AP endonuclease activity under normal physiological condition (236, 242). The enzyme was first identified as a 3' to 5' exonuclease which degraded double-stranded DNA and has an associated 3' phosphatase activity (243, 244). This exonuclease activity specifically requires 3'-OH ends, and thus, combined with 3' phosphatase activity, can attack 3'-phosphate termini in duplex DNA. It has been suggested that Exo III accounts for most of the 3' phosphatase activity present in *E. coli* (245). Additionally, Exo III has been shown to have RNase H-like activity that degrades RNA in a RNA-DNA hybrid (246). The enzyme also has a 3'-phosphodiesterase activity, which removes 3'-phosphoglycolate residues that can be arisen in DNA due to strand breaks that occur by oxygen radical or ionizing radiation (247, 248). This 3'-phosphodiesterase activity is important in BER mediated by bifunctional DNA glycosylases, such as Endo III, since it removes the 3' α,β unsaturated aldehyde residues generated by AP lyase activity after β-elimination at the AP-site (236). This coupled reaction provides the 3'-OH termini required as a primer for DNA polymerase. Mutants of *E. coli* defective in both 3' exonuclease and the associated 3' phosphatase functions of Exo III have been mapped to the *xthA* gene (249), and all of these *xthA* mutants were also found to be defective in AP endonuclease activity (250). This observation led to the discovery of the AP endonuclease activity associated with Exo III. The *xthA* gene has been cloned and sequenced (251, 252), and the deduced
molecular weight of Exo III was determined to be 30,912 (252). Purified native and recombinant enzymes have been shown to catalyze the hydrolysis of DNA containing an AP-site on the 5'-side of the AP-site and have an absolute requirement for double-stranded DNA and a Mg$^{2+}$ cofactor (236, 253).

It has been suggested that a single active site in Exo III catalyzes all the associated reactions with coordination between three distinct structural domains (60, 242). These include an "active site" that catalyzes the cleavage of phosphodiester bonds in one strand of duplex DNA, a "DNA duplex site" that recognizes duplex structure upon the presence of deoxyribose in the strand opposite to that in which the active site works, and a "space site" that recognizes the space in the DNA which can be constituted by a base loss or the space created by the partial denaturation of the DNA duplex at the site of an internal nick. Accordingly, synthetic oligonucleotides containing a modified tetrahydofuran moiety, which is isosteric with 2'-deoxyribose and an analog of the acyclic sugar moiety, has been shown to be cleaved on the 5'-side of AP-site by Exo III (254). Furthermore, the enzyme cleaves the phosphodiester bond 5' to the O-alkylhydroxylamine N-glycosides as well as 5' to the urea N-glycosides in DNA, while thymine glycol N-glycoside and formamidopyrimidine N-glycoside are not substrates for Exo III (198, 255). From these results, it has been suggested that a secondary amine at the N-glycosylic bond and lack of base pairing by the damaged base are required for recognition and cleavage of the AP-site by Exo III (198). The crystal structure of Exo III and a ternary complex of the enzyme with Mn$^{2+}$ and dCMP have been reported (256). Analysis of the tertiary structure of the enzyme suggested that an extra-helical base on the DNA strand opposite to the AP-site plays an important role in substrate recognition by Exo III (256).

E. coli endonuclease IV (Endo IV) has been shown to process a class II AP endonuclease activity. This endonuclease was initially found in xthA mutants deficient in Exo III activity as a residual AP endonuclease activity which was separable from Exo III by chromatographic procedures (257, 258).
Subsequently, the enzyme was extensively purified and characterized (258). Similar to the AP endonuclease activity of Exo III, Endo IV has been shown to attack the phosphodiester bond 5' to the sites of base loss in DNA, leaving 3'-OH groups (241). The enzymatic activity of Endo IV was shown to be inactivated by EDTA in the presence of substrate suggesting that a tightly bound essential metal ion is present in the protein (259). Additionally, Endo IV has been demonstrated to remove phosphoglycoaldehyde, phosphate, deoxyribose-5-phosphate, and 4-hydroxy-2-pentenal residues from the 3' terminus of duplex DNA (236). These activities are collectively referred to as a 3' repair phosphodiesterase. Endo IV also exhibit a 3'-phosphomonoesterase activity that removes 3'-phosphoryl groups (236). Thus, the major difference between Exo III and Endo IV appears to be the absence of exonuclease activity in Endo IV. Cloning and sequencing of the gene encoding Endo IV (nfo) revealed that the gene product corresponding to a polypeptide with a deduced molecular weight of 31,562 (252, 260). Despite the similarity in size and in the reactions catalyzed between Endo IV and Exo III, no significant similarity in amino acid sequences has been observed (252).

Recently, the crystal structure of Endo IV bound to a synthetic AP-site containing DNA molecule revealed several structural features that underlie AP-site recognition and cleavage. In this EndoIV-DNA complex structure, the AP-site containing DNA appeared to be bent by ~90° with both AP-site and its opposite nucleotide flipped out of the DNA helix (261). It has been also observed that binding of the enzyme to the AP-site is mediated by five DNA recognition loops, which arise from the carboxyl-terminal face of the Endo IV β-barrel and form the walls of a positively charged groove that complements the negatively charged phosphate backbone of DNA (261). These recognition loops contact nucleotides on both DNA strands through direct and water mediated interactions (261). Consistent with biochemical results showing that Endo IV has no preference for the base opposite an AP-site (262), structural analysis showed no specific contacts between the enzyme and the orphan base
In addition, the enzyme active site was found to contain three Zn\(^{2+}\) ions that participate directly in a hydrolysis of the phosphodiester bond at 5'-side of the AP-site (261).

Although the level of AP endonuclease activity of Endo IV (~10%) is much lower than that of Exo III under normal condition, it has been shown that the level of Endo IV can be induced by ~20-fold (263). Induction can occur by exposing *E. coli* cells to chemical agents such as methyl viologen, plumbagin, menadione, and phenazine, which enzymatically became reduced *in vivo* via one-electron transfer reactions and then are oxidized to generate superoxide radicals (263). Additionally, *E. coli* that are defective in superoxide dismutase activity displayed an increased level of Endo IV when grown in the presence of pure oxygen (264). Subsequently, this adaptive response of *E. coli* to superoxide was shown to be under the control of the *soxRS* regulatory locus, which contains two regulatory genes producing SoxR and SoxS (264-266). Several studies have established the mechanisms of Endo IV induction which involve the *soxRS* regulon (267-270). SoxR has been shown to contain an iron-sulfur cluster that is sensitive to the prevailing redox potential (267). In addition, oxidized SoxR due to superoxide radical can mediate transcriptional activation of SoxS that binds to the promoter of *soxRS*-regulated genes and facilitates transcription of these genes, including *nfo* (Endo IV) and *sodA* (superoxide dismutase) (268-270). The observation that Endo IV is inducible as well as the occurrence of redundant AP endonuclease activity in *E. coli* suggests that the repair of AP-sites is fundamentally important to viability.

### 1.6.3 Removal of Deoxyribose Phosphate Residues

AP endonuclease incision at an AP-site produces a 3'-OH terminus which can be used as a primer by DNA polymerase, and a 5'-terminal deoxyribose phosphate (dRP) residues which must be removed before ligation of the DNA strand can occur. *In vitro* studies with *E. coli* DNA polymerase I demonstrated that 5'-terminal dRP residues located at strand breaks promote strand
displacement synthesis rather than a DNA polymerase-catalyzed nick translation reaction (271). This was supported by the observation that the 5' to 3' exonuclease activity of DNA polymerase I was unable to excise dRP residues from the 5' termini of incised AP-sites (272). Although DNA polymerase I appeared to be inefficient in removing the terminal dRP residue prior to conducting DNA synthesis, it has been demonstrated that the 5' to 3' exonuclease activity of DNA polymerase I can slowly liberate dRP as part of a small oligonucleotide, mainly as a dimer (273). These observations prompted the search for an enzyme that can efficiently remove the dRP residues, which is referred to as a deoxyribophosphodiesterase (dRPase).

In *E. coli*, the enzymatic activity of dRPase was first identified in a partially purified protein preparation and attributed to a 50-55 kDa protein that required Mg^{2+} for activation (272). The enzyme could excise dRP moieties at incised AP-sites and did not display an AP endonuclease activity or exonuclease activity acting on double-stranded DNA (272). This dRPase activity has been ascribed to *E. coli* exonuclease I (274), which was originally shown to be an exonuclease that degrades single-stranded DNA in the 3' to 5' direction (275). Exonuclease I was later identified as the *sbcB* gene product which had a deduced molecular weight of 53,174 (276). However, further studies showed that dRPase activity was not detected when highly purified exonuclease I preparation was analyzed (277, 278). Subsequently, the relationship between purified dRPase and the *recJ* gene product was recognized following biochemical characterization of these two proteins (277). The *recJ* gene encodes a 60,000 molecular weight enzyme (RecJ) with a 5' to 3' single-strand specific exonuclease activity that was previously implicated in a recombination pathway and in mismatch DNA repair (279). It was demonstrated that the purified dRPase contained a 5' to 3' single-strand specific exonuclease activity while the purified *recJ* gene product possessed both 5' to 3' exonuclease and dRPase activity (277). From this study, it was concluded that the *recJ* gene encoded the dRPase enzyme in *E. coli*. Recently,
the dRPase activities of both RecJ and exonuclease I have been re-investigated using highly purified proteins and various reaction conditions including the original conditions that were used to initially identify the activities (278). The results revealed that both RecJ and exonuclease I were not able to excise dRP residues from the pre-incised AP-sites in any of reaction conditions that were tested (278). Since dRPase activity was not detected even with a great molar excess of enzyme over DNA, the results suggest that both enzymes may not play a significant role in the removal of dRP residues (278). It was also demonstrated that the lack of dRPase activity in both enzymes was not due to the enzyme inactivation since exonuclease I and RecJ displayed robust 3' to 5' and 5' to 3' single-strand specific exonuclease activity, respectively.

Finally, the release of the 5'-terminal dRP moiety from an incised AP-site was demonstrated with Fpg protein (280). As described above, this bifunctional DNA glycosylase has an associated AP lyase activity, which mediates the cleavage of AP-sites using a β-elimination reaction (Section 1.6.1). It has been shown that the mechanism for removing 5'-terminal dRP residues by Fpg also involved a β-elimination reaction, as opposed to hydrolysis (278, 280). Accordingly, other bifunctional DNA glycosylases, such as E. coli Endo III and T4 endonuclease V, have been similarly shown to release dRP residues from incised AP-sites (278). The removal of dRP by Fpg protein appeared to be more efficient than its DNA glycosylase activity on fapy-DNA adducts, since the enzyme has a ~10-fold higher $k_{cat}/K_m$ value for dRP removal than for fapy base excision (280). Recently, it was demonstrated that Fpg protein can be trapped on both preincised and unincised AP-sites using sodium borohydride as the reducing agent (278). This observation further supports the role of Fpg as an AP lyase as well as an dRPase that acts via β-elimination reaction mechanism. Further investigations to elucidate the biological significance of Fpg in dRP removal during the BER pathway remain to be conducted.
1.6.4 Repair DNA Synthesis

DNA synthesis during BER might involve one or more DNA polymerases. In *E. coli*, five DNA polymerases have been found: DNA polymerase I, II, III, IV, and V, which are encoded by the *polA*, *polB*, *polC*, *dinB*, and *umuC* and *umuD* genes, respectively (230). Among these, the involvement of DNA polymerase I in BER has been recognized in a genetic analysis of *E. coli dut* strains that are defective in dUTPase (1, 2). *E. coli dut* strains have increased intracellular dUTP concentrations, which leads to increased incorporation of uracil during DNA synthesis (47). It was observed that *E. coli dut* mutants accumulate DNA fragments that resemble Okazaki fragments, while this accumulation of short DNA fragments was suppressed in *dut ung* double mutants (1, 2), suggesting that an increased level of DNA strand breaks occurred due to uracil excision followed by AP-site incision. These short Okazaki-like fragments appeared to be transiently and readily incorporated into higher molecular weight DNA (2). However, it was observed that the introduction of DNA polymerase I or DNA ligase mutations into *E. coli dut* mutants reduced the rate of the rejoining of short Okazaki DNA fragments (1). Thus, these results implied that Ung-initiated repair of uracil in *E. coli* requires the involvement of DNA polymerase I and DNA ligase. In addition, *in vitro* reconstitution of the uracil-initiated BER pathway has been demonstrated using purified DNA polymerase I and additional *E. coli* enzymes, such as Ung, Endo IV, RecJ, and DNA ligase (182). Taken together, it has been generally accepted that DNA polymerase I occupies the primary role in BER DNA synthesis in *E. coli*.

1.6.4.1 DNA Polymerase I

*E. coli* DNA polymerase I (Pol I) was the first DNA polymerase identified (281). The enzyme is the product of the *polA* gene and has been extensively characterized (282, 283). As deduced from the DNA sequence of the cloned
polA gene, Pol I is composed of a single polypeptide chain of 928 residues with a molecular mass of ~103,000 (284). This monomeric protein displays several discrete catalytic functions that carry out DNA polymerization, pyrophosphorolysis, pyrophosphate exchange, and 3' to 5' and 5' to 3' exonucleolytic degradation reactions (283). Limited proteolysis of Pol I separates the polypeptide chain into two active fragments: (i) a large C-terminal fragment (also called Klenow fragment) composed of 605 amino acids which contains the polymerase and 3' to 5' exonuclease activity and the binding sites for dNTPs (285); (ii) a smaller N-terminal fragment that contains only the 5' to 3' exonuclease activity (286). Like other known DNA polymerases, Pol I catalyzes DNA chain growth in the 5' to 3' direction (283). Pol I has been shown to be processive for 15-20 nucleotides on an activated calf thymus DNA template and on the nicked circular duplex DNA of ColE1 plasmid template (287, 288). However, the processivity of Pol I was increased at least two fold on a gapped ColE1 template and to near 200 nucleotides with a poly (dA/dT) template while this number was significantly reduced at low temperature and high concentration of salt (287, 288). Recently it has been demonstrated that Pol I directly interacts with β-sliding clamp through the protein-protein interaction (289). Using a single-stranded M13 DNA template primed with short oligonucleotide DNA and the γ-complex clamp loader it was shown that the interaction between Pol I and β-sliding clamp could significantly increase the processivity of Pol I (289). The 3' to 5' exonuclease activity of Pol I is a component of the polymerase machinery that recognizes an incorrectly base-paired primer terminus (290). Thus, a mismatched terminal nucleotide on the primer chain binds to the exonucleolytic active site on the enzyme that is required for its hydrolysis and removal. One unique feature of Pol I, observed in no other E. coli DNA polymerase, is its capability to promote replication of DNA at a nick, unaided by other proteins (291). This requires unwinding of the duplex beyond the nick and progressive strand displacement of the 5' chain. Under this mode of action, the 5' to 3'
exonuclease activity functions to degrade DNA in the absence of any DNA damage. In addition, this 5' to 3' exonuclease activity of Pol I has shown to be able to remove small oligonucleotides containing pyrimidine dimers or other forms of bulky base damages from nicked DNA, providing the crucial role in the excision repair mechanism in E. coli (292, 293).

DNA synthesis coupled with degradation by the 5' to 3' exonuclease leads to the transfer of the nick along the template, thereby called "nick translation". It was initially observed that DNA synthesis on the template-primer \((dA_{4000}/[^3H]dT_{300})\) by Pol I was accompanied by a burst of hydrolysis of the template-primer that was approximately equal to the extent of polymerization (294). The rate of hydrolysis was shown to be largely dependent upon the addition of the dNTPs that allowed DNA synthesis to occur (294). Thus, polymerization at a nick is coordinated with the 5' to 3' exonuclease activity of Pol I, so as to move the nick along the helix with 5' to 3' exonuclease activity occurring in advance of a growing chain (295, 296).

However, a detailed investigation of the nick-translation reaction using a circular double-stranded DNA substrate containing a nick revealed that single-stranded overhangs were transiently generated by strand displacement DNA synthesis during the nick translation reaction mediated by Pol I (297). When DNA synthesis was conducted with all four dNTPs present, small oligonucleotide products as large as octamers were generated by Pol I, suggesting that the 5' to 3' exonuclease does not hydrolyze every phosphodiester bond (297). From these observations, it was concluded that short displaced segments of DNA were generated by the polymerization reaction and were removed following 5' to 3' exonuclease activity (297).

The 5' to 3' exonuclease mechanism of action associated with Pol I was later elucidated by the observation that the exonuclease acts as a structure-specific 5' nuclease that cleaves the phosphodiester backbone at the junction between a DNA duplex and a 5'-single-stranded overhang (or flap) (298). This structure specific cleavage did not appear to be coupled to synthesis, although
the presence of primers for polymerization reaction could accelerate the rate of cleavage (298). It was observed that a single-stranded DNA forming a 5'-overhang on duplex DNA up to 200 nucleotides long could be cleaved from the flap structure (298). The position of the 5' nuclease cleavage was shown to be dependent on the substrates. A substrate containing a single-nucleotidic gap with the 5'-overhang was cleaved at several positions along the displaced single-stranded DNA while another substrate containing a nick with the 5'-overhang was cleaved on either side of the first base pair at the junction of the overhang, thereby generating a mixtures of nicked and singly-gapped DNA products (299). The substrate containing a one nucleotide flap on the 3'-side of the nick and 5'-overhang appeared to be cleaved much more rapidly than the other two substrates and most of the cutting occurred between the first two paired bases, resulting in a ligatable nick (299). It was also proposed that the enzyme gains access to the cleavage site by moving from the free end of a 5' overhang (298). In support of this proposition, it was observed that substrates in which the 5' overhanging strand contained a terminal hairpin were refractory to cleavage by the 5' nuclease activity of Pol I (300). However, a dRP residue at either end of the 5' overhang appeared to have very little effect on the 5' nuclease activity of the enzyme (300), suggesting the possible role of this activity in BER.

The fidelity of DNA synthesis catalyzed by Pol I (Klenow fragment) has been measured using enzymes with an inactivated proofreading 3' to 5' exonuclease (301). The results revealed an average base substitution error rate of \( \sim 2 \times 10^{-5} \) and an average frameshift error rate of \( \sim 5 \times 10^{-6} \). In addition, exonucleaseolytic proofreading by the wild-type enzyme appeared to improve the average base substitution fidelity by 4- to 7-fold (301). It has been previously suggested that Pol I discriminates against nucleotide misinsertion by having a lower binding affinity for the incorrect dNTP and a slower catalytic rate for misinsertion than for correct insertion (302). However, the precise values vary from mispair to mispair and the rate of discrimination is
quantitatively larger than the nucleotide binding discrimination (303). The base substitution error specificity of Pol I (Klenow fragment) has been measured using a 361 nucleotide gap-filling TGA reversion assay. It was observed that the T to C transitions by misincorporation of dGTP opposite T were dominant over T to A transversions or T to C transitions (304). In addition, A/dATP errors occurred more frequently than A/dCTP or A/dGTP errors (304). However, the error distribution was altered when the assay was performed with one nucleotide gapped DNA substrate (304), suggesting that the surrounding sequence or nature of template can affect the mutational specificity.

1.6.4.2 Repair Patch Size

The first determination of repair patch size associated with *E. coli* BER was determined using a synthetic oligonucleotide duplex DNA (30-mer) containing a site-specific U/A base paired or U/G mispaired target (305). The complete BER reaction was performed by incubating the substrate with an *E. coli* cell extract. The results demonstrated that the predominant repair product was found to contain a repair patch of one nucleotide (305). From this observation, it was proposed that the 5' to 3' exonuclease or strand displacement activities of Pol I did not significantly participate in the removal of the 5'-terminal dRP residue or additional nucleotides (305). However, in order to suppress generalized degradation of the oligonucleotide substrate, an *E. coli* strain was used that lacked RecBCD nuclease and Exo I activity (305). Since it was later reported that the Exo I may have a role in BER, the BER patch size reported in this investigation may have limited scope (274).

Reconstitution of the uracil-initiated BER pathway was achieved using a uracil-containing duplex oligonucleotide (30-mer) substrate and five purified enzymes isolated from *E. coli*, which were Ung, Endo IV, RecJ, Pol I, and DNA ligase (182). Among these, RecJ was shown not to be absolutely required for the complete repair reaction (182). As observed for BER in cell extracts, *in
vitro DNA repair synthesis primarily involved the incorporation of a single nucleotide that replaced the uracil residue, which was referred to as either one nucleotide or short patch BER pathway (305). An alternative pathway was proposed to explain the ~30% of the repair patches that involved the incorporation of two or more nucleotides; this was referred to as long patch BER pathway (305). The long patch BER pathway likely involved the excision of the dRP residue as part of a small oligonucleotide by the structure-specific 5' nuclease activity of Pol I and generated larger repair patches due to strand displacement DNA synthesis (305). In this study, the presence of RecJ protein appeared to promote short patch BER pathway; however, the proportion of short and long patch size was dependent on the concentration of Pol I (305). Accordingly, the repair of uracil occurred mainly by one-nucleotide replacement when the concentration of Pol I was significantly reduced and the amount of RecJ was maintained at a higher level relative to Pol I (305).

In sharp contrast, another study demonstrated that *E. coli* uracil-initiated BER was accompanied by repair DNA synthesis involving several nucleotides, thereby occurring via the long patch BER pathway (306). A significant difference between this investigation and the one described above is that in this study BER was analyzed using a closed circular duplex uracil-containing DNA substrate. In this investigation, the estimated repair patch size was reported to be <19 nucleotide and >11 nucleotides long, and short patch repair synthesis was not observed (306). This long patch pattern of nucleotide replacement was also observed in *E. coli* cells deficient in exonuclease I and RecJ as well as cells deficient in RecJ and Fpg (306). These observations were significant since each of these proteins had been previously suggested to act as a dRPase activity (274, 277, 280). From these observations, it was suggested that the role of dRPase in BER may be to facilitate the removal of the displaced oligonucleotide by the 5' to 3' exonuclease of Pol I (306). However, it has been recently demonstrated that the presence of 5'-terminal dRP does not interfere with the action of the 5'-nuclease in removing the displaced single-stranded
overhang (300). Taken together, it is still not clear whether the dRPase activity has a significant role in BER and which factors control the length of repair patch during BER in *E. coli*.

### 1.6.5 DNA Ligation

The final step in excision repair involves the joining of the last newly incorporated nucleotide to the parental polynucleotide chain. In *E. coli*, the sealing of the nick following the completion of BER DNA synthesis is catalyzed by the *ligA* gene product, DNA ligase (307). The enzyme is a monomeric protein of a ~77,000 molecular weight and catalyzes phosphodiester bond formation in duplex DNA with juxtaposed nucleotide termini containing 3'-OH and 5'-phosphate groups. The reaction requires Mg$^{2+}$ and nicotinamide adenine dinucleotide (NAD$^+$) (308). Limited proteolysis can divide the enzyme into two functional domains: C-terminal domain that is required for binding to DNA and an N-terminal domain which is responsible for NAD$^+$ binding and for the self-adenylation reaction (309). In addition, the enzyme appeared to bind a zinc ion in the C-terminal domain (309). The requirement of NAD$^+$ appears to be unique in eubacterial DNA ligase since DNA ligases encoded by phages T4 and T7 and those found in eukaryotic cells use ATP (310). During the reaction, the NAD$^+$ is hydrolyzed to yield nicotinamide mononucleotide (NMN) and AMP by *E. coli* DNA ligase, while ATP-dependent DNA ligases break down ATP to AMP and pyrophosphate (PPi) (307). In both types of enzymes, the cleavage of the PPi bond leads to the synthesis of a phosphodiester bond in DNA by a sequence of three discrete steps (307): (i) formation of enzyme-nucleotide intermediate by transfer of the adenine group of NAD$^+$ (or ATP) to the amino group of the conserved lysine residue, resulting in adenylated DNA ligase and NMN (or PPi), (ii) adenylyl activation of the 5'-phosphate terminus of DNA by transfer of the adenylyl group from the enzyme to generate a new PPi linkage between AMP and the 5'-phosphate terminus at the nick, and (iii) formation of the
phosphodiester bond by attack of the 3'-OH terminus of the DNA on the activated 5'-phosphate group, with release of AMP. Consistent with this reaction mechanism, it has been shown that the substitution of the active site lysine completely eliminates the enzyme activity (311, 312).

The number of DNA ligase molecules per *E. coli* cell has been estimated to be ~300 based on the specific activity determinations (283). Considering that *E. coli* DNA ligase has a turnover number of 25 joining events per min per molecule (283), the DNA ligase activity seemed to be relatively abundant in *E. coli* cells. A second *E. coli* NAD+-dependent DNA ligase (LigB) has been recently identified by virtue of its sequence similarity to LigA (313). Purified recombinant LigB may be capable of catalyzing strand joining on a singly-nicked DNA substrate in the presence of a divalent cation and NAD⁺ since it was shown to react with NAD⁺ to form a covalent ligase-adenylate intermediate (313). LigB appeared to differ from LigA in that it lacks the BRCA1 C-terminus domain (BRCT) and two of the four Zn-binding cysteines that are present in LigA and other bacterial NAD⁺ ligases (313). Like LigA, the conserved lysine residue of LigB appeared to be essential for the activity since alanine substitution for the lysine abolished the nick joining activity and the formation of the adenylated enzyme (313). This discovery may raise the prospect that *E. coli*, like eukaryotes, may exploit different DNA ligase species for different physiological functions, such as replication, repair, homologous recombination and non-homologous end-joining. Whether LigB plays any role in the BER pathway remains to be determined.

### 1.7 Research Objectives

At the beginning of this study, it was generally accepted that *Escherichia coli* uracil-initiated base excision DNA repair was initiated only by the *ung* gene product, uracil-DNA glycosylase (49, 305, 306). Given the importance of this cellular defense mechanism in maintaining the genetic stability of the *E. coli* genome, it was important to explore whether an alternative uracil-DNA
glycosylase activity might exist in *E. coli* as a back-up system. To determine if *E. coli* was capable of initiating uracil-DNA repair in the absence of Ung, a strategy was developed using the uracil-DNA glycosylase inhibitor protein to block Ung activity. By using this approach, an Ugi-insensitive uracil-DNA glycosylase activity was discovered in *E. coli* cell extracts, which appeared to act as a double-strand uracil-DNA glycosylase (Dug). Once Dug activity was identified the initial objective was to purify the native enzyme in order to facilitate the cloning of the *dug* gene. Purification of recombinant Dug was undertaken in order to further characterize the enzyme with respect to substrate specificity, DNA binding, and mechanism of action. The final objective of this phase of the research was to determine if Dug plays a role in uracil-initiated BER pathway.

Although the biochemical mechanisms associated with *E. coli* uracil-initiated BER have been investigated in numerous studies, the fidelity of repair DNA synthesis during a complete process of uracil-initiated BER had not been addressed prior to the investigation conducted during this dissertation research. Thus, it was the objective of this study to measure the base substitution error frequency and the mutational specificity that were associated with both Ung- and Dug-mediated uracil-initiated BER. The approach that was developed utilized an M13mp2 *lacZα* DNA-based reversion assay and defined for the first time fidelity of the complete BER reaction.

Based upon the finding that Dug exhibited both uracil- and ethenocytosine-DNA glycosylase activities, several questions arose regarding the relative role and association of this enzyme in BER. The aims of the research under this objective was centered on elucidating the following questions. What is the relative contribution of Ung and Dug to *E. coli* uracil- or ethenocytosine-DNA repair? What is the relative efficiency of the complete BER process initiated at uracil- and ethenocytosine-target sites? What is the DNA repair synthesis patch size distribution for uracil- and ethenocytosine-
initiated BER? In order to address these questions, a lesion specific DNA probe and BER-competition assay were developed to analyze BER in *E. coli* cell-free extracts.

The aim of the final phase of investigation was to determine which step in the *E. coli* BER pathway was rate-limiting. The approach involved enzyme complementation studies using exogenous purified BER enzymes to supplement *E. coli* extracts and then monitoring the influence on the rate and extent of uracil-initiated BER. Studies were also undertaken to examine the specificity of repair DNA synthesis associated with uracil- and ethenocytosine-initiated BER and the results provided the first evidence for repair synthesis involving the incorporation of more than 200 nucleotides which is now referred to as very-long patch BER pathway.
2. MATERIALS AND EXPERIMENTAL PROCEDURES

2.1 Materials

2.1.1 Chemicals

Trizma (Tris-base), Hepes, EDTA, boric acid, ampicillin, tetracycline, rifampicin, streptomycin sulfate, bovine serum albumin (BSA), streptavidin, 2-mercaptoethanol, Tween-20, transfer ribonucleic acid (type X-SA), adenosine triphosphate (ATP), β-nicotinamide adenine dinucleotide (NAD), thiamine, phosphocreatine di-Tris salt were obtained from Sigma. Isopropyl-β-δ-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), dithiothreitol (DTT), 1 kb DNA ladder, ultra-pure cesium chloride, urea, sodium dodecyl sulfate, glycine, phenol, sucrose, ammonium sulfate, and agarose were purchased from Life Technologies. 2'-deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) were obtained from Pharmacia Biotech and 2'-deoxyribonucleoside α-thiotriphosphates (dATP[αS], dGTP[αS], dCTP[αS], and dTTP[αS]) were from Amersham Life Science. Dextrose, sulfuric acid, monobasic and dibasic potassium phosphate, 1-butanol, iso-amyl alcohol, glycerol, and dimethylformamide were obtained from J.T. Baker. Fisher was the source of methanol, toluene, acetic acid, hydrochloric acid, ammonium hydroxide (50%), and trichloroacetic acid. Acrylamide (>99% pure), bis N,N'-methylene-bis-acrylamide, ammonium persulfate, TEMED, bromphenol blue, xylene cyanol FF, Coomassie Brilliant Blue G250, and Bradford reaction dye reagent concentrate used for protein assay were obtained from Bio-Rad.

2.1.2 Radioisotopes

[α-32P]dATP, [α-32P]dCTP, [γ-32P]ATP, and [³H]dTTP were purchased from DuPont-Perkin Elmer.
2.1.3 Bacterial Media

M9 medium contained 1.28 % Na₂HPO₄·7H₂O, 0.3 % KH₂PO₄, 0.05% NaCl, and 0.1 % NH₄Cl. Following sterilization, M9 medium was adjusted to 2 mM MgSO₄, 0.1 mM CaCl₂, 0.01 mM FeCl₃, 0.4 % glucose, and 10 µg/ml thiamine from individual sterile stocks. YT medium was composed of 0.5% yeast extract (Difco), 0.8% tryptone (Difco), and 0.5% NaCl. TYN medium was composed of 1 % yeast extract, 1 % tryptone, and 0.5 % NaCl. B broth was prepared with 1% tryptone and 0.8% NaCl. LB medium was composed of 0.5 % yeast extract, 1 % tryptone, and 1 % NaCl. LC medium was comprised of 0.5% yeast extract, 1 % tryptone, and 0.5% NaCl, adjusted with CaCl₂ to a final concentration of 2 mM. SOC medium contained 0.5 % yeast extract, 2 % tryptone, 0.05 % NaCl, 2.5 mM KCl, 10 mM MgCl₂, and 20 mM glucose and the pH was adjusted to pH 7.0 with 5 M NaOH. SM medium was composed of 50 mM Tris-HCl (pH 7.5) 0.58 % NaCl, 0.2 % MgSO₄·7H₂O, and 1 % gelatin.

Agar plates and top agar were prepared by the addition of 1.5 % and 0.7 % (w/v) bacto-agar (Difco), respectively, to the appropriate liquid media. In order to detect lacZα-complementation, M9 top agar was adjusted to 0.4 mM IPTG and 1 mg/ml X-Gal. Where appropriate, liquid media or agar plates were supplemented with 100 µg/ml ampicillin from a filter-sterilized stock (100 mg/ml) or with 15 µg/ml tetracycline from a 15 mg/ml stock (in 100% ethanol).

2.1.4 Bacterial Strains

The strains of E. coli used in this research and their representative genotypes are listed in Table 1. Strains NR8051, NR8052, NR9162, and CSH50 were provided by T.A. Kunkel (National Institute of Environmental Health Sciences). Strains BH156, BH157, and BH158 were provided from A.S. Bhagwat (Wayne State University). E. coli GM31 was obtained from E. coli Genetic Stock Center (Yale University) and JM109 was purchased from New
England Biolabs. P1 lysate containing mutS::Tn-10 was obtained from J. Hays (Oregon State University), and E. coli mutS strains, NR80511 and 80512 were constructed by the P1 transduction of mutS::Tn-10 into E. coli NR8051 and NR8052, respectively.

Table 1

**E. coli strains and genotypes**

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<tr>
<th>E. coli Strains</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
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<tr>
<td>JM109</td>
<td>recA1 e14′(McrA) Δ(lac-proAB) thi gyrA96 (NaF) endA1 hsdR17 (r6 mC) relA1 supE44/F' traD36 lacI4 Δ(lacZ)M15 proA+B</td>
<td>(315)</td>
</tr>
<tr>
<td>CSH50</td>
<td>ara thi Δ(pro-lac)/F' traD36 proAB lacI4ZΔM15</td>
<td>(225)</td>
</tr>
<tr>
<td>NR9162</td>
<td>hsdR hsdM' araD Δ(ara,leu) Δlac1P0ZY</td>
<td>(316)</td>
</tr>
<tr>
<td>NR8051</td>
<td>Δ(pro-lac) thi</td>
<td>(225, 317)</td>
</tr>
<tr>
<td>NR8052</td>
<td>Δ(pro-lac) thi trpE9777 ung-1</td>
<td>(225, 317)</td>
</tr>
<tr>
<td>NR80511</td>
<td>NR8051 with mutS::Tn10</td>
<td></td>
</tr>
<tr>
<td>NR80521</td>
<td>NR8052 with mutS::Tn10</td>
<td></td>
</tr>
<tr>
<td>GM31</td>
<td>dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78 galK2 xyl-5 thi-1 mtl-1</td>
<td>(318)</td>
</tr>
<tr>
<td>BH156</td>
<td>GM31 with ung-1 tyrA::Tn10</td>
<td>(319)</td>
</tr>
<tr>
<td>BH157</td>
<td>GM31 with mug::Tn10</td>
<td>(319)</td>
</tr>
<tr>
<td>BH158</td>
<td>GM31 with ung-1 tyrA::Tn10 mug::Tn10</td>
<td>(319)</td>
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</table>
2.1.5 Plasmids and Bacteriophage

The expression vector pKK223-3 was obtained from Pharmacia Biotech. Plasmid pKK-Dug, an overexpression vector for double-strand uracil DNA glycosylase (Dug), was constructed as described in this dissertation. Plasmid pGEM-3Zf(+) (pGEM) and helper phage R408 were purchased from Promega and the plasmid pBIG was produced as described in this dissertation. Plasmid pET30a came from Novagen. Bacteriophage M13mp2 was obtained from T.A. Kunkel (National Institute of Environmental Health Sciences) and M13mp2op14 was constructed by Sanderson and Mosbaugh as described previously (314).

2.1.6 Chromatographic Resins

DEAE-Sephadex A50 and Sephadex G-75 were purchased from Pharmacia Biotech. The source for Bio-Gel P-4 gel (130 ± 40 μm), hydroxyapatite Bio-Gel HTP, and AG 501-X8(D) (20-50 mesh) was Bio-Rad.

2.1.7 Oligonucleotides

Oligonucleotides utilized during the course of this research are listed below. The oligonucleotide 34-mers pertaining to preparation of DNA glycosylase substrates, biotinylated 40-mers applied to evanescent wave biosensor, and 33-mer primers employed during construction of pBIG plasmid were synthesized by Midland Certified Reagent Company. Oligonucleotide 23-mers utilized in construction of BER DNA substrates and primers used in Dug cloning were synthesized and gel purified by Oligos Etc. Research Genetics was the source for PCR primers (18-mers) and sequencing primer (21-mer).
• DNA glycosylase substrate oligonucleotides
  C-34-mer: 5'-AGCTTGGCTGCAGGTCGACGGATCCCCGGGAATT-3'
  U-34-mer: 5'-AGCTTGGCTGCAGGTUGACGGATCCCCGGGAATT-3'
  T-34-mer: 5'-AGCTTGGCTGCAGGTTGACGGATCCCCGGGAATT-3'
  εC-34-mer: 5'-AGCTTGGCTGCAGGTεCGACGGATCCCCGGGAATT-3'
  G-34-mer: 5'-AATTCCCGGGGATCCGTCGACCTGCAGCCAAGCT-3'
  A-34-mer: 5'-AATTCCCGGGGATCCGTCGACCTGCAGCCAAGCT-3'

• Biotinylated oligonucleotides
  Biotinylated G-40-mer: 5'-ATTCGGGATCCGTCGACCTGCAGCC
   AAGCTTAATAT-(biotin)-3'
  Biotinylated U-4-mer: 5'-GCTTGGCTGCAGGTUGACGGATCCCCGG
   GAATTTAATAT-(biotin)-3'

• Oligonucleotide primers for M13mp2 BER substrates
  A-23-mer for (A-T) DNA: 5’-CCCAGTCACGTCATTGTAAAACG-3’
  U-23-mer for (U-T) DNA: 5’-CCCAGTCACGTCUTTGTAAAACG-3’
  U-23-mer for (U-G) DNA: 5’-CCCAGTCACGTCUATTGTAAAACG-3’

• Oligonucleotide primers for pGEM and pBiG BER substrates
  C-23-mer for (C-G) DNA: 5’-ATCCTCTAGAGTCGACCTGCAGG-5’
  U-23-mer for (U-G) DNA: 5’-ATCCTCTAGAGTUGACCTGCAGG-5’
  εC-23-mer for (εC-G) DNA: 5’-ATCCTCTAGAGTεCGACCTGCAGG-5’

• PCR primers in pBIG construction
  pET30a-F-33-mer: 5’-CAGAATTCCATGACCCATATTCAACGGGAAA
   CGT-3’
  pET30a-R-33-mer: 5’-CAGAATTCCATGACCCATATTCAACGGGAAA
   CGT-3’

• PCR primers in Dug cloning
  P1-33-mer: 5’-CAGAATTTCATGGTGAGGATATTTTGCTCCAG-3’
  P2-33-mer: 5’-CCAAGCATTCATGGTGAGGATATTTTGCTCCAG-3’
• PCR primers for M13mp2 lacZα gene
  FP-18-mer: 5'-GTGTGGAATTGTGAGCGG-3'
  RP-18-mer: 5'-CGTGCACTCTGCCCAGTTTG-3'

• DNA sequencing primers for M13mp2 lacZα gene
  S-21-mer: 5'-GCACTCCAGCCAGCTTTCCGG-3'

2.1.8 Enzymes and Antiserum

T4 DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, E. coli DNA ligase, E. coli DNA polymerase I, E. coli exonuclease III, Vent DNA polymerase, and restriction endonucleases EcoRI, HindIII, HindIII, Smal, BsaHI, NdeI, and HaeIII, and BsrI were obtained from New England Biolabs. Calf intestine alkaline phosphatase and restriction endonuclease XbaI, AccI, HincII, and PstI were purchased from MBI Fermentas. Proteinase K and creatine phosphokinase (type I, rabbit) were from Sigma and Qiagen was the source of ribonuclease A. E. coli uracil-DNA glycosylase (Ung, fraction V) and uracil-DNA glycosylase inhibitor protein (Ugi, fraction IV) were purified by Sanderson and Mosbaugh as described elsewhere (314, 320). E. coli endonuclease IV (Endo IV, fraction V) and formamidopyrimidine-DNA glycosylase (Fpg, fraction V) was kindly provided by B. Demple (Harvard University) and S. Mitra (University of Texas Medical Branch, Galveston), respectively. Antiserum specific for E. coli DNA polymerase I raised by immunization of rabbits was a gift from L. A. Loeb (University of Washington).
2.2 Experimental Procedures

2.2.1 Preparation of Chromatographic Resins

2.2.1.1 Preparation of DEAE-Sephadex, Hydroxyapatite, Sephadex G-75, and Bio-Gel P4-Resins

The microgranular form of DEAE-Sephadex A-50 was suspended in EQ buffer containing 30 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 1 mM EDTA, 150 mM NaCl, and 20% (w/v) glycerol. The resin was allowed to settle at room temperature, the buffer decanted to remove the fines, and equilibrated in DEAE buffer (30 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 1 mM EDTA, and 20% (w/v) glycerol). The resin was defined 4-5 times in this manner and stored as a 50% (v/v) slurry in the same buffer at 4°C. Sephadex G-75 was equilibrated in UEB buffer containing 10 mM Hepes-KOH (pH 7.4), 10 mM β-mercaptoethanol, 1 mM EDTA, 5% (w/v) glycerol, 1 M NaCl and Bio-Gel P-4 was prepared in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA), as described above. Hydroxyapatite Bio-Gel HTP was defined and equilibrated in HAB buffer (10 mM potassium phosphate (pH 7.4), 1 mM DTT, 200 mM KCl).

2.2.1.2 Preparation of Single-stranded DNA Agarose

Calf thymus DNA (Type I, Sigma) was solubilized at 15 mg/ml in 100 ml of 20 mM NaOH and slowly stirred overnight at room temperature. The DNA solution was incubated at 95°C for 15 min and added to an equal volume of molten 4% agarose equilibrated at 70°C. After mixing thoroughly, the DNA-agarose mixture was poured into an ice cold glass dish (Pyrex, 196 mm x 100 mm) held on ice and allowed to solidify. The solid DNA-agarose mixture was passed twice through a stainless steel sieve (60 mesh) and the resultant gel particles were suspended in 100 ml of resuspension buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl). Each resuspended gel mixture was
placed in a Buchner funnel (17 cm diameter) and washed with approximately 10 L of resuspension buffer at room temperature until the $A_{260\text{ nm}}$ of the filtrate was below 0.02. The single stranded-DNA agarose was stored in the resuspension buffer as a 50 % (v/v) slurry at 4 °C.

2.2.2 Preparation of Oligonucleotide DNA Substrates

2.2.2.1 Gel Purification of Oligonucleotides

Preparations of synthetic oligonucleotides (C-, U-, T-, εC-, G-, and A-34mers) that were deblocked and supplied as a lyophilized powder by the Midland Certified Reagent Company were subjected to further purification using polyacrylamide gel electrophoresis. The powder form of oligonucleotides was resuspended in light TE buffer (5 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) to a final concentration of 10 mM. Sample (100 µl) was removed, mixed with 300 µl of light TE buffer, combined with 200 µl of 3x native sample buffer (150 mM Tris-HCl (pH 6.8), 30 % (w/v) glycerol, and 0.3 % bromphenol blue), and loaded onto nondenaturing 12 % polyacrylamide gels (30 x 40 x 0.16 cm) buffered with TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA). Electrophoresis was carried out with TBE buffer at 1000 V until the tracking dye migrated ~25 cm. After electrophoresis, the polyacrylamide gel was placed on top of a sealed TLC plate (Polygram Cel 300 PEI/UV254) and the oligonucleotide bands were visualized by UV-shadowing. Oligonucleotide DNA bands were excised from the gel using a clean razor blade and the gel slice was placed on the elution chamber of Elutrap apparatus (Schleicher and Schuell) soaked in TAE buffer (40 mM Tris acetate, 1 mM EDTA (pH 8.0)). Electroelution of oligonucleotides was performed at 150 V (50-60 mA) in TAE buffer for ~5 h at room temperature. Following electroelution, the current was reversed for ~5 sec and the DNA solution (~1 ml) was removed from the elution chamber, transferred into SpectraPor dialysis tubing (6,000-8,000 MWCO; 25.5 mm diameter), and dialyzed.
overnight against distilled H₂O at 4 °C. The concentration of oligonucleotide was determined by absorbance spectroscopy (1 OD₂₆₀ₙₘ ≈ 33 μg/ml) and each oligonucleotide sample was aliquoted into 2 nmol quantities, evaporated to dryness using a Speed-Vac Concentrator (Savant), and stored at -80 °C.

2.2.2.2 5'-end Phosphorylation of Oligonucleotides

Gel-purified oligonucleotides (C-, U-, T-, and εC-34-mers) (30 pmol) were 5'-end ³²P-radiolabeled in a reaction mixture (30 μl) containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 65 μCi [γ³²P]ATP (6,000 Ci/mmol), and 10 units of T4 polynucleotide kinase. Samples were incubated for 10 min at 37 °C and supplemented with ATP to 1 μM from a 8.35 μM ATP stock. After incubation for an additional 45 min at 37 °C, reaction mixtures was terminated by adjustment to 11.8 mM EDTA and heated for 10 min at 70 °C. Oligonucleotide A-34-mer and G-34-mer were also 5'-end phosphorylated under similar reaction conditions except the addition of [γ³²P]ATP was omitted. Instead, the reaction mixture was initially adjusted to 1.33 μM ATP. Two terminated reaction mixtures were typically combined and adjusted with TE buffer to a final volume of 150 μl and the phosphorylated oligonucleotides were purified from free ATP by passage through P-4 spun columns containing ~0.5 ml of resin, equilibrated in TE buffer. P-4 spun columns were centrifuged in an IEC clinical centrifuge (setting #4) for 2.5 min at room temperature.

During the large scale 5'-end phosphorylation of oligonucleotides, the reaction mixtures (137.5 μl) contained 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 250 μCi [γ³²P]ATP (6,000 Ci/mmol), 4 nmol oligonucleotides (U-34-mer), and 50 units of T4 polynucleotide kinase. A large scale phosphorylation of oligonucleotide G-34-mer and A-34-mer was also conducted in the similar reaction mixture except that 0.3 μM ATP was included instead of [γ³²P]ATP. Reactions were conducted for 15 min at 37 °C and adjusted to 200 μM ATP using a 5 mM ATP stock. The reaction was
incubated for an additional 45 min at 37 °C, terminated by adjustment to 10.7 mM EDTA, heated for 10 min at 70 °C, and adjusted to 250 µl with TE buffer. Unreacted ATP was removed from the mixture by passage through two consecutive P-4 (1.2 ml) spun columns as described above.

2.2.2.3 Annealing Oligonucleotides

Double-stranded DNA was formed by annealing 2 pmol of ³²P-labeled C-, U-, εC-, and T-34-mer to 4 pmol of A-34-mer or G-34-mer in a reaction mixture (40 µl) containing TE buffer. To construct duplex [³²P]C/G-, [³²P]U/G, [³²P]T/G, and [³²P]εC/G-34-mer DNA substrates, reaction mixtures were placed in a 500 ml beaker containing 70 °C distilled H₂O and allowed to slowly cool to room temperature (~4 h). Single-stranded [³²P]U-34-mer and [³²P]εC-34-mer DNA substrates were prepared using a mock-annealing reaction conducted in the absence of complementary oligonucleotide. A large scale annealing reaction was conducted similarly for the hybridization of 750 pmol of [³²P]U-34-mer to 1,500 pmol of A-34-mer or G-34-mer in a annealing reaction mixture (186 µl) containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, and 50 mM NaCl. Oligonucleotides utilized in evanescent wave biosensor experiments were prepared by annealing 10 µg of 3'-biotinylated G- or A-40-mers to 20 µg of U-34-mer in annealing reaction mixtures (300 µl) containing PBST buffer (10 mM sodium phosphate (pH 7.4), 138 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween 20).

2.2.3 Isolation of M13mp2op14 Single-stranded DNA

The number of plaque forming units per ml (pfu/ml) of M13mp2op14 bacteriophage stocks was determined by titration of the phage stock and infection of mid-log *E. coli* JM109 cells. The M13mp2op14 bacteriophage stock was serially diluted into SM medium and 100 µl aliquots of each diluted sample were placed into sterile 13 x 100 mm glass test tubes. Bacteriophage
dilutions were supplemented with 500 μl of mid-log bacterial culture and gently agitated to mix the bacterial cells and bacteriophage. Prewarmed M9 top agar (2.5 ml) containing 0.4 mM IPTG and 1 mg/ml X-Gal equilibrated at ~45 °C were added to each tube, mixed, and poured onto M9 plates prewarmed at 37 °C. Top agar was allowed to solidify at room temperature and plates were incubated overnight at 37 °C. The concentration of viable M13mp2op14 bacteriophage was calculated by counting the number of discernable plaques obscured at various dilutions of the phage stock.

Single-stranded M13mp2op14 DNA was isolated from bacteriophage grown in E. coli JM109 cells. An overnight culture of E. coli JM109 cells grown in YT medium was used to inoculate, at a 1:10 ratio, 2.5 L of fresh YT medium. Bacterial growth was monitored by absorbance spectroscopy (1 OD595 nm = 8 x 10⁸ cells/ml) until the cell density reached early mid-log phase (~3.0 x 10⁸ cells/ml). The total number of cells in the culture medium was calculated and the bacteriophage stock was added to a multiplicity of infection (m.o.i.) of 0.1. The infected culture was incubated for an additional 12 h with vigorous shaking (250 rpm) at 37 °C. Bacterial cells were harvested by centrifugation in a GSA rotor at 5,000 rpm for 30 min at 4 °C. The supernatant fraction containing the bacteriophage particles was transferred to Sorvall 250 ml centrifuge bottles and supplemented with 5 x PEG/NaCl (15 % PEG 8000, 2.5 M NaCl) to a final 1 x concentration (equal to 25 % of the supernatant volume). The mixture was incubated on ice for 1 h with gentle shaking and precipitated bacteriophage were recovered by centrifugation in a GSA rotor at 7,000 rpm for 15 min at 4 °C. The pellet was resuspended in 2.5 ml of buffer PEB (100 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 1 mM EDTA) and incubated on ice for 1 h with intermittent shaking. Resuspended bacteriophage were pooled, centrifugation performed in a SA600 rotor at 6,000 rpm for 15 min at 4 °C, and the supernatant was adjusted to 0.1% SDS using a 20 % SDS stock. The phage suspension (5 ml aliquots) was extracted twice with an equal volume of phenol equilibrated in PEB buffer and twice with an
equal volume of chloroform:isoamyl alcohol (24:1). All extraction mixtures were rocked by hand for 3 min before phase separation was performed in an IEC clinical centrifuge (setting #6 for 2 min). The recovered aqueous phase containing the single-stranded M13mp2op14 DNA was concentrated using a Centriprep-30 concentrator (Amicon) by centrifugation in a GSA rotor at 4,500 rpm at 4 °C, and buffer exchanged against TE buffer. The concentration of DNA was measured by absorbance spectroscopy (1 \( \text{OD}_{260 \text{ nm}} \) = 36 \( \mu g/\text{ml} \)) and the purity was determined by the ratio of \( \text{OD}_{260 \text{ nm}}/\text{OD}_{280 \text{ nm}} \) (~1.8).

2.2.4 Isolation of Plasmid, pGEM and pBIG, Single-stranded DNA

2.2.4.1 Preparation of Helper Phage R408

Five serial 1/100 dilutions of the supplied R408 phage stock were prepared by adding 0.1 ml of phage into 13 x 100 mm glass test tubes containing 9.9 ml of B broth. In separate sterile tubes, 0.1 ml of each phage dilution was added into 0.2 ml of log phase JM109 cells and incubated for 5 min at room temperature. B top agar (2.5 ml) prewarmed at 45 °C was added into each phage/bacterial cell mixture and poured on each B plate. Plates were incubated overnight at 37 °C, and a single, well-isolated plaque was used to inoculate 250 ml of TYP medium. After incubation of the culture overnight at 37 °C, the bacterial cells were pelleted by centrifugation in a GSA rotor at 8500 rpm for 15 min at 4 °C. The supernatant fraction containing viral R408 was heated at 55 °C for 30 min to kill any remaining bacterial cells and then stored at 4 °C. The concentration (pfu/ml) of helper phage was determined by serial dilution of the phage stock and infection against mid-log \( E. \text{coli} \) JM109 cells as described above. Generally a concentration of 3 to 4 \( x 10^{12} \) pfu/ml of the helper phage stock was obtained and used for the production of phagemid DNA.
2.2.4.2 Preparation of Phagemid Single-stranded DNA

Plasmid (pGEM and pBIG) single-stranded DNA was isolated following growth in *E. coli* JM109 cells with induction by helper phage. *E. coli* JM109 cells containing either pGEM or pBIG plasmid were selected by picking individual ampicillin resistant colonies from M9 plates and grown overnight at 37 °C. The overnight culture was used to inoculate (1:50 dilution) 2 L of TYN medium. After incubation for 1 h, the bacterial culture (OD$_{595}$nm ≈ 0.3) was infected with helper phage R408 at an m.o.i. of 100 and incubation occurred for an additional 14 h with vigorous agitation. Bacterial cells were pelleted by centrifugation and the supernatant containing phagemid was subjected to the CTAB DNA precipitation developed by Del Sal *et al.* (321), with some modification. After phagemid precipitation was conducted using the PEG/NaCl method, as described in Section 2.2.3, the resulting pellet containing phagemid particles was resuspended in high TE buffer (0.1 M Tris-HCl (pH 8.0), 50 mM EDTA) to a final volume of 40 ml and transferred into a sterile polypropylene 50 ml conical centrifuge tube. The phage solution in high TE buffer was incubated on ice for 1 h and the debris was removed by centrifugation in a SS34 rotor at 10,000 rpm for 20 min at 4 °C. The resulting supernatant fraction containing phagemid was transferred to a fresh tube and 0.4 ml of proteinase K was added to a final concentration of 100 μg/ml. After incubation at 37 °C for 1 h with intermittent shaking, the sample was mixed with 4.5 ml of CTAB solution (5% (w/v) cetyltrimethyl ammonium bromide in 0.5 M NaCl) and incubated for 5 min at room temperature. Phagemid single-stranded DNA was recovered by centrifugation in a SS34 rotor at 10,000 rpm for 20 min at 4 °C. The pellet was resuspended in 7.5 ml of 1.2 M NaCl, mixed with 100% ice-cold ethanol (19 ml), incubated at -20 °C for 20 min, and precipitated DNA was recovered by centrifugation in a SS34 rotor at 10,000 rpm for 20 min at 4 °C, followed by washing with ice-cold 75% ethanol. The DNA pellet was dried under room temperature and resuspended in ~3 ml of
TE buffer. The concentration and purity of pGEM or pBIG single-stranded DNA were measured by absorbance spectroscopy as described in Section 2.2.3. Typically, 1-2 mg of plasmid (350-650 μg/ml) single-stranded DNA was obtained by using this method.

2.2.5 Protein Concentration Measurements

The protein concentrations of purified *E. coli* Ung (fraction V) and Dug (fraction V) were determined by absorbance spectroscopy using the molar extinction coefficients $\varepsilon_{280\text{nm}} = 4.2 \times 10^4$ L/mol cm and $\varepsilon_{280\text{nm}} = 2.4 \times 10^4$ L/mol cm, respectively. Determination of the protein concentrations of *E. coli* crude extracts, cell-free extracts, and partially purified Dug preparations was performed with the standard BioRad assay using the Bradford reaction (322). Bovine serum albumin (BSA) was used to produce a standard curve for each assay. The BSA concentration was determined by absorbance spectroscopy using the molecular extinction coefficient ($\varepsilon_{280\text{mm}} = 6.7 \times 10^4$).

2.2.6 Preparation of Competent *E. coli* Cells

Bacterial competent cells were prepared from *E. coli* strain NR9162 for electroporation of M13mp2op14 form I DNA or from JM109 for transformation of pGEM, pBIG, pET30a, pKK223-3, and pKK-Dug plasmid DNA. Overnight cultures of bacterial cells were used to inoculate 1.5 L of 2 x YT medium at a 1:100 ratio and growth continued with vigorous shaking at 37 °C. After the cell growth reached the mid-log phase ($\text{OD}_{595} = 0.6-0.8$), the culture was placed on ice for 10 min. Cells were placed into Sorvall 250 ml centrifuge bottles and harvested by centrifugation in a GSA rotor at 5,000 rpm for 10 min at 4 °C. Cold sterile distilled H$_2$O (250 ml) was added into each cell pellet and the bottle was shaken vigorously until cells were thoroughly resuspended. Centrifugation was repeated and each cell pellet was resuspended with 125 ml of cold sterile distilled H$_2$O as described above.
Centrifugation was again conducted as described above and each pellet was resuspended in 30 ml of cold sterile 10% (w/v) glycerol, transferred to a 50 ml sterile polypropylene conical centrifuge tubes, and centrifugation was performed in a SS34 rotor at 5,000 rpm for 10 min at 4 °C. The supernatant fraction was carefully decanted and remaining bacterial cells (~3 ml) were resuspended in 2 ml of cold sterile 10% (w/v) glycerol, aliquoted (50 μl) into Eppendorf tubes, frozen in liquid nitrogen, and stored at -80 °C.

2.2.7 Isolation and Cloning of *E. coli dug* Gene

The DNA fragment containing an ORF (507-bp) encoding the *dug* gene was obtained by using *E. coli* NR8052 genomic DNA. A single colony of *E. coli* NR8052 was picked with an inoculating loop and diluted in 100 μl of sterile distilled H₂O in a 1.5 ml Eppendorf tube. The cell suspension was boiled for 10 min to lyse the cells and cell debris were removed by centrifugation for 10 min at 13,000 rpm in a Microspin 24S (Sorvall) centrifuge at 4 °C. The supernatant fraction (10 μl) containing *E. coli* genomic DNA was added to a primer annealing reaction mixture (100 μl) containing 20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, and 100 pmol each of P1-33-mer and P2-33-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 carried out by polymerase chain reaction (PCR) in a RoboCycler (Stratagene) using the following cycle parameter: 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min; 72 °C for 10 min.

The PCR reaction product (5 μl) was analyzed using 1.5% agarose gel electrophoresis. Ethidium bromide stained DNA bands were visualized by transillumination (310 nm) and the major 523-bp DNA fragment band was excised from the gel using a clean razor blade. The DNA fragment was isolated and purified by using a QIAquick Gel Extraction Kit (Qiagen) in
accord with the manufacturer’s instruction. The DNA fragment was eluted in 50 μl of 10 mM Tris-HCl (pH 8.5) and 30 μl of the fraction was digested simultaneously with 10 units each of restriction endonuclease EcoRI and HindIII in the reaction buffer containing 10 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 50 mM NaCl, and 1 mM DTT. After incubation for 30 min at 37 °C, the reaction mixture (40 μl) was combined with 10 μl of agarose dye buffer (50 mM EDTA (pH 8.0), 0.5% SDS, 25% (w/v) glycerol, and 0.05% bromphenol blue) and analyzed by 1.5% agarose gel electrophoresis. The 517-bp DNA fragment was isolated from the gel, as before, and eluted in 30 μl of 10 mM Tris-HCl (pH 8.5). EcoRI and HindIII digestion of an overexpression vector, pKK223-3, was also similarly conducted as described above. Following purification, the two DNA fragments containing complementary ends were joined in a ligation reaction carried out overnight at 16 °C. The ligation mixture (10 μl) contained a ~4 fold excess of insert (517-bp fragment containing dug gene) over vector (pKK223-3 EcoRI and HindIII fragment), 100 units of T4 DNA ligase, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, and 25 μg/ml BSA.

Following the ligation reaction, the mixture (1 μl) was used to transform competent E. coli JM109 cells and plated on LB plates containing 0.01% ampicillin. Transformed colonies were isolated and grown overnight in YT containing 0.01% ampicillin and the plasmid DNA was purified using the Wizard Minipreps Kit (Promega). Purified plasmids were screened by the restriction endonuclease (BsaHI, Ndel, and HaeIII) digestion analysis to identify properly cloned molecules. Only the vectors containing dug gene were linearized, designated pKK-Dug, and subjected to DNA sequencing analysis. The primers, P1-33-mer and P2-33-mer, were used to determine the nucleotide sequence of the entire dug gene in pKK-Dug for both DNA strands. DNA sequencing was conducted using an Applied Biosystems model 373A by the Center for Gene Research and Biotechnology (Oregon State University).
2.2.8 Purification of Native Double-strand Uracil-DNA Glycosylase

Native double-strand uracil-DNA glycosylase was purified using a purification scheme similar to that described by Lindahl et al. (49) and modified by Bennett and Mosbaugh (124) for purifying *E. coli* Ung. *E. coli* NR8052 cells were grown at 37 °C in 40 L of YT medium. 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 in a GSA rotor at 6,000 rpm for 15 min at 4 °C, 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 DTT. The cells were then disrupted by sonification using a Dismembrator (Fisher, model 300) and the cell lysate was collected on ice and centrifuged in a SS34 rotor at 13,000 rpm for 20 min at 4 °C to pellet the cellular debris. Following the addition of 2 × 10^5 units of Ugi (fraction IV), the cell-free extract was mixed slowly with an equal volume of 1.6% (w/v) streptomycin sulfate in sonification buffer. After gentle stirring for 30 min at 4 °C, the precipitate was removed by centrifugation in a SS34 rotor at 13,000 rpm 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) and equilibrated for 10 min. Precipitated protein was removed by centrifugation in a SS34 rotor at 13,000 rpm for 20 min at 4 °C. The recovered supernatant fraction was then adjusted to 80% (saturation) with ammonium sulfate, equilibrated for 10 min, 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 cm² x 88 cm) equilibrated in UEB buffer, and eluted with the same buffer at a flow rate of ~20 ml/h. Fractions (5 ml) were collected and assayed for Ugi-insensitive uracil-DNA glycosylase.
activity on the \[^{32}\text{P}]U/G-34-mer heteroduplex DNA substrate. 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 DTT, 200 mM KCl), and designated fraction III. Fraction III (20 ml) was applied to a hydroxyapatite column (4.9 cm\(^2\) x 8 cm) equilibrated with HAB buffer. The column was eluted with the same buffer at a flow rate of ~30-40 ml/h 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 DTT, 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\(^2\) x 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 with a flow rate of ~20-30 ml/h. Fractions (3 ml) were collected and assayed for uracil-DNA glycosylase activity as before. 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 lacking NaCl, the preparation of native Dug (nDug) was designated fraction V, and stored at -80 °C.

2.2.9 Overproduction and Purification of Recombinant Double-strand Uracil-DNA Glycosylase

*E. coli* JM109 cells transformed with pKK-Dug were grown at 37 °C in 9 L of YT medium supplemented with 0.01% ampicillin. After reaching a cell density of ~6.5 \(\times\) 10\(^8\) cell/ml, 100 ml of 100 mM IPTG was added to induce *dug* gene expression and incubation was continued for an additional 4 h at 37 °C. Cells were harvested by centrifugation in a GSA rotor at 6,000 rpm for 15 min at 4 °C, and lysed by sonification as described in Section 2.2.8. The cell lysate was collected, pooled, and centrifuged in a SS34 rotor at 13,000 rpm for 20 min
at 4 °C. Ugi (0.24 ml, 3 x 10^6 units) was added to the cell-free extract (400 ml) to inactivate endogenous E. coli Ung activity. The volume of the supernatant was measured and an equal volume (390 ml) of 1.6 % streptomycin sulfate in the sonification buffer was slowly added by burette with stirring over a period of 30 min. After equilibration for an additional 30 min, precipitated material was removed by centrifugation in a SA600 rotor at 12,000 rpm for 15 min at 4 °C, and the supernatant was recovered and designated fraction I. Powdered ammonium sulfate was slowly added to this supernatant (~770 ml) to 40 % saturation and equilibrated for 10 min. The precipitate was removed by centrifugation in a SA600 rotor at 12,000 rpm for 15 min at 4 °C, and powdered ammonium sulfate was slowly added to the collected supernatant to 80 % saturation and equilibrated for 10 min. The precipitate was recovered by centrifugation as described above and resuspended in ~20 ml of UEB buffer, dialyzed overnight against the same buffer, and designated fraction II. The dialyzed sample was loaded onto a Sephadex G-75 column (6 cm^2 x 88 cm) equilibrated in UEB buffer and fractions (6.48 ml) were eluted with equilibration buffer at a flow rate of ~20 ml/h and assayed for Dug activity. Uracil- and ethenocytosine-DNA glycosylase activity were assayed using [32P]U/G- and [32P]eC/G-34-mer, respectively. Active fractions were pooled (~200 ml), concentrated ~10 fold using Centriprep-10 concentrator, and dialyzed against HAB buffer overnight; fraction III. The dialyzed sample was loaded onto a hydroxyapatite column (19.6 cm^2 x 3.5 cm) equilibrated in HAB buffer, fractions (6.48 ml) were eluted with equilibration buffer at a flow rate of ~30-40 ml/h and assayed for Dug activity. Active fractions were pooled (~200 ml), concentrated (~10 fold), and dialyzed against DAB buffer; fraction IV. The dialyzed sample was applied to a single-stranded DNA-agarose column (4.9 cm^2 x 16 cm) equilibrated in DAB buffer, washed with 150 ml of equilibration buffer, and eluted with a 350 ml of linear gradient of 0 to 600 mM NaCl in DAB buffer at a flow rate of ~20-30 ml/h. Fractions (5 ml) were collected, and 5 µl of aliquots from every three fraction were assayed for Dug.
activity and monitored for conductivity. Active fractions were pooled (~80 ml), concentrated (~8 fold), dialyzed against DEAE buffer (30 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 5% (w/v) glycerol), and designated fraction V. Fraction V (10 ml) was adjusted to 50 mM NaCl and loaded on the DEAE-Sephadex A50 column (4.9 cm² x 6 cm) equilibrated with DEAE buffer containing 50 mM NaCl. The column was washed with the same buffer (60 ml) and eluted with a 100 ml of linear gradient of 150 to 500 mM NaCl in DEAE buffer at a flow rate of ~20-25 ml/h. Fractions (3 ml) were collected, and the active fractions detected in the flow through were pooled (~21 ml), concentrated (~2 fold), dialyzed against DAB buffer, and the resulting preparation was designated Dug (fraction VI).

2.2.10 Electrophoresis

2.2.10.1 Nondenaturing Polyacrylamide Gel Electrophoresis

Analysis of protein and DNA interactions by band mobility shift assay was performed utilizing nondenaturing polyacrylamide gels (17 x 14 x 0.08 cm) composed of 6 % acrylamide and 0.2 % N,N’-methylenebis(acrylamide) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.0)). Polymerization was catalyzed by adjustment to 0.067 % (w/v) ammonium persulfate and 0.012 % (v/v) TEMED. Typically, reaction samples (5 μl) were combined with 1.5 μl of 50% sucrose solution to prepare the loading samples. The samples (5 μl) were loaded into the wells (6 x 8 x 8 mm) slowly to form a thin layer of sucrose/reaction mixtures. Samples (5 μl) containing 0.02% bromphenol blue in TE buffer were also applied on adjacent lanes of the same gel. Electrophoresis was conducted at room temperature and 140 V in TAE buffer until the bromphenol blue reached ~6 cm from the well. The gel was subsequently dried under vacuum and subjected to the autoradiography using X-OMAT AR5 film (Kodak) or PhosphorImager to visualize [³²P]DNA bands.
Nondenaturing 5% polyacrylamide sequencing gels (30 x 40 x 0.08 cm) were similarly prepared and employed in the analysis of *Hinfl* and *BsrI* digestion DNA fragments. The gel was composed of 5% acrylamide and 0.17% N,N'-methylenebis(acrylamide) in TBE buffer (900 mM Tris, 900 mM boric acid, 20 mM EDTA). Samples (10 μl) were mixed with 4 μl of loading buffer (50% sucrose, 0.01% bromphenol blue) prior to being loaded. Samples were electrophoresed at 500-700 V until the bromphenol blue reached 15 cm from the well. Gels were dried under vacuum and 32P radioactivity was monitored using a PhosphorImager.

2.2.10.2 Denaturing Polyacrylamide Sequencing Gel Electrophoresis

Analysis of DNA glycosylase activity and BER patch size distribution was performed using denaturing urea-polyacrylamide gels (30 x 40 x 0.08 cm) composed of 12% acrylamide, 0.40% N,N'-methylenebis(acrylamide), 8.3 M urea, and TBE buffer. Polymerization was catalyzed by adjustment to 0.067% (w/v) ammonium persulfate and 0.012% (v/v) TEMED. Samples were mixed with an equal volume of denaturing formamide dye buffer (95% deionized formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol) and heated at 95 °C for 3 min while the sample wells were washed with the running buffer (TBE buffer). Samples (10-15 μl) were electrophoresed at 1100-1200 V until the bromphenol blue tracking dye migrated ~15-20 cm for DNA glycosylase activity assay or ~20-25 cm during BER patch size analysis. The gels were removed from the glass plates by soaking in water for ~3 minutes to diffuse urea out of the gels and dried under vacuum. [32P]DNA bands were detected using a PhosphorImager.

2.2.10.3 Sodium Dodecyl Sulfate Polyacrylamide Slab Gel Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis was performed essentially as described by Laemmli (323). Slab gels contained
a resolving gel (17 x 12 x 1.5 cm) composed of various concentrations of 12.5% acrylamide and N,N'-methylenebis(acrylamide), 0.1% SDS, and 375 mM Tris-HCl (pH 8.8). The resolving gel was polymerized by adjustment to 0.03% (w/v) ammonium persulfate and 0.075% (v/v) TEMED. The stacking gel (17 x 2 x 1.5 cm) contained 3% acrylamide, 0.08% N,N'-methylenebis(acrylamide), 0.1% SDS, and 125 mM Tris-HCl (pH 6.8). The stacking gel was polymerized by adjustment to 0.10% (w/v) ammonium persulfate and 0.10% (v/v) TEMED. Protein samples were mixed with cracking dye mixture to achieve a final concentration of 250 mM Tris-HCl (pH 6.8), 0.5% SDS, 71.5 mM 2-mercaptoethanol, 5% (w/v) glycerol, and 0.02% bromphenol blue and boiled for 10 minutes before being loaded onto the gel. Electrophoresis was conducted at room temperature at 100 V until the tracking dye reached the resolving gel and then the voltage was increased to 200 V until the tracking dye migrated ~2 cm from the bottom of the gel. The gel running buffer contained 25 mM Trizma base, 192 mM glycine, and 0.1% SDS. Gels were fixed in a solution containing 10% acetic acid and 50% methanol and protein bands were visualized by staining in a solution of 10% acetic acid, 50% methanol, and 0.05% Coomassie brilliant blue G-250. Gels were destained in 7% acetic acid and 5% methanol. When mentioned, protein bands were visualized by silver staining by using ICN Rapid-Ag-Stain Kit according to manufacturer’s protocol.

Activity gel electrophoresis was similarly conducted except that the samples were mixed with cracking dye mixture containing 162 mM Tris-HCl (pH 6.8), 5.1 mM EDTA, 366 mM 2-mercaptoethanol, 18.7% (w/v) glycerol, 2.4% SDS (BDH) and 0.09% bromphenol blue. In addition, 2 mM EDTA was added to both the 3% stacking gel and 12.5% resolving gel and electrophoresis was performed at 4 °C. Following electrophoresis, the gel was cut in half vertically and one half of the gel was subjected to silver staining as before.
2.2.10.4 Agarose Gel Electrophoresis

Analysis of BER reaction products and the examination of plasmid DNA were performed using agarose slab gel electrophoresis. Agarose gel solutions (0.8, 1.0, or 1.5%) were prepared by heating electrophoresis grade agarose powder in a volume of TAE buffer (40 mM Tris-acetate and 1 mM EDTA (pH 8.0)) in a microwave for ~2-4 min. After cooling to the touch, ethidium bromide (500 \( \mu \text{g/ml} \)) was added to 0.2 \( \mu \text{g/ml} \), the mixture was poured into 7 x 8, 12 x 13, or 20 x 24.5 cm casting trays containing 10, 20, or 30 well teflon comb, respectively, and allowed to polymerize. Samples were combined with agarose dye buffer to a final concentration of 10 mM EDTA (pH 8.0), 0.1% SDS, 5% (w/v) glycerol, and 0.01% bromphenol blue prior being loaded onto the gel. Electrophoresis was carried out at 10 V/cm in TAE buffer containing 0.2 \( \mu \text{g/ml} \) ethidium bromide until the bromphenol blue tracking dye migrated ~50-75% of the distance of the gel. DNA bands were visualized by UV-light transillumination (~302 nm) and digital images of the gels were obtained using a Gel Documentation System (Image Store 7500, Ultra Violet Products).

2.2.11 Transfer of DNA to Nitrocellulose Membranes

In order to examine the incorporation of [\( \alpha ^{32P} \)]dAMP into M13mp2op14 DNA during BER, DNA reaction products resolved by 0.8% agarose gel electrophoresis were transferred onto Gene Screen Plus (NEN) membranes utilizing a downward alkaline blotting technique (324). Following electrophoresis, agarose gels (7 x 8 x 0.5 cm) were incubated for 15 min in 50 ml of 0.25 M HCl until the bromphenol blue tracking dye turned yellow and then soaked in 50 ml of 0.4 M NaOH for ~5 min. The transfer apparatus was prepared by placing a stack of paper towels in a Pyrex baking dish. On top of the paper towels, three gel-sized Whatman No. 1 filters were positioned and the top filter was soaked in the transfer solution (0.4 M NaOH). Next, a gel-sized sheet of Gene Screen Plus membrane soaked in 0.4 M NaOH was placed,
followed by the agarose gel. On top of the gel, two additional gel-sized Whatman No. 1 filters soaked in 0.4 M NaOH were placed followed by two long, wet blotting strips of Whatman No. 1 filter paper used to wick the transfer solution from two 0.4 M NaOH reservoirs positioned on either side of the transfer apparatus. A glass plate (19 x 20 cm) was positioned on top to secure the gel and promote even distribution of the transfer solution. Air bubbles were carefully removed from each layer of the transfer apparatus during construction. Transfer of DNA was allowed to proceed overnight, the filter was dried at room temperature, and $^{32}$P]DNA bands were visualized using a PhosphorImager.

2.2.12 Enzyme Assays

2.2.12.1 Uracil-DNA Glycosylase Activity Assay

Ung activity in E. coli cell-free extracts was measured in a reaction mixture (100 μl) containing 70 mM Hepes-KOH (pH 8.0), 1 mM EDTA, 1 mM DTT, 8.2 nmol of activated calf thymus [uracil-$^3$H]DNA (180 cpm/pmol of uracil). E. coli cell extract was serially diluted in R-buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol), and 25 μl of each sample was included in the reaction. The reaction mixture was mixed on ice and incubated for 30 minutes at 37 °C. Reactions were terminated on ice by addition of 250 μl of ice cold 10 mM ammonium formate (pH 4.2). The terminated reaction (300 μl) was applied to a Dowex 1-X8 ion exchange column (0.2 cm$^2$ x 2.0 cm) equilibrated in 10 mM ammonium formate (pH 4.2). The column was washed with 1.7 ml of 10 mM ammonium formate to resolve free $^3$H]uracil from [uracil-$^3$H]DNA and two 1 ml fractions were collected. Fractions were combined with 5 ml of Formula 989 Fluor, mixed by inversion, and $^3$H radioactivity was measured in a Beckman (LS6800) liquid scintillation counter. One unit of uracil-DNA glycosylase activity is defined as the amount
of enzyme, which released one nmol of uracil in 60 min using the standard conditions.

2.2.12.2 Double-strand Uracil-DNA Glycosylase Activity Assay

Uracil- and ethenocytosine-DNA glycosylase activities of Dug were determined utilizing 5'-end $^{32}$P-labeled single-stranded U- and εC-34-mers, and double-stranded U/A-, U/G-, εC/A-, and εC/G-34-mers with the 5'-end $^{32}$P-label positioned in the substrate containing DNA strand. When indicated, 5'-end $^{32}$P-labeled T/G-34-mer was applied to examine thymine-DNA glycosylase activity. Standard reaction mixtures (10 μl) used for detecting DNA glycosylase activity contained 25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 50 mM KCl, 0.01 mM ZnCl$_2$, 0.1 mg/ml acetylated bovine serum albumin, 0.1 (or 0.2) pmol of various 5'-end $^{32}$P-labeled 34-mers, and various amounts of the enzyme (2 μl) as indicated in the Figure Legends. Where appropriate, enzyme samples were diluted with DAB buffer. Reactions were incubated at 30 °C for 30 min and then subjected to treatment with either *E. coli* endonuclease IV or hot alkali to cleave the AP-sites generated by base removal from DNA substrates. To conduct enzymatic cleavage of AP-sites, the reactions were terminated by heating at 70 °C for 3 min and then incubated with 0.1 units of endonuclease IV (1 μl) for 30 min at 30 °C. Endonuclease IV was then inactivated by incubation at 70 °C for 3 min. For the chemical cleavage of AP-sites, 5 μl of each reaction was removed and quenched by supplementation with 2.5 μl of buffer containing 0.3 M NaOH and 30 mM EDTA, and then heated at 90 °C for 30 min. Samples were combined with an equal volume of denaturing formamide dye buffer and analyzed using denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described in Section 2.2.10.2. The gel was then imaged by a PhosphorImager (Molecular Dynamics) and the amount of $^{32}$P-labeled substrate (34-mer) and product (15-mer) oligonucleotide bands were quantified using ImageQuant 5.0 software (Molecular Dynamics) after
subtracting background intensity of the image. 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.

During the purification of native Dug, Ugi-insensitive uracil-DNA glycosylase activity was determined using 5'-end $^{32}$P-labeled U/G-34-mer in the reaction mixture (100 μl) containing 25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 50 mM KCl, 0.01 mM ZnCl$_2$, 0.1 mg/ml acetylated bovine serum albumin, 20 μl of each fraction, and 4 pmol of $^{32}$P[U/G-34-mer. Ugi (1000 units) was also included in the reaction mixtures to inhibit possible Ung activity, and the reactions were incubated at 30 °C for 16 h. During the purification of recombinant Dug, the enzymatic activity was assayed for both uracil- and ethenocytosine-DNA glycosylase using $^{32}$P[U/G- and $^{32}$P]C/G-34-mers, respectively, and the reaction was performed similarly as described above except (i) reaction mixtures contained 5 μl of each fraction; (ii) 0.1 pmol of $^{32}$P34-mer was used as substrates (iii) incubation occurred at 30 °C for 30 min. After incubation, the reaction was terminated with an equal volume of a stop solution containing 2% SDS and 50 mM EDTA. Samples were adjusted to a final concentration of 0.3 mg/ml yeast tRNA and 2 M ammonium acetate, extracted twice with an equal volume of phenol/chloroform (50:50), ethanol-precipitated, and resuspended in 20 μl of distilled H$_2$O. DNA 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.

2.2.12.3 DNA Polymerase Activity Assay

E. coli DNA polymerase I activity was measured in reaction mixtures (100 μl) containing 67 mM Tris-HCl (pH 7.5), 6.7 mM MgCl$_2$, 1 mM DTT, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM each of dATP, dTTP, dCTP, 5 mM phosphocreatine di-Tris salt, 200 units/ml phosphocreatine kinase, 10 μg of activated calf DNA, 24 pmol of $^3$H]dTTP (593 cpm/pmol). Exogenous E. coli
DNA polymerase I (2 μl) was diluted in the enzyme storage buffer (0.1 M potassium phosphate (pH 6.5), 1 mM DTT, 50% (w/v) glycerol) and introduced as the sample (25 μl). Where appropriate, various concentrations of antiserum (8 μl) were introduced into the reaction prior to the addition of substrate and incubated on ice for 5 min. After incubation at 30 °C for 60 min, reactions were terminated on ice by addition of 200 μl of 1 mg/ml BSA in 0.1 M sodium pyrophosphate. DNA was precipitated with 1 ml of 10% (saturated) trichloroacetic acid (0.30 g/ml) and held on ice for 5 min. Acid-insoluble DNA was collected on #30 glass fiber filters (Schleicher and Schuell) pre-soaked in 0.1 M sodium pyrophosphate, washed with 18 ml (six times with 3 ml applications) of 0.1 M sodium pyrophosphate in 1 M HCl, and dried with 95% ethanol. Glass filters were dried under a heating lamp, placed into glass vials containing 10 ml of 0.4 % 2,5-bis-2-(5-tertbutylbenzoaxazolyl)-thiophene (BBOT) in toluene, and [3H]dTMP incorporation into DNA was measured using a Beckman LS6800 liquid scintillation spectrometer.

2.2.13 Determination of Molecular Weight of Double-Strand Uracil-DNA Glycosylase

2.2.13.1 Activity Gel Analysis

Separation of the enzyme by SDS-polyacrylamide gel electrophoresis, extraction of SDS from the gel, and the renaturation of the protein were performed similarly as described by Longley and Mosbaugh (325) with some modifications. Purified native Dug (fraction V, 60 μl, 2.2 μg) or recombinant Dug (fraction VI, 60 μl, 6.3 μg) was combined with 30 μl of cracking dye mixture (162 mM Tris-HCl (pH 6.8), 5.1 mM EDTA, 366 mM 2-mercaptoethanol, 18.7% (w/v) glycerol, 2.4% SDS (BDH) and 0.09% bromphenol blue), and 40 μl of samples were loaded onto a 12.5% SDS-polyacrylamide gel along with prestained protein molecular weight markers containing phosphorylase b (Mr, 111,000), BSA (Mr, 77,000), ovalbumin (Mr,
48,200), carbonic anhydrase ($M_r$ 33,800), trypsin inhibitor ($M_r$ 28,600), and lysozyme ($M_r$ 20,500). Following electrophoresis, the gel was dismantled on the clean glass plate placed on ice and cut in half between the sample sets. The length of the gel to the locations of tracking dye and each of prestained protein marker bands were measured, and one half of the gel was subjected to the silver staining. The other half of the gel was placed in a clean Pyrex dish containing 10 ml of SDS extraction buffer (10 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 25% (v/v) isopropanol). SDS extraction was carried out for 30 min at room temperature with gentle agitation. The buffer was exchanged once and the extraction was continued for an additional 30 min. The gel was then placed on the clean glass plate and sliced horizontally into 3.5 mm segments using Gel Cutter apparatus (Hoefer). Each gel slice was transferred into 12 x 17 mm glass tube, chopped with a glass rod, and soaked in the 500 µl of renaturation buffer (25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 50 mM KCl, 0.01 mM ZnCl$_2$, 0.1 mg/ml acetylated bovine serum albumin). Renaturation of the enzyme was allowed for 14 h at 4 °C with gentle shaking. Samples (40 µl) from each tube were subjected to Dug activity assay. A standard curve was generated based on the relative mobility of prestained molecular weight maker proteins and the molecular weight of the protein, Dug, was determined from the $R_f$ for the mid point of the gel slice that contained Dug activity.

2.2.13.2 Sucrose Density Gradient Centrifugation

The native molecular weight of Dug was determined by centrifugation through a linear 0-30% sucrose gradient containing 30 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 1 mM DTT, and 1 mM EDTA. A sample (200 µl) containing nDug (fraction V, 6.5 ng) and 1 mg of BSA and a control sample containing Ung (5000 units) and 1 mg of BSA were layered onto the gradients. Centrifugation was performed in a SW 50 rotor (Beckman) at 40,000 rpm for 24 hours at 4°C. Following centrifugation, fractions (7 drops, ~200 µl) were collected from the
bottom of the gradient using a Buchler Instruments gradient fractionator. The location of BSA was determined from reading the absorbance at 280 nm with a micro-plate reader (Spectra Max GEMINI, Molecular Devices). The location of the enzyme was determined using the Dug activity assay as described in Section 2.2.12.2. The sedimentation coefficient (S) of Dug was calculated based on that of BSA (4.7 S) and applied to the following formula to determine the molecular weight (MW) of the enzyme: 

\[
\frac{S_{\text{BSA}}^{1.5}}{S_{\text{Dug}}^{1.5}} = \frac{\text{MW of BSA} (68,000)}{\text{MW of Dug}}.
\]

2.2.13.3 Matrix-assisted Laser Desorption/Ionization Mass Spectrometric Analysis

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 recombinant Dug (fraction VI, 0.16 mg/ml) was mixed with 3 µl of 4-hydroxy-o-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.

2.2.14 Analysis of Dug-DNA Interactions

2.2.14.1 Band Mobility Shift Assay

Standard DNA binding reaction mixtures (10 µl) contained 25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 50 mM KCl, 0.01 mM ZnCl₂, 0.1 mg/ml acetylated bovine serum albumin, 10 nM of various ³²P-labeled DNA probes, and various amounts of the enzyme (2 µl). During the competition analysis of Dug binding to DNA, the reaction mixture was
supplemented with 400 nM of various unlabeled oligonucleotide DNA prior to the addition of Dug. DNA binding reaction was carried out at 30 °C for 30 min, samples (5 μl) mixed with 1.5 μl of 50% sucrose, and analyzed by nondenaturing 6% polyacrylamide gel electrophoresis. [32P]DNA bands were detected by a PhosphorImager and the amounts of free (unbound) and mobility-shifted (bound) 32P-labeled oligonucleotide were measured using ImageQuant 5.0 software after subtracting the background value.

2.2.14.2 Evanescent Wave Biosensor Analysis

Evanescent wave biosensor experiment was carried out with IAysys plus optical biosensor (Affinity Sensors) using the following instrument parameters: sampling interval; 0.3 sec, experiment temperature; 24 °C, and stirrer; 100. The IAysys cuvette containing the reaction cell of a biotin-coated sensor chip was placed in the instrument, and the surface was hydrated with PBST buffer (10 mM sodium phosphate (pH 7.4), 138 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween 20) by incubation with 50 μl buffer for 10-15 min. The reaction cell was washed by three repeat replacement with PBST buffer (80 μl) and equilibrated in the same buffer (50 μl) for ~5 min. The quality of the resonance was checked for each experiment by examining the resonance scan plot of a single clear peak with symmetrical skirts.

A streptavidin stock (1 mg/ml) was prepared in PBST buffer, 2 μl of the stock was applied into the reaction cell to a final concentration of 38 μg/ml, and binding to the biotin surface was allowed to occur for 10-15 min. The reaction cell was washed with 80 μl of PBST buffer three times. Typically the response signal of 500-700 units (arc sec) was obtained. The reaction cell containing a streptavidin-coated surface was then equilibrated in 50 μl of PBST buffer and various biotinylated oligonucleotides (9 μl from 100 μg/ml stock) were injected over the streptavidin-coated sensor chip to achieve ~200-300 response units. The reaction cell was washed with DAB buffer, 3 times with 80 μl each, to exchange the binding reaction buffer and then was
equilibrated in the same buffer (50 µl). When indicated, the reaction cell was equilibrated in the 100 mM NaCl DAB buffer. A dilution series of Dug was prepared in the same buffer and various amounts of Dug (2 µl) were injected into the reaction cells. Binding was allowed to continue until the proteins reached equilibrium (saturation) binding with the immobilized oligonucleotide DNA, and then 50 µl of equilibration buffer was added to the reaction cell. Association and dissociation of the Dug-DNA complex were monitored as a real-time change in the refractory index (arc sec) and plotted using IAsys Plot software.

After each round of binding reactions, the protein that remained bound to DNA was removed by washing (3 x 80 µl) with DAB buffer containing 500 mM NaCl to restore the reaction cell surface before the next round of binding reactions. This procedure did not alter, to any measurable extent, the ability of the immobilized DNA to bind the protein in subsequent cycles. Following the experiment using each of immobilized oligonucleotide DNA, the reaction cells were treated with 80 µl of 3 M KOH for 1 min to regenerate the biotin-coated surface, washed extensively with PBST buffer (10 x 80 µl), and subjected to the immobilization of other oligonucleotides as described above.

Analysis of the data was performed using the evaluation software (FASTfit) supplied with the instrument. In this program, a non-linear least squares method was used for the determination of the rate binding constants. The pseudo-first order on-rate ($k_{on}$) values were calculated from the association phases at various concentrations of Dug by the following equation: $R_t = R_{eq} (1 - \exp(-k_{on}t))$, where $R_t$ is the baseline-corrected response and $R_{eq}$ is the response at equilibrium binding. The association and dissociation rate constants ($k_a$ and $k_d$, respectively) were determined from the linear plot of $k_{on}$ versus [Dug], as the slope and the y intercept, respectively. The equilibrium constant ($K_D$) was calculated from the two rate constants by the following equation: $K_D = k_d/k_a$. 
2.2.15 Preparation of E. coli Cell-free Extracts

E. coli cell-free extracts were prepared from E. coli strain NR8051, NR8052, NR80511, NR80521, GM31, BH156, BH157, and BH158. E. coli cells were grown at 37 °C in 500 ml of YT medium containing the appropriate antibiotic to mid-log phase (OD_950nm = 0.8) and harvested by centrifugation in a GSA rotor at 7,000 rpm for 15 min at 4 °C. The cell pellet was resuspended in 20 ml of sonication buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 mM DTT), and cells were lysed by sonification as described in Section 2.2.8. After cell debris was removed by centrifugation in a SS34 rotor at 13,000 rpm for 20 min at 4 °C, protein was precipitated from the supernatant by addition of 0.35 g of powdered ammonium sulfate per ml and recovered by centrifugation in a SS34 rotor at 13,000 rpm for 20 min at 4 °C. The pellet was resuspended in 2-5 ml of R-buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol, transferred into SpectraPor dialysis tubing (6-8,000 MWCO, 6.4 mm diameter), and dialyzed for ~2 h against ~500 ml of R-buffer. The buffer was exchanged once and dialysis was continued for an additional 8-12 h. The dialysate was transferred to Eppendorf tubes (0.5 ml) and stored in small aliquots (100 μl) at -80 °C. Each aliquot was used only once, without refreezing. The concentration of cell-free extract protein was determined by the Bradford reaction using Bio-Rad Protein Assay as described in Section 2.2.5.

2.2.16 Preparation of M13mp2 DNA Base Excision Repair Substrates

2.2.16.1 Primer Extension Reaction of M13mp2op14 DNA

Single-stranded M13mp2op14 DNA was isolated and oligonucleotide primers, U-23-mers (for U-G and U-T DNA) and A-23-mer (for A-T DNA), were 5'-end phosphorylated in a large scale as described previously in Section 2.2.2.2. Each primer was annealed to M13mp2op14 DNA in a reaction mixture
containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, and 50 mM NaCl, 750 pmol of U (U·T or U·G)-23-mer or A-23-mer, and 250 pmol of M13mp2op14 DNA. The mixtures were placed in a 500 ml beaker filled with 70 °C distilled H₂O and slowly cooled to room temperature (~4 h). The heteroduplex U(U·T)-23-mer/M13mp2op14 DNA and U(U·G)-23-mer/M13mp2op14 DNA substrates formed a U/T mispair at nucleotide position 78 and U/G mispair at nucleotide position 79 of the lacZα gene, respectively, whereas the homoduplex A-23-mer/M13mp2op14 DNA substrate formed an A/T basepair. Heteroduplex [³²P]U-23-mer/M13mp2op14 DNA substrate was also prepared by using 5'-end ³²P-labeled U-23-mer followed by hybridization to M13mp2op14 DNA as described above.

Primer extension reaction mixtures (3030 µl) were prepared containing 200 pmol of primed M13mp2op14 DNA, 20 mM Hepes-KOH (pH 7.8), 2 mM DTT, 10 mM MgCl₂, 1 mM ATP, 500 µM each of dATP, dTTP, dCTP, and dGTP, 400 units of T4 DNA polymerase, and 40,000 units of T4 DNA ligase. Individual components of the reaction mixture contributed to a specific percentage of the overall reaction volume and were strictly enforced. The DNA addition constituted 20 % of the reaction mixture volume while the T4 DNA polymerase and T4 DNA ligase additions contributed 8.6 % and 13.2 %, respectively. Enzyme stocks were diluted in T4 DNA polymerase dilution buffer (20 mM Tris-HCl (pH 8.1), 50 mM NaCl, 2 mM DTT, 50 % (w/v) glycerol) or T4 DNA ligase dilution buffer (10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 200 µg/ml acetylated bovine serum albumin, 50 % (w/v) glycerol). After incubation for 5 min on ice and 5 min at 25 °C, the primer extension reaction was carried out for 4 h at 37 °C. The reaction was terminated with the addition of 535 µl of 0.1 M EDTA. An aliquot (2 µl) of the terminated reaction mixture was analyzed using 0.8 % agarose gel electrophoresis, and the efficiency of the reaction was determined from the yield of form I DNA.
2.2.16.2 Isolation of M13mp2op14 Form I DNA by Ethidium Bromide/Cesium Chloride Gradient Centrifugation

Covalently closed circular duplex DNA products (Form I DNA) from the primer extension reactions described above were isolated by ethidium bromide-cesium chloride gradient centrifugation as described elsewhere (314, 326). A sample mixture was prepared by dissolving optical grade CsCl (18.12 g) in ~15 ml of TE buffer, combined with the terminated primer extension reaction (3565 µl), adjusted to a final volume to 23.12 ml with TE buffer, and supplemented with 1.88 ml of ethidium bromide (10 mg/ml) to achieve a final volume of 25 ml. The final concentrations of CsCl and ethidium bromide were 0.725 g/ml and 0.752 mg/ml, respectively.

The sample was divided and transferred into two 12.5 ml polyallomer centrifuge tubes (Beckman) fitted for an SW41 ultracentrifuge rotor (Beckman). Centrifugation was performed at 39,000 rpm for 60 h at 20 °C without braking at the deceleration step, and the form I DNA was collected from the centrifuge tubes using a Precision Glide hypodermic needle (18G1 1/2 gauge) by carefully inserting the needle underneath the form I DNA band through the wall of the tube. The form I DNA (~800 µl) was collected, extracted five times with an equal volume of 1-butanol saturated with 5 M NaCl, concentrated (~10-fold) using a Centricon-30 concentrator, and buffer exchanged into TE buffer. The concentration of purified form I DNA was determined by absorbance spectroscopy (1 OD_{260nm} = 50 µg/ml).

2.2.17 Preparation of Plasmid DNA Base Excision Repair Substrates

2.2.17.1 Construction of pBIG Plasmid DNA

DNA fragment (450-bp) encoding an N-terminal domain of kanamycin resistance gene product was amplified from pET-30a plasmid DNA using the polymerase chain reaction and utilized to construct pBIG plasmid DNA (3.65-kb). PCR reaction mixtures (100 µl) contained 20 mM Tris-HCl (pH 8.8), 2 mM
MgSO$_4$, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 0.1% Triton X-100, 0.2 mM each of dATP, dGTP, dCTP, dTTP, 0.04 pmol of pET-30a plasmid, 2 units of Vent DNA polymerase, 100 pmol each of F-33-mer and R-33-mer oligonucleotide primer. The PCR reaction was conducted by a Stratagene RoboCycler using the following cycle parameter: 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min; 72 °C for 10 min. The resulting PCR reaction product (30 µl) was digested with 60 units of EcoRI at 37 °C for 1 h in a reaction (40 µl) containing 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl$_2$, 0.025% Triton X-100. pGEM plasmid DNA (2 pmol) was also restricted with EcoRI as described above, the reaction (40 µl) was terminated by heating at 70 °C for 5 min, and 5' phosphoryl groups were removed from the plasmid by treatment with calf intestine alkaline phosphatase (1 unit) for 30 min at 37 °C. The resulting PCR and pGEM DNA products were analyzed on the 1.5% and 1% agarose gel, respectively, and isolated from the gels using a QIAquick Gel Extraction Kit (Qiagen). Ligation of the two DNA fragments was performed in the reaction mixture (10 µl) containing a ~3 fold excess of insert (450-bp fragment) over vector (pGEM), as described in Section 2.2.7, and the resulting construct was used to transform competent E. coli JM109 cells and plated on LB plates containing 0.01% ampicillin. Transformed colonies were isolated and grown overnight in LB containing 0.01% ampicillin. The plasmid pBIG DNA was purified using a Stratagene Plasmid Miniprep Kit and size verified using 1% agarose gel electrophoresis analysis.

2.2.17.2 Primer Extension Reaction of pGEM and pBIG DNA

Oligonucleotide primers, U-, εC-, and C-23-mers, were phosphorylated, as described in Section 2.2.2.2, and annealed to the single-stranded pGEM or pBIG phagemid DNA. Annealing reaction was performed in a reaction mixture (758 µl) containing 20 mM Tris-HCl (pH 7.4), 2 mM MgCl$_2$, and 50 mM NaCl, 500 pmol of primers, and 250 pmol of pGEM or pBIG DNA to produce heteroduplex U-23-mer/pGEM, U-23-mer/pBIG, εC-23-mer/pGEM,
and C-23-mer/pBIG, or homoduplex C-23-mer/pGEM and pBIG DNA substrates. [\(^{32}\)P]C-23-mer/pGEM and [\(^{32}\)P]C-23-mer/pGEM substrates were prepared by annealing 5'-[\(^{32}\)P]-end labeled corresponding primer to pGEM or pBIG single-stranded DNA as described above.

Primer extension reactions were performed essentially as described in Section 2.2.16.1 except that T4 DNA ligase (2000 units) was introduced into the primer extension reaction mixtures (3030 \(\mu\)l) as a 50-fold unit excess over T4 DNA polymerase (400 units). Reaction mixtures were prepared on ice and T4 DNA polymerase and T4 DNA ligase were added as the second to last and final additions, respectively. The reactions were incubated for 4 h at 37 °C and terminated with the addition of 535 \(\mu\)l of 0.1 M EDTA. An aliquot (1-2 \(\mu\)l) of the terminated reaction mixture was analyzed using 1% agarose gel electrophoresis.

### 2.2.17.3 Isolation of pGEM and pBIG Form I DNA by Ethidium Bromide/Cesium Chloride Gradient Centrifugation

The terminated primer extension reaction was mixed with 15.16 g of optical grade CsCl and adjusted to a final volume of 19.2 ml with TE buffer. Ethidium bromide (0.8 ml from 10 mg/ml stock) was added to the CsCl mixture to a final concentration of 400 \(\mu\)g/ml. The mixture was divided and loaded onto four 5 ml Quick-Seal polyallomer ultracentrifuge tubes (Beckman) and each tube was then filled with the CsCl solution prepared by dissolving 3.79 g of CsCl in 5 ml of TE buffer. After the tops of centrifuge tubes were sealed using a Seal Former (Beckman), centrifugation was performed in a VTi80 rotor (Beckman) at 50,000 rpm for 14 h at 20 °C and the deceleration step occurred without braking. DNA bands were visualized by brief UV-shadowing, the location of form I DNA was marked on the tube, and form I DNA was collected using a hypodermic needle (22G11/2 gauge). The Form I DNA (~500 \(\mu\)l) was removed, extracted five times with an equal volume of 1-butanol saturated with 5 M NaCl, concentrated (~10-fold) using a Centricon-
30 concentrator, and buffer exchanged into TE buffer. The concentration of purified form I pGEM and pBIG DNA was determined by absorbance spectroscopy (1 OD_{260nm} = 50 \mu g/ml).

2.2.18 Base Excision DNA Repair Reactions

Standard BER reactions were performed in reaction mixtures (100 \mu l) containing 100 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 1 mM DTT, 0.1 mM EDTA, 2 mM ATP, 0.5 mM \beta-NAD, 20 \mu M each of dATP, dTTP, dGTP, and dCTP, 5 mM phosphocreatine di-Tris salt, and 200 units/ml of phosphocreatine kinase. When M13mp2 DNA was employed as the BER reaction substrate, 10 \mu g/ml of M13mp2op14 (U:T or U:G) heteroduplex or (A:T) homoduplex DNA (form I) and 1 mg/ml of E. coli cell-free extract protein were included in the reaction. When pGEM plasmid DNA was utilized as the BER substrate, 2 mg/ml of E. coli cell-free extract protein and 20 \mu g/ml of pGEM form I DNA containing a site-specific U/G or \epsilon C/G base mispair, or C/G base pair were added to the reaction mixtures. For the lesion specific BER-competition assays, similar reaction mixtures were prepared except that 10 \mu g/ml each of pGEM (U:G or \epsilon C:G) and pBIG (U:G or \epsilon C:G) form I DNA was included as substrate. In some experiments, the reaction mixtures were supplemented with various amounts of additional protein (Ugi, E. coli Ung, endonuclease IV, DNA polymerase I, DNA ligase, and Fpg, or antiserum), as indicated in the Figure Legends. In all cases, reaction mixtures were prepared on ice, and the BER reaction was initiated by the addition of DNA substrate and incubation occurred for various times at 30 °C. After incubation, the reactions were terminated by adjustment to 20 mM EDTA (25 \mu l from 100 mM stock) and heated at 70 °C for 3 min. RNase A (1 \mu l) was then added to 80 \mu g/ml, and the reaction mixtures were incubated at 37 °C for 10 min. Each reaction was then adjusted to 0.5% SDS (126 \mu l from 1% stock), proteinase K (5 \mu l from 10 mg/ml stock) was added to 190 \mu g/ml, and incubated for 30 min at 37 °C. The samples were subsequently extracted twice

2.2.19 Analysis of Base Excision DNA Repair Reaction Products

2.2.19.1 Treatment of Base Excision DNA Repair Reaction Products to Remove Unreacted DNA Substrate

BER reaction products (M13mp2op14, pGEM, and/or pBIG DNA) isolated as described in Section 2.2.18 were subjected to the treatment with Ung and endonuclease IV (Ung/Endo IV) for U-G DNA or Dug and endonuclease IV (Dug/Endo IV) for εC-G DNA, and the combined treatment of Ung, Dug, and endonuclease IV (Ung/Dug/Endo IV) during a lesion specific BER-competition assay for both U-G and εC-G DNA. Ung and Dug were prepared in DAB buffer, and endonuclease IV was diluted in a buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% (w/v) glycerol. The treatments were conducted by incubation of 80-100 ng of BER reaction product DNA with an excess amount of the enzymes for 30 min at 37 °C, as indicated in the Figure Legends. Reactions were terminated by heating at 70 °C for 3 min, and the resulting form I and II DNA were resolved by agarose gel electrophoresis.

2.2.19.2 Quantification of Form I and II DNA

Standard M13mp2op14 form I DNA was prepared by serial dilution of M13mp2op14 (U·G or U·T) form I DNA with TE buffer to final concentrations of 0.78, 1.56, 3.13, 6.25, and 12.5 ng/μl. To prepare the standard form II DNA, 0.8-1 μg of M13mp2op14 (U·G or U·T) DNA was treated with Ung (400 units) and endonuclease IV (4 units) at 37 °C for 1 h. The resulting form II DNA was then serially diluted with TE buffer to final concentrations of 0.78, 1.56, 3.13, 6.25, and 12.5 ng/μl. Each form I and II DNA samples (80 μl) were then
combined with 20 µl of agarose dye buffer (50 mM EDTA (pH 8.0), 0.5% SDS, 25% (w/v) glycerol, and 0.05% bromphenol blue), and the mixtures (10 µl) containing 6.25, 12.5, 25, 50, and 100 ng of form I or II DNA were analyzed on the same agarose gel with BER DNA reaction products. Standard pGEM and pBIG form I and II DNA was also prepared similarly except that the dilution of DNA was conducted to generate the final preparations (10 µl) containing 10, 20, 40, and 80 ng of form I and II DNA. For the lesion specific BER-competition assay, DNA standards were produced as before but using the mixture of equal amount of pGEM and pBIG DNA. BER reaction products were analyzed using agarose gel electrophoresis, and the amount of ethidium bromide stained form I and II DNA bands was determined against form I and II standards from the same gel using a Gel Documentation System and ImageQuant software.

2.2.20 Isolation of Repaired Form I DNA

Samples of repaired M13mp2op14 (U·T) DNA were determined from BER reactions and subjected to the Ung/Endo IV treatment as described in Section 2.2.19.1. Form I DNA that was insensitive to the Ung/Endo IV cleavage was isolated by agarose gel electrophoresis. After Ung/Endo IV treatment, DNA samples were combined with agarose dye buffer to a final concentration of 0.1 % SDS, 10 mM EDTA, 5 % (w/v) glycerol, and 0.01 % bromphenol blue and loaded onto two lanes on the 0.8% agarose gel. Following electrophoresis, one-half of the gel containing one sample lane was cut out and the repaired form I DNA was visualized by UV-light transillumination (~302 nm). The form I DNA band was excised to mark the location of the form I DNA. This gel was then aligned to the other half of the gel that was not exposed to the UV light. The repaired form I DNA band on the other half of the gel was excised from the gel with a clean razor blade and placed into the elution chamber of an Elutrap apparatus containing TAE buffer. Electroelution of was performed at 150 V (50-60 mA) in TAE buffer for
3 h at room temperature. Following electroelution, the current was reversed for \(~15\) sec and the DNA solution \((~1 \text{ ml})\) was removed from the elution chamber, transferred into a Centricon-30 (Amicon) concentration device, concentrated \((~5\)-fold\)), and buffer exchanged with distilled H$_2$O.

2.2.21 Transfection of \(E.\ coli\) with M13mp2op14 Form I DNA and Determination of Reversion Frequencies

\(E.\ coli\) NR9162 cells were transfected with repaired and purified Form I DNA recovered from the BER reactions. Form I DNA samples \((0.5\text{-}2.5 \mu l)\), isolated as described in Section 2.2.20, were mixed with 50 \(\mu l\) of competent \(E.\ coli\) or NR9162 cells \((\sim 1\text{-}2 \times 10^{11} \text{ cells/ml})\) in \(10\%\) (w/v) glycerol and incubated on ice for 1-2 min. Transfection was conducted using a Gene Pulser Electroporation System (BioRad) with a single pulse set at \(2.0\) kV, \(25 \mu F\) capacitance, and \(400\ \Omega\). Transfected cells were serially diluted into SOB media \((1/10^2 \text{ to } 1/10^7)\) and 100 \(\mu l\) of each diluted sample was placed into a sterile 13 x 100 mm test tube, mixed with 500 \(\mu l\) of mid-log \(E.\ coli\) CSH50 cells and prewarmed M9 top agar \((2.5 \text{ ml})\) containing 0.4 mM IPTG and 1 mg/ml X-Gal was added. The cells were plated on M9 plates and incubated overnight at \(37\) °C. One diluted sample of each transfection experiment was increased in volume \((~3.0 \text{ ml})\) to allow for the mass plating of \(~25\) individual M9 plates. A separate titration series was prepared for each transfection experiment in order to quantify the number of plaques detected for each mass plating. Typical dilutions for mass platings yielded a concentration of 1,000 to 3,000 plaque forming units per plate. After scoring plaques as either colorless or blue, the reversion frequency was calculated from the ratio of the number of blue plaques to total (colorless plus blue) plaques detected.

2.2.22 Determination of Mutational Spectrum

For the secondary screening of the mutant phages, blue plaques were picked, placed into 200 \(\mu l\) of sterile 0.9 \% NaCl solution, and extracted phage
were diluted into SM media and titered against mid-log E. coli CSH50 cells on M9 plates with IPTG and X-Gal as described in Section 2.2.21. An individual blue plaque was picked by touching a P10 pipet tip (Pipetman) to the surface of the plaque so as to extract a mini-agarose plug approximately 0.3 mm long. This material was then injected into an Eppendorf microcentrifuge tube containing 100 µl of PCR reaction mixture comprising 1 unit of Deep Vent DNA polymerase, Thermopol buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgCl2, 0.1% Triton X-100), 200 µM each of dATP, dTTP, dCTP, dGTP, and 100 pmol each of the lacZα forward primer (FP-18-mer) and reverse primer (RP-18-mer). PCR was carried out in a Hybaid PCR Express Gradient thermocycler under active temperature control (150 µl of mineral oil in the temperature reference tube) using the following cycling parameters: 94 °C (1 min), 30 cycles of 94 °C (1 min), 45 °C (1 min), and 72 °C (3 min), and 72 °C (10 min). PCR products were purified with a StrataPrep PCR Purification Kit (Stratagene) following the manufacturer's instructions. Approximately 70 µl of double-stranded DNA (30-70 µg/ml) was obtained from the material extracted from a single M13 plaque. An aliquot of the purified DNA product (~30 ng) together with 12 pmol of sequencing primer (S-21-mer) complementary to the (+) strand DNA of the M13mp2op14 lacZα gene was used for DNA sequence analysis conducted by the Center for Gene Research and Biotechnology (Oregon State University).

2.2.23 Analysis of Specificity of Repair DNA Synthesis

2.2.23.1 Hinfl Restriction Analysis

Uracil-initiated BER-specific DNA synthesis on M13mp2op14 DNA was determined by utilizing restriction endonuclease Hinfl. Standard BER reactions were conducted in the presence of 400 µCi/ml [α-32P]dATP (6,000 cpm/mmol). DNA reaction products were isolated as described in Section 2.2.18 and subjected to Hinfl restriction analysis. Samples (~100 ng) were
included in a reaction mixture (10 μl) containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 5 units of Hinfl. After incubation for 1 h at 37 °C, the DNA restriction fragments were resolved by 5 % non-denaturing polyacrylamide gel electrophoresis. Following electrophoresis, the gel was dried under vacuum, [%32P]DNA fragment bands were visualized and quantified using a PhosphorImager. The relative intensity of various bands was determined with an ImageQuant program and the amount of [α-32P]dAMP incorporation into the 253-, 261-, 486-, 529-bp fragments was compared after subtracting background values obtained from a blank lane.

2.2.23.2 BsrI Restriction Analysis

Specificity of uracil- and ethenocytosine-mediated repair DNA synthesis on pGEM or pBIG DNA was examined by utilizing restriction endonuclease BsrI. Standard BER reactions supplemented with 10 μCi/ml [α-32P]dCTP (6,000 cpm/mmol) were carried out at 30 °C for various amounts of time as indicated in the Figure Legends. DNA reaction products were isolated, and each DNA sample (100 ng) was treated with excess BsrI restriction endonuclease (2.5-5 units) at 65 °C for 1 h in the reaction (8 μl) containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. The reactions were terminated by the addition of 2 μl of 100 mM EDTA and DNA restriction fragments were analyzed using 5 % non-denaturing polyacrylamide gel electrophoresis. After drying the gel, [%32P]DNA bands were visualized using a PhosphorImager, and the intensity of various [%32P]DNA bands was measured by an ImageQuant software. After subtracting background radioactivity, the [α-32P]dCMP incorporation was normalized for each DNA fragment by dividing the amount of 32P radioactivity detected by the number of cytosine residues located in each corresponding DNA fragment.
2.2.24 Base Excision DNA Repair Patch Size Analysis

2.2.24.1 Exonuclease III Digestion Analysis

Standard BER reaction mixtures were prepared except that 2-deoxyribonucleoside α-thiotriphosphates were used in place of each of the four 2-deoxyribonucleoside triphosphates, and M13mp2op14 (U·T) or pGEM (U·G or εC·G) Form I [$^{32}$P]DNA was used as the BER substrate. For the [$^{32}$P]M13mp2op14 (U·T) DNA, the $^{32}$P-label was located 13 nucleotides upstream of the target uracil and 7 nucleotides downstream of the *SmaI* restriction site on the transcribed strand of the *lacZα* gene sequence. The pGEM DNA substrate contained a $^{32}$P-labeled dAMP residue located 12 nt upstream of target uracil or ethenocytosine and two nucleotides downstream of *BamHI* restriction site on the (-) strand DNA. Following the BER reactions for various times, DNA products were isolated as described in Section 2.2.18. Repaired M13mp2 (200 ng) and pGEM (100 ng) DNA samples were digested with *EcoRI* (10 units) and *HindIII* (5 units) at 37 °C for 30 min, respectively. Following termination at 70 °C for 10 min, samples were incubated in the absence or the presence of various amounts of *E. coli* exonuclease III for 1 h at 37 °C as indicated in the Figure Legends. Following exonuclease III digestion, samples were heated at 70 °C for 10 min, and M13mp2 and pGEM [$^{32}$P]DNA reaction products were then digested with 10 units of *SmaI* for 1 h at 25 °C or with 5 units of *BamHI* for 30 min at 37 °C, respectively. The resulting [$^{32}$P]DNA products were resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis, visualized and quantified using PhosphorImager as described in Section 2.2.10.2. The relative amount of $^{32}$P label 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 particular lane and multiplying by 100.
2.2.24.2 Restriction Endonuclease Analysis

Standard BER reactions were performed with pGEM (U-G or eC-G) DNA in the presence of 40 µCi/ml \([\alpha^{32}\text{P}]d\text{CTP}\) (6,000 cpm/mmol). Reaction products were isolated and subjected to the treatment with various restriction endonuclease. DNA samples (100 ng) were treated with 5 units each of Smal and XbaI, XbaI and AccI, XbaI and HincII, HincII and PstI, PstI and HindIII, or AccI and HindIII in the reaction mixtures (8 µl) containing 20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT. After incubation at 37 °C for 1 h, each reaction was terminated by heating at 70 °C for 10 min, and combined with an equal volume of denaturing formamide dye buffer containing 0.05 fmol of \(^{32}\text{P}\)-labeled C-34-mer (internal standard), and the resulting DNA fragments were analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis. \(^{32}\text{P}\)DNA bands were visualized and quantified using PhosphorImager and ImageQuant program. After subtracting the background value, the highest \(^{32}\text{P}\) radioactivity of internal standard DNA band was designated 100% and the relative amount of \(^{32}\text{P}\) radioactivity for each restriction DNA fragment band in each lane was determined relative to that value.
A distinct feature of *E. coli* Ung is that the uracil-DNA glycosylase activity can be inhibited by irreversible binding to the PBS2 uracil-DNA glycosylase inhibitor (Ugi) protein (123, 124). In the study described in this Chapter, this property of Ugi was exploited to identify a novel Ugi-insensitive uracil-DNA glycosylase activity using *E. coli* cell extracts. The approach utilized synthetic oligonucleotide substrates containing a site-specific uracil residue to detect uracil excision from either a U/A base pair or U/G mispair target in *E. coli* cell extracts. A novel Ugi-insensitive uracil-DNA glycosylase activity was identified and purified to apparent homogeneity. Based on characterization presented in this Chapter, and in compliance with traditional *E. coli* nomenclature, the Ugi-insensitive uracil-DNA glycosylase activity was designated as double-strand uracil-DNA glycosylase, and referred to as Dug.

The *dug* gene was cloned, the gene product was overproduced, and the recombinant protein was purified to apparent homogeneity from *E. coli* that overexpressed *dug*. The substrate specificity of recombinant Dug was examined for uracil- and ethenocytosine-DNA glycosylase activities. The enzyme was further characterized with respect to DNA product binding and the kinetics of interaction between Dug and various DNA substrates were assessed by evanescent wave biosensor. Experiments were also conducted to determine the effect of AP endonuclease on the catalytic activity of Dug.

### 3.1 Results

#### 3.1.1 Detection of Ugi-insensitive Uracil-DNA Glycosylase Activity in *E. coli* Cell-free Extracts

In order to initiate the 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 generated that contained either a site-specific U/A base pair or U/G mispair. *E. coli* cell extracts were prepared from two isogenic *E. coli* strains, NR8051 and NR8052; the latter carries the *ung-1* mutation and has a significantly reduced level of Ung activity (225, 317). 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 reaction 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 [32P]15-mer product (241, 258). 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 2A and 2B, 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
Figure 1. Detection of Ugi-insensitive uracil-DNA glycosylase activity in *E. coli* NR8051 (*ung*) and NR8052 (*ung*) cell-free extracts. Two 5'-end $^{32}$P-labeled duplex oligonucleotides (**[^32P]U/A-34-mer** and **[^32P]U/G-34-mer**) containing a site-specific uracil residue located at position 16 of the radioactively labeled strand were prepared as described under "Experimental Procedures". 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 **[^32P]U/A-** or **[^32P]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 cell extract protein and 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 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". Arrows indicate the location on the autoradiogram of unreacted **[^32P]34-mer** substrate (S) and **[^32P]15-mer** product (P).
Figure 1
Figure 2. Quantification of uracil-DNA glycosylase activity in *E. coli* NR8051 (*ung*') and NR8052 (*ung*') cell-free extracts in the presence or absence of Ugi. \[^{32}\text{P}]\text{DNA bands detected by autoradiography in Figure 1 were quantified using a PhosphorImager and ImageQuant program (Molecular Dynamics). The percentage of product formed was calculated by dividing the amount of \[^{32}\text{P}]15-mer by that of \[^{32}\text{P}]15-mer plus \[^{32}\text{P}]34-mer and multiplying by 100. The percentage of product generated in reactions containing \[^{32}\text{P}]U/A-34-mer (A) or \[^{32}\text{P}]U/G-34-mer (B) substrate and various cell-free extracts is shown as: *E. coli* NR8051 extract plus Ugi (□), NR8051 minus Ugi (○), NR8052 plus Ugi (●), and NR8052 minus Ugi (●).
Figure 2

A. % Product

B. Extract (μg)

Graph A shows the percentage product as a function of extract (μg). Graph B also displays the relationship between these variables but with a different data set.
and preferred the mispaired U/G-34-mer, it was concluded that it could not be synonymous with Ung.

3.1.2 Purification of the Ugi-insensitive Uracil-DNA Glycosylase Activity

The Ugi-insensitive uracil-DNA glycosylase activity was purified from *E. coli* cell extract using three conventional chromatographic steps (Figure 3) as described under "Experimental Procedures". To ensure that the Ugi-insensitive uracil-DNA glycosylase activity could be separated from Ung, *E. coli* strain NR8052 (*ung*) was used, and Ugi (2 x 10⁵ units) was added to the cell extract to inactivate any residual Ung activity. During purification, uracil-excision activity was monitored using the U/G-34-mer cleavage assay, and reactions were carried out in the presence of Ugi to further assure inhibition of any potential Ung activity. In the first chromatographic step, fraction II of the NR8052 (*ung*) cell-free extract was applied to a Sephadex G-75 size exclusion column, calibrated using four protein molecular weight standards, and the Ugi-insensitive U/G mispair-specific activity eluted as a single symmetrical peak (Figure 3A). In the following step, hydroxyapatite chromatography (Figure 3B), the bulk of a contaminating 3' to 5' exonuclease activity, evidenced by the apparent degradation of the cleaved product to shorter oligonucleotides (data not shown), was removed from the Ugi-insensitive uracil-DNA glycosylase preparation. Single-stranded DNA agarose chromatography was performed as the final purification step, and two peaks of enzymatic activity were detected (Figure 3C). The major active peak eluted from the affinity column matrix at a NaCl concentration of ~220 mM, which is higher than that characteristic of *E. coli* Ung (~100 mM) (130). The flow-through fractions containing the minor activity were pooled and re-applied to a single-stranded DNA agarose column; however, most of the activity was found in flow-through fractions again, indicating that the earlier column was not over-loaded and that this activity was distinct from the activity that eluted from the column during the NaCl gradient. Following the single-stranded
Figure 3. Purification of Ugi-insensitive uracil-DNA glycosylase activity from *E. coli* NR8052 cells. (A) Fraction II of the NR8052 (*ung*) cell-free extract was prepared, applied to a Sephadex G-75 size exclusion column (6 cm² x 88 cm), eluted, and assayed for Ugi-resistant uracil-DNA glycosylase activity (●) as described under "Experimental Procedures". The arrow indicates the location of the void volume (V₀) determined using blue dextran 2000 to identify the exclusion volume. Fractions (54-74) containing uracil-DNA glycosylase activity were pooled, concentrated, and dialyzed against HAB buffer containing 10 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol, and 200 mM KCl (fraction III). (B) Fraction III was loaded onto a hydroxyapatite column (4.9 cm² x 8 cm) equilibrated in HAB buffer, and eluted with the same buffer. Active fractions (9-17) were pooled, concentrated, and dialyzed against DAB buffer containing 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 5% (w/v) glycerol (fraction IV). (C) Fraction IV was applied to a single-stranded DNA agarose column (1.8 cm² x 8 cm) equilibrated in DAB buffer. The column was washed and eluted with a linear gradient of 0 to 700 mM NaCl in DAB buffer (■). Fractions were collected, samples (20 μl) were assayed for uracil-DNA glycosylase activity (●), and the autoradiogram from the denaturing 12% polyacrylamide/8.3 M urea gel shown in the insert. Arrows indicate the location of the unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P). The percentage of product generated for the control reaction (lane C) containing both Ung (400 units) and Ugi (1000 units) as well as the assays of each column fraction were determined using a PhosphorImager as described in Figure 2. Active fractions (36-44) were pooled, concentrated, dialyzed against DAB buffer, and designated fraction V.
Figure 3
DNA agarose chromatographic step, fractions containing the major activity were pooled and used for characterization.

3.1.3 Molecular Weight of Ugi-insensitive Uracil-DNA Glycosylase

The Sephadex G-75 column used during the purification of Ugi-insensitive uracil-DNA glycosylase was calibrated by using four protein molecular weight standards including E. coli Ung. In addition, blue dextran 2000 was used to determine the void volume (V₀) of the column. The elution volume (Vₑ) of each protein molecular weight standard was expressed as a fraction of the void volume (Vₑ/V₀). When the logarithm of the molecular weight of the protein standards was plotted against the respective Vₑ/V₀ values, a linear function was obtained (Figure 4). Relative to this standard curve, the Vₑ/V₀ value determined for Ugi-insensitive uracil-DNA glycosylase activity from the Sephadex G-75 column corresponded to a protein of about 18,000 molecular weight (Figure 4).

Purified Ugi-insensitive uracil-DNA glycosylase was analyzed by SDS polyacrylamide gel electrophoresis, and protein bands were then visualized by silver staining. The result indicated that the Ugi-insensitive uracil-DNA glycosylase was purified to apparent homogeneity and consisted of a single polypeptide (Figure 5A, lane I). In order to ensure that the protein band eluting from the single-stranded DNA agarose column represented the Ugi-resistant uracil-DNA glycosylase activity, an activity gel analysis was carried out as described under "Experimental Procedures". Using the [³²P]U/G-34-mer cleavage assay, enzymatic activity was detected which co-migrated with the silver-stained band (Figure 5A and B, lane I). Based on the standard curve of the protein molecular weight standards, the apparent molecular weight of the polypeptide was calculated to be ~21,000 from both the location of the silver-stained band and position of activity gel slice 18 (Figure 5D).
Figure 4. Gel filtration chromatography of Ugi-insensitive uracil-DNA glycosylase. Sephadex G-75 column (6 cm\(^2\) x 88 cm) was equilibrated in buffer containing 10 mM Hepes-KOH (pH 7.4), 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 M NaCl, and 5% (w/v) glycerol. Fraction II of the NR8052 (ung\(^+\)) cell-free extract was loaded onto the column. Samples were collected and assayed for Ugi-resistant uracil-DNA glycosylase activity as described under "Experimental Procedures". The column was calibrated using four molecular weight standards, ovalbumin (O, Mr 45,000), carbonic anhydrase (CA, Mr 31,000), E. coli uracil-DNA glycosylase (Ung, MW 25,563), and cytochrome c (C, Mr 12,500) which eluted as indicated (■); the void volume (V\(_0\)) was determined using blue dextran 2000. The value of the abscissa (V\(_e\)/V\(_0\)) was determined as the quotient of the fraction number representing the peak absorbance determined at 280 nm for ovalbumin and carbonic anhydrase, and at 420 nm for cytochrome c, or the peak activity of Ung divided by the peak fraction number of the blue dextran 2000. The V\(_e\)/V\(_0\) value for the Ugi-insensitive uracil-DNA glycosylase activity was plotted on the standard curve (□).
Figure 4
Figure 5. Determination of the polypeptide molecular weight of Ugi-insensitive uracil-DNA glycosylase by activity gel analysis. (A) Two sets of samples (40 μl each lane, 4 lanes total) of purified Ugi-insensitive uracil DNA glycosylase (nDug, fraction V) and recombinant Dug (rDug, fraction VI) were loaded onto a 12.5% SDS-polyacrylamide gel (lane I and II, respectively) along with molecular weight standards, and electrophoresis was conducted as described under "Experimental Procedures". Following electrophoresis, the gel was cut in half vertically between the sample sets and one half of the gel 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 and C) After electrophoresis, the other half of the gel contained nDug (B) or rDug (C) was vertically cut between the sample lanes, SDS was extracted, and each lane was horizontally sliced into ~3.5 mm segments. Protein was eluted and renatured from the gel slices as described in "Experimental Procedures". Samples (40 μl) were assayed for Ugi-resistant uracil-DNA glycosylase activity, reaction products analyzed on a denaturing 12% polyacrylamide/8.3 M urea gel as described under "Experimental Procedures". The positions of the [32P]U/G-34-mer substrate (S) and [32P]15-mer product (P) are indicated on the autoradiogram by arrows. (D) A standard curve (log M., versus Rf) was generated based on the relative mobility of prestained protein molecular weight markers for phosphorylase b (M. 111,000), BSA (M, 77,000), ovalbumin (M, 48,200), carbonic anhydrase (M, 33,800), trypsin inhibitor (M, 28,600), and lysozyme (M, 20,500). The apparent molecular weight of Ugi-insensitive uracil-DNA glycosylase was determined based on the Rf for the mid-point of the gel slice (fraction 18, horizontal line) as indicated by the vertical arrow.
The native molecular weight of the Ugi-resistant uracil-DNA glycosylase was determined using sucrose density gradient centrifugation. After centrifugation, the location of the enzyme activity in the fractions was determined by the $[^{32}\text{P}]U/G$-34-mer cleavage assay (Figure 6A). Compared with the position of an internal BSA marker (4.7 S, $M_r$ 68,000), the Ugi-resistant uracil-DNA glycosylase had a sedimentation coefficient of 2.1 S (Figure 6B). Calculated according to the equation described by Martin and Ames (327), this coefficient inferred that Ugi-resistant uracil-DNA glycosylase had native molecular weight of ~20,800.

3.1.4 Cloning, Overexpression, and Purification of Recombinant Double-strand Uracil-DNA Glycosylase (Dug)

The molecular weight of the Ugi-resistant uracil-DNA glycosylase determined by various methods described above appeared to be lower than that of *E. coli* Ung and close to the predicted value for the *E. coli* gene encoded by an open reading frame (ORF) of 168 amino acids which was associated with double-strand-specific uracil-DNA glycosylase (dsUDG) activity reported by Gallinari and Jiricny (107). In order to clone this gene, the nucleotide sequence of this ORF was amplified by polymerase chain reaction (PCR) using *E. coli* NR8052 chromosomal DNA as template (Figure 7A). The expected PCR product DNA was anticipated to contain a *Bsa*HI restriction endonuclease cleavage site as determined from the nucleotide sequence of the gene. This site was utilized to verify the identity of the gene insert during the cloning procedure (Figure 7B). The DNA fragment containing the corresponding double-strand-specific uracil-DNA glycosylase gene was cloned into the overexpression vector pKK223-3 under the control of $P_{\text{lac}}$ promoter; the resultant construct was designated pKK-Dug (Figure 7C). The nucleotide sequence of this insert in pKK-Dug was confirmed by DNA sequence analysis for both transcribed and complementary strands (Figure 8).
Figure 6. Native molecular weight of Ugi-insensitive uracil-DNA glycosylase determined by sucrose density gradient centrifugation. (A) A sample mixture (200 μl) containing 6.5 ng of purified Ugi-insensitive uracil-DNA glycosylase (fraction V) and 1 mg of bovine serum albumin (BSA) was layered onto a linear sucrose gradient (0 to 30%) containing 30 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM DTT, and 1 mM EDTA. The centrifugation was conducted in a Beckman SW50 rotor at 40,000 rpm for 24 h at 4°C. Fractions (200 μl) were collected from the bottom of the gradient, each sample (20 μl) was assayed for Ugi-resistant uracil-DNA glycosylase activity, and reaction products analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The location of the unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P) are indicated by arrows. (B) The percentage of product ([³²P]15-mer) measured for each band shown in (A) using a PhosphorImager was plotted (●). A sample from each fraction (100 μl) was also analyzed for absorbance at 280 nm with a micro plate reader for BSA (□). Sedimentation was from right to left.
Figure 6
Figure 7. Scheme for cloning of the *E. coli* double-strand uracil-DNA glycosylase gene. (A) A DNA fragment (523 bp) containing the *E. coli* *dug* gene was obtained by PCR amplification using *E. coli* NR8052 genomic DNA with oligonucleotide primers, P1- and P2-33-mers, as described under "Experimental Procedures". The ATG initiation codon and the TAA termination codon of the *dug* gene were located one nucleotide adjacent to the restriction endonuclease cleavage sites for EcoRI and HindIII, respectively. The positions of BsaHI (193 bp), NdeI (332 bp), and HaeIII (483 bp) restriction sites from the transcription initiation site of the *dug* gene were indicated by arrows. (B) A sample (5 µl) of the PCR reaction product was analyzed by 1.5% agarose gel electrophoresis after no treatment (lane 3), digestion with both EcoRI and HindIII (lane 4), or digestion with BsaHI (lane 5). The samples (1 µg) containing either a 1-kb or 100 bp DNA ladder were used as standards (lane 1 and 2, respectively). The horizontal arrows indicate the size of reference DNA standards expressed in base pairs (bp). (C) The PCR reaction product and pKK223-3 plasmid were digested with both EcoRI and HindIII, resolved on 1.5% and 1% agarose gel, respectively, isolated from the gel, and subjected to a ligation reaction to construct pKK-Dug, as described under "Experimental Procedures".
Figure 7
Figure 8. DNA sequence analysis of double-strand uracil-DNA glycosylase gene insert of pKK-Dug. The DNA fragment coding for double-strand uracil-DNA glycosylase (Dug) was obtained by PCR amplification using *E. coli* NR8052 genomic DNA, and cloned between *EcoRI* and *HindIII* restriction endonuclease cleavage sites of the pKK223-3 plasmid as described under "Experimental Procedures". The resulting construct, pKK-Dug, was isolated and the nucleotide sequence of the entire *dug* gene was determined by the Center for Gene Research and Biotechnology (Oregon State University) for both DNA strands using an Applied Biosystems Model 373A DNA sequencer. The sequences depicted are those of genomic (A) and complementary (B) DNA strands determined in the 5' to 3' direction using sequencing primers, P1- and P2-33 mers, respectively. When the identity of a base was unclear, it was denoted by an 'N' in the DNA sequence.
Attempts to transform *E. coli* NR8052 (ung) with pKK-Dug were unsuccessful; however, *E. coli* JM109, an ung strain, was successfully transformed with pKK-Dug and utilized for overproduction of recombinant protein. In order to separate double-strand uracil-DNA glycosylase activity from the endogenous Ung activity of the host bacterial cells, Ugi (3 × 10^6 units) was added to the cell extracts before performing purification (Figure 9). Recombinant dsUDG was purified according to the same chromatographic procedure used for native Dug, except that a DEAE-Sephadex column was added as the last chromatography step. The recombinant enzyme eluted from the single-stranded DNA agarose column at a NaCl concentration of ~220 mM (Figure 9C), and SDS-polyacrylamide gel electrophoresis analysis of the eluted fractions revealed a protein band that co-eluted with an apparent molecular weight of ~21,000 (Figure 9C, insert). Further purification was conducted, to eliminate minor contaminants, by DEAE-Sephadex chromatography where the activity was detected in the flow-through fractions (Figure 9D). The activity profile again correlated with the protein band detected with SDS polyacrylamide gel electrophoresis of the same fractions, which revealed a single large protein band visualized by silver staining (Figure 9D, insert). This procedure resulted in the isolation of fraction VI that represented an apparently homogeneous protein of Mr ≈ 21,000 (Figure 10). Thus, the observed molecular weight of recombinant dsUDG as determined by SDS polyacrylamide gel electrophoresis appeared to be in excellent agreement with that determined separately for the native Ugi-resistant uracil-DNA glycosylase.

In order 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 5A. Side by side comparison of their electrophoretic mobility showed that the two protein bands co-migrated (Figure 5A, native protein: lane I and recombinant protein: [image description not provided]).
Figure 9. Purification of recombinant double-strand uracil-DNA glycosylase. (A) Recombinant Dug (fraction II) was prepared, loaded onto a Sephadex G-75 column (6 cm² x 88 cm), and eluted as described under "Experimental Procedures". Fractions (6.5 ml) were collected and aliquots (10 µl) were assayed for Dug activity (●) in standard reaction mixtures (100 µl) containing 1000 units of Ugi and 0.1 pmol of [³²P]U/G-34-mer for 30 min at 30 °C. The arrow indicates the location of the column void volume determined using blue dextran 2000. Active fractions (39-69) were pooled, concentrated, and dialyzed against HAB buffer. (B) Recombinant Dug (fraction III) was loaded onto a hydroxyapatite column (19.6 cm² x 3.5 cm) equilibrated in HAB buffer, eluted with the same buffer, and fractions (6.5 ml) were collected. Active fractions (16-50) were pooled, concentrated, and dialyzed against DAB buffer. (C) Recombinant Dug (fraction IV) was applied to a single-stranded DNA agarose column (4.9 cm² x 16 cm) equilibrated in DAB buffer. The column was washed with 150 ml of equilibration buffer and eluted with a 350 ml linear gradient of 0 to 600 mM NaCl in DAB buffer. Fractions (5 ml) were collected, aliquots (5 µl) were assayed for Dug activity (●), and monitored for conductivity (■). Fractions across the activity peak were analyzed on a 12.5% SDS-polyacrylamide gel (insert) and protein bands were detected after silver staining. The location of the molecular weight standards for phosphorylase b (M, 97,400), BSA (M, 66,200), ovalbumin (M, 45,000), carbonic anhydrase (M, 31,000), trypsin inhibitor (M, 21,500), and lysozyme (M, 14,400) are indicated by arrows from top to bottom, respectively. The location of the tracking dye (TD) is indicated by an arrow. Active fractions (80-96) were pooled, concentrated, and dialyzed against DEAE equilibration buffer. (D) Recombinant Dug (fraction V) was loaded onto a DEAE-Sephadex A50 column (4.9 cm² x 6 cm) equilibrated in DEAE buffer. After washing the column with equilibration buffer (60 ml), a 100 ml linear gradient of 0 to 500 mM NaCl in DEAE buffer was applied. Fractions (3 ml) were collected and samples (5 µl) were assayed for Dug activity (●). NaCl concentrations were monitored by measuring conductivity (■). Samples (5 µl) of peak fractions were analyzed by electrophoresis using a 12.5% SDS-polyacrylamide gel (insert) as described in (A). Active fractions (8-14) were pooled, concentrated, dialyzed against DAB buffer, and designated fraction VI.
Figure 9
Figure 10. SDS-polyacrylamide gel analysis of recombinant Dug isolated at various steps during the purification. Protein samples from the purification of recombinant Dug (fraction I-VI) containing 30, 30, 20, 20, 10, and 10 μg of protein, respectively, were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue G-250 as described under 'Experimental Procedures'. The location of the protein molecular weight markers (lane M) that were identified in Figure 9 and tracking dye (TD) are indicated by arrows.
lane II). Activity gel analysis established that the protein band of recombinant dsUDG preparations had almost identical electrophoretic properties with the Ugi-insensitive uracil-DNA glycosylase activity (Figure 5C). These observations inferred that the recombinant dsUDG and the native Ugi-insensitive uracil-DNA glycosylase activity were indistinguishable. During the course of this study, this recombinant protein preparation was referred to as double-strand uracil-DNA glycosylase (Dug).

3.1.5 Molecular Weight Determination of Dug by MALDI Mass Spectrometry

Although the 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 daltons) 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 corresponding to a mass of 18,670 daltons (Figure 11). 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 post-translational modification.

3.1.6 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 E. coli Ung and Dug activities. Standard uracil-DNA glycosylase assays were performed using a $^{32}$P-labeled 34-mer oligonucleotide containing a site-specific U/G target in the presence of various amounts of free uracil or Ugi, and the percent of activity relative to the control was determined (Figure 12). Ung activity determined on $[^{32}$P]U/G-34-mer was inhibited ~50% by 1 mM uracil, whereas Dug activity was not significantly affected by as much as 2 mM uracil (Figure 12A). In addition, Ung was inhibited by Ugi; but, the activity of Dug was not affected by a 4-fold molar
Figure 11. Molecular weight determination of Dug by matrix-assisted laser desorption/ionization mass spectrometry. A sample of Dug (~20 ng) was applied to the probe of a pulsed laser desorption-ionization mass spectrometer as described under "Experimental Procedures". A mass spectrum was produced from 30 individual laser pulses. The relative intensity of charged ions is shown and mass determination was calculated using ion signals from an external calibrant in the same matrix.
Figure 12. Effect of uracil and Ugi on Dug and Ung activity. Two sets of standard uracil-DNA glycosylase reaction mixtures (10 μl) were prepared containing 10 nM [32P]U/G-34-mer and either 5 nM Dug (○) or 5 pM Ung (●) as described under "Experimental Procedures". 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, 0.00025, 0.0005, 0.002, 0.008, 0.016, 0.032, and 20 nM) 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 [32P]DNA bands were quantitatively measured using a PhosphorImager as described in Figure 2. Enzyme activity (%) was determined relative to the control reaction which lacked uracil or Ugi addition: 100% activity corresponded to 61 and 77 fmol of [32P]U/G-34-mer converted to product for Dug and Ung, respectively.
excess of Ugi (Figure 12B). 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.

3.1.7 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 (49, 103, 258). 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 13). 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 the substrates containing either a εC/G or T/G mispair. When taken together, these results clearly illustrate a difference in the substrate specificity of Dug and Ung activities.
Figure 13. DNA substrate specificity of Dug and Ung. Seven sets of standard uracil-DNA glycosylase reaction mixtures (10 µl) containing either single- or double-stranded [³²P]34-mer oligonucleotide substrates (10 nM) with U-, U/A-, U/G-, T/G-, εC-, εC/A-, or εC/G-target residues were prepared as described under "Experimental Procedures". After adding 4 and 800 nM Dug (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 by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The percentage of product ([³²P]15-mer) determined using a PhosphorImager is shown.
3.1.8 Binding Properties of Dug on Various DNA

Band mobility shift assays were conducted to examine whether Dug forms a stable protein-DNA complex. Standard Dug-DNA binding reactions containing 10 nM of [\(^{32}\)P]34-mer oligonucleotide substrates containing a U, U/A, U/G, T/G, eC, eC/A, or eC/G residue and 8 nM of Dug were performed. Samples of each binding reaction were then analyzed by nondenaturing polyacrylamide gel electrophoresis (Figure 14). In the reactions containing double-stranded oligonucleotide substrates, DNA bands with the mobility shifted from free DNA were observed that is due to binding of the DNA by Dug. However, single-stranded DNA substrates were unable to form a stable interaction with Dug in the assay. The formation of protein-DNA complex appeared to occur more efficiently with oligonucleotide duplex containing U/G or eC/G residue than eC/A residue, and much less with U/A- and T/G-34-mer. To further examine the binding properties of Dug on DNA, band mobility shift assays were performed with Dug-DNA binding reactions containing 10 nM [\(^{32}\)P]U/G-34-mer and 10 nM Dug in the presence of 400 nM of various unlabeled competitor DNA-34-mer containing C/G, U, U/A, U/G, T/G, eC, eC/A, or eC/G residues (Figure 15). Dug formed a stable complex with DNA containing the U/G mispair, and competition with a 40-fold excess of unlabeled C/G-homoduplex only partially competed for this complex. However, the formation of this complex was abolished by the competition with excess unlabeled DNA containing U/G, eC/A, or eC/G residue. Competitor DNA duplex containing U/A and T/G residues reduced but did not eliminate the amount of the complex, whereas single-stranded competitor U- and eC-34-mer did not appear to have a significant affect on the Dug-[\(^{32}\)P]U/G-34-mer interaction. These results demonstrated the specificity of Dug to interact with double-stranded DNA.
Figure 14. Band mobility shift analysis of binding affinity of Dug on various DNA. Seven sets of DNA binding reaction mixtures (10 µl) containing either single- or double-stranded [³²P]34-mer oligonucleotide substrates (10 nM) with U-, U/A-, U/G-, T/G-, εC-, εC/A-, or εC/G-target residues were incubated with 8 nM Dug (lanes 2, 4, 6, 8, 10, 12, and 14, respectively) or mock treated (lanes 1, 3, 5, 7, 9, 11, and 13, respectively) at 30 °C for 30 min. After incubation, samples (5 µl) were analyzed by non-denaturing 6% polyacrylamide gel electrophoresis as described under "Experimental Procedures". Autoradiography was performed and the location of the free (F) and bound (B) forms of [³²P]DNA are indicated by arrows.
Figure 15. Competition analysis of Dug binding to various DNA. DNA binding reaction mixtures (10 µl) containing 10 nM of [³²P]U/G-34-mer duplex DNA substrate and Dug (10 nM) were supplemented with unlabeled competitor single- or double-stranded oligonucleotide DNA (400 nM) with C/G-, U/G-, U, U/A-, εC-, εC/A-, or T/G-target residue (lanes 3-10, respectively). After incubation at 30 °C for 30 min, samples (5 µl) were analyzed by non-denaturing 6% polyacrylamide gel electrophoresis as described under "Experimental Procedures". As controls, [³²P]U/G-34-mer substrate was mock treated without competitor DNA in the absence and presence of Dug (lanes 1 and 2, respectively). Arrows indicate the location of the free (F) and bound (B) forms of [³²P]34-mer DNA visualized by autoradiography.
3.1.9 Effect of E. coli Endonuclease IV on Dug Activity

In order to examine the effect of E. coli endonuclease IV on the activity of Dug, the rate of uracil excision was determined for the \(^{32}\text{P}\)U/G-34-mer substrate in the absence or presence of endonuclease IV (Figure 16). Dug activity under standard reaction conditions 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. Also, it seemed unlikely that the rate enhancing effect of endonuclease IV was derived from non-specific protein stabilization, because both reactions were conducted in the presence of 0.1 mg/ml of acetylated BSA. The maximum extent of uracil excision was increased ~5.5-fold relative to the Dug reaction conducted in the absence of endonuclease IV.

3.1.10 Effect of E. coli Endonuclease IV on Dug DNA Binding and Catalysis

Band mobility shift assays were conducted to determine whether endonuclease IV affected the stability of the Dug-DNA complex. Standard Dug binding reactions containing 10 nM \(^{32}\text{P}\)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 non-denaturing polyacrylamide gel electrophoresis (Figure 17A). In the absence of endonuclease IV (Figure 17A, -Endo IV, lanes 1-7), the \(^{32}\text{P}\)U/G-34-mer probe was mobility shifted in a concentration-dependent
Figure 16. Stimulation of Dug activity by *E. coli* endonuclease IV. Standard uracil-DNA glycosylase reaction mixtures (10 µl) containing 10 nM [³²P]U/G-34-mer and 2.5 nM Dug were prepared in the presence (○) and absence (●) of 10 nM *E.coli* endonuclease IV. Control reaction mixtures included 10 nM [³²P]U/G-34-mer and 10 nM endonuclease IV (□). Incubation was conducted at 30 °C and each reaction terminated at the times indicated by heating at 70 °C for 3 min. Following treatment with 0.1 units of endonuclease IV to cleave the generated AP-sites, each sample was analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis, and the amount of [³²P]DNA product formed was quantitatively determined using a PhosphorImager as described under "Experimental Procedures".
Figure 16

Cleaved DNA (nM)

Time (min)
Figure 17. 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 Dug (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 6% polyacrylamide gel electrophoresis as described under "Experimental Procedures". 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 under "Experimental Procedures". 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 \(^{31}\)P radioactivity was measured for each band shown in (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 Dug in each reaction mixture.
Figure 17
manner. When endonuclease IV was included in the binding reaction mixture (Figure 17A, +Endo IV, lanes 1-7), the extent of Dug-DNA complex formation at equivalent Dug concentrations became less intense. These results demonstrated that endonuclease IV destabilized 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 17B). Product analysis of binding reactions carried out in the absence of endonuclease IV (Figure 17B, -Endo IV, lanes 1-7) showed a single $^{32}$P]-15-mer band that increases in intensity with increasing Dug concentration. Analysis of the reactions containing endonuclease IV (Figure 17B, +Endo IV, lanes 1-7) 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 appeared 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 $\beta$-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 $\beta$-elimination reaction ($\delta$ 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 (101, 241). 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 17C). 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 containing the AP-site following uracil excision. This result was consistent with the observation that
a stable Dug-DNA complex formed when DNA substrates contained U/G, eC/G, or eC/A residues since these DNA molecules were efficiently processed by Dug to produce AP-site on the DNA (Figure 13 and 14). In the presence of endonuclease IV, the amount of bound DNA was significantly reduced, but the rate of uracil excision was enhanced (Figure 17C). These results imply that endonuclease IV stimulated the dissociation of Dug from product DNA enabling the enzyme to turnover and participate in further catalytic events.

3.1.11 Effect of EDTA on Endonuclease IV-mediated Dissociation of the Dug/AP-site-DNA Complex

In order to determine if the catalytic activity of endonuclease IV was responsible for stimulating Dug activity, band 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. Previous studies demonstrated that EDTA was a potent inhibitor of endonuclease IV activity (259, 328). Binding reactions containing 20 nM [32P]U/G-34-mer and an equal concentration of Dug resulted in the mobility shift of more than 90% of the [32P]U/G-34-mer DNA probe, both in the presence and absence of EDTA (Figure 18, lane 1). This indicated that the DNA-binding of Dug was insensitive to EDTA. In the absence of EDTA (Figure 18, -EDTA, lanes 1-6), increasing amounts of endonuclease IV in the DNA binding reaction resulted in a linear decline in the amount of bound 32P-labeled probe, consistent with the results obtained in Figure 17. However, in the presence of EDTA (Figure 18, +EDTA, lanes 1-6), 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.
Figure 18. Effect of EDTA on *E. coli* endonuclease IV and Dug binding to DNA. DNA binding reaction mixtures (10 μl) containing 20 nM [³²P]U/G-34-mer, 20 nM Dug, 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 "Experimental Procedures". Autoradiography was performed and the location of the free (F) and bound (B) forms of [³²P]DNA are indicated by arrows.
3.1.12 Ability of Dug to Bind Duplex DNA Containing an Incised AP-site

*E. coli* endonuclease IV allowed Dug to be released from its product DNA and stimulated catalytic activity of Dug. This result suggested that Dug would not efficiently bind DNA containing an incision on the 5'-side of an AP-site that was generated by the AP endonuclease activity of endonuclease IV. To test this proposition, incised AP-site duplex DNA (*AP/G34mer*) was prepared from [32P]U/G-34-mer by treatment with excess Ung and endonuclease IV. Three 32P-labeled DNA duplexes (U/G-, AP/G-, and *AP/G34mer*) were incubated with various amounts of Dug and band 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/G34mer* containing an incision on the 5'-side of the AP-site (Figure 19). These findings supported the hypothesis that the endonuclease IV-mediated stimulation of Dug activity was the result of Dug dissociation from incised AP-sites.

3.1.13 Effect of *E. coli* Fpg protein on Dug/AP-site-DNA complex

Since the stimulatory effect on dissociation of Dug from AP-site-DNA by *E. coli* endonuclease IV appeared to be mediated by incision of AP-site, the interaction between Dug and AP-site-DNA in the presence of another class of AP endonuclease, *E. coli* formamidopyrimidine-DNA glycosylase (Fpg protein) was examined. While endonuclease IV falls into the category of a class II AP endonuclease along with exonuclease III that cleaves the 5'-side of the AP-site, several other DNA glycosylases including Fpg protein associate with intrinsic AP lyase (class I AP endonuclease) activity that cleave the 3'-side of the AP-site in the DNA (180, 236, 329). Standard Dug binding reactions containing 10 nM [32P]U/G-34-mer were incubated without or with 20 nM Dug in the absence and presence of 10 nM endonuclease IV or various amount of Fpg protein, and band mobility shift assays were performed.
Figure 19. 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 Dug (lanes 1-5, respectively), 10 nM of [32P]U/G-, [32P]AP/G-, or [32P]*AP/G-34-mer (pre-treated 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 "Experimental Procedures". The location of the free (F) and bound (B) oligonucleotide probes are indicated by arrows on the autoradiogram.
(Figure 20). In the absence of both endonuclease IV and Fpg (Figure 20, lanes 2 and 5), the majority of DNA molecules appeared to be bound by Dug. The formation of this Dug-DNA complex was markedly reduced in the presence of endonuclease IV (Figure 20, lane 3), which was consistent with the previous observations shown in Figure 17 and 18. Similarly, in the presence of excess Fpg protein, Dug was not found to form a complex with $^{[32P]}\text{U/G-34-mer}$ (Figure 20, lane 4). The amount of DNA bound by Dug was observed to decrease in a concentration dependent manner upon the addition of Fpg protein (Figure 20, lanes 6-9). In addition, when the DNA substrate (U/G-34-mer) was reacted either with endonuclease IV or Fpg protein alone, no detectable protein-DNA interaction was observed in the assay (Figure 20, lanes 1 and 4). This result suggested that the decrease in a Dug-DNA complex formation in the presence of endonuclease IV or Fpg protein was not caused by the competition between Dug and one of these AP endonucleases for the binding to the DNA substrate. Overall the results indicated that the dissociation of Dug from AP-site-DNA occurred due to the incision of AP-site that was mediated by either AP endonuclease or AP lyase activity.

3.1.14 Kinetic Analysis of Dug Binding to DNA by Evanescent Wave Biosensor

Surface mass measurements were performed using an IAsys biosensor to determine the specificity of the interaction between Dug and various DNA oligonucleotides. In this system, biotinylated oligonucleotides were immobilized on the streptavidin-coated surface of the reaction cell, and then subjected to a binding reaction with Dug. In order to ensure that biotin or streptavidin would not affect Dug binding to the DNA, band mobility shift assays were performed using non-biotinylated and biotinylated oligonucleotides in the absence and presence of streptavidin (Figure 21). Control reactions conducted in the absence of protein revealed the differences in the mobility between non-biotinylated and biotinylated DNA (Figure 21,
Figure 20. Effect of *E. coli* Fpg on DNA binding activity of Dug. DNA binding reaction mixtures (10 µl) containing 10 nM of [³²P]U/G-34-mer, 20 nM Dug, and either 10 nM *E. coli* endonuclease IV (*lane 3*) or various amount of *E. coli* Fpg (0, 1, 5, 20, and 50 nM: *lanes 5-9*, respectively) were incubated at 30 °C for 30 min. Control reactions were carried out by incubation of 10 nM of [³²P]U/G-34-mer with either 10 nM endonuclease IV (*lane 1*), 20 nM Dug (*lane 2*), or 50 nM Fpg (*lane 4*). After incubation, samples (5 µl) were analyzed using nondenaturing 6% polyacrylamide gel electrophoresis as described under "Experimental Procedures". *Arrows* indicate the location of the free (F) and bound (B) forms of [³²P]34-mer DNA visualized by autoradiography.
Figure 21. Band mobility shift analysis of Dug interaction with biotinylated DNA and effect of streptavidin on DNA binding. DNA binding reaction mixtures (10 μl) were prepared containing 10 nM each of [\textsuperscript{32}P]AP/G-34-mer (lanes 1-4), [\textsuperscript{32}P]C/G-34-mer (lanes 5-8), [\textsuperscript{32}P]AP/G-biotin-40-mer (lanes 9-12), and [\textsuperscript{32}P]C/G-biotin-40-mer (lanes 13-16). Reaction mixtures were incubated in the absence (-) or presence (+) of Dug (10 nM) and streptavidin (0.2 mg) at 30 °C for 30 min, as indicated, and samples (5 μl) were analyzed using nondenaturing 6% polyacrylamide gel electrophoresis as described under "Experimental Procedures". The location of [\textsuperscript{32}P]DNA probes on the autoradiogram are indicated by arrows as following: unbound [\textsuperscript{32}P]34-mer (F1), unbound [\textsuperscript{32}P]40-mer (F2), [\textsuperscript{32}P]34-mer bound by Dug (B1), [\textsuperscript{32}P]40-mer bound by Dug (B2), and [\textsuperscript{32}P]40-mer bound by Dug and streptavidin (B3).
Figure 21

<table>
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<tr>
<th></th>
<th>Dug</th>
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<td>biotin</td>
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<td>Streptavidin</td>
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Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B3
B2
B1
F2
F1
lanes 1, 5, 9, and 13). As expected, streptavidin bound to the biotinylated but not with non-biotinylated oligonucleotide DNA (Figure 21, lanes 3, 7, 11, and 15). Neither non-biotinylated nor biotinylated homoduplex (C/G) DNA were specifically bound by Dug (Figure 21, lanes 6, 8, 14, and 16), while heteroduplex DNA oligonucleotides containing an AP/G residue were mobility-shifted due to the interaction with Dug (Figures 21, lanes 2 and 10). These interactions were not significantly influenced by the presence of streptavidin (Figure 21, lanes 4 and 12). Furthermore, in the presence of streptavidin, the mobility of the Dug-biotinylated (AP/G) DNA complex was "super" shifted (Figure 21, lane 12). However, the reaction of biotinylated homoduplex (C/G) DNA with both streptavidin and Dug did not display an obvious super-shifted mobility (Figure 21, lane 16), indicating that the interaction was specific for the AP/G residue on the DNA. These results demonstrated that the binding properties of Dug on AP-site-DNA were not altered either by the biotin-DNA substrate or by the streptavidin in the binding reaction. The DNA sequence of the oligonucleotides used in the biosensor experiments was designed to be identical to that of oligonucleotide substrates employed in the band mobility shift assay to avoid a possible sequence context effect on Dug-DNA interaction.

An initial experiment was designed to check the specificity of Dug binding to the single- or double-stranded oligonucleotide containing either a uracil or AP-site target. The same amounts of single-stranded (U-40-mer or AP-site-40-mer) or double-stranded biotinylated oligonucleotides (AP/A-40-mer, U/G-40-mer, or AP/G-40-mer) were immobilized on the reaction cell surface, and 80 nM of Dug was then applied to determine the binding specificity of Dug, as described under the "Experimental Procedures". The refractory index corresponding to the changes in the amount of protein bound to the surface was measured and displayed as real-time binding isotherms as shown in Figure 22. Dug binding to the single-stranded DNA either containing a site-specific uracil or AP-site residue was not significantly
Figure 22. Interaction of Dug with DNA containing uracil or AP-site assessed by evanescent wave biosensor. Biotinylated DNA substrates were immobilized on the surface of streptavidin-coated reaction cells equilibrated in DAB buffer containing 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 5% (w/v) glycerol as described under "Experimental Procedures". The representative binding isotherms shown were obtained using IAsys Plus system (Affinity Sensors) by the addition of Dug (80 nM) to the equilibrated reaction cell coated with immobilized single- or double-stranded DNA containing U, AP, AP/A, U/G, or AP/G target residues as indicated. The response refers to the change in refractory index due to binding which is recorded in arc second.
Figure 22

Response (Arc sec x 10^-2)

Time (sec)

AP/G
U/G
AP/A
AP
U
elevated when compared to the double-stranded DNA reactions. This low affinity for Dug binding was also observed using homoduplex (C/G) oligonucleotide (data not shown). In contrast, oligonucleotide duplex DNA containing an AP-site opposite to adenine or guanine appeared to display increased interaction with Dug. The binding affinity for AP/G-oligonucleotide was ~2.5-fold higher compared with AP/A-oligonucleotide and similar to that for U/G-oligonucleotide. Since the catalytic activity of Dug was able to process uracil efficiently at a U/G mispair to generate an AP-site opposite to guanine, the binding isotherm obtained for U/G-DNA could be considered to be the result of Dug binding largely to AP/G-DNA that was produced during the binding reaction. These results were consistent with the observation obtained by band mobility shift assays that demonstrated stable protein-DNA complex occurred between Dug and DNA containing AP/G residue.

To obtain a quantitative measurement for Dug binding to AP-site-DNA, an appropriate oligonucleotide (AP/G- or AP/A-biotinylated 40-mer) was immobilized on the reaction cell surface, and the binding isotherms at various concentrations of Dug were determined. Analysis of the binding isotherm data using FASTfit software resulted in a calculated kinetic value, $k_{on}$ (pseudo first-order rate constant), and plotting of the $k_{on}$ value against the Dug concentration allowed for the determination of the association rate constant ($k_{ass}$) from the slope and dissociation constant ($k_{diss}$) from the y-intercept. The plot for AP/G-oligonucleotide is shown in Figure 23. From these kinetic constants, the equilibrium dissociation constant ($K_D$) of Dug was calculated for AP/G- and AP/A-DNA by the equation $K_D = k_{diss}/k_{ass}$. As shown in Table 2, binding of Dug to the AP/G-DNA ($K_D = 2.38$ nM) was ~4-fold stronger than to the AP/A-DNA ($K_D = 10.06$ nM). This higher binding specificity of Dug to the AP/G-DNA appeared to be associated with both association and dissociation phases of Dug-DNA interaction because the $k_{ass}$ value was higher (1.5-fold) and $k_{diss}$ value was lower (3-fold) for AP/G- compared to the AP/A-DNA.
Figure 23. Analysis of association kinetics of Dug binding to DNA containing an AP-site by evanescent wave biosensor. Biotinylated oligonucleotide duplex DNA containing an AP/G target site was immobilized on the surface of streptavidin-coated reaction cells equilibrated in DAB buffer containing 100 mM NaCl as described under "Experimental Procedures". Various Dug concentrations (0, 1.9, 3.7, 7.5, 15, 22.2, 29.9, 59.8 nM) were applied to the reaction cells, and the change in refractory index due to binding obtained at different concentrations of Dug were determined and analyzed with the IAsys FASTfit program assuming a single exponential association. A linear plot of $k_{on}$ values at various protein concentrations is shown.
Figure 23
Table 2
Kinetic and equilibrium dissociation constants for the interaction between Dug and oligonucleotide duplex DNA containing either AP/A or AP/G target sites

The determination of the kinetic constants are shown as the average of three independent determinations done at different concentrations of purified recombinant Dug as described under “Experimental Procedures” using the IAsys Plus system (Affinity Sensors). The standard deviations were calculated and provided in the parenthesis. The calculated equilibrium dissociation constant, $K_D$, was obtained from the equation $K_D = k_{dissociation} / k_{association}$ for each experiment and the values were then averaged.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>$k_{ass}$</th>
<th>$k_{diss}$</th>
<th>$K_D$</th>
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<tr>
<td></td>
<td>$M^{-1} S^{-1} \times 10^5$</td>
<td>$S^{-1} \times 10^{-3}$</td>
<td>$M \times 10^{-9}$</td>
</tr>
<tr>
<td>AP/G</td>
<td>4.38 (± 0.32)</td>
<td>1.04 (± 0.81)</td>
<td>2.38 (± 0.96)</td>
</tr>
<tr>
<td>AP/A</td>
<td>2.99 (± 0.49)</td>
<td>3.01 (± 1.19)</td>
<td>10.06 (± 2.61)</td>
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3.2 Discussion

During the course this study a Ugi-insensitive double-strand specific uracil-DNA glycosylase (Dug) was purified to apparent homogeneity from cell extracts of *E. coli*. Several observations led to the conclusion that Dug was distinct from the *E. coli* Ung: (i) Dug activity was identified and purified in the presence of Ugi, a potent and irreversible inhibitor of Ung (124, 130, 330), (ii) the molecular weight of Dug, as estimated by size exclusion chromatography (~18,000), sucrose density gradient centrifugation (~20,800), and SDS-polyacrylamide gel electrophoresis (~21,000) was lower than that obtained for Ung (~25,000), (iii) Dug was inactive on the single-stranded uracil-containing substrates preferred by Ung (56, 68), and (iv) the turnover number of Dug, compared to Ung (~800/min), was extremely low (49, 130). Since many properties of this Ugi-insensitive uracil-DNA glycosylase were similar to the *E. coli* ORF-169 gene product first referred to as dsUDG (107), and subsequently as MUG (113) and εCDG (106), we cloned the gene and purified the overproduced recombinant enzyme, and found it to be indistinguishable from the Ugi-insensitive uracil-DNA glycosylase. Further, the molecular weight of the purified enzyme determined to be 18,670 daltons by mass spectrometry analysis was in excellent agreement with the deduced molecular weight of the amino acid sequence, 18,672 daltons. An examination of substrate specificity of the enzyme revealed that Dug acts on U-A-containing DNA, and has a relatively broad substrate specificity that was restricted to duplex DNA substrate; 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, it is more appropriate 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: (i) Dug may function as a "suicide enzyme" that is inactivated after a single cleavage reaction, such as O\(^6\)-alkylguanine methyltransferase (331) or type I restriction endonucleases (332, 333); (ii) Dug may bind to the AP-site-containing DNA reaction product with sufficient affinity that it is subsequently restricted from engaging new substrate; and (iii) a cofactor(s) essential for efficient Dug function may be absent in the preparation or reaction mixture. DNA-binding experiments involving band mobility 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. Evanescent wave biosensor experiments revealed that Dug had little inherent affinity for single-stranded DNA regardless of the presence of uracil or AP-sites but had nanomolar affinity for AP-site-containing double-stranded DNA. Since the binding affinity of Dug was determined to be ~4-fold higher to AP/G-DNA than AP/A-DNA, the tight binding of Dug to the double-stranded DNA appeared to be dependent on the duplex nature and base opposite to the AP-site. Therefore, the tight binding of Dug to the AP-site after excision of uracil or ethenocytosine opposite to guanine may result in product inhibition through the formation of a stable Dug-DNA complex. Several previous studies have demonstrated that E. coli, as well as, human uracil-DNA glycosylase are subjected to product (AP-site) inhibition using highly sensitive substrate-competition activity assays (59, 89, 334).

Since AP-sites in E. coli DNA are subjected to a variety of AP endonuclease activities (31, 335), it was reasonable to propose that 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 a modest excess (4-fold) of endonuclease IV. Stimulation of Dug
activity was apparently promoted by enzyme dissociation from AP/G-containing DNA, since it was observed that less Dug-DNA complex was detected 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 (236, 259). Interestingly, during the cleavage assay with the reactions including both Dug and endonuclease IV, a second reaction product was observed (Figure 17), one that presumably contained a 3'-terminal hydroxyl rather than the 3'-terminal phosphate created by alkali cleavage of Dug-generated AP-sites (101). 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. These results suggested that it was the interaction of endonuclease IV with DNA, rather than a protein-protein interaction between Dug and endonuclease IV, that promoted displacement of Dug from AP/G-containing DNA. In *E. coli*, endonuclease IV constitutes approximately 5-10 % of total cellular AP endonuclease activity, whereas exonuclease III is thought to comprise 80-90 % (236). Whether exonuclease III activity might also stimulate the activity of Dug remains to be determined. In addition, a number of AP lyases (class I AP endonuclease) exist in *E. coli*, such as Fpg, MutY, exonuclease III, and exonuclease VIII (202, 214, 329, 336). Since the stimulation of Dug activity occurred through AP-site cleavage mediated by endonuclease IV, we tested whether the AP lyase activity that cleaves on the 3'-side of the AP-site has an effect on the Dug/AP-DNA interaction. Similar to endonuclease IV, Fpg protein appeared to provoke dissociation of Dug from AP-site containing DNA. Since these observations demonstrated that the enhancement of catalytic activity of Dug by either class of AP endonuclease was mediated by the incision of the AP-site, it was logical to expect that Dug would not re-bind to the product DNA containing a nick that resulted from
the AP endonuclease induced incision. Indeed, it appeared that Dug did not bind DNA that was nicked by endonuclease IV; this was demonstrated directly in Figure 19.

Waters et al. (112) investigated the effect of human AP endonuclease 1 (HAP1) on the catalytic efficiency of human thymine-DNA glycosylase (hTDG). 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 (105, 113). The presence of a 10-fold excess of E. coli 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 human 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 (112). Interestingly, HAP1-mediated stimulation of hTDG activity was much greater on U/C-containing DNA (112). This finding is consonant with the observation that dissociation of hTDG, in the presence of 2 mM Mg²⁺, is more rapid from DNA containing an AP-site opposite a cytosine or S⁶-methylthioguanine than from AP/G-DNA (112). 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 (107). 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 significant 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 (319) constructed E. coli strains that were 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 (319). In contrast, the reversion frequency of the *E. coli ung* mutant strain was elevated approximately 8-10-fold compared to wild-type (*ung*) strain (319). 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, further elucidation of the role of Dug in base excision DNA repair remains to be elucidated.

Using a rifampicin-resistance forward mutation assay, Lutsenko and Bhagwat (319) 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 (319). In the present study, it was observed that Dug efficiently excises εC from εC/A as well as εC/G, mispairs. Taken together, these data further strengthen the hypothesis of Lutsenko and Bhagwat (319), and Saparbaev and Laval (106), 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. (113) 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. 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 (113). 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 eC/A in this study, nor with the very low activity against T/G-containing DNA observed in this study as well as by Saparbaev and Laval (106). In this regard, it is notable that, following the processing of a eC/A mispair, Dug did not appear to bind specifically to the resultant AP/A-DNA in a band mobility shift assay. 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 that are proposed to interact with the opposite guanine are Gly-143 and Ser-145, which are found in the "wedge" motif (NPSGLSR) (113). 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 eC/G-containing substrates reported by Saparbaev and Laval (106). As demonstrated in this investigation, Dug loses affinity for the "widowed" guanine once the AP-site is 5'-incised by endonuclease IV. The structural basis for this loss of the interaction is not known at this time.

Many DNA glycosylases have overlapping substrate specificities and may provide back-up functions for each other (180). 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 (107). However, in the E. coli ung-1 strain defective in Ung activity, the level of spontaneous G/C to A/T transition mutations was increased ~30-fold relative to the isogenic ung+ strain (48). These results would indicate that in this ung- strain, if the dug gene is indeed wild-type, then either it does not appear to play a significant back-up role in preventing G/C to A/T transition mutations, or the actual G/C to A/T transition mutation rate will be much higher in the ung dug- double mutant. Since Dug has been shown to exhibit a strong preference for ethenocytosine residues in double-stranded DNA, one might
wonder whether an *E. coli* strain defective in *dug* would exhibit an elevated mutation rate upon exposure to the exogenous agents like vinyl chloride. It is important to note that some of the enzymes involved in the repair of DNA damage caused by exogenous agents are induced by the damaging treatment. These include the nucleotide excision repair proteins (337-339), 3-methyl adenine-DNA glycosylase II (340), and endonuclease IV (263). In this study it was demonstrated that Dug activity is stimulated by endonuclease IV. This may be significant since endonuclease IV is inducible under oxidative stress condition in *E. coli* cells. The most common base substitution caused by oxidative damage to DNA is G/C to A/T transition, and one of the main source of this mutation arises from 5-hydroxyuracil, which resulted in DNA through oxidation of cytosine followed by dehydration and deamination (341). Although several *E. coli* proteins have been implicated in the removal of the 5-hydroxyuracil (70, 180), it would be interesting to examine the catalytic activity of Dug on the oxidized cytosine residues such as 5-hydroxyuracil.
4. FIDELITY OF URACIL-INITIATED BASE EXCISION DNA REPAIR IN ESCHERICHIA COLI CELL EXTRACTS

The biochemical mechanisms involved in each step of *E. coli* uracil-initiated BER have been elucidated in substantial detail. However, an assessment of the fidelity of DNA repair synthesis associated with the complete uracil-initiated BER process in *E. coli* has not been investigated. In the study presented in this Chapter, the involvement of both Ung and Dug in uracil-initiated BER was demonstrated in *E. coli* cell extracts utilizing a circular duplex M13mp2 DNA substrate containing a site-specific uracil residue. The use of M13mp2 form I DNA was extended to monitor the relative rate of Ung- and Dug-mediated uracil-DNA repair processes in *E. coli* defective in ung or dug. In addition, the location of repair DNA synthesis was examined to establish that uracil-DNA repair occurred via the BER pathway. Experiments were conducted to evaluate the repair patch size produced during uracil-initiated BER mediated by either Ung or Dug activity. An M13mp2 lacZα DNA-based reversion assay was developed to measure the base substitution error frequency produced during uracil-initiated BER, and the mutational spectrum of base substitution errors introduced by Ung- and Dug-mediated uracil-DNA repair was defined.

4.1 Results

4.1.1 Uracil-initiated Base Excision DNA Repair Assay

An M13mp2 lacZα DNA-based reversion assay was utilized to detect uracil-initiated DNA base excision repair and to determine the base substitution error frequency associated with the completed repair reaction. Briefly, the arginine codon 14 (CGT) of the lacZα gene in M13mp2 DNA was replaced with an opal codon (TGA) by site-directed mutagenesis (314). Circular heteroduplex M13mp2op14 DNA with a site-specific uracil at nucleotide position 78 of the (-) strand DNA was constructed in a primer
extension reaction using T4 DNA polymerase and T4 DNA ligase and Form I DNA was purified. This DNA substrate contained a U·T base mispair at the first position of the opal codon 14 that served as the uracil target for the repair; excision of the uracil residue by uracil-DNA glycosylase initiated the BER pathway (Figure 24A). The uracil residue was strategically located such that faithful and unfaithful uracil-initiated DNA repair synthesis in E. coli cell-free extracts could be distinguished by the lacZα complementation phenotype of the M13mp2 phage genome (Figure 24B). If faithful DNA synthesis occurred during BER opposite the template thymine residue at position 78, a dAMP nucleotide would be incorporated into the (-) strand, and the opal codon would be reestablished in both DNA strands. As the (-) strand DNA serves as the template for production of single-stranded M13 DNA in E. coli, the resulting phage would be expected to be defective in α-complementation. Accordingly, the plaques produced were colorless phenotype when grown on appropriate host indicator plates (Figure 25A). However, if BER DNA synthesis was unfaithful, dCMP, dGMP, or dTMP would be misincorporated. Each of these base substitutions would restore (reverts to wild-type) the α-complementation phenotype; therefore, these mutant phage would produce blue-colored plaques. Misincorporation of either dGMP or dTMP restored the wild type arginine codon that displayed a dark blue plaque phenotype (Figure 25C and D, respectively), whereas dCMP incorporation resulted in a glycine codon, shown to generate a light blue plaque phenotype (Figure 25B). Only reversion at the opal codon resulted in a blue plaque phenotype; misincorporation at other positions in the lacZα gene would not be detected.

Detection of uracil-initiated base excision DNA repair was begun by incubation of form I (U·T) heteroduplex M13mp2op14 DNA with cell extracts that had been supplemented with dNTPs and the components of an ATP regenerating system (Figure 26A). Initially, a time course of incubation was carried out in order to optimize BER reaction conditions and determined the extent of repair. M13mp2op14 DNA was then recovered from the cell extract
Figure 24. Scheme for detecting faithful and unfaithful base excision repair DNA synthesis at the uracil target site. (A) M13mp2op14 DNA (form I) containing a site-specific uracil mispaired with thymine located in opal codon 14 at nucleotide position 78 of the lacZα gene was constructed as described under "Experimental Procedures." The uracil target for uracil-DNA glycosylase (vertical arrow) and direction of DNA repair synthesis on the (-) strand (horizontal arrow) resulting from base excision repair are indicated. EcoRI and Smal endonuclease restriction sites are indicated by vertical lines, respectively. (B) Faithful incorporation of dAMP to replace the uracil residue at position 78 in the (-) strand results in the production of an opal codon. Whereas, incorporation of dCMP, dGMP or dTMP restores a wild-type phenotype and allows α-complementation. Only reversion at the opal codon results in a blue plaque phenotype.
A.

M13mp2
IacZα
Form I

lacZα

EcoR I
Opal Codon 14
Sma I

TGA

5' Primer (-)

UCT

BER DNA Synthesis

Uracil-DNA Glycosylase

B.

<table>
<thead>
<tr>
<th>Incorporation</th>
<th>Sequence (+)</th>
<th>Amino Acid</th>
<th>M13 Plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAMP</td>
<td>TGA</td>
<td>Opal</td>
<td>Clear</td>
</tr>
<tr>
<td>dCMP</td>
<td>GGA</td>
<td>Gly</td>
<td>Light Blue</td>
</tr>
<tr>
<td>dGMP</td>
<td>CGA</td>
<td>Arg</td>
<td>Blue</td>
</tr>
<tr>
<td>dTMP</td>
<td>AGA</td>
<td>Arg</td>
<td>Blue</td>
</tr>
</tbody>
</table>

Figure 24
Figure 25. Plaque phenotype of M13mp2op14 DNA and revertants produced by uracil-initiated BER. The four possible nucleotide incorporations at the uracil target site during BER in *E. coli* cell extracts were analyzed for plaque phenotype color upon transfection and plating of *E. coli* NR9162 cells as described under "Experimental Procedures". M13mp2op14 DNA containing an opal codon (TGA) produced colorless plaques (A) while reversion of the opal codon to a glycine codon (GGA) or an arginine codon (CGA and AGA) produced light blue (B) or dark blue plaques (C and D, respectively), and are represented accordingly. The single-base substitution generated by the original misincorporated nucleotide has been underlined for M13mp2op14 DNA revertants.
Figure 26. Procedure for measuring uracil-initiated BER DNA synthesis fidelity. (A) Uracil-initiated BER reaction was conducted by incubation of form I (U-T) heteroduplex M13mp2op14 DNA with *E. coli* cell extracts in the presence of dNTPs as described under "Experimental Procedures." (B) Reactions were incubated for 0, 15, and 60 min at 30 °C, and the DNA reaction products were recovered and subsequently analyzed by 0.8% agarose gel electrophoresis. In one case, the M13mp2op14 DNA recovered from a 60 min reaction was treated with excess *E. coli* Ung and Endo IV (+) prior to electrophoresis as described under "Experimental Procedures." Form I and II DNA standards were prepared and resolved on the same agarose gel containing the BER reaction products in order to quantify the amount (ng) of form I and II DNA (*horizontal arrows*) produced during BER. (C) Ung/Endo IV-resistant repaired form I DNA was isolated and used to electroporate competent *E. coli* NR9162 (*mutS*) cells. Transfected NR9162 cells are then combined with the indicator *E. coli* strain CSH50 and plated with top agar containing IPTG and X-gal as described under "Experimental Procedures." The frequency of unfaithful DNA synthesis was calculated by dividing the number of blue plaques produced by the sum of clear and blue plaques. The *arrow* indicates the location of a blue M13 plaque.
A. M13mp2 \( \text{lacZ} \alpha \text{ op14} \)

\[ \text{Form I DNA} \]

\[ \text{Cell Extracts} \]

B. 

<table>
<thead>
<tr>
<th>DNA (ng)</th>
<th>Form I</th>
<th>Form II</th>
<th>Ung/Endo IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>25</td>
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<th>60</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Form II</th>
<th>Form I</th>
</tr>
</thead>
</table>

C. Purification

Form I DNA

Electroporation \( E. \text{coli NR9162 (mutS)} \)

\( E. \text{coli CSH50} \)

+ IPTG
+ X-gal

Figure 26
and treated *in vitro* with excess *E. coli* uracil-DNA glycosylase (Ung) and endonuclease IV (Endo IV) in order to convert unrepaired form I DNA to form II DNA (Figure 26B). Since both unreacted substrate DNA and fully repaired M13mp2op14 DNA would migrate as form I molecules during agarose gel electrophoresis, whereas M13mp2op14 DNA containing nicked and/or missing one or more nucleotides (incomplete or aberrant repair) migrated as form II DNA, this Ung/Endo IV treatment would remove unreacted substrate from the pool of repaired molecules by creating a nicked AP-site-containing form II DNA molecule. Therefore, the form I DNA resistant to this Ung/Endo IV treatment would represent bona fide completely repaired DNA. In order to quantify the amount of DNA reaction products, internal standards containing predetermined quantities of form I and II M13mp2op14 DNA were prepared and separately analyzed on the same agarose gel that contained sample lanes (Figure 26B). Since the ethidium bromide staining intensity of form I and II DNA appeared to vary from gel to gel, it was necessary to include a complete set of internal standards in each analysis. Using the ethidium bromide staining intensity of the internal standards, individual standard curves for form I and II DNA were generated to correlate the staining intensity of experimental samples with defined quantities of DNA (Figure 27).

In order to distinguish faithful from unfaithful DNA repair synthesis events, the Ung/Endo IV-resistant form I DNA was extracted from the agarose gel by electroelution and used to electroporate competent *E. coli* NR9162 (*mutS*) cells (Figure 26C). This strain, which is defective for methyl-directed DNA mismatch repair (316, 342), was used in order to avoid *E. coli*-mediated correction of base-base mispairs that might arise due to unfaithful DNA repair reaction during BER. Transfected NR9162 cells were combined with the indicator *E. coli* strain CSH50 and plated with top agar containing IPTG and X-gal. Faithfully repaired M13mp2op14 DNA produced a clear (colorless) plaque phenotype, whereas the unfaithful incorporation of a non-complementary nucleotide in place of the uracil residue could restore the
Figure 27. Standard curves used for quantification of form I and II M13mp2op14 DNA. The ethidium bromide staining intensity of form I and II M13mp2op14 DNA standards obtained similarly to that described in Figure 26B were quantified using a Gel Documentation System (Ultra Violet Products Ltd.) and ImageQuant software. The relative intensity of DNA bands containing 6.25, 12.5, 25, 50, 100 ng of Form I (open circles) and II (closed circles) DNA were used to generate individual standard curves for quantifying the amount (ng) of form I and II DNA in the experimental samples analyzed on the same 0.8% agarose gel. The average ethidium bromide staining intensity of form II DNA was ~3-fold greater than that of an equal amount (ng) of form I DNA.
Figure 27
reading frame of the lacZα gene and through α-complementation resulted in a blue plaque phenotype. The frequency of unfaithful DNA synthesis was calculated by dividing the number of blue plaques produced by the sum of clear and blue plaques.

4.1.2 Detection of Uracil-initiated BER in *E. coli* NR8051 (*ung*⁺) and NR8052 (*ung*⁻) Cell Extracts

Initial experiments were conducted to detect uracil-initiated BER in *E. coli* extracts of Ung-proficient (NR8051) and Ung-deficient (NR8052) cells. M13mp20p14 (U·T) DNA (form I) was incubated with each cell-free extract for various times in the absence and presence of Ugi, and the reaction products were isolated, subjected to the Ung/Endo IV treatment, and resolved by agarose gel electrophoresis (Figure 28). Mock reacted M13mp20p14 (U·T) DNA molecules in the absence of cell extract were also treated with Ung/Endo IV and migrated (>95%) as form II DNA (Figure 28, lanes C), whereas the unreacted control DNA substrate was found to contain almost exclusively (>95 %) form I DNA (Figure 28, lanes S). These results validated that the Ung/Endo IV treatment was effective in determining the complete repair of uracil-DNA by conversion of unrepaired form I DNA substrate to form II DNA. Inspection of Ung/Endo IV resistant form I DNA revealed that the repair of uracil occurred efficiently and in a time dependent manner in Ung-proficient cell extract (Figure 28A, lanes 1-6). However, when the reaction was conducted in the presence of Ugi, a significantly reduced level of repaired form I DNA observed (Figure 28B, lanes 1-6). A similar extent of repair was also detected in the reaction conducted in Ung-deficient cell extracts in both absence and presence of Ugi (Figure 28C and D, lanes 1-6). These results indicated that the majority of uracil repair events were initiated by a Ugi-sensitive uracil-DNA glycosylase that was associated with the activity of *E. coli* Ung and implied involvement of the BER pathway.
Figure 28. Detection of uracil-initiated BER in *E. coli* NR8051 (ung*) or NR8052 (ung') cell extracts using M13mp2op14 (U·T) DNA. Standard BER reaction mixtures (100 μl) containing 1 μg of M13mp2op14 (U·T) DNA, 0.1 mg of *E. coli* NR8051 (A and B) or NR8052 (C and D) cell extracts were incubated for 0, 10, 20, 30, 40, and 60 min at 30 °C (lanes 1-6, respectively) in the absence (A and C) and presence (B and D) of 1000 units of Ugi. Each reaction was then terminated by addition of 25 μl of 0.1 M EDTA and the samples heated at 70 °C for 3 min. The M13mp2op14 DNA was recovered following the BER reactions, subjected to Ung/Endo IV treatment, and analyzed by 0.8% agarose gel electrophoresis as described under "Experimental Procedures." As a control, M13mp2op14 (U·T) DNA (1 μg) was mock reacted and then treated Ung and Endo IV (lane C). Untreated M13mp2op14 (U·T) DNA (100 ng) and a sample containing 1 μg of a 1-kb DNA ladder were employed 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.
The percentage of repaired form I DNA detected in each lane was determined and is represented in Figure 29. The results showed quantitatively that during the first 20 min of the reaction, the initial rate of repair was ~5.5-fold slower in Ung-deficient (NR8052) compared to the Ung-proficient (NR8051) cell extracts. The addition of Ugi did not appear to affect the rate or extent of repair in extracts of *E. coli* NR8052 cells. However, uracil-initiated BER was substantially diminished in extracts of the Ung-proficient strain NR8051 when supplemented with Ugi. The level of repaired DNA, detected after 60 min, in the Ugi-supplemented Ung-proficient cell extract was comparable to that observed in both the Ung-deficient and Ugi-supplemented Ung-deficient cell extracts. These results implied that the majority of uracil-initiated BER involved the uracil-DNA glycosylase activity associated with the *ung* gene product; however, some repair of uracil occurred without the involvement of Ung activity.

4.1.3 Involvement of Dug in Uracil-initiated BER in *E. coli* NR8052 (*ung*) Cell Extract

The major Ugi-insensitive uracil-excision activity in *E. coli* cell has been demonstrated to be associated with Dug as reported during the course of this study (343) and discussed in Chapter 3. In addition, it has been reported that the excision of uracil by Dug occurs efficiently from U/T as well as U/G and U/C mispairs, but with significantly reduced efficiency from U/A base pair (106, 343). Based on these observations, it was proposed that the complete repair of uracil in the absence of Ung activity might be mediated by the uracil-DNA glycosylase activity of Dug. Initially an experiment was conducted to determine whether the Ung-independent repair of uracil also occurred from U/G mispairs by utilizing the M13mp2op14 (U·G) DNA substrate. Time course BER reactions were conducted by incubation of M13mp2op14 (U·G) DNA with *E. coli* NR8051 (*ung*) or NR8052 (*ung*) cell extract in the absence and presence of Ugi. Analysis of the repaired DNA product by agarose gel
Figure 29. Analysis of *E. coli* uracil-initiated BER of M13mp2op14 (U-T) DNA in the presence and absence of Ugi. Standard BER reaction mixtures (100 µl) was conducted, and the M13mp2op14 DNA was recovered, subjected to Ung/Endo IV treatment, analyzed by 0.8% agarose gel electrophoresis as described in Figure 28. DNA bands detected by ethidium bromide staining were quantitatively measured relative to the corresponding DNA standards (6.3-100 ng) using a Gel Documentation System. The percentage of form I DNA in each sample was calculated by dividing the amount of form I DNA (ng) by that of form I plus II DNA and multiplying by 100. The percentage of repaired (form I) DNA in each sample was plotted as a function of time for the following reactions: (●) *E. coli* NR8051 minus Ugi, (○) NR8051 plus Ugi, (■) NR8052 minus Ugi, and (□) NR8052 plus Ugi. Mean values and standard deviations of three experiments are represented.
electrophoresis revealed that the repair of uracil from U/G mispairs occurred in the reactions deficient in Ung activity but at a slower rate relative to the reaction in Ung-proficient cell extract (Figure 30). Evaluation of the repair kinetics with M13mp2op14 (U·G) DNA in each reaction revealed that the initial rate of repair was ~4-fold lower in the absence than in the presence of Ung activity, which was similar to the observation that obtained using M13mp2op14 (U·T) DNA (Figure 31). These results demonstrated that the Ung-independent uracil repair event also occurred from U/G mispairs that were most likely processed by Dug (107, 343).

In order to ascertain whether Dug may participate in uracil-initiated base excision repair in E. coli, BER assays were conducted using M13mp2op14 (U·G) DNA (form I). To eliminate Ung-mediated repair of uracil, 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 products isolated, and treated with excess Ung/Endo IV in order to convert unrepaired DNA molecules to form II DNA. Analysis of reaction products by agarose gel electrophoresis revealed the time dependent appearance of repaired form I DNA (Figure 32A, lanes 1-7, -Dug). To examine whether Dug was indeed involved in this repair reaction, the BER reactions were supplemented with purified Dug. Under this condition, the amount of Ung/Endo IV-resistant form I DNA formed was significantly increased (Figure 32A, lanes 1-7, +Dug). 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 32B). 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 in the absence of Ung activity.
Figure 30. Detection of uracil-initiated BER in E. coli NR8051 (ung⁺) or NR8052 (ung⁻) cell extracts using M13mp2op14 (U·G) DNA. M13mp2op14 (U·G) DNA was prepared containing a uracil mispaired with guanine located in opal codon 14 at nucleotide position 79 of the lacZα gene as described under "Experimental Procedures". Standard BER reaction mixtures (100 µl) containing 1 µg of M13mp2op14 (U·G) DNA, 0.1 mg of E. coli NR8051 (A and B) or NR8052 (C and D) cell extracts were incubated for 0, 10, 20, 30, 40, and 60 min at 30 °C (lanes 1-6, respectively) in the absence (A and C) and presence (B and D) of 1000 units of Ugi. The M13mp2op14 DNA was recovered following the BER reactions, subjected to Ung/Endo IV treatment, and analyzed by 0.8% agarose gel electrophoresis as described in Figure 28. As a control, M13mp2op14 (U·G) DNA (1 µg) was mock reacted and then treated Ung and Endo IV (lane C). Untreated M13mp2op14 (U·G) DNA (100 ng) and a sample containing 1 µg of a 1-kb DNA ladder were employed 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.
Figure 31. Analysis of *E. coli* uracil-initiated BER of M13mp2op14 (U·G) DNA in the presence and absence of Ugi. M13mp2op14 form I and II DNA bands detected in Figure 30 were quantified using a Gel Documentation System as described in Figure 29, and the percentage of form I DNA in each sample was calculated by dividing the amount of form I DNA (ng) by that of form I plus II DNA and multiplying by 100. The percentage of repaired (form I) DNA in each sample was plotted as a function of time for the following reactions: (●) *E. coli* NR8051 minus Ugi, (○) NR8051 plus Ugi, (■) NR8052 minus Ugi, and (□) NR8052 plus Ugi.
Figure 31
Figure 32. Involvement of Dug in uracil-initiated BER in *E. coli* NR8052 (ung) cell extracts. (A) Two BER 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 Dug (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 excess *E. coli* Ung and Endo IV, and then analyzed by 0.8% agarose gel electrophoresis as described under "Experimental Procedures". 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 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 staining. (B) DNA bands detected (A) were quantitatively measured as described in Figure 29, and the percentage of repaired form I DNA detected in samples with (●) and without (○) Dug addition was calculated by dividing the amount of form I (ng) by that of form I plus form II DNA and multiplying by 100.
Figure 32

A. - Dug

+ Dug

C 1 2 3 4 5 6 7 M C 1 2 3 4 5 6 7

Form II

Form I

B. Form I DNA (%)

Time (min)

Figure 32
4.1.4 Evidence for Uracil-initiated BER DNA Synthesis in *E. coli* NR8051 (*ung*) and NR8052 (*ung*) Cell Extracts

To determine if uracil-initiated BER DNA synthesis was involved in the production of Ung/Endo IV-resistant form I DNA, standard BER reactions were conducted using M13mp2op14 (U·T) DNA (form I) in the presence of [α-[^32]P]dATP. After agarose gel electrophoresis of the BER reaction products, examination of the PhosphoImages showed the incorporation of[^32]P]dAMP into the Ung/Endo IV-resistant form I DNA recovered from reactions containing cell extracts of *E. coli* NR8051 (*ung*) (Figure 33A) as well as NR8052 (*ung*) (Figure 33B). Notably, the intensity of form I[^32]P]DNA generated in the Ung-proficient cell extract was considerably greater than that produced in the Ung-deficient extract, although the amount of[^32]P radioactivity incorporated into the total DNA (form I + form II) in each reaction was similar. Quantification of the[^32]P radioactivity associated with the form I DNA band revealed that the amount of DNA synthesis generated in Ung-deficient cell extracts was 8-fold less than that produced in Ung-proficient extracts after 60 min (Figure 33C). When taken together with the findings in Figure 29 that illustrated the extent of complete BER, the results suggest that the reduced level of DNA synthesis observed in Figure 33C likely resulted from an overall reduction in the level of BER.

4.1.5 Specificity of Uracil-initiated DNA Repair Synthesis in *E. coli* NR8051 (*ung*) Cell Extracts

To determine the distribution of[^32]P]dAMP incorporation associated with the repaired DNA molecules,[^32]P]DNA isolated from the BER reaction time course using *E. coli* NR8051 cell extracts, as described in Figure 33, was subjected to *Hinfl* restriction endonuclease digestion and resolved by nondenaturing polyacrylamide gel electrophoresis (Figure 34). While there are 26 *Hinfl* recognition sites in the M13mp2op14 DNA sequence, *Hinfl* digestion produces but 16 DNA fragments in excess of 200 bp. Among these,
Figure 33. Incorporation of $[\alpha^{32P}]dAMP$ into M13mp2op14 DNA during uracil-initiated BER. Two sets of standard BER reaction mixtures (100 μl) containing 1 μg of M13mp2op14 (U·T) DNA, 40 μCi of $[^{32P}]dATP$, and 0.1 mg of *E. coli* NR8051 (A) or NR8052 (B) cell extracts were incubated for 0, 5, 10, 30, and 60 min at 30 °C (lanes 1-5, respectively). The reactions were terminated, M13mp2op14 $[^{32P}]$DNA isolated, treated with *E. coli* Ung and Nfo, analyzed by 0.8% agarose gel electrophoresis, and the $[^{32P}]$DNA fragments were blotted from the agarose gel to a Gene Screen Plus membrane as described under "Experimental Procedures." The arrows indicate the location of form I and II DNA bands visualized by PhosphorImager. (C) The relative amount of $[^{32P}]dAMP$ incorporated into repaired form I DNA was determined for reactions containing NR8051 (■) and NR8052 (●) cell extracts and plotted after subtracting background values. The results shown represent the mean and standard deviation of three experiments.
Figure 34. Specificity of BER DNA synthesis in *E. coli* NR8051 cell extracts. (A) *Hinfl* restriction endonuclease cleavage map of M13mp2op14 DNA indicating restriction sites (*hash marks*) and the location of the 253-, 529-, 261-, and 486-bp DNA fragments. The uracil (U) residue targeted for base excision repair is located at position 78 in the (−) strand of the *lacZα* gene. (B) After conducting standard BER reactions with *E. coli* NR8051 cell extracts, DNA was isolated at various times, as described under "Experimental Procedures." DNA samples obtained from reactions conducted for 0, 5, 10, 30, and 60 min (*lanes 1-5*, respectively) were subjected to digestion with 10 units of *Hinfl* for 1 h at 37 °C and resolved by 5% nondenaturing polyacrylamide gel electrophoresis. The location of the DNA fragment (U-529) that contained the site-specific uracil is indicated by an arrow, as are the locations of three other fragments. (C) [³²P]dAMP incorporation into the DNA fragments 253- (*striped bar*), 529- (*black bar*), 261- (*white bar*), and 486-bp (*stippled bar*) was determined using a PhosphorImager and ImageQuant software. The relative intensity of each DNA fragment was measured at the time point indicated, after subtracting background values. Error bars represent the standard deviation of three experiments.
Figure 34
the 529 bp fragment containing the uracil site is bordered by 261 bp and 253 bp fragments positioned 5' and 3', respectively (Figure 34A). Following electrophoresis, inspection of the PhosphoImage showed that \( {^{32}}P \)dAMP incorporation occurred preferentially in the 529 bp fragment in a time-dependent manner (Figure 34B and C). The minor amount of incorporation detected on the neighboring fragments (261- and 253-bp) was assumed to correspond to non-specific background, since a similar level of incorporation was observed associated with a 486 bp fragment located on the opposite side of the M13mp2op14 DNA molecule (Figure 34C).

4.1.6 Uracil-dependent BER DNA Synthesis in *E. coli* NR8051 (*ung*) and NR8052 (*ung*) Cell Extracts

In order to examine whether the preferential incorporation of \( {^{32}}P \)dAMP into the 529 bp fragment was the result of BER DNA repair synthesis instigated by uracil excision, standard BER reactions were conducted in the presence of \( [\alpha-^{32}P] \)dATP as before, but the M13mp2op14 DNA substrate contained either a U·T or A·T base pair at the first position of the *lacZ\alpha* opal codon 14. After incubation for 1 h, the reaction products were recovered, and Hinfl \( {^{32}}P \)DNA fragments were resolved by nondenaturing polyacrylamide gel electrophoresis (Figure 35). Inspection of the PhosphoImage revealed that preferential incorporation of \( {^{32}}P \)dAMP into the 529 bp fragment of the (UT) DNA occurred relative to \( {^{32}}P \)dAMP incorporation into the same 529 bp fragment of the (AT) DNA in both the *E. coli* NR8051 (Figure 35A, insert) and NR8052 (Figure 35B, insert) cell extracts. In addition, corresponding non-uracil containing fragments between (U·T) and (A·T) DNA substrates appeared to accumulate similar levels of \( {^{32}}P \)dAMP incorporation in both *E. coli* cell extracts. The level of specificity was evaluated after quantifying the amount of \( {^{32}}P \)dAMP incorporation into individual DNA fragments. The results indicated that a uracil residue located in the target uracil DNA fragment stimulated DNA synthesis by 17.2- and 5.8-fold above background
Figure 35. Uracil-dependent BER DNA synthesis in *E. coli* NR8051 and NR8052 cell extracts. Standard BER reaction mixtures (100 μl) containing 0.1 mg of *E. coli* NR8051 (A) or NR8052 (B) cell extract protein were incubated with 1 μg of M13mp2op14 (A·T) DNA (lanes 1) or (U·T) DNA (lanes 2) in the presence of 40 μCi of [³²P]dATP. After incubation for 1 h at 30 °C, the DNA reaction products were isolated, subjected to *Hinfl* restriction endonuclease digestion, and the [³²P]DNA fragments were then analyzed by 5% nondenaturing polyacrylamide gel electrophoresis as described under "Experimental Procedures". The location of the 253-, U-529-, 261-, and 486-bp *Hinfl* DNA fragments is indicated by arrows on the autoradiogram shown in the inserts. [³²P]dAMP incorporation into these DNA fragments was determined as described in Figure 34, and plotted for (A·T) DNA (white bars) and (U·T) DNA (black bars), respectively.
Figure 35 (continued)
for the reactions containing *E. coli* NR8051 (Figure 35A) and NR8052 (Figure 35B) cell extracts, respectively. When taken together, these results indicate that the vast majority of DNA synthesis on the 529-bp fragment was most likely uracil initiated and was limited to this fragment. Thus, most of the DNA synthesis observed in both Ung-proficient and Ung-deficient *E. coli* cells was consistent with the occurrence of a uracil-initiated base excision repair mechanism.

4.1.7 Analysis of Uracil-initiated BER in Cell Extract of *E. coli* Defective in Ung and Dug

To further assess the role of the *dug* gene product in uracil-initiated BER, standard BER reactions were conducted using the three *E. coli* isogenic strains BH156 (ung·dug·), BH157 (ung·dug·), and BH158 (ung·dug·). Examination of the uracil-initiated BER reaction time course by agarose gel electrophoresis revealed a time-dependent accumulation of Ung/Endo IV-resistant form I DNA in both the *E. coli* BH156 (Figure 36, lanes 1-6) and BH157 cell extracts (Figure 36, lanes 7-12). However, the rate of repair appeared to be 5-fold greater in the reactions containing *E. coli* BH157 cell extract. In contrast, Ung/Endo IV-resistant form I DNA was not observed in BER reactions with BH158 cell extracts (Figure 36, lanes 13-18). This experiment indicated that uracil-initiated BER occurred in the absence of either Ung or Dug but at different rates. Since the repair of uracil in *E. coli* cell extract deficient in both Ung and Dug activities was reduced to the undetectable level, this result implied that, in the absence of Ung, uracil-initiated BER in *E. coli* was initiated by Dug.

4.1.8 Analysis of Patch Size Distribution of Uracil-initiated BER in *E. coli* NR8051 and NR8052 Cell Extracts

In order to determine the patch size distribution of DNA repair synthesis associated with uracil-initiated BER using the M13mp2op14 DNA substrate,
Figure 36. Analysis of uracil-initiated BER in *E. coli* BH156 (ung), BH157 (dug), and BH158 (ung, dug) cell extracts. Three sets of standard BER reaction mixtures (100 µl) containing 1 µg of M13mp2op14 (U·T) DNA were incubated for 0, 5, 10, 20, 30, and 60 min at 30 °C with 0.1 mg of cell extract protein from *E. coli* BH156 (lanes 1-6, respectively), BH157 (lanes 7-12, respectively), or BH158 (lanes 13-18, respectively). Form I DNA reaction products were isolated, treated with *E. coli* Ung and Endo IV, and resolved by 0.8% agarose gel electrophoresis (inset) as described under "Experimental Procedures". Untreated M13mp2op14 (U·T) DNA (100 ng) was used as a reference standard (lanes S). As a control, M13mp2op14 (U·T) DNA (1 µg) was mock-reacted, isolated, and then subjected to Ung/Endo IV treatment (lane C). The location of form I and II DNA bands is indicated by arrows. The amount of form I and II DNA detected by ethidium bromide staining was quantitatively measured with a gel documentation system and the percentage of form I DNA was determined as described under "Experimental Procedures". The results of two independent experiments are plotted for *E. coli* BH156 (●), BH157 (■), and BH158 (▲).
an approach was developed similar to that described by Huang et al. (344) and Gish and Eckstein (345), and modified as previously described (346). As shown in Figure 37, this approach relies on the resistance of DNA containing 2'-deoxyribonucleoside α-thiolmonophosphates to degradation by E. coli exonuclease III (Exo III). For this purpose, the M13mp20p14 (U-T) DNA substrate was modified to include a site-specific [32P]dCMP residue located at nucleotide position 90 on the (-) strand situated between the uracil-target and the SmaI restriction endonuclease recognition site. Using this M13mp20p14 [32P]DNA substrate, standard BER reactions were conducted in E. coli cell extracts as before except that the four complementary 2'-deoxyribonucleoside triphosphates were replaced with the corresponding 2'-deoxyribonucleoside α-thioltriphosphates. As a result, various amounts of [α-S]dNMP were incorporated into the [32P]DNA during DNA repair synthesis, and the extent of [α-S]dNMP incorporation per DNA molecule corresponded to the repair patch size. In order to locate the 3'-boundary of the repair patch produced during the uracil-initiated BER DNA synthesis, the recovered M13mp20p14 [32P]DNA was cleaved with EcoRI to generate linear DNA with a recessed 3' terminus located on the (-) strand 20 nucleotides downstream from the uracil-target residue. The (-) strand of the linearized [32P]DNA was then digested in the 3' to 5' direction by Exo III. It was anticipated that the 3' to 5' degradation of the (-) strand would terminate at the 3' border of the repair patch, since it had been reported that Exo III does not efficiently hydrolyze phosphorothiol DNA linkages (347). Subsequent to Exo III treatment, the [32P]DNA was restricted with SmaI to generate a DNA fragment containing a 32P-labeled oligodeoxynucleotide the length of which was diagnostic of the BER patch size (Figure 37). For example, a repair patch size of one nucleotide would be expected to produce a [32P]oligodeoxynucleotide 20-mer, since the incorporation of a single dAMP[α-S] at the uracil-target site would block digestion by Exo III at a location 20 nucleotides from the 5'-end created by SmaI restriction. Similarly, repair patches consisting of two or more
Figure 37. Scheme for determining uracil-initiated BER patch size with M13mp2op14 (U·T) DNA using *E. coli* exonuclease III digestion analysis. The $^{32}$P-labeled M13mp2op14 (U·T) form I DNA was constructed, as described under "Experimental Procedures", and contained a $^{32}$P radiolabel (*) located 13 nucleotides upstream of the target uracil and 7 nucleotides downstream of the *SmaI* restriction site on the (-) strand DNA. Using this $^{32}$P DNA substrate, standard BER reactions were conducted with *E. coli* cell extracts in the presence of the four complementary 2'-deoxyribonucleoside α-thioltriphosphates. In order to locate the 3'-boundary of the repair patch, the recovered M13mp2op14 $^{32}$P DNA was cleaved with *EcoRI* to generate linear DNA with a recessed 3' terminus located on the (-) strand, and then digested in the 3' to 5' direction by *E. coli* exonuclease III. Subsequent to exonuclease III treatment, the $^{32}$P DNA was restricted with *SmaI* to generate a DNA fragment containing a $^{32}$P-labeled oligodeoxynucleotide the length of which was diagnostic of the BER patch size. The various $^{32}$P-labeled DNA fragments produced in the patch size assay were resolved by denaturing 12% polyacrylamide gel electrophoresis as described under "Experimental Procedures".
Figure 37
dNMP[α-S] incorporations were expected to produce $^{32}$P-labeled oligodeoxynucleotides of increasing size (i.e., 21-, 22-, 23-mer). The various $^{32}$P-labeled DNA fragments produced in the patch size assay were resolved by denaturing polyacrylamide gel electrophoresis and detected using a PhosphorImager.

To optimize the amount of Exo III required to measure the 3' base excision repair patch boundary, DNA samples (~200 ng) recovered from either a BER reaction containing E. coli NR8052 cell extracts, $[^{32}$P]$M_{13}$mp2op14 (U-T) DNA, and dNTP[αS]s or a mock reaction conducted without cell extract were linearized with EcoRI and digested with various amounts of Exo III (0.002-20 units). After incubation with SmaI, $[^{32}$P]DNA fragments were resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis and visualized using a PhosphorImager. The result showed that the mock-reacted DNA was susceptible to the digestion with Exo III in a concentration dependent manner and completely degraded following treatment with 2 units of Exo III (Figure 38, lanes 4-8). This result was expected since no incorporation of [α-S]dNMP could occur due to the lack of repair in the absence of cell extract. In contrast, distinctive Exo III-resistant DNA bands of 20 to 40 nucleotides in length were observed following treatment of BER reaction DNA product with Exo III (Figure 38, lanes 10-14). However, the distribution of these bands was not constant at low concentrations (0.002-0.2 units) of Exo III, which suggested that incomplete digestion had occurred (Figure 38, lanes 10-12). After treatment with 2 units of Exo III, the banding pattern became stable and remained uniform following 20 units of Exo III treatment (Figure 38, lanes 13 and 14, respectively), indicating that complete digestion of the substrate had occurred using an Exo III:DNA ratio of greater than 0.1:1 (units: μg DNA). Therefore, $[^{32}$P]DNA bands remained to be resistant to Exo III (> 2 units) treatments implied that [α-S]dNMP incorporation occurred during repair DNA synthesis.
Figure 38. Effect of exonuclease III concentration on determining uracil-initiated BER patch size using M13mp2op14 DNA. Two standard BER reaction mixtures (400 µl) containing 4 µg of [32P]M13mp2op14 (U·T) DNA and 20 µM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] were incubated with 0.4 mg of E. coli NR8052 cell extract protein (lanes 9-14) or mock treated without cell extract (lane 3-8) at 30°C for 60 min. DNA products were isolated and, samples (~200 ng) were digested with EcoRI and then treated with 0, 0.002, 0.02, 0.2, 2, and 20 units of exonuclease III (lanes 3-8 and 9-12, respectively) for 1 h at 37°C. Following exonuclease III digestion, reaction products were restricted with SmaI and DNA reaction products were resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The DNA size markers, 40-mer (lanes 1) generated by digesting 200 ng of M13mp2op14 (U·T) [32P]DNA with EcoRI and SmaI, and the 19-mer (lanes 2) produced by additional treatment with Ung and Endo IV, are indicated by arrows.
Figure 38
Experiments were conducted to determine the size and distribution of the DNA repair patches produced by uracil-initiated DNA repair synthesis during Ung-proficient and Ung-deficient BER by examining M13mp2op14 \[^{32}\text{P}]\text{DNA} recovered from BER reactions conducted with \textit{E. coli} NR8051, NR8052, and mock treatments. In each case, the \[^{32}\text{P}]\text{DNA} was linearized with \textit{EcoRI} and then digested in the 3'-5' direction with excess Exo III (2 and 20 units). Subsequent cleavage with \textit{SmaI} produced a \[^{32}\text{P}]\text{DNA} fragments, the length of which indicated the BER patch size, analyzed by denaturing 12\% polyacrylamide/8.3 M urea gel electrophoresis (Figure 39). Two reference standards were prepared and analyzed on the same gel following treatment of the M13mp2op14 (U-T) \[^{32}\text{P}]\text{DNA} substrate with \textit{EcoRI} and \textit{SmaI} alone (Figure 39A, lanes 1 and 13) or in conjunction with Ung and Endo IV (Figure 39A, lanes 2 and 12), which produced the expected \[^{32}\text{P}]\text{DNA} fragments of 40 and 19 nucleotides, respectively. The 19-mer corresponded to the BER intermediate formed immediately prior to DNA synthesis and defined the 5'-boundary of the repair patch (346). As before, exonuclease III digestion of the mock treated \[^{32}\text{P}]\text{DNA} (Figure 39A, lanes 4 and 5) did not produce a distribution of fragments smaller than the 40-mer control (Figure 39A, lane 3) since BER had not occurred and \[^{\alpha}\text{-S}]\text{dNMP} was not incorporated into the \[^{32}\text{P}]\text{DNA} substrate. In contrast, a discrete set of fragments was generated following Exo III digestion of repaired M13mp2op14 \[^{32}\text{P}]\text{DNA} produced in BER reactions containing \textit{E. coli} NR8051 (Figure 39A, lanes 6-8) and NR8052 (Figure 39A, lanes 9-11) cell extracts. The amount of each \[^{32}\text{P}]\text{DNA} fragment was quantitatively determined and the distribution of the repair patch size was plotted in Figure 39B. In both cell extracts, the vast majority of BER occurred via a long patch repair mechanism, whereas short patch (1 nucleotide) repair accounted for ~7\% of the BER events. While the repair patch size distribution was similar in each reaction, the Ung-proficient BER reaction was somewhat biased toward longer repair patches.
Figure 39. Analysis of DNA repair patch size associated with uracil-initiated BER in *E. coli* NR8051 and NR8052 cell extracts. (A) Standard BER reaction mixtures (100 µl) containing 1 µg of M13mp2op14 (U·T) [³²P]DNA, 20 µM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] and 0.1 mg of cell extract protein of *E. coli* NR8051 (lanes 6-8) and NR8052 (lanes 9-11) were incubated for 60 min at 30 °C. As a control, M13mp2op14 (U·T) [³²P]DNA (1 µg) was mock-reacted in the absence of cell extract protein (lanes 3-5). DNA products were isolated, samples (~200 ng) were digested with EcoRI, and then incubated with 0 (lanes 3, 6, and 9), 2 (lanes 4, 7, and 10), and 20 (lanes 5, 8, and 11) units of *E. coli* exonuclease III. Following exonuclease III digestion, the DNA was cleaved with *Smal*, and then resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures." The DNA size markers, 40-mer (lanes 1 and 13) generated by digesting 200 ng of M13mp2op14 (U·T) [³²P]DNA with EcoRI and *Smal*, and the 19-mer (lanes 2 and 12) produced by additional treatment with Ung and Endo IV, are indicated by arrows. (B) The amount of ³²P radioactivity detected in each band in (A) was quantitatively measured using a PhosphorImager and the results for the *E. coli* NR8051 (white bars) and NR8052 (black bars) reactions digested with 20 units of *E. coli* exonuclease III are plotted. The [³²P]DNA bands of 20 to 40 nucleotides in length corresponded to BER repair patches of 1 to 21 nucleotides in length, respectively. The relative amount of ³²P label in each band (% distribution) was determined by dividing the amount of ³²P radioactivity detected per band by the total ³²P signal detected for all bands and multiplying by 100. Mean values and standard deviations for the distribution of four experiments are indicated.
Figure 39
4.1.9 Determination of Error Frequency Associated with *E. coli* Uracil-initiated BER

Utilizing the M13mp2op14 lacZα DNA-based reversion assay, the fidelity of uracil-initiated BER synthesis in *E. coli* cell extracts was examined. Initially, the background reversion frequency of the M13mp2op14 (A·T) DNA was determined in extracts of *E. coli* NR8051 and NR8052 cells. In each case, a similar reversion frequency, 0.14 x 10⁻⁴ (NR8051) and 0.21 x 10⁻⁴ (NR8052), was observed (Table 3). Next, the reversion frequency associated with uracil-initiated BER of M13mp2op14 (U·T) DNA was determined to be 5.5 x 10⁻⁴ and 19.7 x 10⁻⁴ for reactions conducted with *E. coli* NR8051 and NR8052 cell extracts, respectively (Table 3). Thus, the absence of Ung promoted an apparent increase in the reversion frequency of 3.6-fold. Similar results were obtained when BER of M13mp2op14 (U·T) DNA was performed using the analogous cell extracts (NR80511 and NR80521) that contained a mutS mutation. Therefore, methyl-directed mismatch repair did not seem to influence the fidelity of the BER reaction. Since Dug has been shown to carry out uracil-initiated BER in the absence of Ung (Figure 32 and 36), these results implied that the Dug-mediated uracil-initiated BER might be responsible for the increased mutation frequency in *E. coli* cell extracts deficient in Ung activity.

In order to clarify the role of Dug as a potential mediator of the elevated error frequency associated with Ung-deficient uracil-initiated BER, repair reactions containing cell extracts of *E. coli* NR8051 were supplemented with Ugi or Dug protein, and reactions containing NR8052 cell extracts were supplemented with Ugi or Ung protein. Following the recovery of repaired DNA, the reversion frequency of the M13mp2op14 (U·T) DNA substrate was determined (Table 4). Supplementation of NR8051 extracts with Ugi gave rise to a reversion frequency (40.3 x 10⁻⁴) elevated 7.3-fold relative to that measured for NR8051 extracts alone (5.5 x 10⁻⁴). Conversely, when extracts of NR8052 were supplemented with Ung, the reversion frequency was reduced ~8-fold.
Table 3

Frequency of mutations produced by uracil-initiated BER in
*E. coli* NR8051 and NR8052 cell-free extracts

Standard BER reaction mixtures (500 µl) were prepared that contained 0.5 mg of *E. coli* NR8051, NR80511 (*mutS*), NR8052, or NR80521 (*mutS*) cell extract and 5 µg of M13mp2op14 (U•T) or (A•T) DNA. After incubation at 30 °C for 60 min, the reactions were terminated, DNA products were recovered, and form I DNA resistant to Ung/Endo IV treatment was isolated by 0.8% agarose gel electrophoresis as described under "Experimental Procedures." *E. coli* NR9162 cells were then transfected with the form I DNA and the M13mp2 lacZα DNA-based reversion assay was performed as described under "Experimental Procedures."

<table>
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<th><em>E. coli</em> Extract</th>
<th>DNA Substrate</th>
<th>Plaques Scored</th>
<th>Reversion Frequencya</th>
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<td>Total</td>
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<tr>
<td>-/+ b</td>
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<td></td>
<td>U•T</td>
<td>107,500</td>
<td>212</td>
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<tr>
<td>NR80521 (<em>ung-1, mutS</em>)</td>
<td>U•T</td>
<td>329,460</td>
<td>712</td>
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a Reversion frequencies were calculated by dividing the number of blue plaques scored by the total number of blue plus colorless plaques. Revertants included dark blue and light blue phenotypes.
b The (-) and (+) strand nucleotide at the target site.
Table 4

Frequency of mutations produced by uracil-initiated BER in E. coli NR8051 and NR8052 cell-free extracts supplemented with purified Ung, Dug, or Ugi protein

Standard BER reaction mixtures (500 µl) were prepared that contained 5 µg of M13mp2op14 (U•T) DNA and 0.5 mg of E. coli cell extract, as indicated. After incubation at 30 °C for 60 min, the reactions were terminated, DNA products were recovered, and Ung/Endo IV-resistant form I DNA was isolated by 0.8% agarose gel electrophoresis as described under "Experimental Procedures." The form I DNA was then used to transfect E. coli NR9162 cells, and the M13mp2 lacZa DNA-based reversion assay was performed as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>E. coli Extract</th>
<th>Allele</th>
<th>Protein Additiona</th>
<th>Plaques Scored</th>
<th>Reversion Frequencyb</th>
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<td></td>
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<tr>
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<td></td>
<td>524,716</td>
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<tr>
<td>BH157 ung+ dug-</td>
<td>--</td>
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<td>1,300,550</td>
<td>741</td>
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</table>

a Ugi (1000 units), Dug (20 pmol), or Ung (4 units) was included in the standard BER reaction as indicated.
b Reversion frequencies were calculated by dividing the number of blue plaques scored by the total number of blue plus colorless plaques. Revertants included dark blue and light blue phenotypes.
relative to that obtained for NR8052 extracts alone. Taken together, these results suggest that uracil-initiated BER conducted in the absence of Ung was more mutagenic than Ung-initiated BER. Consistent with this interpretation, addition of Dug to NR8051 extracts resulted in an increase (~2-fold) in the frequency of opal codon 14 reversion, whereas the addition of Ugi to NR8052 extracts did not appreciably augment the elevated mutation frequency (Table 4).

The reversion frequency of uracil-initiated BER at opal codon 14 was also determined for *E. coli* of a different genetic background (Table 4). Experiments conducted with *E. coli* BH156 (*ung* *dug*+) exhibited a reversion frequency of $25.9 \times 10^{-4}$. This value was similar to those obtained for *E. coli* NR8052 ($19.7 \times 10^{-4}$), NR8052 supplemented with Ugi ($39.4 \times 10^{-4}$), and NR8051 supplemented with Ugi ($40.3 \times 10^{-4}$). In the complementary experiment, uracil-initiated BER in extracts of *E. coli* BH157 (*ung* *dug*-) resulted in a relatively low reversion frequency of $5.7 \times 10^{-4}$ which compared favorably to that obtained for extracts of NR8051 ($5.5 \times 10^{-4}$). Determination of the reversion frequency associated with the *E. coli* BH158 (*ung* *dug*-) was not possible, as the production of Ung/Endo IV-resistant Form I DNA was not detected as shown in Figure 36. When taken together, these results demonstrated that *E. coli* uracil-initiated BER mediated by Dug was more error prone than that mediated by Ung.

4.1.10 Mutational Spectrum of Uracil-initiated BER in *E. coli*

In order to define the type of misincorporation that occurred during uracil-initiated base excision DNA repair synthesis, the mutational analysis was performed on the revertant M13 phage DNA as described under "Experimental Procedures". Briefly, DNA from individual revertant M13mp2op14 phage plaques was amplified and the nucleotide sequence of the *lacZα* gene encompassing the opal codon 14 reversion target was determined to define the nature and the distribution of base substitutions
introduced during the process of BER. Examples of DNA sequence analysis for three revertants representing each of the three possible nucleotide misincorporation events at the site of the uracil are shown in Figure 40. Since the sequencing primer used in DNA sequence analysis was complementary to the (+) strand of revertant M13mp2op14 DNA, the sequence displayed in each electropherogram corresponded to the sequence of (-) strand in the 5' to 3' direction and indicated misincorporation of dCMP (Figure 40A), dGMP (Figure 40B), or dTMP (Figure 40C) at the site of uracil during the base excision DNA repair synthesis.

First, the spontaneous mutational spectrum was determined for M13mp2op14 (A·T) DNA obtained from the BER reactions containing E. coli NR8051 cell extracts. Inspection of the mutational distribution showed that of the 30 mutants sequenced, 20 (67 %) reverted by base substitutions in the third nucleotide position of the opal codon (Figure 41A). Of these 20, 12 were A to C transversions, and 8 were A to G transition mutations. A relatively minor class of mutations occurred in the first position, 8 mutations were detected; 7 of these were T to A transversions. A similar bias for mutations at the third position was also observed for M13mp2op14 (A·T) DNA incubated in a BER reaction containing extracts of NR8052 (Figure 41B).

Second, the mutational spectrum of uracil-initiated BER, obtained using the M13mp2op14 (U·T) DNA substrate and E. coli NR8051 cell extract, revealed that 80 of the 82 mutants sequenced reverted at the first nucleotide position of the opal codon (Figure 41C); thus, these mutations occurred almost exclusively at the uracil target site. The large majority of these uracil-initiated BER mutations were T to G transversions (70 %) and T to A transversions (29 %).

Third, the mutational spectrum of uracil-initiated BER in E. coli NR8051 and NR8052 cell extracts supplemented with Ugi was compared using the M13mp2op14 (U·T) DNA substrate. From the BER reaction containing NR8051 cell extracts, 90 revertants were analyzed and 98 % (88 of 90) of the
Figure 40. DNA sequence analysis of M13mp2op14 DNA lacZα gene revertants. Isolated light blue and dark blue plaques were used to procure single-stranded M13mp2op14 DNA and the DNA sequences were determined by the Center for Gene Research and Biotechnology (Oregon State University) using an Applied Biosystems Model 373A DNA sequencer as described under "Experimental Procedures". The DNA sequence analysis of M13mp2op14 DNA containing a glycine codon (GGA) or an arginine codon (CGA, AGA) are indicative of T→C (A), T→C (B), and T→G (C) mutations at the site of the uracil, respectively. The codon containing the single-base substitution generated by the original mispair is indicated by underline for each revertant. The sequences depicted are those of the (-) DNA strand determined in the 5' to 3' direction. Unidentified bases are denoted by an 'N' in the DNA sequence.
Figure 41
Figure 41 (continued)
Figure 41 (continued)
I0 CD 33.
- o CD dGTP (Arg)
dCTP (Gly)
dTTP (Arg)
dATP (Stop)
(t dGTP (Ser)
dCTP (Stop)
dTTP (Stop)
dATP (Leu)
dGTP (Cys)
dCTP (Trp)
dTTP (Stop)
dATP (Cyc)

Number of Mutants

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<th>T</th>
<th>G</th>
<th>A</th>
</tr>
</thead>
<tbody>
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Figure 41 (continued)
Figure 41 (continued)
base substitutions occurred in the first nucleotide position (Figure 41D). The majority of these mutations (73%) were determined to be T to G transversions (64 of 88); T to A transversions accounted for the remainder. The spectra of mutations obtained from the BER reaction containing NR8052 extracts also consisted almost exclusively of first nucleotide base substitution mutations (94 out of 95) (Figure 41E). Of these, 60% (56 of 94) were T to G transversions, while 40% were T to A transversions.

4.2 Discussion

The ability of Ung-proficient or Ung-deficient E. coli cell extracts to carry out uracil-initiated BER of a covalently circular M13mp2 lacZα DNA-derived substrate was examined for the purpose of determining the fidelity associated with complete BER reaction. Several previous studies have implicated Dug as participating in uracil-DNA repair in Ung-deficient cells (106, 107, 343). However, a report by Lutsenko and Bhagwat concluded that Dug plays no role in E. coli uracil-initiated BER (319). An additional observation from the results of Sandigursky et al. (306) showed that the repair of 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, they suggested that Ung was absolutely required for repair of uracil-DNA in E. coli cells. Contrary to these observations, the results presented in this study support the proposition that Dug can participate in uracil-DNA repair, and provide the first line of evidence that Dug is primarily responsible for uracil-mediated BER in the absence of Ung. These findings reinforce the observations of Gallinari and Jiricny (107), who originally identified the double-strand uracil-DNA glycosylase activity in cell-free extracts of E. coli NR8052 that carried the ung-1 mutation; however, they do not exclude the possibility that Dug may also play a role in the repair of ethenocytosine residues, as has been suggested by other investigators (106, 319).
Several lines of evidence support the interpretation that the uracil-mediated repair observed in this study occurred via a BER pathway. Firstly, upon conclusion of the BER reaction, form I DNA was generated that was resistant to cleavage by the combined treatment of Ung and Endo IV. This finding indicated that all steps of uracil-DNA repair had been completed. Secondly, DNA synthesis during the repair reaction occurred preferentially in the *Hinfl* DNA fragment (529 bp) encompassing the uracil target. Furthermore, DNA synthesis within the 529 bp fragment was almost exclusively dependent on the presence of a uracil residue. Thirdly, the addition of Ugi to cell extracts of *E. coli* NR8051 substantially inhibited the formation of Ung/Endo IV resistant form I DNA. However, complete inhibition was not observed, as *E. coli* NR8051 is proficient for Dug activity, which is insensitive to inhibition by Ugi (107, 343). Fourthly, DNA synthesis was mostly associated with a repair patch involving ≤20 nucleotides that oriented 3' to the uracil target. These results were not characteristic of the *E. coli* methyl-directed DNA mismatch repair pathway, where DNA repair synthesis tracts of 1 kb or more can occur (348). Lastly, formation of Ung/Endo IV-resistant form I DNA was not observed in BER reactions containing extracts of *E. coli* BH158, in which both ung and dug are inactivated. This observation strongly suggests that Ung and Dug are the predominant, if not exclusive, uracil excision activities in wild type *E. coli*, and that BER was initiated by one or the other uracil-DNA glycosylase.

Examination of the kinetics of BER in extracts of NR8051 (*ung*<sup>+</sup>) cells showed that 60% of the M13mp2op14 (U·T) DNA substrate was repaired after a 20 min reaction. In contrast, the rate of BER in extracts of NR8052 (*ung*) cells was ~5.5-fold lower. A similar result was also observed when M13mp2op14 (U·G) DNA was utilized as the BER substrate. To ensure that the repair of uracil in Ung-deficient cell extracts was Ung-independent uracil-initiated BER, an excess of Ugi was added to the reaction mixtures. In this case, the rate and extent of uracil repair appeared to be similar to regardless of the presence of
Ugi. Thus, these results indicated that ~20% of the uracil-DNA repair in *E. coli* cell extracts involved a Ugi-insensitive BER system; Dug-mediated BER. Consistent with this interpretation, the addition of purified Dug to the Ung-deficient reaction led to an increased rate of repair early in the reaction time course and enhanced the extent of repair by ~2.5 fold. However, the BER reaction that was supplemented with Dug did not display the initial burst that was typically observed in Ung-mediated uracil-initiated BER. One interpretation of these data is that Ung rapidly turns over during BER, whereas Dug has a low rate of turnover. Since strong binding by Dug to its reaction product AP-site/G DNA has been demonstrated in a previous study (343) and also in Chapter 3, it is tempting to speculate that Dug binding hinders efficient processing of the abasic site, and thus, impedes completion of the BER pathway. While *E. coli* endonuclease IV was shown to stimulate the catalytic turnover of Dug, Dug-initiated BER remained significantly less efficient than Ung-initiated BER, even under the stimulated condition (343). Thus, the role of Dug in uracil-DNA repair conducted via the BER pathway may be to provide an auxiliary repair system that serves as a secondary line of defense against uracil-provoked mutagenesis.

What influence does the fidelity of BER have on uracil-initiated mutagenesis in *E. coli*? Based on the results reported in Table 3, the error frequency associated with Ung-mediated BER in cell-free extracts was determined to be $5.5 \times 10^{-4}$ per repaired uracil residue. Under normal conditions, the vast majority of uracil in *E. coli* DNA results from dUMP incorporation during replication (2, 72). Tye *et al.* (2) reported that one uracil residue was introduced per 1200 nucleotides polymerized. Accordingly, one would anticipate that ~4000 uracil residues are incorporated per round of chromosomal DNA replication (72). Based on these reports, one can extrapolate that *E. coli* Ung-proficient BER could generate ~2 mutations per cycle of semi-conservative DNA replication, providing that error correction did not occur prior to mutation fixation. Addition of Ugi to the Ung-proficient
BER reactions resulted in a \(~7\)-fold increase in reversion frequency without an accompanying change in the mutational specificity. Thus, Ugi produced an \textit{ung} phenotype that reflected the elevated reversion frequency and mutational specificity associated with Dug-mediated BER. The \textit{ung} mutator phenotype was reproduced in strains sharing the \textit{E. coli} GM31 genetic background, namely, BH156 and BH157. Taken together, these results indicate that Ung-mediated BER occurs with higher fidelity than that initiated by Dug.

The mutational specificity of \textit{E. coli} uracil-initiated BER repair observed in the opal codon 14 TGA reversion assay appeared distinct from that observed in other fidelity assays conducted with purified \textit{E. coli} DNA polymerase I (large fragment). In extracts of Ung-proficient \textit{E. coli} cells (NR8051), our mutational analysis revealed that T to G transversions, resulting presumably from T·C mispairs, were dominant (56 of 79), while T to A transversions, the likely result of T·T mispairs, comprised the remainder (23 of 79). In contrast, Minnick \textit{et al.} (304), using a 361-base gap-filling TGA reversion assay and 3' to 5' exonuclease-deficient DNA polymerase I (large fragment), observed that the error rate of dGTP incorporation opposite T was \(~49\)-fold greater than that of dCTP incorporation opposite T, and \(~10\)-fold greater than dTTP incorporation opposite T. Perhaps it is not surprising that these results differ from those reported by Minnick \textit{et al.} (304), since we have utilized a system in which all steps of the BER pathway are represented. The mutational specificity of uracil-mediated BER is the end result of DNA repair synthesis, which includes misincorporation, proofreading, and/or misextension, and must be followed by ligation. On the other hand, the gap-filling assay is restricted to measurement of the accuracy of the polymerization step in the absence of competing reactions, and does not require ligation.

Patch size distribution of DNA synthesis was measured for uracil-initiated BER associated with Ung- \textit{versus} Dug-mediated repair. In both cases, the patch size was heterogeneous, ranging from 1 to \(~20\) nucleotides in length, although the size of the repair patch produced in Dug-mediated BER reactions
was consistently somewhat shorter. Quantification of the distribution of the repair patches showed that the mean patch size was 11 nucleotides in Ung-mediated BER compared to 7 nucleotides in Dug-mediated BER. A small amount (~7%) of one nucleotide replacement synthesis was observed in both systems; however, the predominant type of DNA repair synthesis was long-patch. The latter observation is consistent with the results of Sandigursky et al. (306), who found that repair of a U·G base pair in a closed circular plasmid involved replacement of ~15 nucleotides downstream of the uracil target. The first experiments conducted to elucidate the repair patch size associated with BER in *E. coli* cell extracts utilized a duplex oligodeoxynucleotide 30-mer DNA with a single U·G base pair located approximately in the middle of the substrate (305). Interestingly, under these conditions, more than 70% of DNA repair synthesis involved incorporation of a single nucleotide (305). As previously pointed out, the size of the repair patch may be greatly influenced by the nature of the DNA repair substrate (306, 349). Thus, short oligodeoxynucleotide substrates may not provide a platform sufficient for interaction with DNA polymerase and accessory repair proteins.

It is generally accepted that DNA polymerase I occupies the primary role in uracil-mediated DNA repair synthesis in *E. coli* (1, 31). Our analysis of the mutational spectra derived from Ung-proficient and Ung-deficient extracts suggests that the specificity of misinsertion remains essentially the same regardless of the uracil-DNA glycosylase involved; accordingly, one might infer that the same DNA polymerase is involved in Ugi-resistant as well as in Ugi-sensitive uracil-DNA repair. Given the relatively high mutation frequencies we observed in this study, a role for Pol IV and/or Pol V DNA polymerases in BER cannot be formally excluded. These DNA polymerases have been described as low fidelity enzymes that exhibit error rates of ~10³ to 5 x 10⁴ when copying undamaged DNA *in vitro* (350); however, experiments to assess the contribution of these enzymes to BER have not yet been carried out. The molecular mechanisms underlying the mutational specificity of
uracil-initiated BER in *E. coli* and the increased reversion frequency associated with the Dug-mediated BER pathway await further elucidation.
5. ANALYSIS OF 3,N\textsuperscript{1}-ETHENOCYTOSINE- AND URACIL-INITIATED BASE EXCISION DNA REPAIR IN *E. coli*

While Ung is the principal activity responsible for uracil-initiated BER in *E. coli*, involvement of Dug in the uracil-initiated BER process has been demonstrated in *E. coli* cells that are deficient in Ung activity (Chapter 4). In addition to the uracil-DNA glycosylase activity, Dug exhibits an activity that excises ethenocytosine from double-stranded DNA. The study (Chapter 3) conducted with purified Dug revealed an apparent lack of turnover for the uracil-DNA glycosylase activity of Dug and that the enzyme appeared to be more active on the ethenocytosine-containing DNA than on the uracil-containing DNA. Additionally, the efficiency of uracil excision by Dug was shown to be influenced by the nature of the opposite base in the duplex DNA substrates, whereas ethenocytosine-DNA glycosylase activity was not dramatically affected by the opposite base (Chapter 3).

In the present Chapter, the ethenocytosine-DNA glycosylase activity of Dug was further characterized with respect to product binding and catalytic turnover. In addition, the relative contribution of Ung and Dug toward the excision of uracil and ethenocytosine bases from DNA was evaluated using a synthetic oligonucleotide duplex DNA and *E. coli* cell extracts. Experiments were conducted to examine the participation of Dug in a complete ethenocytosine-initiated BER using a closed circular duplex DNA substrate. The relative efficiency of the repair was determined in *E. coli* cell-free extracts utilizing two form I plasmid DNA substrates each containing a site-specific uracil or ethenocytosine residue. Using this "lesion specific BER-competition assay" the rate and extent of uracil and ethenocytosine-DNA repair were measured using isogenic *E. coli* cells that were deficient in Ung and/or Dug. This Chapter also presents the results of the repair patch size analysis for ethenocytosine-initiated BER and the comparison with the patch distribution observed in uracil-initiated BER mediated by Ung or Dug.
5.1 Results

5.1.1 Comparison of Substrate Specificity of Dug on U/G and εC/G DNA

Three studies have demonstrated that Dug exhibits both uracil- and ethenocytosine-DNA glycosylase activities (106, 319, 343). In order to further assess the substrate specificity of Dug activity at U/G and εC/G mispaired sites, 5’-end 32P-labeled synthetic oligonucleotides containing either a uracil or ethenocytosine residue at position 16 were prepared. The target containing oligonucleotides were annealed to the complementary oligonucleotide (G-34-mer) creating either the U/G or the εC/G double-stranded DNA substrate. U/G- and εC/G-34-mers (20 nM) were separately incubated with various amount of Dug to allow the DNA glycosylase reaction to occur and the Dug-generated AP-sites were cleaved by Endo IV treatment. Analysis of the reaction products by denaturing polyacrylamide gel electrophoresis revealed that Dug was considerably more active on εC/G-34-mer relative to the U/G-34-mer (Figure 42A). Quantification of the results revealed a linear dependence of the extent of uracil-DNA glycosylase activity of Dug (0 to 20 nM) on 20 nM U/G-34-mer, suggesting that the enzyme did not efficiently turnover (Figure 42B). This result was consistent with the previous observation that Dug tightly bound to the AP/G-DNA produced after removal of uracil from U/G-DNA (Chapter 3). In contrast, Dug was observed to generate a significantly increased amount of cleaved product from the εC/G-34-mer substrate over the amount of added enzyme (Figure 42B).

To further investigate whether enzyme turnover occurred during the ethenocytosine-DNA glycosylase activity of Dug, the DNA glycosylase reactions were conducted with 5’-end 32P-labeled U/G- or εC/G-34-mer and various amount of Dug, and subjected to band mobility shift analysis. In addition, to determining if Dug stably bound to the DNA substrate, a cleavage assay was conducted to determine the catalytic activity of uracil- and
Figure 42. Comparison of the relative efficiency of excision of uracil and ethenocytosine by Dug. (A) DNA glycosylase reaction mixtures (10 μl) were prepared containing 20 nM of [³²P]U/G- or [³²P]eC/G-34-mer as indicated, and either 0, 0.63, 1.25, 2.5, 5, 10, 20, or 40 nM of Dug (lanes 1-8, respectively). Control reactions were also prepared that lacked Dug (lane N) or contained 1000 units of Ung (lane U). After incubation at 30 °C for 30 min, each reaction was terminated by heating at 70 °C for 3 min, treated with 1 unit of Endo IV, and analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". Unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P) DNA bands visualized by PhosphorImager are indicated by arrows. (B) [³²P]DNA bands detected in (A) were quantified using a PhosphorImager and ImageQuant program, and the percentage of product formed was calculated by dividing the amount of [³²P]15-mer by that of [³²P]15-mer plus [³²P]34-mer and multiplying by 100. The percentage of product generated from each reaction contained [³²P]U/G-34-mer (□) or [³²P]eC/G-34-mer (○) is plotted as a function of Dug concentration.
Figure 42
ethenocytosine-DNA glycosylase. The results of binding and cleavage assays were quantified and plotted in Figure 43. Similar to the previous observation described in Chapter 3, the amount of Dug-bound DNA formed in the reaction containing the U/G-34-mer was approximately equal to the amount of AP-site-containing DNA (product DNA), and to the amount of Dug in the reactions. Interestingly, an increased amount of DNA was bound by Dug in the reaction conducted with εC/G-34-mer than with U/G-34-mer. Since Dug displayed a strong binding affinity \(K_D = 2.38 \text{ nM}\) for the duplex DNA containing AP-site/G-residue (Table 2), this increased level of Dug-DNA complex indicated that more AP-site-DNA was generated from εC/G-DNA than from U/G-DNA by same amount of Dug. Indeed, the DNA glycosylase activity of Dug appeared to process more ethenocytosine than uracil from DNA during an equivalent reaction. Moreover, the amount of ethenocytosine determined to be removed form the DNA was in excess of the amount of DNA bound by Dug in the reaction performed with εC/G-34-mer. These results indicated that the ethenocytosine-DNA glycosylase activity of Dug was able to process more than a stoichiometric amount of substrate, unlike the uracil-DNA glycosylase activity of Dug which exhibited near stoichiometric levels. However, the amount of εC/G-34-mer processed by 5 nM Dug consumed only \(~60\%\) of total substrates and the majority of the enzyme \(~4.5 \text{ nM}; 90\%\) appeared to bind to DNA (AP-site/G). These results suggested that Dug formed a protein-DNA complex with AP-site/G-DNA but seemed to be unable to participate efficiently in the ethenocytosine processing from εC/G-DNA.

5.1.2 Analysis of Uracil- and Ethenocytosine-DNA Glycosylase Activities in E. coli Cells Deficient in ung and/or dug

The relative activities of endogenous uracil- and ethenocytosine-DNA glycosylases were determined in cell-free extracts prepared from four isogenic E. coli strains, GM31, BH156 (ung), BH157 (dug), and BH158 (ung, dug).
Figure 43. DNA binding and catalytic activity of Dug on uracil- and ethenocytosine-containing DNA. (A) Two sets of standard DNA glycosylase reaction mixtures (10 μl) containing 0, 0.16, 0.32, 0.63, 1.25, 2.5, and 5 nM Dug and 10 nM of \([^{32}\text{P}]U/G\)- or \([^{32}\text{P}]\varepsilon C/G\)-34-mer were incubated at 30 °C for 30 min. Following incubation, one-half of each reaction mixture (5 μl) was mixed with 1.5 μl of 50% sucrose, and samples (5 μl) were subjected to band mobility shift assay as described under "Experimental Procedures". The amount of DNA bound to Dug was determined for each reaction contained \([^{32}\text{P}]U/G\)-34-mer (□) or \([^{32}\text{P}]\varepsilon C/G\)-34-mer (○) using PhosphorImager and plotted as a function of Dug concentration. The other portion (5 μl) of reaction was terminated by heating at 70 °C for 3 min, treated with 1 unit of Endo IV, and analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". \([^{32}\text{P}]\)-34-mer substrate and \([^{32}\text{P}]\)-15-mer product bands were quantified using a PhosphorImager, the amount of cleaved product was calculated for the reactions conducted with \([^{32}\text{P}]U/G\)-34-mer (■) or \([^{32}\text{P}]\varepsilon C/G\)-34-mer (●), and plotted as a function of Dug concentration.
Figure 43

**Bound DNA (nM)**

**Cleaved DNA (nM)**

![Graph showing the relationship between Dug (nM) and Bound DNA (nM) and Cleaved DNA (nM).](image-url)
Reaction time courses were conducted with *E. coli* GM31 cell extracts and DNA substrates, \([^{32}P]U/G-\) and \([^{32}P]eC/G-34\)-mer, to determine the initial rates of excision of uracil and ethenocytosine from the DNA, respectively. Following the incubation, DNA products were isolated and the AP-sites were incised by Endo IV treatment. Product and substrate DNA were resolved by denaturing polyacrylamide gel electrophoresis and the amount of radioactivity associated with the \([^{32}P]DNA\) bands was quantified to assess the extent of uracil and ethenocytosine removal (Figure 44). Control reactions were conducted with or without excess Ung and Dug to determine the efficiency of Endo IV treatment that resulted in AP-site cleavage (Figure 44A, lanes N, U, and D). In each case, >98% of the \([^{32}P]U/G-\) and \([^{32}P]eC/G-34\)-mer was shown to be susceptible to cleavage by the combination of Ung/Endo IV and Dug/Endo IV treatment, respectively. In addition, the reaction conducted with \([^{32}P]C/G-34\)-mer homoduplex DNA showed that substrate was not subjected to any detectable degradation in the reaction containing the GM31 cell extract (Figure 44A, lanes 1 and 2). As anticipated, U/G-34-mer was efficiently processed and most of uracil was removed from DNA substrate after 5 min incubation (Figure 44A, lanes 4-8). The removal of ethenocytosine from eC/G-34-mer occurred with a reduced rate compared to the excision of uracil from U/G-34-mer (Figure 44A, lanes 10-14, and Figure 44B). The extent of uracil and ethenocytosine excision in the Ung and Dug proficient *E. coli* GM31 cell extracts was further analyzed in a similar reaction time-course except that a 20-fold reduced amount of cell extract protein was utilized (Figure 45). The relationship between the extent of ethenocytosine excision and the amount of cell extract protein was determined for 60 and 90 min reactions. Under these conditions, the activity of GM31 cell extract protein for removing ethenocytosine from eC/G-34-mer was determined to be 80 and 100 fmol of residues removed per \(\mu g\) of extract protein for 60 and 90 min reactions, respectively (Figure 45B). A comparison of the initial velocities calculated from the 10 min reactions revealed that the DNA substrate containing the
Figure 44. Analysis of uracil- and ethenocytosine-DNA glycosylase activities in E. coli GM31 cell-free extracts. (A) Reaction mixtures (100 μl) containing Dug buffer (25 mM Hepes-KOH (pH 7.9), 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.1 mM ZnCl₂, and 0.1 mg/ml acetylated BSA) and 10 μg of E. coli GM31 cell extract protein were incubated at 30 °C for various times with 0.2 pmol of [³²P]DNA substrate as following: 0 and 90 min with [³²P]C/G-34-mer (lanes 1 and 2, respectively) and 0, 5, 10, 30, 60, and 90 min with [³²P]U/G-34-mer (lanes 3-8, respectively) or [³²P]eC/G-34-mer (lanes 9-14, respectively), as indicated. As controls, the reactions containing each [³²P]DNA substrate were incubated at 30 °C for 90 min without cell extract protein (lane N) but in the presence of 100 units of Ung (lane U) or 8.3 pmol of Dug (lane D). Reaction products were isolated, AP-sites hydrolyzed, and resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". Arrows indicate the location of the unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P) DNA bands visualized by PhosphorImager. (B) [³²P]DNA bands detected in (A) were quantified using a PhosphorImager and ImageQuant program. The percentage of product formed was calculated by dividing the amount of [³²P]15-mer by that of [³²P]15-mer plus [³²P]34-mer and multiplying by 100, and plotted as a function of incubation time: O, [³²P]U/G-34-mer; ●, [³²P]eC/G-34-mer; ▲, [³²P]eC/G-34-mer.
Figure 45. Comparison of the relative efficiency of uracil- and ethenocytosine-DNA glycosylase activities in E. coli GM31 cell-free extracts. Reaction mixtures (100 μl) containing Dug buffer and 0.5 μg of E. coli GM31 cell extract were incubated at 30 °C for 0, 5, 10, 30, 60, and 90 min with 0.2 pmol of [32P]U/G-34-mer (lanes 1-6, respectively) or [32P]εC/G-34-mer (lanes 7-12, respectively). As controls, similar reactions were also incubated at 30 °C for 90 min without cell extract protein (lane N) but in the presence of 100 units of Ung (lane U) or 8.3 pmol of Dug (lane D). Arrows indicate the location of the unreacted [32P]34-mer substrate (S) and [32P]15-mer product (P) DNA bands visualized by a PhosphorImager. (B) [32P]DNA bands detected in (A) were quantified using a PhosphorImager and ImageQuant program. The percentage of product formed was calculated as described in Figure 44 and plotted for each time point in the reaction sequence containing [32P]U/G-34-mer (□) or [32P]εC/G-34-mer (■).
Figure 45
U/G target was preferred ~19-fold over the εC/G-containing DNA (Figure 45B). The majority of this efficient uracil excision appeared to be mediated by Ung, since the rate and extent of uracil excision was found to be significantly reduced in the *E. coli* BH156 cell extract that is deficient in Ung activity (Figure 46). On the other hand, the *E. coli* BH156 cell extract exhibited a similar rate and extent of ethenocytosine excision activity (Figure 46B) as compared to that observed in wild-type *E. coli* GM31 cell extract (Figure 44B). However, detectable ethenocytosine-DNA glycosylase activity was not observed in *E. coli* BH157 cell extracts that are deficient in dug (Figure 47). Thus, Dug appeared to constitute the principal ethenocytosine-DNA excision activity in *E. coli* cells. In addition, no detectable uracil-DNA glycosylase activity was observed in *E. coli* BH158 (*ung, dug*) cell extracts (Figure 48), inferring that Dug was most likely responsible for the residual uracil-DNA glycosylase activity observed in the cell extracts that were deficient in Ung activity (Figure 46).

These results were consistent with the previous observations demonstrating that Dug played major role in uracil-initiated BER in the absence of Ung (Chapter 4). Taken together, these results suggested that the uracil and ethenocytosine excision activities observed in *E. coli* BH156 cell extracts represented Dug-mediated uracil- and ethenocytosine-DNA glycosylase activities, respectively. The extent of Dug-mediated excision of uracil in the *E. coli* BH156 (*ung*) cell extract was determined to be 3.5 and 5 fmol of uracil removed per μg of cell extract protein for 60 and 90 min reactions, respectively (Figure 46B). This was ~20-fold lower than the amount of ethenocytosine (80-100 fmol/μg of cell extract protein) excised by the Dug-mediated ethenocytosine-DNA glycosylase activity. Moreover, an examination of the initial rates of uracil and ethenocytosine excision mediated by Dug in *E. coli* BH156 (*ung*) cell extracts revealed that the action of ethenocytosine-DNA glycosylase activity occurred ~23-fold faster than that of uracil-DNA glycosylase activity (Figure 46B).
Figure 46. Analysis of uracil- and ethenocytosine-DNA glycosylase activities in *E. coli* BH156 (*ung*) cell-free extracts. (A) Reaction mixtures (100 µl) containing Dug buffer and 10 µg of *E. coli* BH156 cell extract protein were incubated at 30 °C for various times with 0.2 pmol of [³²P]DNA substrate as following: 0 and 90 min with [³²P]C/G-34-mer (*lanes 1 and 2, respectively*) and 0, 5, 10, 30, 60, and 90 min with [³²P]U/G-34-mer (*lanes 3-8, respectively*) or [³²P]eC/G-34-mer (*lanes 9-14, respectively*), as indicated. As controls, the reactions without cell extract protein (*lane N*) but in the presence of 100 units of Ung (*lane U*) or 8.3 pmol of Dug (*lane D*) were also incubated at 30 °C for 90 min. Reaction products were isolated, AP-sites hydrolyzed, and resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". Arrows indicate the location of the unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P) DNA bands visualized by a PhosphorImager. (B) [³²P]DNA bands detected in (A) were quantified using a PhosphorImager and ImageQuant program. The percentage of product formed was calculated as described in Figure 44 and plotted as a function of incubation time: O, [³²P]U/G-34-mer; ▲, [³²P]eC/G-34-mer; A, [³²P]C/G-34-mer.
Figure 46
Figure 47. Analysis of uracil- and ethenocytosine-DNA glycosylase activities in *E. coli* BH157 (*dug*) cell-free extracts. (A) Reaction mixtures (100 μl) containing Dug buffer and 10 μg of *E. coli* BH157 cell extract protein were incubated at 30 °C for various times with 0.2 pmol of [³²P]DNA substrate as following: 0 and 90 min with [³²P]C/G-34-mer (lanes 1 and 2, respectively) and 0, 5, 10, 30, 60, and 90 min with [³²P]U/G-34-mer (lanes 3-8, respectively) or [³²P]eC/G-34-mer (lanes 9-14, respectively), as indicated. As controls, the reactions without cell extract protein (lane N) but in the presence of 100 units of Ung (lane U) or 8.3 pmol of Dug (lane D) were also incubated at 30 °C for 90 min. Reaction products were isolated, AP-sites hydrolyzed, and resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". Arrows indicate the location of the unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P) DNA bands visualized by a PhosphorImager. (B) [³²P]DNA bands detected in (A) were quantified using a PhosphorImager and ImageQuant program. The percentage of product formed was calculated as described in Figure 44 and plotted as a function of incubation time: ○, [³²P]U/G-34-mer; ●, [³²P]eC/G-34-mer; Δ, [³²P]C/G-34-mer.
Figure 47
Figure 48. Analysis of uracil- and ethenocytosine-DNA glycosylase activities in E. coli BH158 (ung, dug) cell-free extracts. (A) Reaction mixtures (100 µl) containing Dug buffer and 10 µg of E. coli BH158 cell extract protein were incubated at 30 °C for various times with 0.2 pmol of [³²P]DNA substrate as following: 0 and 90 min with [³²P]C/G-34-mer (lanes 1 and 2, respectively) and 0, 5, 10, 30, 60, and 90 min with [³²P]U/G-34-mer (lanes 3-8, respectively) or [³²P]eC/G-34-mer (lanes 9-14, respectively), as indicated. As controls, the reactions without cell extract protein (lane N) but in the presence of 100 units of Ung (lane U) or 8.3 pmol of Dug (lane D) were also incubated at 30 °C for 90 min. Reaction products were isolated, AP-sites hydrolyzed, and resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under “Experimental Procedures”. Arrows indicate the location of the unreacted [³²P]34-mer substrate (S) and [³²P]15-mer product (P) DNA bands visualized by a Phosphorlmager. (B) [³²P]DNA bands detected in (A) were quantified using a Phosphorlmager and ImageQuant program. The percentage of product formed was calculated as described in Figure 44 and plotted as a function of incubation time: O, [³²P]U/G-34-mer; ●, [³²P]eC/G-34-mer; △, [³²P]C/G-34-mer.
5.1.3 Lesion Specific BER-competition Assay

In order to determine the relative efficiency of the complete BER process associated with uracil- and ethenocytosine-DNA base excision repair in *E. coli* cells, a lesion specific BER-competition assay was developed. This assay utilized two form I DNA substrate molecules each containing a site-specific uracil or ethenocytosine residue so that the relative activity associated with each type of BER could be simultaneously determined in the same repair reaction (Figure 49). The initial BER DNA substrate, pGEM plasmid (3.2 kb), was utilized to create a second BER substrate, pBIG plasmid (3.65 kb), by inserting a 450 bp DNA fragment encoding an N-terminal domain of the kanamycin resistance gene product at the *Eco*RI restriction endonuclease cleavage site of pGEM DNA. Thus, the DNA sequence contexts of both pGEM and pBIG plasmids were identical around nucleotide position 40, where the mispaired U/G or εC/G residue was to be introduced. Circular heteroduplex pGEM and pBIG DNA substrates with a site-specific uracil or ethenocytosine were then constructed using T4 DNA polymerase and T4 DNA ligase in a primer extension reaction containing defined oligonucleotides (Figure 49D). In addition, both pGEM and pBIG circular duplex DNA containing a C/G at the target site was constructed and each preparation of form I DNA was purified. The experimental logic of the lesion specific BER-competition assay is described below. To allow the competition between uracil-initiated BER and ethenocytosine-initiated BER to occur, a pair of form I DNA substrates comprising of pGEM (U-G) and pBIG (εC-G) DNA, or pGEM (εC-G) and pBIG (U-G) was introduced into the BER reaction mixture containing one of several *E. coli* cell extracts. Therefore, both uracil- and ethenocytosine-DNA were simultaneously repaired under the same reaction conditions; however, the extent of complete BER was assumed to reflect the efficiency of competing repair reactions. Following the BER reactions, reaction products (both pGEM and pBIG DNA) were recovered and treated *in vitro* with *E. coli* Ung, Dug, and
Figure 49. Scheme for measuring uracil- and ethenocytosine-initiated BER in *E. coli* cell-free extracts using a lesion specific BER-competition assay. (A) The DNA fragment (450-bp) encoding an N-terminal domain of the kanamycin resistant gene product of pET30a plasmid (Novagen) was amplified using the polymerase chain reaction. (B) After isolating the 450-bp DNA fragment, it was inserted into the *Eco*RI restriction endonuclease cleavage site of pGEM plasmid (3.2-kb) to generate pBIG (3.65-kb) plasmid DNA. (C) Single-stranded DNA of pGEM and pBIG was isolated separately, annealed to the 5'-end-phosphorylated oligonucleotide, C-, U-, or εC-23-mer, and then subjected to a primer extension reaction to construct each BER substrate: pGEM (C·G), (U·G), or (εC·G) and pBIG (C·G), (U·G), or (εC·G) DNA. (D) The resulting covalently closed circular duplex DNA reaction products were isolated by ethidium bromide cesium chloride gradient centrifugation. (E) Mixture of form I DNA substrates containing pGEM uracil-DNA and pBIG ethenocytosine-DNA, or vice versa, was subjected to the BER assay using *E. coli* cell-free extracts. This allowed for the competition between uracil- and ethenocytosine-initiated BER in the same reaction. (F) DNA reaction products were isolated, subjected to the combined treatment with Ung, Dug, and Endo IV to convert unrepaired form I DNA to form II DNA. Following this reaction, the DNA was analyzed by 1% agarose gel electrophoresis, and quantified to determine the rate and extent of uracil- and ethenocytosine-initiated BER.
A. pET30a
B. EcoRI
C. pGEM 3.2 kb
D. pBIG 3.65 kb
E. pBIG 3.65 kb
F. E. coli Cell Extracts
G. Ung / Dug / EndoIV
H. 1% Agarose Gel Electrophoresis

Figure 49
Endo IV to remove unreacted uracil- and ethenocytosine-containing DNA from the pool of repaired form I DNA molecules by conversion to form II DNA molecules. Therefore, the relative proficiency of BER could be determined by measuring the amount of pGEM and pBIG form I DNA that were insensitive to cleavage by the combined treatment of Ung/Dug/EndoIV. The DNA substrate, pBIG, was designed to have a different molecular size from pGEM in order to facilitate the resolution of form I and II DNA molecules using 1% agarose gel electrophoresis. A significant advantage of this experimental design is that the relative efficiency of the complete repair process for each of uracil- and ethenocytosine-initiated BER could be determined in the same reaction.

5.1.4 Preparation and Characterization of Repair DNA Substrates

In order to optimize the primer extension reaction conditions for producing the form I DNA substrates, 3.3 pmol of single-stranded pGEM DNA was annealed to a two-fold molar excess of 5'-end phosphorylated primer (U-23-mer) and incubated with T4 DNA polymerase at ratios of 0.5:1, 2:1, and 4:1 (polymerase unit to pmol primed template). Following DNA synthesis reactions, the reaction products were analyzed using agarose gel electrophoresis (Figure 50, lanes 4-6). As a control, the 3.3 pmol of heteroduplex U-23-mer/pGEM DNA was analyzed to indicate the position of the unextended primer/template DNA (Figure 50, lane 3). Additionally, 1 kb DNA ladder and a homoduplex pGEM form I DNA were analyzed on the same gel (Figure 50, lane 1 and 2, respectively). The addition of T4 DNA polymerase and T4 DNA ligase resulted in the extension of the primer to yield various ratios of covalently closed circular DNA (form I), nicked circular DNA (form II), and linearized DNA (form III) products. The helper phage DNA (6.4 kb) that was employed for the production of plasmid single-stranded DNA was also detected in the primer extension reaction products. Optimal production of form I DNA was determined to occur at a ratio of 2:1 of DNA
Figure 50. Effect of T4 DNA polymerase concentration on the efficiency of the primer extension reaction. Pilot primer extension reaction mixtures (50 μl) containing 3.3 pmol of pGEM single-stranded DNA annealed to 5'-end phosphorylated U-23-mer, 500 μM each of dATP, dTTP, dCTP, and dGTP, and various amounts of T4 DNA polymerase were prepared as described under "Experimental Procedures". A 50-fold unit excess of T4 DNA ligase over T4 DNA polymerase was included in each reaction. After incubation for 4 h at 37°C and the reactions were terminated by adjustment to 15 mM EDTA, samples (1 μl, ~100 ng DNA) from reactions originally containing 1.7, 6.6, and 13.2 units of T4 DNA polymerase (lanes 4-6, respectively) were analyzed using 1 % agarose gel electrophoresis as described under "Experimental Procedures". As controls, untreated pGEM single-stranded DNA primed with U-23-mer (150 ng) (lane 3), a sample containing pGEM homoduplex plasmid DNA (100 ng) (lane 2), and a 1-kb ladder (lane 1) were also analyzed as reference standards. The location of ethidium bromide stained DNA bands corresponding to form I, II, and III DNA bands, the primed template, and the helper phage (R408) single-stranded DNA are indicated by arrows.
Figure 50
polymerase to primer/template DNA as determined by ethidium bromide staining intensity. Similar results were also obtained with pBIG DNA annealed to 5'-end phosphorylated U-23-mer (data not shown). Thus, this ratio of 2:1 (polymerase unit to pmol primed template) was applied to preparative primer extension reaction mixtures in order to generate consummate amounts of pGEM and pBIG form I DNA.

To isolate form I DNA from form II and III by-products as well as from helper phage DNA, the terminated reaction mixture was subjected to ethidium bromide-cesium chloride gradient centrifugation. After centrifugation the form I DNA band was isolated and analyzed using agarose gel electrophoresis to assess the purity of pGEM (U.G), pGEM (εC.G), pBIG (U.G), and pBIG (εC.G) DNA preparations (Figure 51). Each form I DNA preparation was determined to contain >98% form I DNA.

Experiments were then conducted to determine the susceptibility of the uracil-containing DNA substrates to the treatment with Ung and Endo IV and the ethenocytosine-containing DNA substrates to the treatment with Dug and Endo IV. Isolated pGEM and pBIG DNA (form I) were combined to prepare the substrate mixture containing the same target residue (C/G, U/G, or εC/G) on both DNA substrates. Each of the three DNA substrate mixtures (80 ng each of pGEM and pBIG) were then subjected to the treatment with Ung, Dug, and/or Endo IV, and the resulting form I and II DNA molecules were resolved by agarose gel electrophoresis (Figure 52). Treatment with Ung alone did not apparently affect the form I DNA containing C/G, U/G, and εC/G residues (Figure 52, lanes 1-3), whereas the combined treatment with Ung and Endo IV converted almost all of U/G-containing pGEM and pBIG substrates from form I to form II DNA molecules (Figure 52, lane 5). As expected, the majority (> 95%) of C/G- and εC/G-containing DNA substrates were insensitive to Ung/Endo IV treatment (Figure 52, lanes 4 and 6, respectively). In contrast, pGEM and pBIG form I substrates containing an εC/G residue were efficiently converted to form II DNA by combined
Figure 51. Analysis of pGEM and pBIG form I DNA isolated using ethidium bromide-cesium chloride gradient centrifugation. A primer extension reaction (3030 µl) containing either 200 pmol of pGEM (lanes 2 and 3) or pBIG (lanes 4 and 5) DNA annealed to the 5'-end phosphorylated U-23-mer (lane 2 and 4) or εC-23-mer (lane 3 and 5), 500 µM of each dATP, dGTP, dCTP and dTTP, 400 units of T4 DNA polymerase, and 20,000 units of T4 DNA ligase was conducted and the resulting pGEM and pBIG DNA (Form I) molecules were isolated using ethidium bromide-cesium chloride gradient centrifugation as described under "Experimental Procedures". A sample (1 µl) of each purified form I DNA (~100 ng) was analyzed by 1% agarose gel electrophoresis (lanes 3 and 4, respectively), and a 1-kb ladder (lane 1) was also analyzed as a reference standard. The location of ethidium bromide-stained heteroduplex pGEM and pBIG form I DNA bands are indicated by arrows.
Figure 51
Figure 52. DNA substrate verification using a Dug- and Ung-mediated cleavage assay. Each of the DNA substrate mixtures of pGEM and pBIG (80 ng each) that constitute homoduplex (lanes 1, 4, 8, 11, and 14) or heteroduplex form I DNA containing either U/G (lanes 2, 5, 9, 12, and 15) or εC/G (lanes 3, 6, 10, 13, and 16) was subjected to the treatment with Ung (100 units), Dug, and/or Endo IV (1 unit), as indicated. After incubation at 30 °C for 30 min, the reactions were terminated by heating at 70 °C for 3 min and DNA products were analyzed using 1% agarose gel electrophoresis as described under "Experimental Procedures". The arrows indicate the location of form I and II DNA bands for pGEM and pBIG plasmids. A sample containing 500 ng of a 1-kb DNA ladder was analyzed as a reference standard (lane 7).
<table>
<thead>
<tr>
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![Image of gel electrophoresis](image)

- Lane 1-16
- M: Marker
- pBIG Form I
- pBIG Form II
- pGEM Form I
- pGEM Form II

Figure 52
treatment with Dug and Endo IV (Figure 52, lanes 10, 13, and 16). The production of form II DNA also occurred with the U/G-containing DNA (Figure 52, lanes 9, 12, and 15), but not from C/G-containing DNA upon Dug/Endo IV treatment (Figure 52, lanes 8, 11, and 14). However, a minor amount of form II DNA was observed at the highest concentration for the C/G-DNA substrate. In each case, the form I to form II DNA conversion occurred in a Dug concentration dependent manner. These results were consistent with the substrate specificity of Dug observed using oligonucleotide DNA substrates, as shown in Figure 42. Overall these results indicated that the treatment of pGEM and pBIG DNA substrates with Ung/Endo IV and Dug/Endo IV was effective in eliminating unreacted U/G- and εC/G-containing DNA from the pool of form I DNA molecules.

5.1.5 Detection of Uracil- and Ethenocytosine-DNA Repair in E. coli Cell Extracts

Initial experiments were conducted using both pGEM and pBIG DNA substrates to detect uracil- and ethenocytosine-DNA repair in various E. coli cell extracts that were proficient and/or deficient in Ung and Dug activities. Form I DNA substrates (pGEM and pBIG) harboring the same target residue (U/G or εC/G) on both substrates were incubated with E. coli cell extracts for various reaction times. The DNA was isolated, and then subjected to the Ung/Endo IV treatment for UG-DNA or Dug/Endo IV treatment for εC-G-DNA. As demonstrated in Figure 52, these treatments effectively converted unrepaiored form I DNA to form II DNA, and repaired form I DNA was then resolved from form II DNA by agarose gel electrophoresis. In order to quantify the amount of each DNA reaction product, internal standards containing predetermined quantities of form I and II (pGEM and pBIG) DNA were separately analyzed on the same agarose gel as the experimental samples (Figure 53A). Standard curves were generated for pGEM form I, pBIG form I, pGEM form II, and pBIG form II DNA molecules based on the ethidium
Figure 53. Standard curves used for quantification of form I and II pGEM and pBIG plasmid DNA. (A) Sample mixtures containing 10, 20, 40, and 80 ng each of form I (lanes 2-5) and form II (lanes 6-9) pGEM and pBIG plasmid DNA were prepared and analyzed using 1% agarose gel electrophoresis as described under "Experimental Procedures". A sample containing 500 ng of a 1-kb DNA ladder was also analyzed as a reference standard (lane 1). The arrows indicate the location of each form I and II DNA band detected by ethidium bromide staining. (B) The intensity of form I and II DNA bands in (A) were quantified using a Gel Documentation System and ImageQuant software. The relative intensity for each set of DNA band was used to generate individual standard curves: pGEM form I DNA; ○, pGEM form II DNA; □, pBIG form I DNA; •, and pBIG form II DNA; ■. The amount of form I and II DNA in the experimental samples analyzed on the same gel was determined according to the linear fit of these standard curves.
Figure 53
bromide staining intensity of each sample using defined quantities of DNA (Figure 53B). Typically, the corresponding standard curves of pGEM and pBIG DNA appeared to be nearly identical for both form I and II DNA while the ethidium bromide staining intensity of DNA was reduced by ~0.37-fold for form I DNA relative to form II DNA.

A time course analysis of the repair reactions using *E. coli* GM31 cell extracts and uracil- or ethenocytosine-DNA resulted in the time dependent appearance of form I DNA molecules that were resistant to Ung/Endo IV or Dug/Endo IV treatment, respectively (Figure 54A, lanes 1-6). The rate and extent of ethenocytosine-DNA repair was determined and compared to that observed for uracil-DNA (Figure 54B). The results showed that the repair of ethenocytosine residues occurred nearly as efficiently as uracil-DNA repair using wild-type *E. coli* cells proficient in Ung and Dug activities. In addition, the results indicated that both uracil- and ethenocytosine-DNA repair was detected with essentially identical efficiency using either the pGEM (3.2 kb) or pBIG substrate (3.65 kb). This indicated that the difference in the molecular size of these two DNA substrates did not affect the repair rate detected under the conditions used in this study.

To determine whether and to what extent a deficiency of Ung or Dug might influence the repair of uracil- and ethenocytosine-DNA, similar time course repair reactions were performed with cell extracts prepared from *E. coli* BH156 or BH157 cells that were genetically defective in ung or dug, respectively. As expected, the extent of uracil-DNA repair was markedly reduced in cell extracts of the Ung-deficient strain BH156 (Figure 55, U/G, lanes 1-6). However, the presence of the Ung defect did not significantly alter the rate and extent of ethenocytosine-DNA repair when compared to that detected using *E. coli* GM31 cell extracts (Figure 54 and 55, eC/G, lanes 1-6). In contrast, the repair reactions conducted using cell extracts that were deficient in Dug activity revealed that repaired form I DNA was undetectable for the ethenocytosine-DNA substrate (Figure 56, eC/G, lanes 1-6) whereas
Figure 54. Analysis of uracil- and ethenocytosine-initiated BER in *E. coli* GM31 cell-free extracts. (A) Standard BER reaction mixtures (100 µl) containing 1 µg each of pGEM and pBIG form I DNA with an individual site-specific U/G or εC/G mispair in both DNA substrates, as indicated, were incubated with 0.2 mg of *E. coli* GM31 cell extracts at 30 °C for 0, 5, 10, 30, 60, and 90 min (lane 1-6, respectively). The DNA reaction products were recovered following BER, and samples (~160 ng) were removed, treated with either 100 units of Ung and 1 unit of Endo IV (U-G-DNA) or 0.1 pmol Dug and 1 unit of Endo IV (εC-G-DNA) at 30 °C for 30 min, and analyzed using 1% agarose gel electrophoresis as described under "Experimental Procedures". As controls, mixtures of pGEM and pBIG (1 µg each) were mock-reacted and then treated with Ung/Endo IV or Dug/Endo IV (lane C) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (lane S and M, respectively). Arrows indicate the location of the form I and II DNA bands of pGEM (G) and pBIG (B) DNA detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively measured relative to the corresponding DNA standards (10-80 ng) using a Gel Documentation System and ImageQuant program. The amount of repaired DNA in each sample was calculated by dividing the amount of form I DNA (ng) by that of form I plus II sample and plotted as the percentage of form I DNA corresponding to the following: pGEM(U-G) (○), pBIG(U-G) (●), pGEM(εC-G) (□), and pBIG(εC-G) (■). Mean values and standard deviations of three experiments are represented.
Figure 54
Figure 55. Analysis of uracil- and ethenocytosine-initiated BER in *E. coli* BH156 (*ung*) cell-free extracts. (A) Standard BER reaction mixtures (100 µl) containing 1 µg each of pGEM and pBIG form I DNA with an individual site-specific U/G or εC/G mispair in both DNA substrates, as indicated, were incubated with 0.2 mg of *E. coli* BH156 cell extracts at 30 °C for 0, 5, 10, 30, 60, and 90 min (lane 1-6, respectively). The DNA reaction products recovered following BER were subjected to either Ung/Endo IV (U·G-DNA) or Dug/Endo IV (εC·G-DNA) treatment and analyzed using 1% agarose gel electrophoresis as described in Figure 54. As controls, mixtures of pGEM and pBIG (1 µg each) were mock-reacted and then treated with Ung/Endo IV or Dug/Endo IV (lane C) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (lane S and M, respectively). Arrows indicate the location of the form I and II DNA bands of pGEM (G) and pBIG (B) DNA detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively analyzed as described in Figure 54, and the amount of repaired DNA in each sample was plotted as the percentage of form I DNA corresponding to the following: pGEM(U·G) (O), pBIG(U·G) (●), pGEM(εC·G) (□), and pBIG(εC·G) (■). Mean values and standard deviations of three experiments are represented.
Figure 55
Figure 56. Analysis of uracil- and ethenocytosine-initiated BER in *E. coli* BH157 (*dug*) cell-free extracts. (A) Standard BER reaction mixtures (100 µl) containing 1 µg each of pGEM and pBIG form I DNA with an individual site-specific U/G or εC/G mispair in both DNA substrates, as indicated, were incubated with 0.2 mg of *E. coli* BH157 cell extracts at 30 °C for 0, 5, 10, 30, 60, and 90 min (*lane 1-6*, respectively). The DNA reaction products recovered following BER were subjected to either Ung/Endo IV (U·G-DNA) or Dug/Endo IV (εC·G-DNA) treatment and analyzed using 1% agarose gel electrophoresis as described in Figure 54. As controls, mixtures of pGEM and pBIG (1 µg each) were mock-reacted and then treated with Ung/Endo IV or Dug/Endo IV (*lane C*) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (*lane S* and *M*, respectively). *Arrows* indicate the location of the form I and II DNA bands of pGEM (G) and pBIG (B) DNA detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively analyzed as described in Figure 54, and the amount of repaired DNA in each sample was plotted as the percentage of form I DNA corresponding to the following: pGEM(U·G) (○), pBIG(U·G) (●), pGEM(εC·G) (□), and pBIG(εC·G) (■). Mean values and standard deviations of three experiments are represented.
Figure 56
the level of uracil-DNA repair appeared to be similar to that observed in *E. coli* GM31 cell extracts (Figure 54 and 56, U/G, lanes 1-6). Further analysis of repair reactions using *E. coli* BH158 cell extracts defective in both ung and dug resulted in no measurable amount of repaired form I DNA for both uracil- and ethenocytosine-DNA (Figure 57). These results demonstrated that the repair of uracil could be mediated by Ung and/or Dug. On the other hand, ethenocytosine-DNA glycosylase activity of Dug seemed to be absolutely required for the repair of ethenocytosine-DNA since the both *E. coli* BH157 and 158 cell extracts that were defective in Dug activity were not apparently capable of repairing the ethenocytosine-DNA substrate.

5.1.6 Specificity of Uracil- and Ethenocytosine-DNA Repair Synthesis in *E. coli* Cell Extracts

In order to localize the uracil- and ethenocytosine-DNA repair synthesis, pGEM and pBIG form I DNA containing either a U/G or εC/G residue were reacted with *E. coli* GM31, BH156 (ung), or BH157 (dug) cell extracts in the presence of [α-32P]dCTP. Control reactions were also performed with pGEM (C-G) and pBIG (C-G) homoduplex DNA to examine the extent of nonspecific DNA synthesis that occurred during the reaction. [32P]DNA reaction products were isolated and digested with the restriction endonuclease BsrI, which was expected to generate 9 and 10 DNA fragments in excess of 100 bp for pGEM and pBIG DNA, respectively. Among these fragments, the target uracil or ethenocytosine residue was located in the 272-bp and 490-bp DNA fragments of pGEM and pBIG DNA, respectively (Figure 58A). Following the BsrI digest, DNA fragments were resolved by nondenaturing polyacrylamide gel electrophoresis and [32P]DNA bands were visualized by PhosphorImager to determine the location of [32P]dCMP incorporation (Figure 58B). While low level [32P]dCMP incorporation into pGEM and pBIG homoduplex DNA was detected in many of the fragments (Figure 58B, lanes 1, 4, and 7), the preferential incorporation of [32P]dCMP into the heteroduplex substrates
Figure 57. Analysis of uracil- and ethenocytosine-initiated BER in *E. coli* BH158 (*ung* *dug*) cell-free extracts. (A) Standard BER reaction mixtures (100 μl) containing 1 μg each of pGEM and pBIG form I DNA with an individual site-specific U/G or εC/G mispair in both DNA substrates, as indicated, were incubated with 0.2 mg of *E. coli* BH158 cell extracts at 30 °C for 0, 5, 10, 30, 60, and 90 min (lane 1-6, respectively). The DNA reaction products recovered following BER were subjected to either Ung/Endo IV (U-G-DNA) or Dug/Endo IV (εC-G-DNA) treatment and analyzed using 1% agarose gel electrophoresis as described in Figure 54. As controls, mixtures of pGEM and pBIG (1 μg each) were mock-reacted and then treated with Ung/Endo IV or Dug/Endo IV (lane C) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (lane S and M, respectively). Arrows indicate the location of the form I and II DNA bands of pGEM (G) and pBIG (B) DNA detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively analyzed as described in Figure 54, and the amount of repaired DNA in each sample was plotted as the percentage of form I DNA corresponding to the following: pGEM(U-G) (O), pBIG(U-G) (●), pGEM(εC-G) (□), and pBIG(εC-G) (■). Mean values and standard deviations of three experiments are represented.
Figure 57
Figure 58. Regional specificity of BER DNA synthesis associated with uracil-and ethenocytosine-mediated DNA repair in various *E. coli* cell-free extracts. (A) *BsrI* restriction endonuclease cleavage maps of pGEM and pBIG indicating restriction sites (hash marks) and the location of uracil (U) or ethenocytosine (εC) residue in the (-) strand DNA of each plasmid are shown. The size (bp) of each DNA restriction fragment is indicated. (B) Standard BER reaction mixtures containing [³²P]dCTP (40 μCi) and 2 μg of either pGEM or pBIG DNA substrates, as indicated, with a site-specific C/G (lanes 1, 4, and 7), U/G (lanes 2, 5, and 8), or εC/G (lanes 3, 6, and 9) were incubated at 30 °C for 30 min with various cell-free extract proteins (0.2 mg) obtained from *E. coli* strain GM31 (lanes 1-3), BH156 (lanes 4-6), and BH157 (lanes 7-9). DNA products were isolated, and each DNA sample (100 ng) was treated with 5 units of *BsrI* for 1 h at 65 °C. The resulting DNA restriction fragments were then resolved by 5% nondenaturing polyacrylamide gel electrophoresis and visualized using a PhosphorImager as described under "Experimental Procedures". The locations of the DNA fragments (*) originally containing the uracil and ethenocytosine residue are indicated by arrows, as are the locations of other fragments. The amount of [³²P]dCMP incorporation into each DNA band was determined using an ImageQuant program, and normalized for each DNA fragment by dividing by the number of cytosine residues located in each corresponding DNA fragment. The highest normalized value was then designated 100% and the percentage of the [³²P]dCMP incorporation into other DNA fragments was calculated relative to that value. The relative [³²P]dCMP incorporation into the DNA fragments generated from (C) pGEM DNA; 445- (white bars), *272- (black bars), and 603-bp (striped bars), and (D) pBIG DNA; 255- (white bars), *490- (black bars), and 603-bp (striped bars) are plotted for each reaction.
Figure 58
Figure 58 (continued)
appeared to occur in the DNA fragments that initially contained a target U/G or εC/G residue, but only in the presence of the activity of uracil-DNA glycosylase and/or ethenocytosine-DNA glycosylase (Figure 58B, lanes 2, 3, 5, 6, and 8). In the reactions containing GM31 cell extracts, the incorporation of $[^{32}P]dCMP$ into the target fragments was significantly increased for both pGEM and pBIG DNA substrates containing U/G or εC/G residue (Figure 58B, lanes 2 and 3). The level of this specific incorporation of $[^{32}P]dCMP$ into the target fragments was significantly reduced for the U/G-containing DNA but not for the εC/G-containing DNA when reacted with *E. coli* BH156 cell extracts (Figure 58B, lane 5 and 6, respectively). Moreover, the analysis of $[^{32}P]DNA$ product recovered from the reactions containing the U/G- or εC/G-DNA and *E. coli* BH157 cell extracts revealed that the specific incorporation of $[^{32}P]dCMP$ into the target fragments was almost abolished for εC/G-DNA but not for the U/G-DNA (Figure 58B, lane 9 and 8, respectively). Thus, the incident of the specific incorporation into the target fragments was in accord with the occurrence of uracil- and ethenocytosine-DNA repair, which appeared to be mediated by Ung and/or Dug as demonstrated in Figures 54-57.

Quantitative analysis of $[^{32}P]dCMP$ incorporation into the *BsrI* DNA fragments of both pGEM and pBIG DNA was performed (Figure 58C and D, respectively). When compared to the background level of $[^{32}P]dCMP$ incorporation observed in the homoduplex (C/G) DNA, the results indicated that the uracil-containing target DNA fragment (272-bp and 490-bp fragment for pGEM and pBIG, respectively) stimulated DNA synthesis by ~20-fold in both reactions conducted with GM31 and BH157 cell extracts. Whereas, the stimulation was reduced to 4-fold in the reaction containing *E. coli* BH156 cell extract that exhibited Dug but not Ung activity. A similar level of the $[^{32}P]dCMP$ incorporation was observed in both corresponding pGEM and pBIG DNA substrates, indicating that the difference in molecular size of DNA substrate did not influence the repair process. Introduction of the
ethenocytosine residue in both pGEM and pBIG DNA also promoted increased repair DNA synthesis in a target fragment (Figure 58C and D). The reaction containing the Dug-proficient cell extracts exhibited ~20-fold \[^{32}\text{P}]\text{dCMP incorporation above background. When taken together, these results indicated that the vast majority of uracil- and ethenocytosine-DNA repair synthesis was instigated by the uracil and ethenocytosine residue, respectively, and was largely limited to the target \textit{BsrI} restriction DNA fragment. In addition, the occurrence of repair synthesis was dependent on uracil- or ethenocytosine-DNA glycosylase activity. Thus, most of the uracil- and ethenocytosine-repair DNA synthesis appeared to be mediated through the BER pathway.

5.1.7 Analysis of Relative Efficiency of \textit{E. coli} Uracil- and Ethenocytosine-initiated BER

A lesion specific BER-competition assay was developed by utilizing two form I plasmid DNA substrates to assess the relative efficiency of uracil-initiated BER \textit{versus} ethenocytosine-initiated BER. Moreover, the rate and extent of Ung-mediated BER on uracil, Dug-mediated BER on uracil, and Dug-mediated BER on ethenocytosine-containing DNA were determined for each substrate using various cell extracts prepared from \textit{E. coli} proficient and/or deficient in Ung and Dug. Two DNA substrate mixtures were prepared that contained a 1:1 molar ratio of pGEM (U.G) and pl3IG (C.G) or pGEM (\(\varepsilon\text{C}.\text{G}\)) and pBIG (U.G) DNA. The initial experiment was carried out utilizing two sets of time course reaction that contained each substrate mixture with the wild type \textit{E. coli} GM31 cell extract. Under this reaction condition, uracil-initiated BER was expected to be mediated by the combined action of Ung and Dug whereas ethenocytosine-initiated BER was anticipated to be dependent on Dug activity. Following each reaction, the DNA products were subjected to the combined treatment with an excess amount of Ung, Dug, and Endo IV to convert unreacted and unrepaired form I to form II DNA, and then the
DNA was analyzed using agarose gel electrophoresis (Figure 59A). A time dependent appearance of repaired form I DNA was observed in both sets of reactions containing pGEM and pBIG DNA. Upon examination of the percentage of form I DNA in each reaction set, no particular distinction in the rate and extent of BER between pGEM and pBIG was observed for both uracil- and ethenocytosine-DNA (Figure 59B). This result was consistent with the observation described in Figure 54. Determination of a linear rate for the appearance of form I DNA during the initial time periods (0-10 min) revealed that the efficiency of complete repair process appeared to be similar between uracil- and ethenocytosine-initiated BER (15 and 12 fmol/min, respectively). Thereafter, a very similar extent of the repair was reached (~80%) after 90 min for both uracil- and ethenocytosine-containing DNA.

An analogous experiment was performed with the *E. coli* BH156 cell extract to compare the relative efficiency of Dug-mediated BER of uracil- and ethenocytosine-DNA. Agarose gel analysis of Ung/Dug/Endo IV resistant form I DNA from each set of time course reactions revealed that the complete BER of uracil-DNA occurred albeit at a much reduced rate compared to that of ethenocytosine-DNA (Figure 60A). Quantitative analysis revealed that the rate of Dug-mediated ethenocytosine-BER was ~8-fold greater than that of Dug-mediated uracil-BER during the initial 30 min period (Figure 60B). In addition, the efficiency of uracil-initiated BER in the BH156 cell extract was significantly reduced (Figure 60B) compared to that observed in GM31 cell extract (Figure 59B). On the other hand, analysis of the BER efficiency, in the *E. coli* BH157 cell extract deficient in Dug but proficient in Ung (Figure 61B), resulted in a similar rate and extent of uracil-initiated BER compared to that observed in the GM31 cell extract (Figure 59B). These results implied that the contribution of Dug to complete uracil-initiated BER was minimal when in the presence of Ung. As expected, no measurable repair of ethenocytosine-DNA was observed in BH157 cell extract (Figure 61), implicative of the essential role of Dug in ethenocytosine-initiated BER.
Figure 59. Analysis of the relative efficiency of uracil- and ethenocytosine-initiated BER in E. coli GM31 cell-free extract. (A) The lesion specific BER-competition assay was performed using standard BER reaction conditions with a heteroduplex plasmid mixture (1 μg each) of pGEM(U·G) and pBIG(εC·G) or pGEM(εC·G) and pBIG(U·G), as indicated. BER reaction mixtures (100 μl) were incubated with E. coli GM31 cell extract protein (0.2 mg) at 30 °C for 0, 5, 10, 30, 60, and 90 min (lane 1-6, respectively). After the reaction products were isolated, each DNA sample (~160 ng) was subjected to the combined treatment with Ung (100 units), Dug (0.1 pmol), and Endo IV (1 unit) at 30 °C for 30 min and analyzed using 1% agarose gel electrophoresis as described under ‘Experimental Procedures”. As controls, mixtures of pGEM and pBIG (1 μg each) were mock-reacted and then treated with Ung, Dug, and Endo IV (lane C) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (lane S and M, respectively). Arrows indicate the location of the form I and II DNA bands detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively analyzed as described in Figure 54, and the amount of repaired DNA in each sample was plotted as the percentage of form I DNA corresponding to the following: pGEM(U·G) (○), pBIG(U·G) (●), pGEM(εC·G) (□), and pBIG(εC·G) (■). Mean values and standard deviations of three experiments are represented.
Figure 59
Figure 60. Analysis of the relative efficiency of uracil- and ethenocytosine-initiated BER in *E. coli* BH156 (*ung*) cell-free extract. (A) The lesion specific BER-competition assay was performed with standard BER reaction mixtures (100 µl) containing plasmid mixtures (1 µg each) of pGEM(U·G) and pBIG(εC·G) or pGEM(εC·G) and pBIG(U·G), as indicated, and 0.2 mg of *E. coli* BH156 cell extract protein. After the reactions were incubated 30 °C for 0, 5, 10, 30, 60, and 90 min (lane 1-6, respectively), the reaction products were isolated, each DNA sample (~160 ng) was subjected to the combined treatment with Ung (100 units), Dug (0.1 pmol), and Endo IV (1 unit) at 30 °C for 30 min and analyzed by 1% agarose gel electrophoresis as described under "Experimental Procedures". As controls, mixtures of pGEM and pBIG (1 µg each) were mock-reacted and then treated with Ung/Dug/Endo IV (lane C) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (lane S and M, respectively). Arrows indicate the location of the form I and II DNA bands detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively analyzed as described in Figure 54, and the amount of repaired DNA in each sample was plotted as the percentage of form I DNA corresponding to the following: pGEM(U·G) (○), pBIG(U·G) (●), pGEM(εC·G) (□), and pBIG(εC·G) (■). Mean values and standard deviations of three experiments are represented.
Figure 60
Figure 61. Analysis of the relative efficiency of uracil- and ethenocytosine-initiated BER in *E. coli* BH157 (*dug*) cell-free extract. (A) The lesion specific BER-competition assay was performed with standard BER reaction mixtures (100 μl) containing plasmid mixtures (1 μg each) of pGEM(U·G) and pBIG(εC·G) or pGEM(εC·G) and pBIG(U·G), as indicated, and 0.2 mg of *E. coli* BH157 cell extract protein. After the reactions were incubated 30 °C for 0, 5, 10, 30, 60, and 90 min (lane 1-6, respectively), the reaction products were isolated, each DNA sample (~160 ng) was subjected to the combined treatment with Ung (100 units), Dug (0.1 pmol), and Endo IV (1 unit) at 30 °C for 30 min and analyzed by 1% agarose gel electrophoresis as described under "Experimental Procedures". As controls, mixtures of pGEM and pBIG (1 μg each) were mock-reacted and then treated with Ung/Dug/Endo IV (lane C) as described above. Untreated pGEM and pBIG (80 ng each) and a sample containing 500 ng of a 1-kb DNA ladder were also employed as reference standards (lane S and M, respectively). Arrows indicate the location of the form I and II DNA bands detected by ethidium bromide staining. (B) DNA bands shown in (A) were quantitatively analyzed as described in Figure 54, and the amount of repaired DNA in each sample was plotted as the percentage of form I DNA corresponding to the following: pGEM(U·G) (○), pBIG(U·G) (●), pGEM(εC·G) (□), and pBIG(εC·G) (■). Mean values and standard deviations of three experiments are represented.
Figure 61
5.1.8 Analysis of the BER Patch Size Distribution of Ung- and Dug-mediated BER

BER patch size distributions associated with Ung-mediated uracil-initiated BER and Dug-mediated uracil- and ethenocytosine-initiated BER were determined in various E. coli cell extracts. During the course of these experiments, two types of methods were employed which are referred to as; the exonuclease III digestion analysis and the restriction endonuclease analysis. Since the occurrence and efficiency of BER was very similar between the two plasmid substrates, pGEM DNA containing a U/G or εC/G target site was utilized as a model BER substrate in both types of experimental analysis, as described below.

The method involving exonuclease III digestion was performed similarly to that described in Chapter 4 except that the target residue was a U/G or εC/G mispair and unique restriction endonuclease sites, HindIII and BamHI, were utilized to identify the BER patch size as illustrated in Figure 62. In addition, the pGEM DNA substrate contained a site-specific [32P]dAMP residue located between the target base and the BamHI site (three nucleotides downstream from BamHI site) on the (-) strand. To detect the 3' boundary of the repair patch, BER reactions were performed in the presence of four dNTP[αS]s and the recovered DNA products were restricted with HindIII to generate linear DNA. Digestion of linear DNA with Exo III and then BamHI was expected to generate a set of discrete [32P]DNA fragments. These [32P]DNA fragments were analyzed using denaturing polyacrylamide gel electrophoresis and the length of each fragment was indicative of the BER patch size (i.e. 14-, 15-, and 16-mers corresponded to a repair tract of 1, 2, and 3 nucleotides, respectively). The basis of this analysis depended upon the inability of Exo III to hydrolyze dNTP[αS] nucleotides incorporated during BER DNA synthesis as recognized previously (344, 347). As a pilot experiment, the amount of Exo III required to define the 3' border of the repair patch was determined using [32P]pGEM (U-G) DNA (Figure 63). [32P]DNA
Figure 62. Scheme for determining uracil- and ethenocytosine-initiated BER patch size with pGEM DNA by exonuclease III digestion analysis. The $^{32}$P-labeled pGEM(X-G) (X=U or εC) form I DNA was constructed, as described under "Experimental Procedures", and contained a $[^{32}$P]dAMP residue (*)& located 12 nucleotides upstream of the target site and 2 nucleotides downstream of the BamHI restriction site on the (-) strand DNA. Standard BER reactions were performed with pGEM $[^{32}$P]DNA containing either U/G or εC/G mispair and various E. coli cell extracts in the presence of each of the four complementary 2'-deoxyribonucleoside α-thioltriphosphates. In order to locate the 3'-boundary of the repair patch, the recovered pGEM $[^{32}$P]DNA was cleaved with HindIII to generate linear DNA with a recessed 3' terminus located on the (-) strand, and then digested in the 3' to 5' direction by E. coli exonuclease III. Subsequent to exonuclease III treatment, the $[^{32}$P]DNA was restricted with BamHI to generate a DNA fragment containing a $^{32}$P-labeled oligodeoxynucleotide the length of which was diagnostic of the BER patch size. The various $^{32}$P-labeled DNA fragments produced in the patch size assay were resolved by denaturing 12 % polyacrylamide gel electrophoresis as described under "Experimental Procedures".
Figure 62
Figure 63. Effect of exonuclease III concentration on determining the BER patch size distribution using the pGEM DNA substrate. Two standard BER reaction mixtures (100 µl) containing 2 µg of pGEM (U·G) DNA and 20 µM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] was incubated with 0.2 mg of E. coli GM31 cell extract protein (lanes 8-14) or mock treated without cell extract (lane 1-7) at 30°C for 60 min. DNA products were isolated, each DNA sample (~100 ng) was digested with HindIII and then treated with 0, 0.02, 0.2, 2, 20, 50, and 100 units of exonuclease III (lanes 1-7 and 8-14, respectively) for 1 h at 37°C. Following exonuclease III digestion, reaction products were restricted with SmaI and DNA reaction products were resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The DNA size markers, the 30-mer generated by digesting 100 ng of pGEM (U·G) [32P]DNA with HindIII and BamHI, and the 13-mer produced by additional treatment with Ung and Endo IV, are indicated by arrows.
Figure 63
products were recovered following BER from the *E. coli* GM31 cell extract (Figure 63, lanes 9-14), or mock-reaction conducted without cell extract (Figure 63, lanes 2-7), and each DNA sample (100 ng) was subjected to the treatment with various amounts of Exo III (0.02-100 units). Analysis of the resulting \[^{32}P\]DNA fragments by denaturing polyacrylamide gel electrophoresis revealed that the treatment of repaired pGEM DNA with 20 to 100 units of Exo III produced a similar distribution of Exo III resistant \[^{32}P\]DNA bands (Figure 63, lanes 12-14) and indicated that complete digestion for the repaired \[^{32}P\]DNA molecules occurred at an Exo III: DNA ratio of greater than 0.2:1 (units: \(\mu\)g DNA). In contrast, DNA samples recovered from the mock-reaction and treated with more than 2 units of Exo III were completely susceptible to exonucleolytic degradation (Figure 63, lanes 3-6), indicating that this DNA lacked incorporation of \([\alpha-S]\)dNMP.

Patch size analysis was conducted following uracil-initiated BER in *E. coli* GM31, BH156 (ung), and BH157 (dug) cell extracts. As controls, mock-reacted pGEM (U.G) \[^{32}P\]DNA was examined. For each of the four reaction sets, BER reaction products were linearized by *Hind*III, mock-digested or digested with 20 and 100 units of Exo III as indicated in the figure legend, reacted with *Bam*HI, and the resulting \[^{32}P\]DNA products were analyzed by denaturing 12 % polyacrylamide/8.3 M urea gel electrophoresis. Incubation of the mock-reacted control with Exo III resulted in the complete digestion of the 30-mer \[^{32}P\]DNA fragment (Figure 64A, lanes 1-3). Thus, the incorporation of dNMP\([\alpha S]\)s at and after the uracil-target site was required for resistance to Exo III digestion. Exo III-resistant DNA bands of 14 to 30 nucleotides in length were observed in the reactions conducted with the *E. coli* GM31, BH156, and 157 cell extract and indicated that repair synthesis occurred with a repair patch size of 1-17 nucleotides (Figure 64A, lanes 5 and 6, 8 and 9, 11 and 12, respectively). The reduced level of Exo III resistant \[^{32}P\]DNA observed in the Ung-deficient BH156 cell extract reflected the decreased extent of uracil-initiate BER mediated by Dug and was consistent with previous observations.
Figure 64. Patch size distribution of uracil-initiated BER in various E. coli cell-free extracts determined by exonuclease III digestion analysis. (A) Standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (U-G) [32P]DNA and 20 μM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] were incubated at 30 °C for 30 min with 0.2 mg of cell extract protein of E. coli GM31 (lanes 4-6), BH156 (lanes 7-9), and BH157 (lanes 10-12). As a control, pGEM (U-G) [32P]DNA (2 μg) was mock-reacted in the absence of cell extract protein (lanes 1-3). Reaction products were isolated, samples (~100 ng) were digested with HindIII, and then incubated with 0 (lanes 1, 4, 7, and 10), 20 (lanes 2, 5, 8, and 11), and 100 (lanes 3, 6, 9, and 12) units of E. coli exonuclease III. Following exonuclease III digestion, the DNA was cleaved with BamHI, and then resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures." The locations of the DNA size markers, 30- and 13-mers, generated as described in Figure 63, are indicated by arrows. (B) The amount of 32P radioactivity detected in each band in (A) was quantitatively measured using a PhosphorImager and the results for the E. coli GM31 (black bars), BH156 (gray bars), and BH157 (striped bars) reaction products digested with 100 units of exonuclease III are plotted. The [32P]DNA bands of 14 to 30 nucleotides in length corresponded to BER repair patches of 1 to 17 nucleotides in length, respectively. The relative amount of 32P label in each band (% distribution) was determined by dividing the amount of 32P radioactivity detected per band by the total amount of 32P radioactivity detected for all bands and multiplying by 100.
Quantitative determination of $[^{32}\text{P}]\text{DNA}$ band intensity was performed, and the patch size distribution was expressed for each reaction as a percentage of the total BER patch size detected in each lane (Figure 64B). The results showed that uracil-initiated BER conducted by *E. coli* GM31 (Ung- and Dug-mediated), BH156 (Dug-mediated), and BH157 (Ung-mediated) mainly occurred as long patch repair events. While the overall patch size distribution was similar in each case, Dug-mediated uracil-initiated BER was somewhat biased toward shorter repair patches. In order to determine the patch size distribution associated with ethenocytosine-initiated BER, an analogous experiment was performed except that pGEM (εC·G) $[^{32}\text{P}]\text{DNA}$ was utilized as the BER substrate. Exo III digestion of the reaction products obtained from the *E. coli* GM31 and BH156 cell extracts revealed that the ethenocytosine-initiated BER DNA synthesis occurred with 1-17 nucleotide repair patches (Figure 65A, lanes 4-6 and 7-9, respectively). In contrast, Exo III digestion of the DNA sample recovered from the reaction containing BH157 cell extract did not produce a detectable repair patch (Figure 65A, lanes 11 and 12), indicating that no incorporation of $[\alpha\text{-S}]\text{dNMP}$ occurred. This result was consistent with earlier observations indicating the requirement for Dug in ethenocytosine-initiated BER (Figures 56 and 61). The vast majority of ethenocytosine-DNA repair synthesis was observed to occur via a long patch mechanism using both the wild type and ung deficient cells, while short patch (1 nucleotide) repair accounted for ~15% of the BER events (Figure 65B). In addition, the presence of Ung did not appear to influence the patch size distribution of ethenocytosine-initiated BER (Figure 65B).

Next, the patch size determination for uracil- and ethenocytosine-initiated BER was assessed by the method involving restriction endonuclease analysis that was developed by Frosina et al. (183) and subsequently modified (179, 351). This approach utilized various unique restriction endonuclease recognition sites located near the target residue to determine the localization and extent of repair DNA synthesis accompanied with the incorporation of
Figure 65. Patch size distribution of ethenocytosine-initiated BER in various *E. coli* cell-free extracts determined by exonuclease III digestion analysis. (A) Standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (εC-G) \[^{32}P\]DNA and 20 μM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] were incubated at 30 °C for 30 min with 0.2 mg of cell extract protein of *E. coli* GM31 (lanes 4-6), BH156 (lanes 7-9), and BH157 (lanes 10-12). As a control, pGEM (εC-G) \[^{32}P\]DNA (2 μg) was mock-reacted in the absence of cell extract protein (lanes 1-3). Reaction products were isolated, samples (~100 ng) were digested with HindIII, and then incubated with 0 (lanes 1, 4, 7, and 10), 20 (lanes 2, 5, 8, and 11), and 100 (lanes 3, 6, 9, and 12) units of *E. coli* exonuclease III. Following exonuclease III digestion, the DNA was cleaved with BamHI, and then resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures." The location of the DNA size markers, 30- and 13-mers, generated as described in Figure 63, are indicated by arrows. (B) The amount of \[^{32}P\] radioactivity detected in each band in (A) was quantitatively measured using a PhosphorImager and the results for the *E. coli* GM31 (black bars) and BH156 (gray bars) reaction products digested with 100 units of exonuclease III are plotted. The \[^{32}P\] DNA bands of 14 to 30 nucleotides in length corresponded to BER repair patches of 1 to 17 nucleotides in length, respectively. The relative amount of \[^{32}P\] label in each band (% distribution) was determined by dividing the amount of \[^{32}P\] radioactivity detected per band by the total amount of \[^{32}P\] radioactivity detected for all bands and multiplying by 100.
Figure 65
[\textsuperscript{32}P]dNMP. As illustrated in Figure 66A, the pGEM DNA substrate contained a target residue of either U/G or eC/G mispair that was designed to be located in the DNA region where the digestion with restriction endonucleases SmaI and XbaI, XbaI and AccI, XbaI and HindIII, HincII and PstI, PstI and HindIII, or AccI and HindIII generated various sizes of DNA fragments. BER reactions were performed in the presence of [\textsuperscript{32}P]dCTP, therefore resulting in the incorporation of [\textsuperscript{32}P]dCMP during repair DNA synthesis, and the analysis of the restriction DNA fragments was expected to define the DNA fragment containing 3'-end of the repair DNA synthesis by locating which restriction DNA fragment(s) was \textsuperscript{32}P-labeled. Assuming no repair DNA synthesis occurred on the 5'-side of the target site, a repair patch size of 1-3 nucleotides would result in a XbaI-HincII [\textsuperscript{32}P]DNA fragment (fragment C), but no further \textsuperscript{32}P-labeled restriction fragments would be produced by any of the other restriction endonuclease digestions (Figure 66B, fragment C). On the other hand, the occurrence of a longer repair patch of 3-9 nucleotides or >10 nucleotides would generate \textsuperscript{32}P-labeled restriction fragments D and E in addition to [\textsuperscript{32}P]DNA fragment C, by HincII-PstI and PstI-HindIII digestions, respectively. Thus, the total amount of [\textsuperscript{32}P]dCMP incorporation that occurred during both short-and longer-patch BER was calculated from AccI-HindIII [\textsuperscript{32}P]DNA fragment (Figure 66, fragment F) and the portion of BER events associated with 1-3 nucleotide repair patch sizes was determined from the amount of \textsuperscript{32}P-radioactivity in DNA fragments D, E, and F by the following equation: [F-(D+E)]/F \times 100.

To perform the patch size analysis for uracil-initiated BER, reactions were conducted that contained pGEM (U-G) DNA and various E. coli cell extracts in the presence of [\textsuperscript{32}P]dCTP. After the reaction, each DNA product was recovered, subjected to the treatment with various restriction endonucleases, and analyzed by denaturing polyacrylamide gel electrophoresis (Figure 67A). As anticipated, the incorporation of [\textsuperscript{32}P]dCMP was not detected in DNA fragments A and B, since no repair DNA synthesis occurred upstream of the
Figure 66. Scheme for determining the patch size distribution of uracil- and ethenocytosine-initiated BER by restriction endonuclease analysis. (A) pGEM (X·G) DNA (X=U or εC) containing a site-specific uracil or ethenocytosine at position 40 of the plasmid (-) strand DNA was constructed as described under "Experimental Procedures". The direction of DNA synthesis (horizontal arrow) during BER and the recognition sequences of various restriction endonucleases (vertical arrows) are indicated. Digestion of repaired pGEM DNA with Smal and XbaI (A), XbaI and AccI (B), XbaI and HincII (C), HincII and PstI (D), PstI and HindIII (E), or AccI and HindIII (F) generates 9-, 7-, 8-, 8-, 8-, or 17-bp DNA fragments, respectively. (B) Standard BER reactions were performed with either pGEM (U·G) or pGEM (εC·G) DNA in the presence of various E. coli cell extracts. In order to monitor the repair DNA synthesis, [32P]dCTP was also included in the BER reaction mixtures. The sites of possible [32P]dCMP incorporation (*) generated for various BER patch size lengths are depicted according to the direction of repair DNA synthesis shown in (A) along with the restriction endonuclease cleavage sites. Repair DNA synthesis of 1 to 3, 1 to 9, and 1 to 17 nucleotide patches generates [32P]DNA fragments of C, D, and E, respectively, after digestion of repaired pGEM DNA with appropriate restriction endonucleases indicated by vertical arrows. The DNA products of each restriction endonuclease reaction (A-F) were resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis, and the amount of 32P-label in each DNA bands was quantified using a PhosphorImager. The percentage of short patch repair events (1-3 nucleotides) was calculated from the amount of 32P radioactivity associated with various DNA fragments as follows: [F-(D+E)]/F × 100. The percentage of long patch repair events (4-17 nucleotides) was similarly determined by the following formula: [1-(F-(D+E))]/F × 100.
Figure 66
Figure 67. Patch size distribution of uracil-initiated BER in various E. coli cell-free extracts determined by restriction endonuclease analysis. (A) Standard BER reaction mixtures (100 µl) containing heteroduplex pGEM (U-G)DNA (2 µg) and [³²P]dCTP (40 µCi) were incubated at 30 °C for 30 min with 0.2 mg of E. coli GM31, BH156, and BH157 cell extract protein, as indicated. DNA reaction products were isolated, samples (~100 ng) were subjected to the restriction endonuclease analysis described in Figure 66, and the resulting DNA fragments (A-F) were resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The location of 8-mer and 17-mer DNA products, as well as the 34-mer internal standard DNA (IS) bands visualized by PhosphorImager is indicated by arrows. (B) The relative amount of ³²P-label in each DNA band shown in (A) was measured using a PhosphorImager and ImageQuant software. The resulting value was corrected for the loading error relative to the intensity of the IS band detected in each lane. The distribution of repair patch size was calculated as described in Figure 66 and plotted as the percentage of short patch (black bars) and long patch (white bars) repair events.
uracil target site. In contrast, [\textsuperscript{32}P]DNA bands corresponding to restriction fragment C, D, and E were observed in each of the reaction conducted with *E. coli* GM31, BH156, and BH157 cell extracts. A reduced level of total \textsuperscript{32}P-radioactivity was observed for Ung-deficient (BH156) BER reaction and this was consistent with the previous observation (Figure 64), indicating a reduced extent of BER for Dug-mediated uracil-DNA repair. An internal standard C-34-mer [\textsuperscript{32}P]DNA (IS) was also resolved on the same gel and employed to correct the possible loading error for the quantification analysis (Figures 67 and 68). Quantitative measurement of the patch size distribution showed that the repair patch size of less than 4 nucleotides constituted ~30% of total uracil-initiated BER patch and this distribution was similar in each reaction (Figure 67B). Patch size analysis for ethenocytosine-initiated BER was similarly performed except using pGEM (eC-G) DNA and the results are shown in Figure 68. As expected, no detectable incorporation of [\textsuperscript{32}P]dCMP into the restriction fragments was observed in the reaction containing the BH157 cell extract due to the lack of Dug activity. The majority (~75%) of the ethenocytosine-DNA repair appeared to occur via long patch repair with more than 3 nucleotides repair patch size in both *E. coli* GM31 and BH156 cell extracts. When taken together, these results implied that the patch size distribution of *E. coli* ethenocytosine-initiated BER mediated by Dug was very similar to that of uracil-initiated BER mediated by Ung.

5.2 Discussion

In this Chapter, results were presented that analyzed the relative contribution of two genetically distinct DNA-glycosylases involved in two *E. coli* DNA repair pathways, uracil- and ethenocytosine-initiated BER. A prerequisite to this study was the identification of the activity of Dug as uracil- and ethenocytosine-DNA glycosylases (106, 107, 343). Dug was initially isolated from *E. coli* cells deficient in Ung as a Ugi-insensitive uracil-DNA glycosylase activity that was restricted to duplex DNA substrates (107, 343).
Figure 68. Patch size distribution of ethenocytosine-initiated BER in various E. coli cell-free extracts determined by restriction endonuclease analysis. (A) Standard BER reaction mixtures (100 μl) containing heteroduplex pGEM (eC-G)DNA (2 μg) and [32P]dCTP (40 μCi) were incubated at 30 °C for 30 min with 0.2 mg of E. coli GM31, BH156, and BH157 cell extract protein as indicated. DNA reaction products were isolated, samples (~100 ng) were subjected to the restriction endonuclease analysis described in Figure 66, and the resulting DNA fragments (A-F) were resolved by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The location of 8-mer and 17-mer DNA products, as well as the 34-mer internal standard DNA (IS) bands visualized by PhosphorImager is indicated by arrows. (B) The relative amount of 32P-label in each DNA band shown in (A) was measured using a PhosphorImager and ImageQuant software. The resulting value was corrected for the loading error relative to the intensity of the IS band detected in each lane. The distribution of repair patch size was calculated as described in Figure 66 and plotted as the percentage of short patch (black bars) and long patch (white bars) repair events.
Furthermore, a previous report by Saparbaev and Laval (106) showed that DNA glycosylase activity in *E. coli* that excises ethenocytosine residues from double-stranded DNA was associated with Dug. Indeed, purified recombinant Dug has been shown to remove ethenocytosine residues efficiently from double-stranded DNA but not from single-stranded DNA (106, 343). In addition, it has been previously reported that the rate of excision of ethenocytosine residues from duplex DNA was not influenced by the base on the opposite DNA strand, whereas the efficient excision of uracil was found to be dependent on the opposite base being guanine in a U/G mispair (106, 343). This *in vitro* substrate preference of purified Dug appeared to be closely related to the Dug-DNA interaction since Dug binds tightly to the AP-site/G product and is unable to react with other U/G target sites; therefore, each molecule of Dug appears to remove only one molecule of uracil from U/G-containing DNA (343). In contrast to the uracil-DNA glycosylase activity of Dug, the ethenocytosine-DNA glycosylase activity of purified Dug can remove more than a stoichiometric amount of ethenocytosine bases from εC/G-containing DNA. These observations raise the possibility that the fundamental process involved in the recognition of the modified bases could be different. For example, the ethenocytosine residue may be recognized by chemical constituents of the base whereas the uracil residue may be recognized through the structure of the mismatch or more general DNA distortion (113). It should be noted that the efficiency of the excision of ethenoadenine residues by *E. coli* 3-methyladenine-DNA glycosylase does not depend on the nature of the opposite base (176). It may be reasonable to speculate that Dug directly recognizes the etheno-adduct and flips out the ethenocytosine residue into a specific pocket within the active site, similar to mechanism described for the Ung protein (87, 90).

The relative efficiency of endogenous uracil- and ethenocytosine-DNA glycosylase activities was examined using defined uracil- and ethenocytosine-containing oligonucleotide DNA (U/G- and εC/G-34-mer) substrates.
Processing of ethenocytosine bases was not detected in the *E. coli* cell extracts defective in Dug activity which was consistent with previous reports suggesting that Dug may be the only enzyme in *E. coli* that can remove this mutagenic adduct (319). On the other hand, the uracil-DNA glycosylase activity of Dug appeared to be the principal uracil excision activity in the absence of Ung. Therefore, the determination of uracil- and ethenocytosine-DNA glycosylase activities in *E. coli* cell extracts deficient in Ung allowed for a comparison of the relative efficiency of Dug-mediated uracil-excision to the Dug-mediated ethenocytosine-excision. The rate of base excision was ~23-fold greater by the ethenocytosine-DNA glycosylase activity than by the uracil-DNA glycosylase activity associated with Dug. This result is comparable to the report by Saparbaev and Laval (106) who showed that Dug to be ~50-fold more catalytically efficient on εC/G than on U/G in studies that measured the kinetic parameters of purified Dug using oligonucleotide substrates. However, it should be pointed out that, in the study by Saparbaev and Laval (106), an excess amount of Fpg protein was routinely included in the glycosylase reaction to cleave AP-sites generated by Dug. Since the stimulation of uracil-DNA glycosylase activity of Dug can occur through AP-site cleavage mediated by *E. coli* endonuclease IV (343), the addition of Fpg protein may also result in a stimulated catalytic response. In this regard, Saparbaev and Laval's (106) observation that turnover occurred for the uracil-DNA glycosylase activity of Dug most likely was attributed to the influence of the Fpg protein on the mode of the reaction. The observation that Fpg protein caused Dug to dissociated from the U/G-DNA substrate as determined by band mobility shift assays (Figure 20) further supports this notion. In addition, the data presented in this Chapter further supports that the ethenocytosine-DNA glycosylase activity of Dug is more efficient than uracil-DNA glycosylase activity of Dug.

Having observed that Dug is essential for removing ethenocytosine residues from DNA in *E. coli* cell extracts, the results also demonstrated the
involvement of Dug in initiating ethenocytosine-initiated BER in E. coli cell-free extract. An examination of the repair reaction in E. coli proficient in Dug activity revealed that ~80% of the ethenocytosine-containing DNA substrates was converted to the completely repaired form I DNA after 90 min of incubation. Under the conditions examined, most of the ethenocytosine-DNA repair occurred via the BER pathway. Several observations support this conclusion: (i) the repair was dependent on the presence of ethenocytosine-DNA glycosylase activity, (ii) the occurrence of repair DNA synthesis was specific to the target ethenocytosine-containing DNA, and (iii) patch size of repair DNA synthesis in ethenocytosine-DNA repair was similar to that observed in uracil-initiated BER. To our knowledge, this study is the first to report on the complete ethenocytosine-initiated DNA repair using the BER mechanism. Previously, the uracil-DNA glycosylase activity of Dug had been shown to mediate uracil-initiated BER from U/G and U/T residues in the absence Ung activity, as described in Chapter 4 and reported previously by Sung et al (343, 352). Since the major uracil-excision activity in E. coli cells was attributed to Ung, a back-up role for Dug in uracil-initiated DNA repair has been proposed (107, 343, 352). The data presented in this study further support that Dug activity with overlapping substrate specificity on uracil- and ethenocytosine-DNA indeed participated in the BER pathway. A recent study by Mokkapati et al. (353) reported that Dug is fairly abundant in stationary-phase E. coli cells and has a significant anti-mutator role at this stage of cell growth. However, the exact function of Dug in mutation avoidance was not investigated in this study. Given the finding that Dug participates in the BER pathway, it is speculated that the anti-mutator function of Dug might result from Dug-mediated uracil- and ethenocytosine-initiated BER.

To investigate the relative efficiency of uracil- and ethenocytosine-initiated BER, a lesion specific BER-competition assay was developed. The assay was performed by utilizing two form I plasmid DNA substrates each containing a site-specific uracil or ethenocytosine residue as the target lesion.
The molecular size difference between the two plasmid substrates allowed the analysis of two DNA repair reactions under same condition but did not affect the proficiency of the BER. Using this lesion specific BER-competition assay, the rate and extent of uracil and ethenocytosine-DNA repair were measured using isogenic *E. coli* cells that were Ung and/or Dug deficient. The results indicated that (i) ethenocytosine-initiated BER occurred as efficiently as uracil-initiated BER in the presence of Ung and Dug; (ii) the rate of Dug-mediated ethenocytosine-initiated BER was ~8-fold greater than that of Dug-mediated uracil-initiated BER; and (iii) ethenocytosine-initiated BER did not occur in the absence of Dug. Since the uracil-excision occurred ~23-fold faster than ethenocytosine-excision in cell extracts proficient in both Ung and Dug, the similar repair efficiency observed between uracil-DNA and ethenocytosine-DNA suggested that the first step involving uracil- and ethenocytosine-excision may not be a rate-limiting step in a complete BER process. Similarly, the difference (~50-fold) in the Dug catalyzed base excision between uracil- and ethenocytosine-DNA was much higher than that observed by comparison of Dug-mediated uracil- and ethenocytosine-initiated BER. In the human uracil-initiated BER pathway, it has been reported that the rate-limiting step appears to involve the removal of 5'-deoxyribose phosphate residue that is catalyzed by the 8 kDa domain of DNA polymerase β (354). This suggestion was made based on *in vitro* studies of the BER reaction using a reconstituted enzyme system composed of purified human enzymes (uracil-DNA glycosylase, AP endonuclease 1, DNA polymerase β, and DNA ligase I) (354). It should be noted that, in *E. coli*, the major BER DNA polymerase, DNA polymerase I, is unable to hydrolyze the 5'-deoxyribose phosphate residue generated after the cleavage of AP-site (271-273). Hence, additional studies will be required to define the rate-limiting step and enzyme that influences on the efficiency of uracil- and ethenocytosine-initiated BER in *E. coli*.

The repair patch size distribution of uracil- and ethenocytosine-initiated BER was assessed by two different approaches; exonuclease III digestion
analysis (346, 352) and restriction endonuclease analysis (179, 183, 351). The exonuclease III digestion analysis revealed that the predominant type of repair synthesis occurred by a long patch mechanism in both Ung- and Dug-mediated uracil-initiated BER. The replacement of only one nucleotide (short patch BER DNA synthesis) contributed only a small portion of the total BER DNA synthesis for both Ung- and Dug-mediated BER. This observation was consistent with previous results obtained using an M13mp2op14 (U-T) DNA substrate (as shown in Chapter 4) instead of the pGEM DNA used in this Chapter. Furthermore, a similar patch size distribution was also observed in Dug-mediated ethenocytosine-initiated BER. In all cases, the repair patch of 12 nucleotides was the single most prevalent patch size. It should be noted that Lundquist and Olivera (297) previously reported that an average of ≥12 nucleotides is generated by DNA polymerase I during DNA synthesis from a nicked DNA substrate prior to the action of 5' to 3' exonuclease activity. The data presented in this study might therefore suggest that the majority of repair DNA synthesis during uracil- and ethenocytosine-initiated BER occurs by a DNA polymerase I-mediated strand displacement DNA synthesis mechanism. In order to validate the observed repair patch size results, a second method was used to examine the BER reaction products using restriction endonuclease analysis. Although this approach has a significant limitation in defining the discrete distribution profile of repair patch size, the results showed that ~20-30% of the repair patches were shorter than 4 nucleotides for both uracil- and ethenocytosine-initiated BER. This result was in good agreement with the sum of the percentage of 1, 2, and 3 nucleotide patch sizes determined by the method involving exonuclease III analysis. Taken together, these results indicated that long patch BER is the predominant pathway over short patch BER during Dug- and Ung-mediated uracil- and ethenocytosine-initiated DNA repair. Additional experiments will be required to determine which DNA polymerase is involved in short and long patch BER pathways.
6. VERY-LONG PATCH BASE EXCISION DNA REPAIR IN 
ESCHERICHIA COLI: MODULATION OF PATCH SIZE DISTRIBUTION 
BY DNA LIGASE AND DNA POLYMERASE I

Previous studies using *E. coli* cell extracts and short uracil-containing oligonucleotide substrates have led to the proposal of a model for the uracil-initiated BER pathway that mainly involves the replacement of a single nucleotide at the target site, which is referred to as short patch BER (182, 305). However, a second pathway, long patch BER, was also recognized but considered to be a minor component compared with short patch BER pathway (182). In contrast, another study involving the use of closed circular DNA substrates revealed that uracil-initiated BER almost exclusively occurred with the replacement of several (~15) nucleotides (306). The results presented in Chapters 4 and 5 confirm that BER occurs primarily by a long patch mechanism when a duplex circular DNA substrate was used to analyze repair. Furthermore, the analysis of the repair patch size distribution presented in this study revealed that uracil-DNA repair in *E. coli* can be initiated by both Ung and Dug and that BER primarily generates long patches ranging 2 to 20 nucleotides. Although the biochemical mechanism for each step associated with both short and long patch BER have been presented elsewhere, the factors that determine the rate of complete BER process and the choice of BER pathway (short *versus* long) have not been elucidated.

In this Chapter, the rate limiting step associated with uracil-initiated BER conducted in *E. coli* cell extracts was identified. Experiments were performed that involved supplementation of *E. coli* cell extracts with purified BER proteins and the rate of the complete BER was assessed using form I plasmid DNA containing a site specific uracil or ethenocytosine residue. By this approach the limiting enzyme was identified since its addition stimulated the BER reaction. A detailed analysis of the repair DNA synthesis associated with both uracil- and ethenocytosine-initiated BER revealed evidence for a previously unidentified BER pathway that involved the DNA synthesis of
more than 200 nucleotides. This pathway is referred to as "very-long patch BER". Experiments were also performed to examine whether Pol I was the major DNA polymerase that participated in the very-long patch BER pathway. In addition, the effects of DNA ligase and DNA polymerase I in modulating the repair patch size distribution of uracil- and ethenocytosine-initiated BER were evaluated in detail.

6.1 Results

6.1.1 Influence of DNA Ligase and Fpg Protein on Uracil-initiated BER in *E. coli* Cell Extracts

In order to detect uracil-initiated BER in *E. coli* cell extract, this study employed a closed circular duplex DNA substrate containing a site-specific uracil that served as the target for the uracil-DNA glycosylase mediated BER pathway. Experiments were conducted using pGEM (U-G) DNA and *E. coli* GM31 cell extracts. Following BER, the product DNA was isolated and treated with excess *E. coli* Ung and Endo IV to convert uracil- or AP-site-containing form I DNA to form II molecules. This treatment effectively eliminated unreacted and incompletely repaired substrates from the pool of fully repaired DNA molecules that were resistant to Ung/Endo IV treatment. An initial experiment was performed to examine whether the uracil-initiated BER in the *E. coli* cell extract was affected by supplementation with *E. coli* DNA ligase or Fpg protein. *E. coli* DNA ligase catalyses the NAD-dependent formation of phosphodiester linkages between 5'-phosphoryl and 3'-hydroxyl groups at the nick of BER intermediates in the final step of BER (307, 310). Whereas, the intrinsic lyase activity of Fpg can remove an abasic 5'-deoxyribose phosphate (dRP) residues from the BER intermediates generated by AP-site cleavage (278, 280). Purified *E. coli* DNA ligase or Fpg protein was added to the standard BER reactions, and the DNA reaction products produced after various times (0-90 min) were resolved by agarose gel
electrophoresis (Figure 69A). Inspection of the ethidium-bromide stained gel revealed that addition of DNA ligase provoked an increased rate of repaired form I DNA in early time points (0-30 min) compared to the reaction carried out without supplementation (Figure 69A, -Addition versus +DNA Ligase, lanes 1-4). The initial rate of repair was 3-fold greater in the presence of exogenous DNA ligase, and the maximum extent of BER was achieved after a 10 min reaction compared to almost 90 min in the absence of added DNA ligase (Figure 69B). On the other hand, the addition of Fpg protein did not alter the rate or extent of BER (Figure 69B). This inability of Fpg to affect the BER was not due to enzyme inactivity. It was experimentally determined that the Fpg preparation acted efficiently as an AP lyase since >95% of AP-site-containing form I DNA was converted form II DNA after a 30 min reaction. Taken together, these results imply that the ligation step rather than the step involving dRP removal is the rate-limiting step in E. coli uracil-initiated BER.

6.1.2 Detection of Very-long Patch Repair DNA Synthesis Mediated by Uracil- and Ethenocytosine-initiated BER in E. coli Cell Extracts

In order to determine the distribution of DNA synthesis associated with repaired DNA molecules, standard BER reactions were performed with E. coli GM31 cell extract and pGEM (U-G) DNA in the presence of [α-32P]dCTP. At various times, the [32P]DNA product isolated from the BER reaction was digested with restriction endonuclease BsrI and resolved by nondenaturing polyacrylamide gel electrophoresis. While there are 12 BsrI recognition sites in the pGEM DNA sequence, BsrI digestion produces 9 DNA fragments in excess of 100 bp (Figure 70). Among these, the 272-bp DNA fragment contained the target uracil residue, 205 nucleotides upstream from the BsrI restriction site positioned between 272- and 603-bp restriction fragments. Following electrophoresis, inspection of the phosphor image revealed that [32P]dCMP incorporation preferentially occurred in the 272-bp fragment in early times and increased in a time-dependent manner (Figure 71A, U/G,
Figure 69. Effect of DNA ligase and Fpg protein supplementation on the rate and extent of uracil-initiated BER in *E. coli* GM31 cell extracts. (A) Three sets of standard BER reaction mixtures (100 µl) containing 2 µg of pGEM (U·G) DNA and 0.2 mg of *E. coli* GM31 cell extract protein were prepared as described under the "Experimental Procedures" except that *E. coli* DNA ligase (0.15 µM) or Fpg (0.2 µM) was added to some reaction mixtures as indicated. Incubation was carried out for 0, 5, 10, 30, 60, and 90 min at 30 °C (lanes 1-6, respectively). After terminating the reactions, pGEM DNA was isolated, subjected to Ung/Endo IV treatment, and analyzed by 1% agarose gel electrophoresis as described under "Experimental Procedures". As a control, pGEM (U·G) DNA was mock-reacted and treated with excess Ung and Endo IV (lane C). Untreated pGEM (U·G) DNA (80 ng) and a sample containing 1 µg of a 1-kb DNA ladder 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 in (A) was quantified using a Gel Documentation System and the ImageQuant program, and 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 (□).
Figure 69
Figure 70. *BsrI* restriction endonuclease cleavage map of pGEM DNA. The relative locations of *BsrI* restriction endonuclease recognition sites are illustrated by hash marks on the pGEM DNA molecule. DNA fragments produced by *BsrI* digestion that are in excess of 100 bp of the size in base pairs, and the number of cytosine residues (*parentheses*) located in each DNA fragment are denoted. The location of the uracil (*U*) or ethenocytosine (*eC*) residue in the (-) strand of pGEM DNA and the direction of DNA repair synthesis (*arrow*) during BER are shown.
Figure 70
Figure 71. Specificity of uracil-initiated BER DNA synthesis in E. coli GM31 cell extracts. (A) Two sets of standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (C-G) or (U-G) DNA, 0.2 mg of E. coli GM31 cell extract protein, and 10 μCi/ml of [32P]dCTP were prepared. The reactions were incubated at 30 °C for 0, 5, 10, 30, 60, and 90 min (lanes 1-6, respectively). The reaction mixtures for lane 7-9 were similarly constructed with pGEM (U-G) DNA except that 1 μl of Ugi dilution buffer (lane 7), or 1 μl of Ugi (1000 units) was included (lane 8), or E. coli BH158 (ung, dug) cell extract replaced the GM31 cell extract (lane 9) and incubated at 30 °C for 90 min. Following each reaction, DNA products were isolated, and each DNA sample (100 ng) was treated with 2.5 units of BsrI for 1 h at 65 °C. The resulting DNA restriction fragments were then resolved by 5% nondenaturing polyacrylamide gel electrophoresis and visualized using a Phosphorlmager as described under "Experimental Procedures". 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. (B) The amount of [32P] radioactivity of each DNA band shown in (A) was determined using the ImageQuant program. After subtracting the background radioactivity, the [32P]dCMP incorporation was normalized for each DNA fragment by dividing the amount of [32P] radioactivity detected by the number of cytosine residues located in each corresponding DNA fragment. The highest normalized value was then designated 100% and the percentage of the [32P]dCMP incorporation in other DNA fragments was calculated relative to that value. The data for DNA fragments of 272-bp (red bars), 603-bp (blue bars), 117-bp (green bars), and 445-bp (gray bars), and plotted for each reaction described in (A).
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lanes 1-6). Interestingly, an increased level of $[^{32}P]dCMP$ incorporation was also observed in the 603-bp fragment after 30 min (Figure 71A, U/G, lanes 4-6). This fragment was located just downstream from the uracil containing 272-bp fragment. Control reactions were conducted using homoduplex pGEM DNA containing a C/G base pair at the target site to determine whether this elevated level of $[^{32}P]dCMP$ incorporation specifically occurred due to the uracil containing DNA. The corresponding DNA fragments (272- and 603-bp fragments) in C/G DNA appeared to accumulate only a background level of incorporation (Figure 71A, C/G, lanes 1-6). Quantitative analysis indicated that the presence of uracil residue stimulated DNA synthesis in the 272- and 603-bp DNA fragments by 20- and 8-fold, respectively, in the 60 min reaction, compared to the background level determined in C/G DNA (Figure 71B, C/G versus U/G, lane 5). Similarly, after 90 min, the level of $[^{32}P]dCMP$ incorporation into the 117-bp fragment located just downstream of the 603-bp fragment was increased ~2-fold in the U/G DNA compared to the C/G DNA (Figure 71B, C/G versus U/G, lane 6). However, the specific incorporation of $[^{32}P]dCMP$ into these DNA fragments was significantly reduced when the reaction was conducted in the presence of Ugi or in the cell extract deficient in Ung and Dug activities (Figure 71, lanes 8 and 9) compared to the control (Figure 71, lane 7). When taken together, these results indicated that most of DNA synthesis occurred during the reaction was uracil-initiated and instigated by uracil-DNA glycosylase activity. Thus, the repair DNA synthesis observed with the 603-bp restriction fragment was presumably associated with more than a 205 nucleotide of uracil-initiated BER synthesis, and hereafter this is referred as very-long patch BER.

The majority of uracil-initiated BER observed in the *E. coli* GM31 cell extract was shown to be mediated by Ung, as described in Chapter 5. However, it remained to be determined if the very-long patch repair DNA synthesis observed in Figure 71 was mainly associated with Ung-mediated BER versus Dug-mediated BER or some other pathway. Thus, experiments
were conducted utilizing various *E. coli* cell extracts to examine the occurrence of the very-long patch BER DNA synthesis during both Ung- and Dug-mediated BER. pGEM DNA containing either a U/G or eC/G target site was reacted with *E. coli* GM31, BH156 (ung), and BH157 (dug) cell extracts for 90 min. Additional reactions were also conducted using homoduplex pGEM (eC-G) DNA to further assess Dug-mediated BER. The reaction products were then recovered and subjected to the *BsrI* digestion analysis. The result revealed that incorporation of [\(^{32}\)P]dCMP into pGEM (eC-G) DNA occurred predominantly in the 272-bp and 603-bp fragments and this occurred only in the cell extract proficient in Dug (Figure 72, lanes 3 and 6). The reaction conducted using the *E. coli* BH157 cell extract that was deficient in dug showed only background levels of [\(^{32}\)P]dCMP incorporation (Figure 72, lane 9). Furthermore, the incorporation appeared to be specific to the ethenocytosine-containing DNA, since only a background level of [\(^{32}\)P]dCMP incorporation was detected in the DNA fragments generated from the pGEM (C-G) DNA (Figure 72, lanes 1, 4, and 7). In addition, uracil-initiated BER conducted in the absence of Ung but presence of Dug (BH156 cell extract) resulted in the specific incorporation of [\(^{32}\)P]dCMP into the 603-bp fragment as well as the 207-bp fragment that initially contained the uracil residue (Figure 72, lane 5). These results indicated that the very-long patch BER occurred during Dug-mediated BER of uracil- and ethenocytosine-DNA as well as during Ung-mediated BER of uracil-DNA.

### 6.1.3 Alteration of Uracil-initiated BER Patch Size Distribution by Supplementing DNA Ligase

It was postulated that very-long patch BER DNA synthesis occurred because of a delay in the ligation step. This was consistent with the observation that ligation appeared to be the rate-limiting step during uracil-initiated BER in *E. coli* cell extract (Figure 69). Thus, it was reasonable to examine whether the addition of exogenous DNA ligase affected the
Figure 72. Specificity of uracil- and ethenocytosine-initiated BER in various E. coli cell-free extracts. Standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (C-G), (U-G), or (εC-G) DNA and 10 μCi/ml of [32P]dCTP were incubated with 0.2 mg of E. coli GM31 (lanes 1-3), BH156 (lanes 4-6), and BH157 (lanes 7-9) cell extract protein at 30 °C for 90 min. DNA reaction products were isolated, and each DNA sample (100 ng) was treated with 2.5 units of BsrI for 1 h at 65 °C. The resulting DNA restriction fragments were then resolved by 5% nondenaturing polyacrylamide gel electrophoresis and visualized using a PhosphorImager as described under "Experimental Procedures". 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.
Figure 72
distribution of repair DNA synthesis and influenced the frequency of very-
long patch repair. To examine this proposition, purified *E. coli* DNA ligase or
Fpg was added to standard BER reaction mixtures containing *E. coli* GM31 cell
extract and [\(^{32}\)P]dCTP. Reaction products produced after various times were
subjected to the BsrI treatment and resolved by nondenaturing
polyacrylamide gel electrophoresis (Figure 73A). During the reaction time
course, the addition of DNA ligase greatly reduced the overall amount of
[\(^{32}\)P]dCMP incorporation into the DNA substrate, while Fpg supplementation
did not dramatically affect the amount of repair DNA synthesis (Figure 73A).
This decrease in [\(^{32}\)P]dCMP incorporation was not the result of an inefficient
repair process because supplementation with DNA ligase was previously
shown to stimulate the overall extent of BER (Figure 69). A more likely
interpretation would suggest that the repair efficiently occurred but that DNA
synthesis was more restricted to the vicinity of the uracil target when DNA
ligase was added. In support of this interpretation, the incorporation of
[\(^{32}\)P]dCMP into the 603-bp fragment was observed to be reduced to the
background level in the presence of DNA ligase. Thus, indicating that the
addition of DNA ligase essentially abolished very-long patch BER pathway.
Quantitative analysis revealed that the level of [\(^{32}\)P]dCMP incorporation into
the 272-bp fragment reached a plateau level after 10 min in the presence of
exogenous DNA ligase, whereas it occurred after 30 min in the reaction
conducted without supplementation (Figure 73B). Taken together, these
results indicated that the addition of DNA ligase to the *E. coli* GM31 cell
extract limited the extent of uracil-initiated repair DNA synthesis but
stimulated the completion of BER.

DNA repair synthesis was further characterized to determine whether
the patch size distribution associated with short-patch uracil-initiated BER
was altered by addition of DNA ligase or Fpg protein. The approach
described previously in Chapter 5 was utilized that relies on the incorporation
of 2'-deoxyribonucleoside α-thiolmonophosphates during BER to render the
Figure 73. Effect of DNA ligase and Fpg protein on the specificity of uracil-initiated BER DNA synthesis. (A) Three sets of standard BER reaction mixtures (100 µl) with pGEM (U-G) DNA were prepared as described in Figure 69 except that 10 µCi/ml of [³²P]dCTP was added with or without the addition of exogenous E. coli DNA ligase (0.15 µM) and Fpg (0.2 µM) as indicated. Following incubation at 30 °C for 0, 5, 10, 30, 60, and 90 min (lanes 1-6, respectively), DNA samples (100 ng) were isolated, subjected to BsrI digestion, and resolved by 5% non-denaturing polyacrylamide gel electrophoresis as described under "Experimental Procedures". [³²P]DNA fragments (arrows) were identified using a PhosphorImager and the 272-bp DNA fragment that contained uracil residue was located (*272). (B) The percentage of relative [³²P]dCMP incorporation into the DNA fragments of *272-bp (red bars), 603-bp (blue bars), 117-bp (green bars), and 445-bp (gray bars) was determined as described in Figure 71, and plotted for each lane shown in (A).
Figure 73
repaired DNA strand resistant to *in vitro* digestion with *E. coli* Exo III. The pGEM DNA substrate was again used that contained a uracil target located on the (−)-strand, 16 nucleotides upstream (5'-side) of a unique HindIII site, and 13 nucleotides downstream (3'-side) of a unique BamHI site. In order to measure the patch size, a site-specific [32P]dAMP residue was introduced between the uracil target and BamHI site and at 12 nucleotides downstream from the target. The repair patch size was determined by monitoring the length of the [32P]DNA remaining after the sequential treatment of the BER reaction products with HindIII, Exo III, and BamHI. As described in Chapter 5, the 3'-5' exonuclease activity of Exo III was expected to terminate upon 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 in *E. coli* GM31 cell extracts in the absence and presence of DNA ligase or Fpg protein (Figure 74A). As expected, Exo III digestion of [32P]DNA recovered from mock reaction and 0 min reaction did not produce a detectable repair patch since the incorporation of 2'-deoxyribonucleoside α-thiolmonophosphates could not occur due to the absence of cell extract or incubation, respectively (Figure 74A, U and 0 min). On the other hand, following digestion with Exo III, each BER reaction set yielded discrete [32P]DNA fragments which were determined by the location of the 3'-end of repair patches (Figure 74A). The distribution of these fragments was evaluated for each reaction time course. Quantitative determination of BER repair patch size was performed, and the distribution of repair patch size was plotted for the 5 min and 60 min reaction (Figure 74B and C, respectively). In the reaction conducted for 5 min without supplementation, one-nucleotide repair patch appeared be the most prevalent patch size (Figure 74B, white bars). However, the majority of product DNA in this reaction was not associated with complete repair process since less than 20% of DNA molecules appeared as repaired form I DNA (Figure 69). Indeed, when this reaction product was not treated with Exo III, most of [32P]DNA
Figure 74. Effect of DNA ligase and Fpg protein on the uracil-initiated BER patch size distribution. (A) Three sets of standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (U-G) [³²P]DNA, 20 μM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] and 0.2 mg of E. coli GM31 cell extract protein were incubated in the presence and absence of exogenous E. coli DNA ligase (0.15 μM) or Fpg (0.2 μM) at 30 °C for 0, 5, 10, 30, 60, and 90 min as indicated. As a control, pGEM (U-G) [³²P]DNA (2 μg) was mock-reacted in the absence of cell extract proteins (U). Following the BER reaction, DNA products were isolated, each DNA sample (100 ng) was digested with 5 units of HindIII and then treated with 100 units of exonuclease III (+) or mock treated (-) as indicated. After these treatments, the DNA was cleaved with 5 units of BamHI, and the [³²P]-labeled DNA fragments were resolved by 12% polyacrylamide, 8.3 M urea gel electrophoresis as described under "Experimental Procedures". The location of [³²P]DNA size markers are indicated by arrows. The markers were generated by digesting pGEM (U-G) [³²P]DNA (100 ng) with excess HindIII and BamHI (30-nt) and by subsequently treating the [³²P]-labeled oligonucleotide (30-mer) with excess Ung and Endo IV (13-nt). The relative amount of [³²P] radioactivity detected in each DNA band corresponding to repair patch sizes of 1-17 nucleotides was determined for the 5 min (B) and 60 min (C) reactions shown in (A), using the method illustrated in Figures 62 and described in Figure 64. The frequency of each BER patch size 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 bars), with DNA ligase (black bars), and with Fpg (striped bars).
appeared to be BER intermediates (i.e. 13-mer corresponds to the DNA cleaved at 5'-site of AP-site, and 14 to 29-mer represents DNA molecules that underwent repair DNA synthesis, but were not ligated at the nick) (Figure 74A). As the reaction was continued, these BER intermediates disappeared and the predominant DNA fragment detected in the absence of Exo III treatment appeared as 30-mer which represents completely repaired DNA, including the ligation step (Figure 74A, 30, 60, and 90 min). Analysis of the repair patch distribution after a 60 min reaction revealed that the vast majority of BER occurred via the long patch mechanism, whereas short patch (1-nucleotide) repair accounted for ~12% of BER events (Figure 74C, white bars). This observation with E. coli GM31 was consistent with the previous result described in Chapter 5. These results implied that the majority of BER intermediates observed in the initial phase of the reaction were subjected to further extension by repair DNA synthesis; thereby resulting in longer repair patches following the completion of BER. The supplementation of Fpg protein did not show any perceivable affect on the repair patch size distribution in either the 5 min or 60 min reactions (Figure 74 B and C, striped bars). However, the addition of DNA ligase in the reaction conducted for 60 min drastically altered the overall patch distribution toward short patch products and increased the percentage of the one-nucleotide repair patch events by ~4-fold (Figure 74C, black bars). Moreover, the repair patch distribution was very similar between the 5 min and 60 min reactions which were both conducted in the presence of exogenous DNA ligase (Figure 74B and C, black bars). These results suggested that the majority of BER intermediates produced in the initial phase of the reaction were subjected to the completion of BER by the addition of DNA ligase; thus leading to the shorter patch BER pathway.
6.1.4 Influence of DNA Ligase and Fpg on Dug-mediated BER at Ethenocytosine Residues

In order to examine the effect of exogenous *E. coli* DNA ligase and Fpg protein on the efficiency of Dug-mediated BER in the *E. coli* GM31 cell extract, pGEM form I DNA substrate containing a site-specific ethenocytosine was used in place of pGEM (U-G) DNA. Following the BER reaction, DNA products were subjected to the combined treatment with Dug and Endo IV to convert un-repaired form I DNA to form II DNA. Since the repair of ethenocytosine residues was not detected in the *E. coli* BH157 and BH158 cell extracts that were deficient in Dug activity (see Chapter 5.1.5), the appearance of form I DNA resistant to Dug/Endo IV treatment was attributed exclusively to Dug dependent ethenocytosine-initiated BER. Analysis of the ethenocytosine-initiated BER reaction time course conducted in *E. coli* GM31 cell extracts revealed that the initial rate of repair was ~3-fold greater in the presence of exogenous DNA ligase, whereas the rate and extent of BER were similar in the presence of Fpg protein (Figure 75). These results were consistent with the effect of exogenous DNA ligase that was observed in uracil-initiated BER. In addition, very-long patch repair DNA synthesis was also detected during ethenocytosine-initiated BER, but abolished by the supplementation of DNA ligase (Figure 76). In contrast, the addition of Fpg protein did not affect either the efficiency or specificity of repair DNA synthesis associated with ethenocytosine-initiated BER (Figure 75 and 76).

Repair patch size analysis was conducted to further examine the influence of DNA ligase and Fpg on the distribution of Dug-mediated ethenocytosine-initiated BER DNA synthesis (Figure 77). In the reaction conducted for 5 min, the occurrence of a single nucleotide replacement appeared to be the most prevalent events during the repair DNA synthesis and this was consistently observed when the reaction was supplemented with exogenous DNA ligase or Fpg (Figure 77B). In contrast, the vast majority of ethenocytosine repair appeared to occur via the long patch pathway as
Figure 75. Effect of DNA ligase and Fpg supplementation on the rate and extent of ethenocytosine-initiated BER in E. coli GM31 cell extracts. (A) Three sets of standard BER reaction mixtures (100 µl) containing 2 µg of pGEM (εC-G) DNA and 0.2 mg of E. coli GM31 cell extract protein were incubated for at 30 °C for 0, 5, 10, 30, 60, and 90 min (lanes 1-6, respectively) in the presence and absence of the exogenous E. coli DNA ligase (0.15 µM) or Fpg (0.2 µM) as indicated. The DNA reaction products were isolated, subjected to Dug/Endo IV treatment, and analyzed using 1% agarose gel electrophoresis as described under "Experimental Procedures". As a control, pGEM (εC-G) DNA (100 ng) was mock-reacted and treated with excess Dug and Endo IV (lane C). Untreated pGEM (εC-G) DNA (80 ng) and a sample containing 1 µg of a 1-kb DNA ladder were utilized as reference standards (lanes S and M, respectively). (B) The percentage of repaired form I DNA in each sample was determined as described in Figure 69, and plotted as a function of incubation time for the following reactions: E. coli GM31 cell extract without exogenous protein (○); supplemented with E. coli DNA ligase (●); or Fpg (□).
Figure 75
**Figure 76.** Effect of DNA ligase and Fpg on the specificity of ethenocytosine-initiated BER DNA synthesis. (A) Three sets of standard BER reaction mixtures (100 µl) with pGEM (εC-G) DNA were prepared as described in Figure 75 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. Following incubation at 30 °C for 0, 5, 10, 30, 60, and 90 min (lanes 1-6, respectively), DNA samples (100 ng) were isolated, subjected to *Bsr*I digestion, and resolved by 5% nondenaturing polyacrylamide gel electrophoresis as described under "Experimental Procedures". $[^{32}P]DNA$ fragments (*arrows*) were identified using a PhosphorImager and the 272-bp DNA fragment that contained uracil residue was located (*272*). (B) The percentage of relative $[^{32}P]dCMP$ incorporation into the DNA fragments of *272*-bp (*red bars*), 603-bp (*blue bars*), 117-bp (*green bars*), and 445-bp (*gray bars*) was determined as described in Figure 71, and plotted for each lane shown in (A).
Figure 77. Effect of DNA ligase and Fpg on the ethenocytosine-initiated BER patch size distribution. (A) Three sets of standard BER reaction mixtures (100 µl) containing 2 µg of pGEM (εC-G) [32P]DNA, 20 µM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] and 0.2 mg of E. coli GM31 cell extract protein were incubated in the presence and absence of exogenous E. coli DNA ligase (0.15 µM) or Fpg (0.2 µM) at 30 °C for 0, 5, 10, 30, 60, and 90 min as indicated. As a control, pGEM (εC-G) [32P]DNA (2 µg) was mock-reacted in the absence of cell extract proteins (U). Following the BER reaction, DNA products were isolated, each DNA sample (100 ng) was digested with 5 units of HindIII and then treated with 100 units of exonuclease III (+) or mock treated (-) as indicated. After these treatments, the DNA was cleaved with 5 units of BamHI, and the [32P]-labeled DNA fragments were resolved by 12% polyacrylamide/8.3 M urea gel electrophoresis as described under "Experimental Procedures". The location of [32P]DNA size markers (30-nt and 13-nt) prepared as described in Figure 74 are indicated by arrows. The relative amount of [32P] radioactivity detected in each DNA band corresponding to repair patch sizes of 1-17 nucleotides was determined for the 5 min (B) and 60 min (C) reactions shown in (A), using the method illustrated in Figures 62 and described in Figure 64. 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 bars), with DNA ligase (black bars), and with Fpg (striped bars).
illustrated in the 60 min reaction conducted without supplementation of either DNA ligase or Fpg (Figure 77C, white bars). However, the addition of exogenous DNA ligase was observed to stimulate short-patch repair about 4-fold, whereas the patch distribution was not affected by the addition of exogenous Fpg protein (Figure 77C, black bars and striped bars, respectively). When taken together, these results implied that, after the initial repair DNA synthesis of 1 or 2 nucleotides, the pathway of the BER was determined based on the efficiency of nick sealing mediated by DNA ligase. Since these observations regarding ethenocytosine-initiated BER were similar to that in uracil-initiated BER, this suggests that the very-long patch BER as well as the effect of DNA ligase on the patch distribution might be a general mechanism governing *E. coli* BER.

6.1.5 Attenuation of the Inhibitory Effect of DNA Polymerase I on Uracil-initiated BER by DNA Ligase

In order to determine the effect of various BER components on the efficiency of uracil-initiated BER, experiments were conducted whereby increased amounts of *E. coli* Ung, Endo IV, or Pol I were introduced into the BER reaction containing the *E. coli* GM31 cell extract. While the addition of exogenous Ung and Endo IV did not influence the efficiency of uracil-initiated BER, adding increasing amounts of Pol I resulted in a reduced extent of BER (Figure 78A). However, this inhibitory effect of Pol I on BER was shown to be attenuated by the presence of exogenous *E. coli* DNA ligase and the level of attenuation appeared to be dependent on the molar ratio of DNA ligase to Pol I (Figure 78B). These results implied that the efficiency of the ligation step that determined the rate of *E. coli* uracil-initiated BER was modulated by competition between DNA ligase and Pol I. Analysis of the uracil-initiated BER reaction time course using agarose gel electrophoresis revealed that the addition of Pol I led to ~30% reduction in the extent of repaired form I DNA after 60 min (Figure 79). This observation supported the notion that the
Figure 78. Influence of *E. coli* uracil-DNA glycosylase, endonuclease IV, DNA polymerase I, and DNA ligase on uracil-initiated BER. (A) Four sets of standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (U·G) DNA and 2 mg 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 of Ung (○); 0, 1, 2, 4, 8 units of Endo IV (□); 0, 5, 10, 20, 40 units of DNA polymerase I (●); 0, 5, 10, 20, 40 units of DNA polymerase I plus 20 units of DNA ligase (■). After incubation for 60 min at 30 °C, the pGEM DNA was recovered, subjected to Ung/Endo IV treatment, and analyzed using 1% agarose gel electrophoresis as described under "Experimental Procedures". The fluorescent intensity of ethidium bromide-stained DNA bands (form I and II) was determined as described in Figure 69, 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 bars) or absence (black bars) of exogenous DNA ligase.
Figure 78
Figure 79. Effect of DNA polymerase I supplementation with or without DNA ligase on the rate and extent of ethenocytosine-initiated BER in E. coli GM31 cell extracts. (A) Three sets of standard BER reaction mixtures (100 µl) containing 2 µg of pGEM (U.G) DNA and 0.2 mg of E. coli GM31 cell extract protein were incubated at 30 °C for 0, 5, 10, 30, 60, and 90 min (lanes 1-6, 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/Endo IV treatment, and analyzed using 1% agarose gel electrophoresis as described under "Experimental Procedures". Untreated pGEM (U.G) DNA (lanes S), mock-reacted DNA treated with Ung and Endo IV (lane C), and a 1-kb DNA ladder (lanes 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 69, 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 (□).
### Figure 79

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efficiency of BER was influenced by the DNA ligase to Pol I ratio. The addition of DNA ligase to the reaction containing the same amount of Pol I abrogated the inhibitory effect of Pol I on the BER (Figure 79B). Moreover, the initial rate was increased and the time necessary to achieve the maximum extent of repair was significantly decreased in the reaction containing both Pol I and DNA ligase, compared to the control BER reaction conducted in the absence of protein supplementation (Figure 79B). Given that the nick-containing BER intermediates are a substrate for both Pol I and DNA ligase (283, 307, 310), the balance between DNA ligation and DNA polymerization activity likely determines the efficiency of the complete BER process.

6.1.6 Influence of DNA Polymerase I and DNA Ligase on Uracil-initiated BER Patch Size Distribution

In order to investigate whether the rate between DNA ligase and Pol I affects the repair patch size distribution, patch size analysis was performed following uracil-initiated BER reactions in E. coli cell extracts supplemented with Pol I in the presence and absence of exogenous DNA ligase. For each reaction time course, the HindIII-BamHI 32P-labeled DNA restriction fragments produced with and without Exo III treatment were analyzed (Figure 80A). Examination of 32P-labeled 30-mer after digestion with HindIII-BamHI, but not with Exo III, revealed that the occurrence of BER intermediates observed in the initial phase of the reaction conducted without exogenous enzyme were markedly reduced by the addition of exogenous Pol I (Figure 80A, -Addition versus +Pol I). This result suggested that the nick on the BER intermediates may have been subjected to the further extension of repair DNA synthesis due to increased activity of Pol I. The BER reaction conducted for 5 min in the E. coli cell extract supplemented with both Pol I and DNA ligase also resulted in intact 30-mer band as a major product after HindIII-BamHI digestion. Since the complete BER process occurred rapidly in this reaction (Figure 80, +Pol I +Lig), the results indicated that the nick on the BER intermediates was sealed
Figure 80. Effect of DNA polymerase I supplementation with or without DNA ligase on the *E. coli* uracil-initiated BER patch size distribution. (A) Three sets of standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (U-G) \[^{32}P\]DNA, 20 μM each of dATP[αS], dTTP[αS], dGTP[αS], and dCTP[αS] and 0.2 mg of *E. coli* GM31 cell extract protein were incubated in the presence and absence of exogenous *E. coli* DNA polymerase I (0.2 μM) and DNA ligase (0.15 μM) at 30 °C for 0, 5, 10, 30, 60, and 90 min as indicated. 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, 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. 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 "Experimental Procedures". The location of \[^{32}P\]DNA size markers (30-nt and 13-nt) prepared as described in Figure 74 are indicated by arrows. The relative amount of \(^{32}P\) radioactivity detected in each band (13-30 nt) was determined for the 60 min reactions in (A), using the method illustrated in Figure 62 and described in Figure 64. 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).
by increased activity of DNA ligase. Inspection of the BER patch size distribution revealed that addition of Pol I stimulated the generation of longer repair patches (2-17 nucleotides) and reduced the occurrence of short patch (1-nucleotide) repair by ~3-fold (Figure 80B and C). However, this Pol I effect was attenuated in the presence of exogenous DNA ligase (Figure 80D), resulting in a pattern of patch size distribution similar to that observed in the absence of exogenous protein (Figure 80B). Specifically, the repair DNA synthesis involving one and two nucleotides replacement contributed to ~10% of total repair events in the presence of exogenous Pol I (Figure 80C), while these shorter (1-2 nucleotides) repair patches constituted ~20% in both the reaction conducted without additional protein and the reaction supplemented with Pol I and DNA ligase simultaneously (Figure 80B and D, respectively). Thus, these results suggest that the ratio between DNA ligase and Pol I is the key factor in determining the BER patch size distribution.

6.1.7 Influence of DNA Polymerase I and DNA Ligase on Very-long Patch BER DNA Synthesis

BsrI restriction analysis of the BER DNA products was conducted to determine whether the specificity of repair DNA synthesis in very-long patch BER was dependent on the balance between Pol I and DNA ligase. As before, [32P]dCMP incorporation into pGEM (U·G) DNA by E. coli GM31 cell extracts was monitored by using non-denaturing polyacrylamide gel electrophoresis following BsrI digestion of the product DNA produced from a time course reaction. An examination of BsrI digested DNA fragments revealed that supplementation of BER reaction with excess Pol I provoked a readily discernible increase in the incorporation of [32P]dCMP into the 603-bp fragment in early time points compared to the control reaction conducted without supplementation with exogenous protein (Figure 81A, +Addition and +Pol I). After the BER reaction containing exogenous Pol I proceeded for 90 min, significantly increased incorporation of [32P]dCMP was observed in all of
Figure 81. 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) containing 2 µg of pGEM (U-G) DNA, 2 mg of E. coli GM31 cell extract protein, and 10 µCi/ml of [³²P]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. BER reactions were incubated at 30 °C for 0, 5, 10, 30, 60, and 90 min (lanes 1-6, respectively), DNA products were isolated, DNA samples (100 ng) were subjected to BsrI digestion, and resolved by 5% nondenaturing polyacrylamide gel electrophoresis as described under "Experimental Procedures". The locations of [³²P]-labeled restriction DNA fragments were visualized by PhosphorImager and are indicated by arrows. The [³²P]DNA fragment containing the uracil residue is indicated (*272). The percentage of relative [³²P]dCMP incorporation into the DNA fragments of *272-bp (red bars), 603-bp (blue bars), 259-bp (yellow bars), and 445-bp (gray bars) was calculated as described in Figure 71, and plotted for each of the 60 min reactions (lanes 5) shown in (A). The data are 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 81
the *BsrI*-DNA fragments including the 445-bp fragment that was located upstream from uracil containing the 272-bp fragment (Figure 81A, +Pol I, lane 6). The results for each of the three reaction sets were quantitatively analyzed and plotted showing the incorporation associated with the 272-, 603-, 259-, and 455-bp DNA fragments (Figure 81B-D). Specific incorporation of $[^{32}P]$dCMP in the reaction supplemented with Pol I appeared to occur after 5, 10, 30, and 60 min for the 272-, 603-, 259-, and 455-bp fragments, respectively (Figure 81C). This time-dependent sequence of $[^{32}P]$dCMP incorporation into the series of DNA fragments corresponded to the 5' to 3' direction of DNA synthesis initiated from the uracil target. Thus, it appeared that the accumulation of $[^{32}P]$dCMP resulted from extension of DNA synthesis induced by Pol I that significantly elongated the uracil-initiated BER patch. However, the addition of exogenous DNA ligase along with Pol I significantly suppressed the extension of repair DNA synthesis observed with added Pol I (Figure 81C versus D). Increased levels of $[^{32}P]$dCMP incorporation into the 272-bp fragment was observed in earlier time points when both Pol I and DNA ligase were added to the reaction compared to the control reaction without addition (Figure 81B versus D). Moreover, the increase (~3-fold) of $[^{32}P]$dCMP incorporation reflected the fold stimulation of the percentage of repaired form I DNA in the reaction supplemented with both Pol I and DNA ligase (Figure 79). These results indicated that the increased level of DNA repair synthesis observed by the addition of both Pol I and DNA ligase was associated with an increased efficiency of complete BER. When taken together, these results indicated that the intervention of DNA ligation brought about the final stage of BER was dependent on the ratio between Pol I and DNA ligase. Under conditions of excess Pol I, repair DNA synthesis tends to escape the ligation step which promotes a larger patch size.
6.1.8 Influence of DNA Polymerase I Activity on the Specificity of Uracil-initiated BER DNA Synthesis

In order to investigate whether Pol I is the major DNA polymerase responsible for the repair DNA synthesis in very-long patch BER, antiserum raised against Pol I was utilized to inhibit the uracil-initiated BER reaction of *E. coli* GM31 cell extract. To validate the approach, DNA polymerase assays were conducted to ascertain whether the Pol I antiserum neutralized the activity of Pol I. Purified *E. coli* Pol I was preincubated with various amounts of the Pol I antiserum and then the DNA polymerase activity was measured by monitoring the incorporation of [³H]dTMP into activated calf thymus DNA. The results indicated that increasing amounts of Pol I antiserum caused a decrease in the DNA polymerase activity of purified Pol I (Figure 82). Maximal inhibition (96%) was achieved with the addition of 8 µl of Pol I antiserum. Next, [³²P]DNA produced from BER reactions conducted with *E. coli* GM31 cell extracts in the absence and presence of Pol I and anti-Pol I antiserum was subjected to the *Bsr*I restriction analysis to determine the distribution of repair DNA synthesis (Figure 83). As a control, the effect of adding pre-immune rabbit serum to the BER reaction was analyzed. The result indicated that the pre-immune serum had no apparent influence on [³²P]dCMP incorporation into DNA (Figure 83A, lane 1 *versus* lane 2). In contrast, addition of neutralizing antiserum resulted in a discernible decrease in [³²P]dCMP incorporation into the 603-bp fragment that was indicative of very-long patch BER (Figure 83A, lanes 3 and 4). Relative [³²P]dCMP incorporation into the 272-bp fragment was also reduced by the addition of 2 and 4 µl of neutralizing antiserum (Figure 83A, lanes 3 and 4, respectively). This result suggested that the inactivation of Pol I activity resulted in an overall reduction in repair DNA synthesis. Thus, the end result of the action of neutralizing antiserum in BER appeared to be similar to that of exogenous DNA ligase that caused shortening the repair patch size (Figure 74). However, the additional supplementation of this reaction with 1 unit of Pol I
Figure 82. Effect of DNA polymerase I specific antiserum on the purified *E. coli* DNA polymerase I activity. DNA polymerase activity was determined under the standard BER reaction condition in the reaction mixtures (100 µl) containing 1 unit of *E. coli* DNA polymerase I, 10 µg of activated calf thymus DNA, and [³H]dTTP (593 cpm/pmol). Reaction mixtures were pre-incubated with 0, 0.25, 0.5, 1, 2, 4, and 8 µl antiserum raised against DNA polymerase I for 5 min on ice. After incubation for 60 min at 30 °C, reaction mixtures were subjected to DNA polymerase activity assay as described under "Experimental Procedures". The percentage of polymerase activity was determined as the amount of [³H]dTMP incorporated in the presence of corresponding amount of antiserum divided by the amount of [³H]dTMP incorporated in the absence of antiserum and then multiplied by 100. Incorporation of 42 pmol of [³H]dTMP represents 100% polymerase activity. The mean value from two independent experiments are plotted. No variation exceeded more than 2% of polymerase activity.
Figure 82
Figure 83. Influence of the DNA polymerase I specific neutralizing antiserum on the specificity of uracil-initiated BER DNA synthesis. (A) Standard BER reaction mixtures (100 μl) containing 2 μg of pGEM (U·G) DNA and 2 mg of E. coli GM31 cell extract protein were prepared with the following; no addition (lane 1); 2 μl rabbit pre-immune serum (lane 2); 2 and 4 μl of antiserum (lanes 3 and 4, respectively); 1, 2, and 4 units of E. coli 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, DNA samples (100 ng) were subjected to BsrI digestion, and resolved by 5% non-denaturing polyacrylamide gel electrophoresis as described under "Experimental Procedures". The locations of 32P-labeled restriction DNA fragments were visualized by PhosphorImager and are indicated by arrows. (B) The percentage of relative [32P]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 71 and plotted for each reaction shown in (A).
Figure 83
resulted in a restoration of the very-long patch repair DNA synthesis (Figure 83A, lane 5). Moreover, the addition of increased amounts of purified Pol I stimulated the extension of repair DNA synthesis throughout other DNA fragments (Figure 83A, lanes 5, 6 and 7). The incorporation of $[^{32}\text{P}]\text{dCMP}$ into each of the 272-, 603-, and 259-bp fragments was quantitatively analyzed and plotted for each reaction (Figure 83B). Addition of the neutralizing antiserum (2 μl) to the reaction reduced the amount of incorporation into the 272- and 603-bp fragment by 1.4- and 3-fold, respectively (Figure 83B, lanes 3 and 4). In contrast, addition of increased amounts of purified Pol I to the reaction containing the neutralizing antiserum resulted in an increase in the level of $[^{32}\text{P}]\text{dCMP}$ 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 for 1, 2, and 4 units of purified Pol I, respectively (Figure 83B, lanes 5-7). Thus, the overall stimulation of DNA synthesis was greater in the 259-bp fragment than in the 272-bp fragment. These results also indicated that the length of DNA repair patch was modulated depending on the level of Pol I and implied that Pol I was the major DNA polymerase associated with very-long patch BER DNA synthesis.

6.2 Discussion

In this Chapter, the specificity of repair DNA synthesis in uracil-initiated BER in *E. coli* cell extracts using a plasmid DNA substrate containing a site-specific U-G base mispair was examined. Unlike several previous investigations (182, 305, 354) that have utilized oligonucleotides containing damaged base to characterize repair patch associated with BER, a covalently closed circular DNA substrate with a defined uracil target was employed in this study to extend the detection of repair DNA synthesis and mimic the physiological substrate. Under the conditions examined, it was demonstrated that uracil-mediated repair events can result in a very large repair patch of at least 200 nucleotides. Several observations support the interpretation that the
observed very-long patch repair DNA synthesis was associated with uracil-initiated BER. First, DNA synthesis involving these large repair patch events was detected in form I DNA that was resistant to cleavage by the combined treatment with Ung and Endo IV. Second, repair DNA synthesis within the 603-bp fragment was dependent on the presence of a uracil residue located in the 272-bp fragment. Third, specific incorporation of [\textsuperscript{32}P]dCMP into the 603-bp fragment was significantly reduced by the addition of Ugi, a potent and irreversible inhibitor of Ung (130, 330). Fourth, repair DNA synthesis associated with the 603-bp fragment was significantly diminished in E. coli BH158 cell extract, in which both ung and dug were inactivated (319). Furthermore, ethenocytosine-initiated BER was also associated with the very large repair patches which were dependent on Dug activity. These observations strongly suggest that the very large repair patches resulted from BER DNA synthesis. This report is the first to present evidence for a very-long (> 200 nucleotides) repair patch generated by the BER pathway. Previous studies with oligonucleotide substrates (30-mer) have suggested that the major pathway for the repair of uracil is achieved by a short patch repair process that entails the replacement of just a single nucleotide (182, 305). In addition, a minor alternative BER pathway was also reported that results in a slightly longer repair patch size of ≥2 nucleotides (182). In contrast, recent studies utilizing closed circular DNA substrates have demonstrated that most uracil-initiated BER is achieved via long patch repair that involves repair patches of 2 to ~20 nucleotides that reside on the 3'-side of the uracil residue (306, 352). As previously discussed, short oligonucleotide substrates may not provide a platform sufficient for interaction with the DNA polymerase and accessory repair proteins used in vivo (352). Thus, the size of the repair patch detected in vitro may be influenced by the nature and length of DNA repair substrate (306, 349).

In vitro uracil-initiated BER was previously reconstituted with five purified enzymes from E. coli: Ung, Endo IV, RecJ protein, Pol I, and DNA
ligase (182). In this system, RecJ protein was not essential for completing the repair of uracil-containing DNA. However, the addition of RecJ protein altered repair DNA synthesis favoring single nucleotide replacement over a longer repair patch. Two types of *E. coli* dRPase activity have been reported previously. One is a hydrolytic dRPase that has been proposed to be associated with *E. coli* RecJ and exonuclease I (272, 274, 277). However, the detection of 5'-dRPase activity associated with either RecJ or exonuclease I was unable to be reproduced by other investigations under various reaction conditions (277, 278). The other type is a AP lyase activity associated with *E. coli* Fpg protein (280). Fpg protein is able to remove a 5'-dRP residue by employing β-elimination as opposed to hydrolysis. Recent study demonstrated that Fpg exhibited a robust dRPase activity that is active in the presence and absence of Mg^{2+} (278). Thus, in this Chapter the effect of dRPase on the efficiency and patch size distribution of BER was examined. Under the BER reaction conditions, the addition of Fpg protein did not affect the rate or extent of either uracil- or ethenocytosine-initiated BER. It was also observed that the supplementation of Fpg in both repair reactions did not alter the profile of repair DNA synthesis associated with either short patch, long patch or very-long patch BER pathways. Similarly, Sandigursky et al. (306) reported that the pattern of nucleotide replacement during BER in *E. coli* cell deficient in exonuclease I and RecJ or in RecJ and Fpg was identical to the repair patch produced in wild type *E. coli* cell extract.

Examination of the kinetics of BER in the time course reactions conducted with *E. coli* cell extracts supplemented with each of essential BER protein revealed that the presence of exogenous *E. coli* DNA ligase elevated the initial rate of complete repair by ~3-fold in both uracil- and ethenocytosine-initiated BER. In contrast, the addition of exogenous Ung or Endo IV did not affect the efficiency of BER. The addition of Pol I was observed to inhibit the production of completely repaired form I DNA. These
observations strongly suggested that the rate-limiting step in *E. coli* BER is the ligation event.

The majority of repair DNA synthesis associated with *E. coli* BER occurs via the long-patch repair mechanism (106, 352). Hence, the vast majority of BER intermediate molecules produced prior to ligation might be generated due to the coupled functions of Pol I; (i) the strand displacement DNA synthesis mediated by polymerase activity of the large domain (283, 298), and (ii) the cleavage of resulting single-stranded displaced oligonucleotides through 5'-nuclease (originally called 5'-3' exonuclease) activity associated with the small domain (182, 355). A previous study by Lundquist and Olivera (297) suggested that either the DNA polymerase activity or the exonuclease activity associated with Pol I may compete on the displaced single-stranded DNA with a probability of 55% that the DNA polymerase acts before the exonuclease at each phosphodiester bond. During long patch BER, the cleavage of the single-stranded flap DNA at the junction of the double-stranded DNA is a prerequisite for DNA ligation (182, 300). Thus, a delay in the action of the 5'-nuclease due to the further polymerization would be expected to impede the ligation step. As a result, the rate of complete BER would be correspondingly reduced. Since both the polymerase domain of Pol I and DNA ligase recognize the 3'-OH of the 5'-end of the nick produced as a BER intermediate (283, 307), it is not unreasonable to assume that an excess of DNA ligase over Pol I may increase the probability for ligation over both polymerase and 5'-nuclease action. Thus, an excess of DNA ligase might be anticipated to stimulate the completion of BER. In this case, the increased level of BER would be predicted to be accompanied by limited repair DNA synthesis resulting in a decrease in the repair patch size of BER. Indeed, this result was observed in that the addition of DNA ligase to the *E. coli* GM31 cell extracts resulted in a drastic diminution of long-patch repair DNA synthesis and an increase (~4-fold) in the one-nucleotide replacement synthesis in both uracil- and ethenocytosine-initiated BER. In contrast, supplementation of the
"E. coli cell extracts with Pol I altered the repair patch size distribution to favor long patch sizes. Furthermore, the tendency of Pol I to stimulate extended repair synthesis resulting in larger repair patches was alleviated by adding additional DNA ligase. These observations strongly suggest that the balance between Pol I and DNA ligase is a key factor in determining both the overall rate and patch size of repair associated with the BER pathway. Previous estimates of the number of DNA ligase molecules per E. coli cell was determined to be about 300, a value close to that estimated (~400) for Pol I (60, 283). The $k_{cat}$ value for DNA polymerase activity of E. coli Pol I was determined to be ~6 sec$^{-1}$ using poly d(A) as a template (356). On the other hand, the turnover number for E. coli DNA ligase is about 0.4 sec$^{-1}$ (283). Considering the number of each enzyme molecules in an E. coli cell, the comparison of the turnover number of Pol I to that of DNA ligase suggests that DNA synthesis events are 15-fold more likely to occur than the ligation. Thus, this might explain why ligation was rate-limiting in E. coli BER. However, a simple calculation based on the ratio between Pol I and DNA ligase is unlikely to totally reflect the constraints that act in vivo. For example, the level of DNA base damage to the genome may affect the relative efficiency of the enzyme to process a given site.

The efficiency of Dug-mediated repair of ethenocytosine residues also appeared to be dependent on the ligation step. Unlike Ung-mediated BER, Dug binds tightly after excision of uracil or ethenocytosine to the product DNA, but the cleavage of the AP-site by AP endonuclease (Endo IV) and AP lyase (Fpg protein) stimulates catalytic turnover of Dug, as described in Chapter 3. However, the rate of repair during ethenocytosine-initiated BER was not changed by the addition of E. coli Fpg protein while the repair was significantly stimulated in the presence of exogenous E. coli DNA ligase. This could be explained by the fact that most AP-sites generated by the action of Dug can be processed efficiently by the abundant AP endonuclease activity of exonuclease III (Exo III) which is responsible for ~90% of AP-site cleavage in
E. coli cells (236). Thus, although purified Dug does not efficiently turn over in vitro, the enzyme turnover is likely facilitated by the next enzyme in the pathway (AP endonuclease) that eliminates Dug activity as being the rate-limiting step during the series of sequential BER reactions.

During BER in E. coli, the AP-site cleavage by a class II AP endonuclease (i.e. Exo III and Endo IV) produces a 5'-dRP residue that is not efficiently removed by the 5'-3' exonuclease activity of Pol I (271-273). However, a previous study has demonstrated that this sugar phosphate residue can be slowly released as part of a short oligonucleotide (2 nucleotides) upon incubation with Pol I (273). The major path for DNA synthesis has been shown to occur via strand displacement of the DNA strand containing 5'-dRP moiety (271). Subsequent cleavage of this flap DNA is conducted by the 5'-nuclease activity of Pol I, which is tolerant of a 5'-terminal AP-site containing DNA (297, 300). On the other hand, various oxidative base DNA damages are mainly repaired by bifunctional DNA glycosylases that exhibit an intrinsic AP lyase activity (60, 180). In this case, the concerted hydrolysis of N-glycosyl bonds and cleavage of resulting AP-sites at 3'-termini through β-elimination would be expected to generate 5'-phosphoryl and 3' unsaturated aldehyde termini. Alternatively, in the case of β,δ-elimination reaction, 3'-phosphoryl termini would be produced which are refractory to further processing unless the phosphate group is removed by the phosphatase function of exonuclease III (198, 247, 248). After processing of the aberrant 3'-termini, the resulting BER intermediates would contain one nucleotide gaps at the sites where damaged bases were located. Thus, BER initiated by a bifunctional DNA glycosylase does not involve the 5'-dRP residue unlike monofunctional DNA glycosylase-mediated BER (i.e. uracil- and ethenocytosine-initiated BER). Further study will be required to determine the rate-limiting step and the repair patch distribution that results from bifunctional DNA glycosylase-mediated BER pathway. It would not be surprising if the results of
bifunctional DNA glycosylase-mediated BER differed from the results of this study that were observed from monofunctional DNA glycosylase-mediated BER.

What DNA polymerase is the major enzyme involved in very-long patch BER in *E. coli*? The results presented here indicated that neutralizing the endogenous Pol I activity by Pol I-specific antisera abolished the very-long patch repair DNA synthesis associated with uracil-initiated BER. Moreover, the very-long patch repair was restored upon addition of exogenous purified Pol I. These results strongly suggest that Pol I plays an essential role in *E. coli* during very-long patch repair DNA synthesis. In *E. coli* nucleotide excision repair (NER), several earlier observations demonstrated that Pol I was responsible for the major repair DNA synthesis although *in vitro* all three DNA polymerases (Pol I, II, and III) could utilize gapped DNA substrates of the type generated during repair process (357-360). While the majority of repair synthesis during *E. coli* NER occurs with gap filling DNA synthesis of ~12 nucleotides (361), a small fraction (~1%) of the repair synthesis has been shown to result from longer than 1,500 nucleotides of synthesis. The latter type of NER was referred to as long-patch excision repair (362, 363). This long-patch repair appeared to differ from the other major NER pathway in that it absolutely requires the SOS inducible response (362). This observation led to speculation for the involvement of other DNA polymerases that were induced by the SOS system. However, the requirement of Pol I in the long-patch repair was demonstrated by utilizing *E. coli* strains defective in Pol II or Pol III (364). Furthermore, the frequency of long patch repair during NER was shown to be increased in mutants defective in the 5' to 3' exonuclease function of Pol I (364). By analogy, such a function of the 5'-nuclease of Pol I might be the important player in determining the repair patch in very-long patch BER in *E. coli*.

When exogenous DNA ligase was added to *E. coli* cell extracts, the complete inhibition of repair DNA synthesis associated with very-long patch
BER was observed. One possible explanation for this observation might result from the competition between DNA ligase and Pol I at the nick after each round of strand displacement synthesis during BER. Indeed, results were presented that demonstrated that the length of repair synthesis of very-long patch BER could be manipulated by altering the balance between Pol I and DNA ligase. However, another possibility cannot be excluded. The addition of DNA ligase may result in a disruption of certain protein-protein interactions of various BER proteins including DNA ligase, Pol I, and additional possible auxiliary factors. In the mammalian BER pathway, the ligation step has been shown to be well coordinated through complex protein-protein interactions; XRCC1 and DNA ligase III, DNA polymerase β and DNA ligase I, and PCNA and DNA ligase I (365-367). Several studies have suggested that the sliding clamp replication protein, PCNA, acts as a scaffold protein that promotes assembly of BER proteins at an incised abasic site and stimulates the activity of DNA ligase I and flap endonuclease 1 (FEN1) during long patch BER in mammalian cells (368-370). A recent study reported that the *E. coli* β clamp interacts with DNA ligase and Pol I, and increases the processivity of Pol I (289). Further investigation will be requested to determine whether these protein-protein interactions might affect the specificity of BER DNA synthesis. Additional experiments will be needed to define the molecular mechanisms of very-long patch repair DNA synthesis associated with BER. Moreover, the biological significance of the very-long patch BER pathway *in vivo* awaits further elucidation.


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