Processivity of *Escherichia coli* and Rat Liver Mitochondrial Uracil−DNA Glycosylase Is Affected by NaCl Concentration†

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ABSTRACT: *Escherichia coli* uracil−DNA glycosylase was shown to catalyze the hydrolysis of a site-specific uracil residue from a defined single-stranded oligonucleotide (25-mer). With duplex 25-mer, the rate of uracil removal from double-stranded DNA containing a U•G mispair was ~2-fold greater than a U•A base pair. The mechanism by which *E. coli* and rat liver mitochondrial uracil−DNA glycosylase located sequential uracil residues within double-stranded DNA was investigated. Two concatemeric polynucleotide substrates were constructed by ligation of homologous 5′-end ^{32}P-labeled 25-mer double-stranded oligonucleotides that contained either a site-specific U•G or U•A target site at intervals of 25 nucleotides along one strand of the DNA. Reaction of uracil−DNA glycosylase with these concatemeric DNAs, followed by alkaline hydrolysis of the resultant AP-sites, would produce predominantly [^{32}P]25-mer products, if a processive mechanism was used to locate successive uracil residues, or oligomeric multiples of [^{32}P]25-mer, if a distributive mode was exhibited. Both the bacterial and the mitochondrial enzymes were found to act processively on U•A- and U•G-containing DNA in the absence of NaCl, based on the initial rate of 25-mer produced relative to the total amount of uracil excised. Approximately 50% of the total uracil excised resulted in the release of 25-mer product. The addition of NaCl (≥50 mM) caused reduced processivity on both U•A- and U•G-containing DNA substrates. The mode of action of uracil−DNA glycosylase was very similar to that observed for the *Eco*RI endonuclease cleavage of restriction sites contained in the same DNA substrate which was used as a positive control.

Uracil residues may accumulate in prokaryotic and eukaryotic DNA by the incorporation of dUMP in place of dTMP and by the deamination of existing cytosine residues in DNA (Schlomai & Kornberg, 1978; Lindahl & Nyberg, 1974; Frederico et al., 1990; Duncan & Miller, 1980). While uracil can successfully replace thymine as genetic information by forming a U•A base pair, it may adversely affect specific protein−DNA interactions (Verri et al., 1990; Duncan et al., 1978). In contrast, the deamination of cytosine in duplex DNA results in the formation of U•G mispairs which precipitate G•C to A•T transition mutations (Duncan & Miller, 1980; Duncan et al., 1978). Because U•G mispairs represent premutagenic lesions, it has been suggested that uracil−DNA excision repair evolved to reduce cytosine deamination-induced mutation rates (Duncan et al., 1978).

Uracil−DNA excision repair has been identified in many biological systems ranging from bacteria to human cells (Sancar & Sancar, 1988; Sakumi & Sekiguchi, 1990; Mosbaugh & Bennett, 1994). Uracil−DNA glycosylase initiates repair by excising uracil from deoxyribose in DNA (Lindahl et al., 1977). The minimum substrate recognized by the enzyme is a trimer, providing the 5′-end of the oligonucleotide is phosphorylated (Varshney & van der Sande, 1991). *Escherichia coli* uracil−DNA glycosylase (Ung)[1] shares substantial amino acid sequence homology with uracil−DNA glycosylases isolated from a wide variety of biological sources, including human (Olsen et al., 1989; Upton et al., 1993). *E. coli* Ung is a monomeric polypeptide of 228 amino acids that has a molecular weight of 25 558 (Bennett et al., 1994), a turnover number of 800 uracil residues released per minute (Lindahl et al., 1977), and an isoelectric point of 6.6 (Bennett & Mosbaugh, 1992). Mitochondrial uracil−DNA glycosylase (form I), purified to apparent homogeneity from rat liver, shares similar properties with *E. coli* Ung (Olsen et al., 1989; Upton et al., 1993). Like Ung, the mitochondrial enzyme is a single polypeptide that has an apparent molecular weight of 24 000 and a turnover number of ~1000 min^{-1}; however, it exhibits a higher isoelectric point of 10.3 (Domena et al., 1988). Both enzymes show ~2-fold preference for single-stranded uracil−DNA over a duplex substrate and are inhibited by the reaction products, free uracil and apyrimidinic site DNA (Lindahl et al., 1977; Domena et al., 1988; Bennett et al., 1993). The effect of nucleotide sequence context on catalysis of the target uracil has been examined for both the *E. coli* Ung (Eftedal et al., 1993) and the mitochondrial enzyme (Domena et al., 1988). Eftedal et al. (1993) reported that 5′-A/TUAA/T-3′ forms the consensus sequence for efficient recognition of uracil by *E. coli* Ung, whereas uracil in 5′-G/CUT-3′ and

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[1] Abbreviations: Ung, *E. coli* uracil−DNA glycosylase; AP, apurinic/apyrimidinic; Ugi, bacteriophage PBS2 uracil−DNA glycosylase inhibitor protein; IPTG; isopropyl β-D-thiogalactopyranoside; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate adjusted to pH 8.0.

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5′-G/CUG/C-3′ sequences is recognized with ~10-fold lower efficiency.

Proteins that recognize a specific sequence or site on DNA may locate their target by random three-dimensional diffusion or by facilitated diffusion (Berg et al., 1981). The search mechanisms of several DNA-recognizing proteins have been examined, including the *E. coli* lac repressor (Winter et al., 1981), bacteriophage λ cro repressor (Kim et al., 1987), DNA polymerase (Bambara et al., 1978; Maki & Kornberg, 1988; Studwell & O'Donnell, 1990; Sabatino et al., 1988; Bonner et al., 1992), RNA polymerase (Yankulov et al., 1994; Martin et al., 1988), *Eco*RI (Jack et al., 1982; Terry et al., 1985), and *Bam*HI endonuclease (Nardone et al., 1986). Regulatory proteins, like lac repressor, locate the operator binding site by facilitated diffusional translocation along the DNA backbone (Winter et al., 1981). This process involves an initial DNA interaction with a nontarget sequence and a series of diffusion-driven translocation events within the domain of the lac repressor protein until the operator site is reached or the protein dissociates into solution (Berg et al., 1981).

Recently, the processive properties of DNA repair enzymes such as T4 endonuclease (Lloyd et al., 1980; Ganesan et al., 1986; Gruskin & Lloyd, 1986; Dowd & Lloyd, 1990), *E. coli* photolyase (Gruskin & Lloyd, 1988), UvrABC endonuclease (Gruskin & Lloyd, 1988), and uracil−DNA glycosylase (Higley & Lloyd, 1993; Purmal et al., 1994) have been examined. In contrast to site-specific DNA binding proteins, uracil−DNA glycosylases must perform the following repetitive sequence of events: (i) locate the uracil target site; (ii) cleave the N-glycosylic bond between uracil and the deoxyribose DNA backbone; and (iii) either dissociate from the metabolized target site or scan the DNA for additional uracil residues. Thus, the process of searching for successive uracil residues may involve either a distributive or a processive mechanism. If the enzyme dissociates from the DNA following catalysis, the search for the next uracil target will become positionally uncorrelated relative to the location of the previous target site (Berg et al., 1981). If, on the other hand, the enzyme remains associated with the DNA following catalysis, it is likely to carry out a positional correlated search for a neighboring uracil residue (Berg et al., 1981). A processive mechanism will be established if uracil−DNA glycosylase locates sequential uracil residues prior to dissociation from the DNA. Various degrees of processivity may be achieved depending on the ability of the enzyme to translocate and maintain prolonged contact with nontarget DNA sequences. For example, *Eco*RI was found to use a positionally correlated mode of search at low ionic strength (25 mM NaCl) to cleave ~80% of the restriction sites that were located 50−300 bp apart (Terry et al., 1985). A hallmark of processive enzymes is that the degree of processivity is decreased by increasing ionic strength (Terry et al., 1985; Ganesan et al., 1986; Gruskin & Lloyd, 1986; Dowd & Lloyd, 1990). Thus, at 200 mM NaCl, the processive action of *Eco*RI is largely eliminated, presumably due to reduced nonspecific electrostatic binding to nontarget DNA sequences (Terry et al., 1985).

Recently, conflicting reports have appeared in the literature that describe the mechanism by which *E. coli* uracil−DNA glycosylase locates uracil residues within duplex DNA (Higley & Lloyd, 1993; Purmal et al., 1994). In an attempt to resolve this conflict, we designed an assay to determine if uracil−DNA glycosylases use a distributive or processive search mechanism for locating sequential uracil residues on the same DNA strand. In this study, we have examined the mechanism that both *E. coli* and rat liver mitochondrial uracil−DNA glycosylase use to locate successive uracil residues in DNA. A double-stranded concatemeric polynucleotide substrate was synthesized using a repeating 25 nucleotide monomeric unit. Each unit contained either a U•A pair or a U•G mispair in a defined sequence context. Thus, a 25-mer was released when adjacent uracil residues were successively excised and the phosphodiester bond adjacent to the apyrimidinic sites cleaved by alkali treatment. By comparing the amount of 25-mer released relative to the total amount of uracil excised during the initial stages of the reaction, the processivity of each enzyme was measured. Furthermore, we investigated the substrate specificity of the *E. coli* enzyme using the uracil-containing 25-mer DNA substrate.

EXPERIMENTAL PROCEDURES

Materials

Ampicillin and Hepes were purchased from Sigma, and isopropyl β-D-thiogalactopyranoside and dithiothreitol were from Life Technologies. [γ-^{32}P]ATP was obtained from DuPont−New England Nuclear. Calf thymus [*uracil*-^{3}H]-DNA was prepared as previously described (Domena & Mosbaugh, 1985) using [^{3}H]dUTP from Amersham.

E. coli uracil−DNA glycosylase (fraction VI) was purified to apparent homogeneity from the *ung* overexpression strain *E. coli* JM105/pSB1051 as described previously (Bennett et al., 1993). Rat liver mitochondrial uracil−DNA glycosylase (fraction Va) was purified as previously described by Domena et al. (1988). The bacteriophage PBS2 uracil−DNA glycosylase inhibitor protein was overproduced in *E. coli* JM105/pZWtacI and purified to apparent homogeneity as fraction IV (Bennett & Mosbaugh, 1992). T4 polynucleotide kinase and restriction endonucleases *Eco*RI and *Hpa*II were purchased from New England Biolabs; T4 DNA ligase was obtained from Life Technologies.

Methods

Preparation of Oligonucleotides. Oligonucleotides (U-25-mer) GGGGCTCGTAUAAGGAATTCGTACC containing a uracil residue at nucleotide position 11 and two others (A-25-mer and G-25-mer) CCCCGGTACGAATTCCTTX-TACGAG containing either an adenine or a guanine residue at position 19 (X = A or G, respectively) were synthesized on an Applied Biosystems 380B DNA synthesizer (Center for Gene Research, Oregon State University). Deblocked and deprotected oligonucleotides were prepared as described by Longley and Mosbaugh (1993).

5′-End Phosphorylation Reaction with U-25-mer. Oligonucleotide U-25-mer was 5′-end ^{32}P-labeled in a reaction mixture (1.2 mL) containing buffer K [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.1 mM EDTA], 170 nmol of U-25-mer,[2] 1 mCi of [γ-^{32}P]ATP (6000 Ci/mmol), and 700 units of T4 polynucleotide kinase. After incubation at 37 °C for 45 min, 3.7 μmol of ATP was added and incubation continued for an additional 45 min. The

[2] Concentrations of U-, A-, and G-25-mer represent moles of oligonucleotide.

reaction was then quenched by adjustment to 10 mM EDTA and heated to 70 °C for 15 min. Oligonucleotides A-25-mer and G-25-mer were similarly 5′-end-phosphorylated in separate reactions which lacked ^{32}P-labeled ATP. Aliquots (250 μL) of each reaction mixture were purified through P-4 (BioRad) spun columns (1.4 mL) equilibrated in DAB buffer [30 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 5% (w/v) glycerol] as described by Mosbaugh and Linn (1983). Oligonucleotide concentrations were determined spectrophotometrically using the molar extinction coefficient $\epsilon_{280nm} = 2.75 \times 10^5$ (U-25-mer), 2.62×10^5 (A-25-mer), and 2.58×10^5 (G-25-mer).

Annealing Reaction of U/A- and U/G-25-mer Duplex DNA. Hybridization of 30 nmol of [^{32}P]U-25-mer to 45 nmol of either A-25-mer or G-25-mer was conducted by heating to 95 °C followed by slow-cooling to room temperature. The double-stranded [^{32}P]U/A-25-mer contained a U·A base pair whereas [^{32}P]U/G-25-mer contained a U·G mispair at the location of the uracil residue.

Preparation of Double-Stranded Uracil-Containing Concatemeric Polynucleotide Substrates. Synthetic oligonucleotides (U-, A-, and G-25-mer) were separately purified by polyacrylamide gel electrophoresis. Each of the three oligonucleotides (1.8 mg) was combined with native sample buffer to a final concentration of 50 mM Tris-HCl (pH 6.8), 10% (w/v) glycerol, and 0.1% bromphenol blue. Samples were loaded onto a nondenaturing 12% polyacrylamide gel (30 × 40 × 0.3 cm), and electrophoresis was carried out using TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) at 1000 V until the tracking dye had migrated ~20 cm. The 25-mer oligonucleotide bands were visualized by UV-shadowing and excised from the gel using a razor blade. Following electroelution of 25-mer into buffer containing 40 mM Tris−acetate, 0.1 mM EDTA (pH 8.0), the sample was dialyzed into distilled water and vacuum-evaporated to dryness.

Oligonucleotide U-25-mer 5′-end ^{32}P-labeling was performed similarly to that described above, except that the phosphorylation reaction mixtures (550 μL) contained 20 nmol of polyacrylamide gel-purified U-25-mer, 0.9 mCi of [γ-^{32}P]ATP, and 200 units of T4 polynucleotide kinase. After 15 min at 37 °C, ATP was added to 4 mM; reactions were terminated following 60 min total incubation and processed as previously described. Oligonucleotide [^{32}P]U-25-mer (5 nmol) was annealed to both A-25-mer and G-25-mer (5 nmol each) in separate hybridization reactions by incubating the mixtures for 2 min at 85 °C, 15 min at 65 °C, 15 min at 37 °C, 15 min at 25 °C, and 15 min on ice (Sambrook et al., 1989). Duplex oligonucleotides ([^{32}P]U/A-25-mer and [^{32}P]U/G-25-mer) were joined by ligation in identical reactions (675 μL) containing DAB buffer, 5 mM $MgCl_2$, 1 mM ATP, 10 nmol of duplex 25-mer, 100 units of T4 polynucleotide kinase and 14 units (Weiss) of T4 DNA ligase. After a 2 h incubation at 16 °C, the reactions were supplemented with ATP (360 nmol) and T4 DNA ligase (14 units), and incubation was continued for 18 h. The ligation reactions were then applied to a Sepharose CL-4B gel filtration column (1.8 cm^2 × 47 cm) equilibrated in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], and fractions (600 μL) were analyzed for ^{32}P radioactivity by Cerenkov counting in a liquid scintillation spectrometer (Beckman, LS6800). Portions (3 μL) of the gel filtration fractions were combined with 3 μL of denaturing sample buffer (95% deionized formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol), and heated at 95 °C for 3 min. Samples (5 μL) were then examined by denaturing 4% polyacrylamide gel electrophoresis. Fractions containing high molecular weight (>250 nucleotides) [^{32}P]DNA were pooled, ethanol-precipitated, and resuspended in TE buffer.

Uracil−DNA Glycosylase Reactions. Uracil−DNA glycosylase activity was measured as previously described (Bennett & Mosbaugh, 1992). Enzyme dilutions were made in UDB buffer [50 mM Hepes−KOH (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 50 μg/mL acetylated bovine serum albumin]. One unit of uracil−DNA glycosylase is defined as the amount that releases 1 nmol of uracil per hour under standard conditions.

Activity on single- and double-stranded [^{32}P]U-25-mer substrates was measured in standard reaction mixtures (25 μL) containing 70 mM Hepes−KOH (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 95 pmol of [^{32}P]U-25-mer, [^{32}P]U/A-25-mer, or [^{32}P]U/G-25-mer (14.1×10^3 cpm/pmol), and various amounts of uracil−DNA glycosylase (0.003−1.8 units) as indicated in the figure legends. After incubations for 30 min at 37 °C, the reactions were terminated on ice with 1 μL (1 μg) of uracil−DNA glycosylase inhibitor protein. Apyrimidinic sites (AP-sites) generated by uracil removal in the DNA substrate were hydrolyzed by the addition of 3 M K_2HPO_4 (pH 13.7) to 10% (v/v) and incubation at 55 °C for 3 h. Following the hydrolysis reaction, samples were neutralized with 6 μL of 1.5 M KH_2PO_4, and combined with 33 μL of denaturing sample buffer in preparation for electrophoresis.

Ung activity on the double-stranded uracil-containing concatemeric DNA polynucleotides was measured in reaction mixtures (100 μL) containing 135 pmol of [^{32}P]U/A- or [^{32}P]U/G-DNA[3] (48.4×10^3 cpm/pmol), 0.09 unit of enzyme, and various NaCl concentrations (0−300 mM) as indicated. Incubation was performed at 37 °C, and samples (10 μL) were withdrawn at the times indicated (0−90 min). Reactions were stopped on ice with 1 μg of Ugi. Each sample was divided into two (5.5 μL) parts. The first part was supplemented with 5 μL of TE buffer, and reserved for alkaline hydrolysis. The second part was combined with *Hpa*II to form a reaction mixture (10.5 μL total volume) containing 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, and 4.8 units of *Hpa*II that was incubated at 37 °C for 1 h. AP-sites in both samples were then hydrolyzed as described above, immediately combined with an equal volume of denaturing sample buffer, and analyzed by denaturing 12% polyacrylamide gel electrophoresis.

EcoRI Restriction Endonuclease Reactions. The processivity of *Eco*RI was analyzed in reaction mixtures (100 μL) containing 20 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 5 mM $MgCl_2$, 50 μg/mL acetylated bovine serum albumin, 135 pmol of [^{32}P]U/A-DNA, 40 units of *Eco*RI, and 25 or 200 mM NaCl. Incubation was at 37 °C for 0−30 min. Samples (10 μL) were removed and placed on ice, 1 μL of 250 mM EDTA was added, and the enzyme was heat-inactivated by treatment at 75 °C for 10 min. Subsequently, each sample was divided into two aliquots. The first aliquot was supplemented with TE buffer (5 μL), combined with an equal volume of denaturing sample buffer, and analyzed by

[3] Concentrations of concatemeric DNA polynucleotide (U/A- and U/G-DNA) represent moles of [^{32}P]25-mer units.

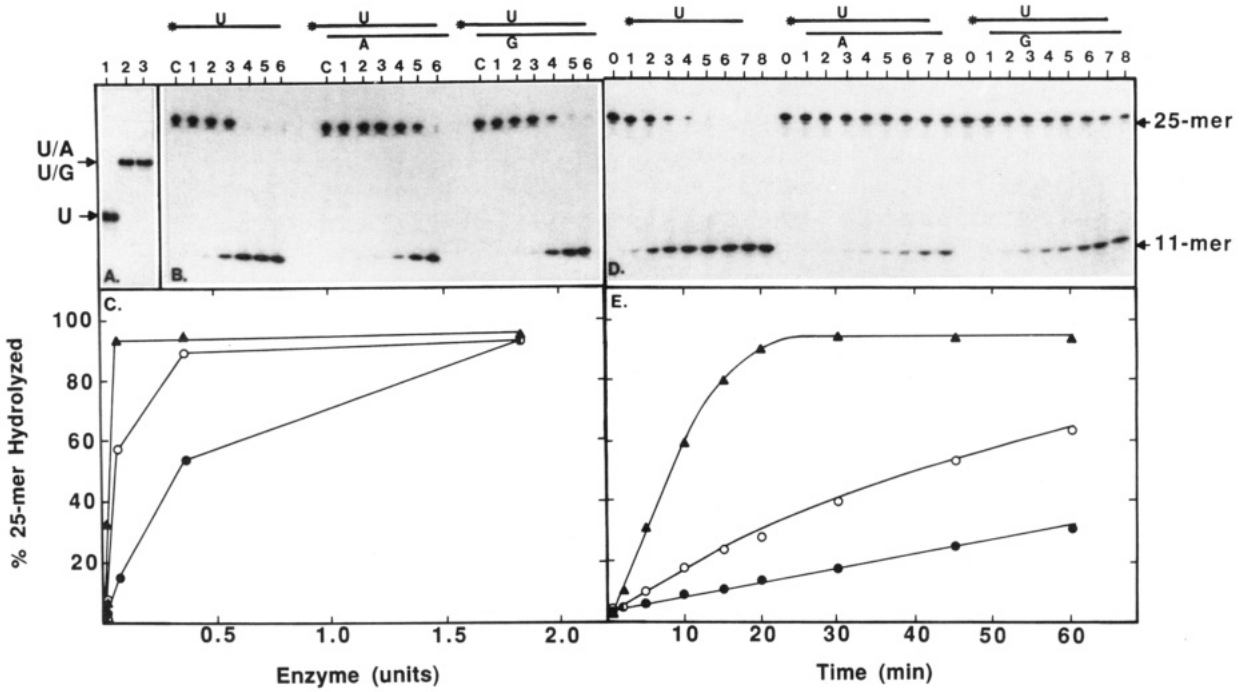

FIGURE 1: Substrate specificity of uracil−DNA glycosylase. (A) Three hybridization reaction mixtures (775 μL) each containing 30 nmol of [^{32}P]U-25-mer were prepared with either no addition (control), 45 nmol of A-25-mer or 45 nmol of G-25-mer as described under Experimental Procedures. Following the annealing reaction, a portion of each preparation (2.5 μL) was diluted 10-fold in a mock uracil−DNA glycosylase reaction buffer and incubated at 37 °C for 1 h. Samples (6 μL) were combined with an equal volume of tracking dye buffer containing 100 mM Tris-HCl (pH 6.8), 20% (w/v) glycerol, and 2% bromphenol blue, and 3 μL was analyzed by nondenaturing 12% polyacrylamide gel electrophoresis. The gel was dried and subjected to autoradiography. Lanes 1−3 correspond to U-, U/A-, and U/G-25-mer reactions, respectively. The locations of single-stranded (U) and duplex (U/A and U/G) oligonucleotides are indicated by arrows. (B) Standard uracil−DNA glycosylase reaction mixtures (25 μL) containing 0, 0.003, 0.015, 0.075, 0.37, and 1.8 units of Ung (lanes 1−6, respectively) and 95 pmol of [^{32}P]U-25-mer as either single-stranded (U) or duplex (U/A or U/G) as indicated were incubated at 37 °C for 30 min. A control reaction (lane C) was prepared which was incubated without Ung on ice for 30 min. After incubation, each reaction was terminated with excess Ugi, placed on ice, and subjected to alkaline hydrolysis, and samples (3 μL) were analyzed by denaturing 12% polyacrylamide/8.3 M urea gel electrophoresis as described under Experimental Procedures. Arrows denote the location on the autoradiogram of unreacted substrate (25-mer) and product (11-mer). (C) Radioactive bands were excised from the dried gel (B), the amount of ^{32}P radioactivity was measured, and the percent 25-mer hydrolyzed was calculated as [^{32}P]11-mer divided by [^{32}P]25-mer plus [^{32}P]11-mer and plotted for each reaction mixture using symbols defined below. (D) Three standard uracil−DNA glycosylase reaction mixtures (250 μL) contained 950 pmol of [^{32}P]U-25-mer as single-stranded (U) or duplex (U/A or U/G) as indicated, and 0.73 unit of Ung. Incubation occurred at 37 °C; samples (25 μL) were removed at the times indicated in (E), subjected to alkaline hydrolysis, and analyzed on a denaturing 12% polyacrylamide gel as described in (B). (E) The percent 25-mer hydrolyzed was determined for each reaction time course in (D) and [^{32}P]U-25-mer (▲), [^{32}P]U/A-25-mer (●), and [^{32}P]U/G-25-mer (○) are plotted.

denaturing 12% polyacrylamide gel electrophoresis. The second aliquot was combined with 5 μL of Ung (2900 units), incubated at 37 °C for 30 min to completely excise the uracil residues, and alkali-treated to cleave the DNA at AP-sites as described above.

Electrophoresis. Analysis of uracil−DNA glycosylase reaction products was performed using denaturing polyacrylamide gels (30 × 40 × 0.08 cm) containing 12% acrylamide, 0.41% *N,N′*-methylenebis(acrylamide), 8.3 M urea, and TBE buffer. Polymerization was catalyzed by the addition of 0.067% ammonium persulfate and 0.012% TEMED. Samples from Ung reactions were combined with an equal volume of denaturing sample buffer and heated at 95 °C for 3 min, and typically 10 μL of sample mixture was loaded into each well. Electrophoresis was carried out in TBE buffer at 1200 V until the tracking dye had migrated ~20 cm (~30 cm for denaturing 4% polyacrylamide gels). The gels were dried under vacuum, and autoradiography was performed with X-OMAT AR5 film (Kodak). Bands containing ^{32}P radioactivity were excised from the dried gels, placed into vials with 5 mL of Formula 989 Fluor (DuPont), and measured for radioactivity in a liquid scintillation spectrometer.

Nondenaturing 12% polyacrylamide gels were similarly prepared but without urea to assess the duplex nature of the annealed 25-mer DNA substrate. DNA samples (6 μL) were mixed with 6 μL of nondenaturing sample buffer containing 100 mM Tris-HCl (pH 6.8), 20% (w/v) glycerol, and 0.2% bromphenol blue, and 3 μL of sample mixture was loaded for analysis. Electrophoresis was performed at 500 V, and autoradiography was conducted as described above.

RESULTS

Substrate Specificity on U-, U/A-, and U/G-Containing [^{32}P]DNA. In order to assess the substrate specificity of *E. coli* uracil−DNA glycosylase acting at U·A base-paired and U·G mispaired sites, a 5′-end ^{32}P-labeled synthetic oligonucleotide (25-mer) with a single uracil residue at position 11 was prepared. The uracil-containing oligonucleotide (U-25-mer) was annealed to one of two complimentary oligonucleotides (A-25-mer or G-25-mer), identical in sequence except at the position opposite the uracil, creating either the U·A or the U·G double-stranded DNA substrate. To verify that these substrates (U/A-25-mer, U/G-25-mer) remained double stranded during a typical Ung reaction, their mobility relative to the single-stranded U-25-mer was examined by

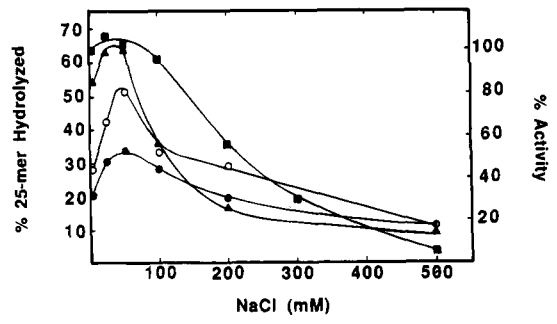

FIGURE 2: Effect of [NaCl] on uracil–DNA glycosylase activity. Four sets of standard reaction mixtures (25 μL) were prepared containing either 0.037 unit of Ung and 95 pmol of [^{32}P]U-25-mer (▲), 0.12 unit of Ung and 95 pmol of [^{32}P]U/A-25-mer (●) or [^{32}P]U/G-25-mer (○), or 0.37 unit of Ung and 2 nmol of calf thymus [*uracil*-^{3}H]DNA (■), and each reaction was supplemented with NaCl as indicated. After incubation at 37 °C for 30 min, reactions containing [^{32}P]DNA substrates were subjected to alkaline hydrolysis and analyzed by denaturing 12% polyacrylamide gel electrophoresis to determine the percent 25-mer hydrolyzed as described in Figure 1. The percent Ung activity on [*uracil*-^{3}H]DNA was determined relative to the 0 mM NaCl control.

nondenaturing polyacrylamide gel electrophoresis following a 1 h incubation at 37 °C (Figure 1A). The mobility of the U/A- and U/G-25-mer was virtually identical and distinctly different from that of single-stranded U-25-mer, implying that the double-stranded oligonucleotide substrates remain duplex under standard reaction conditions.

The three 25-mer substrates (U, U/A, U/G) were incubated with various amounts of Ung to catalyze uracil excision. The Ung-generated apyrimidinic sites were hydrolyzed by alkali treatment that cleaved the deoxyribose–phosphate bond on the 3′-side of the AP-site (Lindahl & Anderson, 1972; Teebor & Brent, 1981). Together these reactions produced an AP-site-containing [^{32}P]11-mer as the secondary product of Ung activity. Analysis of Ung reactions by denaturing polyacrylamide gel electrophoresis revealed that Ung was considerably more active on single-stranded U-25-mer, and somewhat more active on the mispaired U/G-25-mer relative to the paired U/A-25-mer duplex (Figure 1B,C). The rate of uracil excision was examined for each of the three uracil-containing 25-mer substrates (Figure 1D,E). Initial velocities calculated from the first 20 min of the Ung reaction revealed 108, 22.8, and 9.5 fmol/min for U-, U/G-, and U/A-25-mer, respectively. Thus, the DNA substrate containing U/G was preferred 2.4-fold over U/A whereas single-stranded U-25-mer was preferred 4.7- and 11.4-fold over both U/G and U/A substrates, respectively.

Effect of Ionic Strength on Substrate Specificity. To investigate the influence of monovalent ions on substrate specificity, Ung reactions were conducted with increasing concentrations of NaCl (Figure 2). Ung activity on all four substrates (U-, U/A-, and U/G-25-mer and calf thymus uracil-containing DNA) was stimulated to varying degrees by 25–100 mM NaCl. However, stimulation was greatest for U/G-25-mer (1.82-fold) and U/A-25-mer (1.68-fold) relative to Ung reactions in the absence of NaCl. Interestingly, activity on single-stranded U-25-mer and calf thymus uracil-containing DNA was significantly inhibited at 200 mM NaCl (69 and 44%, respectively) whereas activity on the U/A-and U/G-25-mers was virtually the same as that in reactions without NaCl.

Processivity Analysis of E. coli Uracil–DNA Glycosylase. Substrate Construction for Processivity Analysis. In order to determine the mechanism by which uracil–DNA glycosylase locates successive uracil residues in DNA, a defined concatemeric DNA substrate was constructed from duplex [^{32}P]U/A- or [^{32}P]U/G-25-mer units. Each end of the duplex 25-mer was designed with a four nucleotide complementary overlapping 5′-sequence that formed a *Hpa*II restriction endonuclease site upon self-hybridization (Figure 3). Concatemeric [^{32}P]DNA substrates with uracil residues at regular intervals (25 nucleotides) along one strand of the duplex polymer were formed in a DNA ligation reaction. Analysis of the ligation products by nondenaturing as well as denaturing polyacrylamide gel electrophoresis revealed that >90% of the polymeric [^{32}P]U/A-DNA and [^{32}P]U/G-DNA was ≥250 bp. Gel filtration chromatography was conducted to isolate high molecular weight [^{32}P]U/A- and [^{32}P]U/G-DNA concatemers (data not shown). Fractions containing DNA whose average size was 800–1100 bp were pooled and used as the substrate for processivity reactions. The average length of the concatenated [^{32}P]DNA substrates was also determined based on the percent of [^{32}P]11-mer produced after subjecting each substrate to excess Ung and alkali-treatment (Figure 3, reaction 1). The ratio of [^{32}P]-11-mer to total [^{32}P]25-mer plus [^{32}P]11-mer is dictated by the length of the concatemeric unit, since every uracil residue is spaced 25 nucleotides apart except at the 5′-end. A complete Ung digestion and subsequent alkaline treatment was found to produce 2.9% and 2.7% of the U/A- and U/G-DNA as 11-mer, respectively. Thus, the average concatemeric unit was found to be ~850 bp (U/A-DNA) and ~950 bp (U/G-DNA).

Validation of Processivity Assay by EcoRI. In order to validate the processivity assay, the properties of *Eco*RI endonuclease were analyzed using the concatemeric [^{32}P]U/A-DNA substrate. This substrate was designed to contain regularly spaced *Eco*RI recognition sites. If *Eco*RI acted processively, the major restriction endonuclease product generated should have been a [^{32}P]25-mer (Figure 3, reaction 3). As expected, when *Eco*RI digestion products from reactions containing 25 mM NaCl were analyzed by denaturing polyacrylamide gel electrophoresis, 73% of the initial reaction products were 25-mer oligonucleotide. In contrast, only 28% of the initial products appeared as 25-mer in reactions containing 200 mM NaCl. In each case, the extent of *Eco*RI reactions was determined after a combined treatment with excess Ung and alkali to cleave the DNA at every uracil residue. This treatment produced [^{32}P]25-mer oligonucleotide from the unrestricted DNA substrate and [^{32}P]-21-mer oligonucleotide from the *Eco*RI digestion products (Figure 3, reactions 3 and 4). These results confirmed the processive properties of *Eco*RI and validated the assay.

Processivity of E. coli Uracil–DNA Glycosylase. In designing experiments to assess processivity, a high molar ratio of substrate to enzyme was used to ensure that on average no more than one Ung molecule was bound initially to a particular concatemeric DNA molecule. Standard processivity analysis was performed in reactions containing 1.35 μM uracil residues and 38.3 pM *E. coli* Ung. This allowed for a ~1/35 250 molar ratio of Ung to uracil residues and ~1/1000 Ung to DNA ratio for each concatemeric substrate.

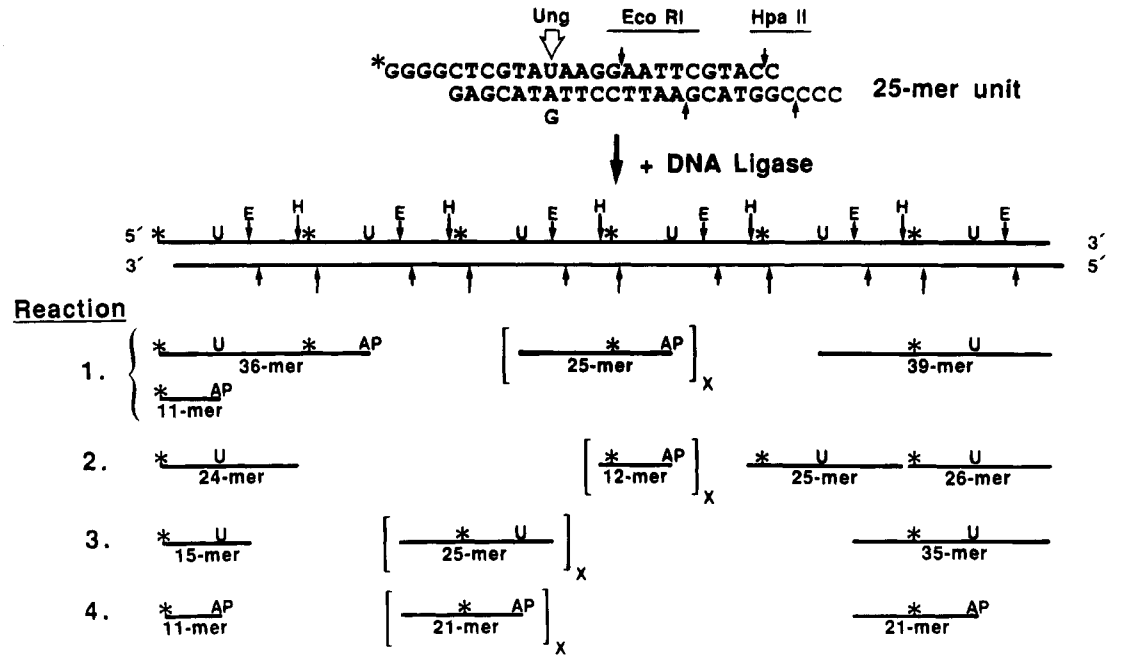

FIGURE 3: ^{32}P-Labeled oligonucleotide products generated from the uracil-containing polymeric [^{32}P]DNA substrate. Polymeric [^{32}P]DNA substrates were prepared by ligation of double-stranded 25-mer units. Each 25-mer contained a uracil residue at position 11 in the upper strand opposite adenine or guanine in the lower complementary strand; only the uracil-containing strand was 5′-end ^{32}P-labeled. An *Eco*RI endonuclease cleavage site is situated 3′ to nucleotide 15 of the uracil-containing strand. The 25-mer unit was designed with self-complementary 5′-overhanging ends of four nucleotides that form a *Hpa*II endonuclease recognition site upon hybridization. Ligation of annealed 25-mer units created concatemers in which each uracil−DNA glycosylase, *Eco*RI, and *Hpa*II recognition site was separated by 25 nucleotides. Reaction 1: Sequential uracil excision by uracil−DNA glycosylase followed by alkaline hydrolysis of the resultant AP-site produces 25-mers containing a 3′ AP-site and an internal ^{32}P-label. Excision of the 5′-uracil, the penultimate 5′-uracil, or the 3′-uracil residue produces an 11-, 36-, or 39-mer, respectively. Reaction 2: *Hpa*II endonuclease digestion of uracil−DNA glycosylase-treated [^{32}P]DNA and subsequent alkaline hydrolysis generate 12-mer from AP-site 25-mer (uracil excised) and 25-mer from DNA with intact uracil. Cleavage of the 5′ and 3′ *Hpa*II sites yields 24-mer and 26-mer, respectively. Reaction 3: *Eco*RI endonuclease digestion of [^{32}P]DNA produces 25-mer except at the 5′-and penultimate 3′-sites which yield 15- and 35-mer, respectively. Reaction 4: *Eco*RI-treated DNA subjected to digestion by uracil−DNA glycosylase and subsequent alkaline hydrolysis produces 21-mer from *Eco*RI-cleaved 25-mer, 11- and 21-mer from *Eco*RI 15- and 35-mer produced in reaction 3, and 25-mer from DNA containing intact *Eco*RI sites.

If Ung locates successive uracil residues by a processive mechanism, then [^{32}P]25-mer units, generated from one-dimensional diffusion along the DNA chain, would be the expected predominant reaction product. Consequently, 25-mer products would be detected from the onset of the reaction and would appear as the major species throughout the reaction time course. In contrast, if Ung locates uracil targets by a distributive mechanism, then at early points in the extent of the reaction 25-mer products would not predominate since successive excision of uracil residues would be expected to occur randomly on successive DNA molecules. In this case, the accumulation of [^{32}P]25-mer products in large amounts would appear only late in the reaction time course. After conducting a reaction time course at 0 mM NaCl, the products from uracil excision and AP-site hydrolysis were analyzed by denaturing polyacrylamide gel electrophoresis (Figure 4A). During the early stages of reactions ($\leq$20 min) containing either the U/A- or the U/G-DNA substrate, only the 25-mer reaction product was visible. As the reaction progressed, partially digested oligonucleotides became apparent but at relatively low levels (Figure 4B,C). After 20 min, the 25-mer represented 85% of the lower molecular weight oligomeric products (25- to 125-mers) for the reaction with U/A-DNA and 83% for the U/G-DNA reaction. These results suggested that Ung was acting in a processive manner.

In order to interpret these results more fully, it is important to understand what percentage of uracil-excision events resulted in the production of 25-mer oligonucleotides; therefore, the products of each reaction time point were divided into two aliquots. One aliquot was used to determine the percentage of total [^{32}P]DNA substrate that appeared as 25-mer units, as described above, while the other aliquot was used to determine the percentage of uracil residues in the [^{32}P]DNA substrate that were excised, regardless of position. This was measured by treating the Ung reaction products with excess *Hpa*II restriction endonuclease. The concatameric DNA substrate was designed so that a *Hpa*II recognition sequence was formed upon ligation of individual 25-mer duplexes and thus repeated every 25 bp (Figure 3). As a control, both the U/A- and U/G-DNA substrates were digested with excess *Hpa*II and shown to almost exclusively produce 25-mer units (Figure 5A,C, lane 1). If the uracil residue within the *Hpa*II 25-mer was previously excised by Ung, alkaline hydrolysis of the resultant AP-site was expected to release a [^{32}P]12-mer; if the uracil was not excised, the [^{32}P]25-mer oligonucleotide remained intact (Figure 3, reaction 2). As expected, when U/A- and U/G-DNA substrates were treated with excess Ung, and the products treated with *Hpa*II and AP-sites cleaved by alkaline hydrolysis, ~95% of the products were [^{32}P]12-mer oligonucleotides (Figure 5A,C, lane 2). Thus the extent of Ung digestion at each point in the reaction time course (Figure 4) was determined as the percentage of [^{32}P]12-mer relative to total ^{32}P-labeled DNA product ([^{32}P]25-mer plus [^{32}P]12-mer) in each reaction aliquot (Figure 5B,D). A small amount ($\leq$5%) of the *Hpa*II reactions was resolved as 50-mer and

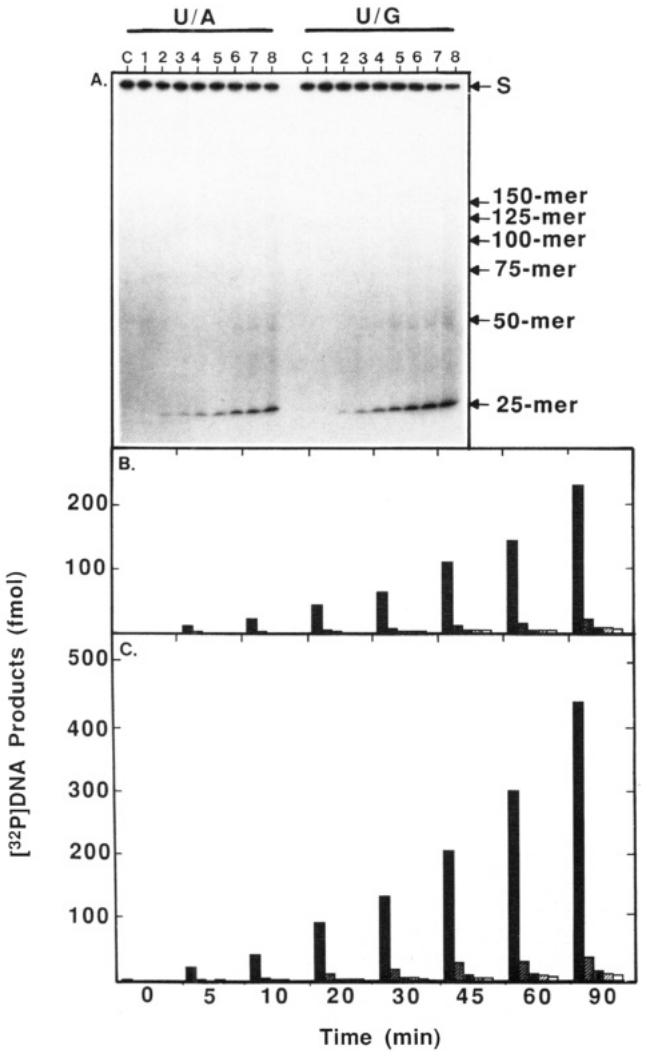

FIGURE 4: Analysis of reaction products generated by Ung on concatemeric uracil-containing [^{32}P]DNA. (A) Two standard reaction mixtures (100 μL) containing 0.09 unit of Ung and 135 pmol of [^{32}P]U/A-DNA or [^{32}P]U/G-DNA were incubated at 37 °C. Aliquots (10 μL) were removed at 0, 5, 10, 20, 30, 45, 60, and 90 min (lanes 1−8). A control reaction (lane C) containing 13.5 pmol of the appropriate [^{32}P]DNA substrate was incubated on ice for 90 min. After terminating the reactions on ice with excess Ugi, each sample was divided into two aliquots, designated Ung reaction and *Hpa*II reaction. The Ung reaction aliquot was subjected to alkaline hydrolysis, and analyzed by denaturing 12% polyacrylamide gel electrophoresis as described under Experimental Procedures. The positions of the 25-mer and partially digested oligomer are indicated on the autoradiogram by arrows along with the unreacted [^{32}P]-DNA substrate (S). Following autoradiography, radioactive bands (25-mer through 125-mer) were individually excised from the gel and the ^{32}P radioactivity was measured. The molar quantities of the five digestion products, 25-mer, 50-mer, 75-mer, 100-mer, and 125-mer (respective bars from left to right at each time), were determined for the [^{32}P]U/A-DNA (B) and [^{32}P]U/G-DNA (C) reactions.

was constant over the course of the Ung reaction. It appeared that a minor percentage of the concatemeric substrates was intractable to *Hpa*II cleavage, but the extent of digestion calculations was not substantially affected. The emergence of the 12-mer band (Figure 5) appeared commensurate with the production of the 25-mer band, the Ung reaction product, in Figure 4. Quantitation of the gel results revealed a linear dependence of the extent of Ung digestion with time and that uracil residues in the concatemeric U/G-DNA were released 2.2-fold faster than in U/A-DNA (Figure 5E).

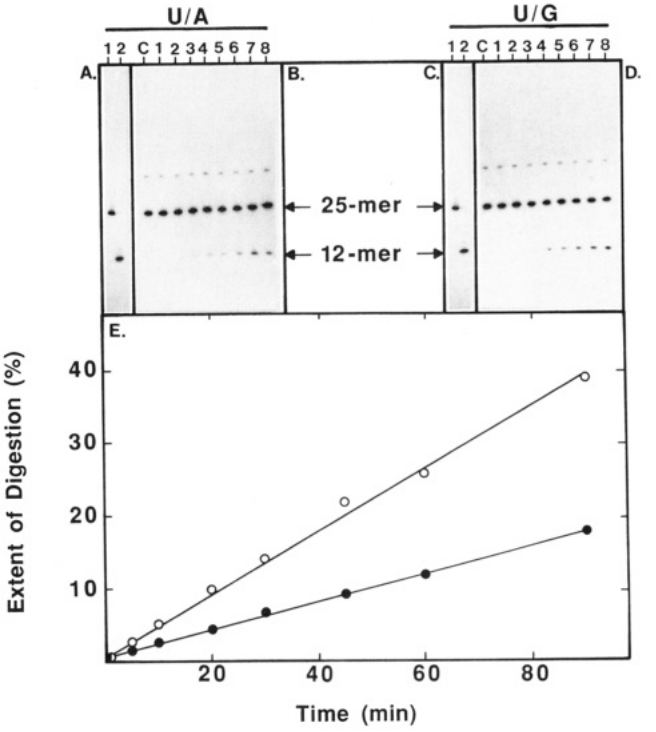

FIGURE 5: *Hpa*II digestion of Ung reaction products generated from the concatemeric uracil-containing [^{32}P]DNA substrate. Standard uracil−DNA glycosylase reaction mixtures (10 μL) were prepared containing either 13.5 pmol of (A) [^{32}P]U/A-DNA or (C) [^{32}P]U/G-DNA and 3.7 units of Ung. After incubation at 37 °C for 1 h, each reaction was stopped on ice with excess Ugi and divided into two equal parts. One sample (A and C, lane 1) was processed as indicated for Ung reactions in Figure 5. The other sample (A and C, lane 2) was treated with 4.8 units of *Hpa*II for 1 h at 37 °C. Following the Ung and/or *Hpa*II reactions, the AP-sites previously produced by Ung were cleaved by alkaline hydrolysis, denaturing sample buffer was added, electrophoresis on denaturing 12% polyacrylamide gels was conducted and autoradiography was performed as described under Experimental Procedures. The locations of the 25-mer and 12-mer reaction products are located by arrows. (E) The extent of uracil excision after various incubation times (0, 5, 10, 20, 30, 45, 60, and 90 min) was determined as the percent of total ^{32}P label (25-mer + 12-mer) represented by [^{32}P]-12-mer and plotted as a function of reaction time for (B) [^{32}P]U/A-DNA (●) and (D) [^{32}P]U/G-DNA (○) in lanes 1−8, respectively.

Effect of NaCl on Processivity of E. coli Uracil−DNA Glycosylase. The effect of NaCl on the nature and distribution of reaction products was determined using both U/A-DNA and U/G-DNA at 300 mM NaCl (Figure 6). Examination of the autoradiogram revealed that the intensity of the bands of partially digested oligomer clearly increased relative to that observed at 0 mM NaCl (Figure 4). The rate of activity at 300 mM NaCl was inhibited 24% (U/A-DNA) and 30% (U/G-DNA) compared to the reaction at 0 mM NaCl.

When the percentage of 25-mer produced in each reaction was plotted as a function of the total extent of uracil excision, the processivity can be assessed. It is important to examine only data taken early in the progression of the reaction, since (1) the 25-mers would be least likely to accumulate if a distributive mechanism were acting, (2) 25-mer would be most likely to appear if uracil excision was processive, and (3) the inhibitory effect of AP-sites produced from uracil excision would be least likely to interfere by changing the character of the substrate. The relationship between the amount of 25-mer produced and the total extent of uracil

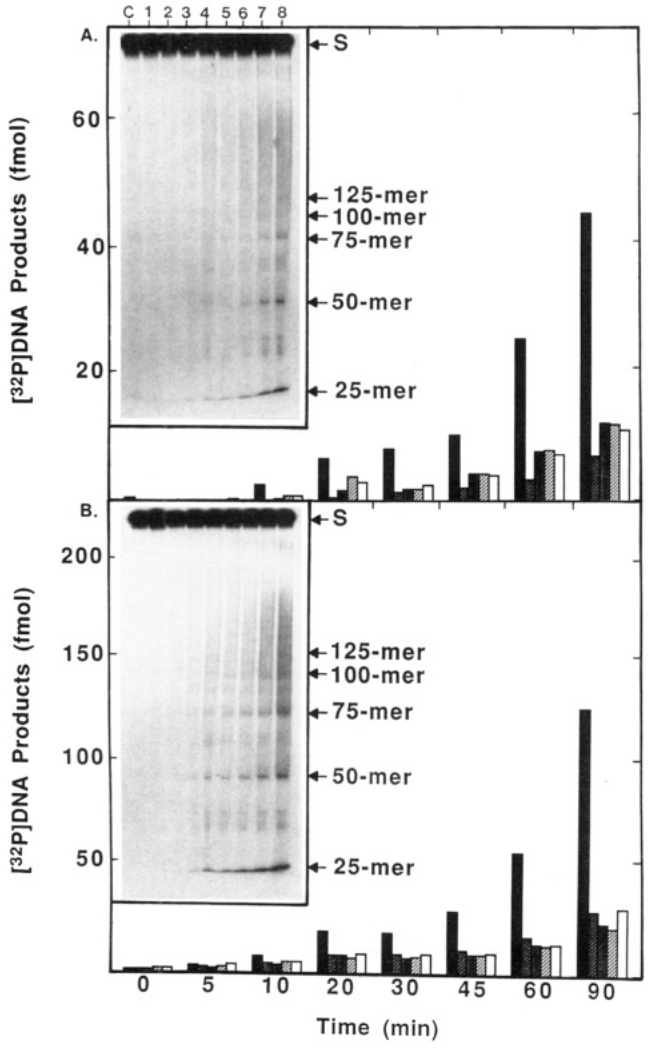

FIGURE 6: Ung processivity reactions conducted at 300 mM NaCl on polymeric [^{32}P]DNA. Standard uracil−DNA glycosylase reaction mixtures (100 μL) containing 0.09 unit of Ung and 135 pmol of either (A) [^{32}P]U/A-DNA or (B) [^{32}P]U/G-DNA were prepared but with the addition of 300 mM NaCl. Incubation occurred at 37 °C for 0, 5, 10, 20, 30, 45, 60, and 90 min (lanes 1−8, respectively). A control reaction (lane C) containing 13.5 pmol of the appropriate [^{32}P]DNA substrate was incubated on ice for 90 min. Samples (10 μL) were withdrawn and treated as described in Figures 4 and 5. Autoradiograms are shown as inserts for each reaction time course. Arrows indicate the positions of Ung reaction products. The location of the unreacted polymeric [^{32}P]DNA substrate (S) is indicated. Following autoradiography, the various bands were excised, ^{32}P radioactivity was measured, and molar quantities of the five smallest digestion products (arrows) were determined as described under Experimental Procedures. The respective amounts of 25-mer, 50-mer, 75-mer, 100-mer, and 125-mer are shown in each histogram (bars left to right at each time).

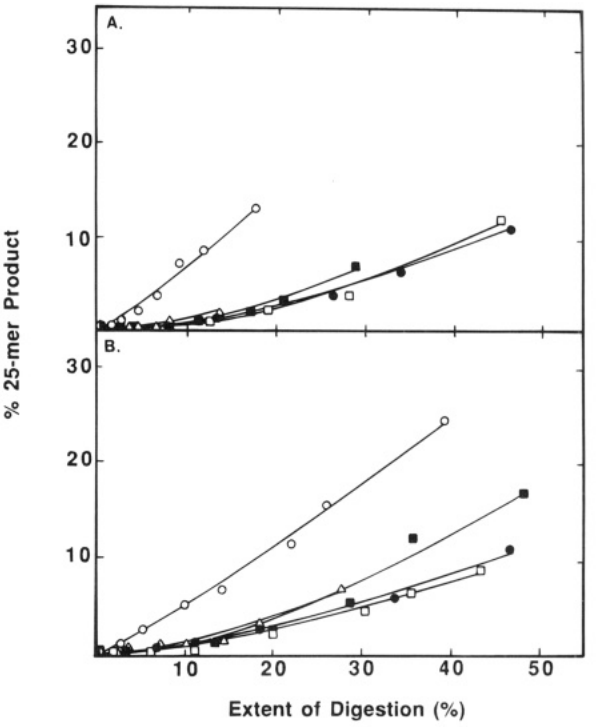

FIGURE 7: Effect of NaCl concentration on the processivity of Ung. Five sets of standard uracil−DNA glycosylase reactions (100 μL) were prepared containing 0.09 unit of Ung and 135 pmol of (A) [^{32}P]U/G DNA which were supplemented with 0 mM (○), 50 mM (●), 100 mM (□), 200 mM (■), or 300 mM (△) NaCl. After incubation at 37 °C for various times, the amount of [^{32}P]25-mer produced following uracil excision and subsequent alkaline hydrolysis was determined by denaturing polyacrylamide gel electrophoresis as described in Figures 4 and 5. The amount of 25-mer detected was determined relative to the total ^{32}P radioactivity at each respective time point (*Hpa*II reaction, [^{32}P]25-mer + [^{32}P]-12-mer) and designated as percent 25-mer product. Similarly, the extent of total uracil excised was determined after treatment with excess *Hpa*II as described in Figure 5. The amount of [^{32}P]12-mer generated divided by the [^{32}P]25-mer plus [^{32}P]12-mer for each time point measured the overall extent of the Ung reaction and was designated Extent of Digestion (%).

excision was considered essentially linear over the initial 20% progression of the reactions containing 0 and 300 mM NaCl (Figure 7). However, the value of the slopes was very different. At 0 mM NaCl, the slope values for the reaction containing U/A-DNA and U/G-DNA were 0.56 and 0.50, and at 300 mM NaCl, they were 0.11 and 0.12, respectively. Under ideal conditions, an enzyme of unlimited processivity acting on a substrate of infinite length should release 25--mer for virtually every excision, and the slope of the processivity plot would approach unity. In contrast, a distributive enzyme would release almost no 25-mer and demonstrate a slope of approximately zero. Hence, the results suggest that Ung acts with processive character in the absence of NaCl and with reduced processivity at 300 mM NaCl (Figure 7).

In light of these observations, the processivity of Ung was studied at NaCl concentrations ranging from 0 to 300 mM (Figure 7). At all NaCl concentrations (≥50 mM), the average slope of the processivity plot was 0.11 ± 0.015 and 0.15 ± 0.026 for U/A-DNA and U/G-DNA, respectively. These results indicate that the addition of ≥50 mM NaCl brings about a decreased processivity of Ung. Interestingly, Ung was equally processive on the two DNA substrates, although ~2-fold more active on the mispaired U/G-DNA. At moderate NaCl concentrations (50−200 mM), Ung activity was stimulated but showed decreased processivity.

Processivity of Rat Liver Mitochondrial Uracil−DNA Glycosylase. Homogeneous rat liver mitochondrial uracil−DNA glycosylase was examined to determine whether it also used a processive mechanism to locate successive uracil residues in duplex DNA. As before, reactions were carried out on concatemeric U/A-DNA and U/G-DNA, reaction products were analyzed by denaturing polyacrylamide gel electrophoresis, and the results were evaluated using a processivity plot (Figure 8). Inspection of the graphs revealed that reactions containing 0 mM NaCl exhibited initial slope values of 0.45 and 0.66 for the U/A-DNA and

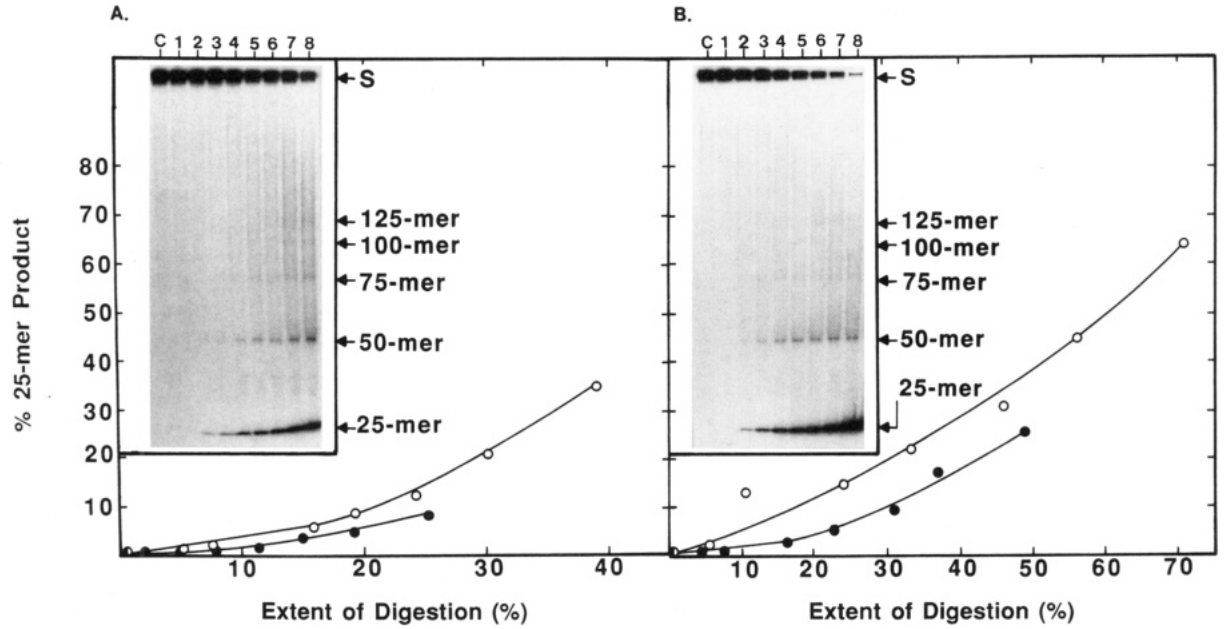

FIGURE 8: Analysis of processivity for rat liver mitochondrial uracil−DNA glycosylase. Two sets of standard reaction mixtures (100 μL) were prepared containing 0.08 unit of rat liver uracil−DNA glycosylase and 135 pmol of (A) [^{32}P]U/A-DNA or (B) [^{32}P]U/G-DNA and supplemented with either 0 mM (○) or 100 mM (●) NaCl. Aliquots (10 μL) were removed at various times and processed to determine the percent 25-mer produced and extent of digestion (%) as described in Figure 7. Autoradiograms of the 0 mM reaction products are shown in the inserts, and arrows indicate the position of the 25-mer, partially digested oligomer, and unreacted [^{32}P]DNA substrate (S). Lanes 1−8 correspond to 0, 5, 10, 20, 30, 45, 60, and 90 min of reaction, respectively; lane C was incubated for 90 min without enzyme.

U/G-DNA, respectively. These values stand in contrast to the slopes calculated for the 100 mM NaCl reaction: 0.14 and 0.23 for the respective reactions. Thus, the processivity of mitochondrial uracil−DNA glycosylase is decreased ~3-fold at 100 mM NaCl. Like the *E. coli* Ung, the mitochondrial enzyme was more active (~1.8-fold) on the mispaired U/G-DNA; however, it was inhibited 30−35% by 100 mM NaCl.

DISCUSSION

We have demonstrated that both *E. coli* and rat liver mitochondrial uracil−DNA glycosylases preferentially hydrolyze uracil residues from U·G mismatched over U·A matched base pairs in DNA, and that uracil residues in single-stranded oligonucleotide are recognized more efficiently than in duplex oligonucleotide. The observed preference for single-stranded DNA agrees with several previous studies using other DNA substrates and the *E. coli* (Lindahl et al., 1977), yeast (Crosby et al., 1981), rat liver nuclear and mitochondrial (Domena & Mosbaugh, 1985; Domena et al., 1988), and human placenta uracil−DNA glycosylase (Olsen et al., 1989). Curiously, Verri et al. (1992) reported that while uracil−DNA glycosylase from HeLa cells, herpes simplex virus I, and *E. coli* also removed uracil more efficiently from an oligonucleotide (26-mer) containing a U·G versus U·A site, all three enzymes strongly preferred the double-stranded U·G-containing substrate over the single-stranded U-26-mer (Verri et al., 1992). However, the single-stranded oligonucleotide substrate was preferred over the U·A-containing duplex DNA by each enzyme (Verri et al., 1992). On the other hand, Eftedal et al. (1993) suggest that partially purified calf thymus uracil−DNA glycosylase exhibits a 1.7-fold preference for uracil in a single-stranded 22-mer over the duplex DNA substrate. Unlike the uracil−DNA glycosylases in the report of Verri et al. (1992), the calf thymus enzyme removed uracil residues from U·A base pairs with a slightly faster rate than U·G mispairs (Eftedal et al., 1993). These differences could be explained by different enzyme properties, reaction conditions, variations in DNA substrate sequence context, or inefficient annealing of duplex DNA during substrate preparation. We feel that it is critically important that the duplex nature of the U·A- and U·G-containing DNA substrate preparations be analyzed for completeness of hybridization. Since single-stranded DNA is generally recognized as the preferred substrate (Lindahl et al., 1977; Mosbaugh & Bennett, 1994), contaminating unhybridized uracil-containing oligonucleotides in duplex preparations could produce erroneous substrate specificity results. We have been careful to demonstrate that both U/A- and U/G-25-mer substrate preparations were exclusively double-stranded prior and subsequent to the enzyme incubation period.

Two defined concatemeric polynucleotide substrates were synthesized with repeating uracil residues and *Eco*RI and *Hpa*II endonuclease recognition sites at nonoverlapping 25 nucleotide intervals. One DNA substrate that was ~850 bp contained equally spaced U·A sites, and the other that was ~950 bp contained repeating U·G mispairs. To validate the processivity assay, we analyzed the processivity of *Eco*RI endonuclease using the concatemeric U·A-DNA substrate: the results were in good agreement with those reported by Terry et al. (1985). We interpreted these findings as indicating that the polynucleotide substrate constructed and the method employed to analyze the reaction mechanism were appropriate for measuring the extent of processive hydrolysis.

We conclude that both *E. coli* and rat liver mitochondrial uracil−DNA glycosylases can utilize a processive mechanism to locate adjacent uracil residues. Very similar degrees of processivity were found for the U·A- and U·G-containing DNA substrates, indicating that the nature of the uracil target site does not influence the search mechanism. As expected,

the processivity was affected by NaCl concentration, which is consistent with the premise that nonspecific protein/DNA interactions play a significant role in the enzyme locating uracil residues (Gruskin & Lloyd, 1986). In the absence of NaCl, approximately 50% of the reaction originated from adjacent target sites for both the *E. coli* and rat liver mitochondrial enzymes. Thus, the initial rates at which 25-mer was generated were disproportionately higher than expected for a distributive mode of action. Those results clearly demonstrate that sequential uracil residues were hydrolyzed by a positionally correlated mode of action, considering that *E. coli* Ung was present in a molar ratio of ~1:1000 DNA molecules, and that, on average, each U/A- and U/G-DNA substrate contained 34 and 38 uracil sites, respectively. In contrast, at ≥50 mM NaCl concentrations, less than 15% of the uracil excised by either enzyme stemmed from adjacent target sites. It was not surprising that the bacterial and the mitochondrial uracil−DNA glycosylase utilized an analogous mechanism of action, as these two types of enzymes appear to be highly conserved polypeptides (Olsen et al., 1989; Upton et al., 1993). Furthermore, UV-catalyzed cross-linking of *E. coli* Ung to DNA has demonstrated that highly conserved amino acids reside in or near the DNA binding site (Bennett et al., 1994).

During the course of this investigation, Purmal et al. (1994) reported that *E. coli* Ung utilized a distributive mechanism of uracil removal. Several significant differences exist between these two studies. (1) Unlike the synthetic polynucleotide DNA substrate used in this study, Purmal et al. (1994) constructed two duplex DNA molecules that contained uracil residues on both DNA strands. While the spacing between uracils on the same strand was 10 and 20 nucleotides for polymers I and II, respectively, the spacing between uracils in both strands was actually 3 and 7 nucleotides. Thus, it was possible that two uracil target sites may have simultaneously occupied the DNA binding site of the enzyme. (2) The uracil residues located in the DNA substrate designed by Purmal et al. (1994) were in a 5′-CUT-3′ sequence context, which reportedly represents a poor consensus sequence for catalysis (Eftedal et al., 1993). In contrast, we have placed the uracil residue within a 5′-AUAA-3′ sequence where it is reportedly efficiently recognized (Eftedal et al., 1993). (3) The analysis by which the mechanism of uracil removal was determined by Purmal et al. (1994) was performed, in large part, after significant levels of uracil had been hydrolyzed from the substrate. Under such conditions, the enzyme may be more likely to encounter an AP-site than a dUMP target residue. Since AP-sites inhibit uracil−DNA glycosylases, presumably by binding to the enzyme (Domena et al., 1988), they could influence the extent of protein/DNA translocation. It is not known what effect AP-sites in close proximity on opposite strands may have on enzyme/DNA dissociation. It is conceivable that the enzyme may distinguish between the AP-site generated within the active site during a catalytic event and one that is encountered along the DNA during nonspecific translocation. The effect of AP sites may be significant, since the K_i of AP-site DNA is approximately equal to the K_m of uracil−DNA for rat liver mitochondrial uracil−DNA glycosylase (Domena et al., 1988). Our results are consistent with those reported by Higley and Lloyd (1994), who concluded that Ung acts with partial processivity on pBR322 (form I) DNA containing randomly located uracil residues and that NaCl concentrations ≥50 mM caused reduced processivity of Ung.

What is the biological significance of the extended interaction of uracil−DNA glycosylase with its reaction product, the AP-site, and DNA? Pausing by uracil−DNA glycosylase at an AP-site could act to nucleate other proteins involved in the uracil-excision DNA-repair pathway. In *E. coli* and human cell extracts, one-nucleotide repair patches have been observed in double-stranded oligonucleotides that contained U·A base pairs or U·G mispairs (Dianov et al., 1992). Thus, uracil-excision repair seems to involve a very concerted reaction mechanism. The influence of other proteins involved in uracil-excision repair on the concerted character and processivity of DNA repair remains to be examined.

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