Mammalian 5-Formyluracil−DNA Glycosylase. 1. Identification and Characterization of a Novel Activity That Releases 5-Formyluracil from DNA†

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ABSTRACT: 5-Formyluracil (fU) is a major oxidative thymine lesion produced by reactive oxygen species and exhibits genotoxic and cytotoxic effects via several mechanisms. In the present study, we have searched for and characterized mammalian fU−DNA glycosylase (FDG) using two approaches. In the first approach, the FDG activity was examined using purified base excision repair enzymes. Human and mouse endonuclease III homologues (NTH1) showed a very weak FDG activity, but the parameter analysis and $NaBH_4$ trapping assays of the Schiff base intermediate revealed that NTH1 was kinetically incompetent for repair of fU. In the second approach, FDG was partially purified (160-fold) from rat liver. The enzyme was a monofunctional DNA glycosylase and recognized fU in single-stranded (ss) and double-stranded (ds) DNA. The most purified FDG fraction also exhibited monofunctional DNA glycosylase activities for uracil (U), 5-hydroxyuracil (hoU), and 5-hydroxymethyluracil (hmU) in ssDNA and dsDNA. The fU-excising activity of FDG was competitively inhibited by dsDNA containing U·G, hoU·G, and hmU·A but not by intact dsDNA containing T·A. Furthermore, the activities of FDG for fU, hmU, hoU, and U in ssDNA and dsDNA were neutralized by the antibody raised against SMUG1 uracil−DNA glycosylase, showing that FDG is a rat homologue of SMUG1.

Reactive oxygen species generated by aerobic metabolism and exogenous agents induce structurally diverse oxidative damage to DNA (*1*, *2*). If left unrepaired, DNA damage arrests DNA synthesis or induces DNA replication errors, causing deleterious effects on living organisms such as cell death and mutations (*3*). To cope with the harmful effects of DNA damage, cells have several DNA repair mechanisms including base excision repair (BER),[1] nucleotide excision repair, mismatch repair, and recombination repair (*4*). The primary mechanism involved in restoration of oxidative DNA damage is BER, which is well conserved from prokaryotic to eukaryotic organisms (*5*). In the first step of BER, a damage-specific DNA glycosylase removes an aberrant base from DNA by hydrolyzing the N-glycosidic bond, generating an apyrimidinic/apurinic (AP) site. The enzyme with this activity alone is called a monofunctional DNA glycosylase. Alternatively, a bifunctional DNA glycosylase has an additional AP lyase activity, converting an AP site into a nicked form in a concerted reaction. The intact or nicked AP site is subsequently processed by sequential actions of AP endonuclease, gap-filling DNA polymerase, and DNA ligase. In *Escherichia coli*, oxidative pyrimidine lesions such as thymine glycols (Tg) are primarily excised from DNA by endonuclease (Endo) III and Endo VIII and oxidative purine lesions such as 7,8-dihydro-8-oxoguanine (8oxoG) and formamidopyrimidine are removed by formamidopyrimidine−DNA glycosylase (Fpg) (*5*). Mammalian cells have a homologue of Endo III (NTH1) and a functional homologue of Fpg (OGG1) (*4*). Human homologues of Endo VIII (NEH1/NEI1, NEH2/NEI2, and NEI3) have been recently identified (*6*−*10*).

5-Formyluracil (fU) is a major oxidative thymine lesion formed by the Fenton reaction, ionizing radiation, and photosensitized reactions (*11*−*17*). Although the C5-formyl group in fU is not directly involved in base pairing, substitution of the methyl group by an electron-withdrawing formyl group increases the acidity of the amide proton at N3 and promotes ionization of the base moiety (*18*, *19*). The ionized form of fU in a DNA template directs misincorporation of dGMP (*20*). In addition, a dNTP containing an ionized form of fU is misincorporated opposite template G

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[1] Abbreviations: BER, base excision repair; AP, apurinic/apyrimidinic; U, uracil; hoU, 5-hydroxyuracil; Tg, thymine glycol; fU, 5-formyluracil; hmU, 5-hydroxymethyluracil; 8oxoG, 7,8-dihydro-8-oxoguanine; 7mG, 7-methylguanine; FDG, 5-formyluracil−DNA glycosylase; Endo III (VIII), endonuclease III (VIII); Fpg, formamidopyrimidine−DNA glycosylase; NTH1, endonuclease III homologue; NEH1/NEI1 and NEH2/NEI2, endonuclease VIII homologues; OGG1, 8-oxoguanine−DNA glycosylase; MPG, methylpurine−DNA glycosylase; TDG, thymine−DNA glycosylase; MBD4, methyl-CpG binding domain; SMUG1, single-strand-selective monofunctional uracil−DNA glycosylase; Ung (UNG), *Escherichia coli* (mammalian) uracil−DNA glycosylase; Ugi, uracil−DNA glycosylase inhibitor, ss, single stranded; ds, double stranded.

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during DNA synthesis (*18*, *21*). The fU lesion site-specifically incorporated in plasmid vectors induces mutations in *E. coli* and mammalian cells, although there are some discrepancies between the in vitro and in vivo mutation spectra (*22*, *23*). In addition, the 2′-deoxyribonucleoside form of fU (fdU) added in culture media exerts genotoxic effects on bacterial and mammalian cells (*11*, *24*, *25*). Free fdU also forms covalent adducts with cysteamine derivatives (*26*), and fU in oligonucleotides forms a Schiff base with lysine residues in oligopeptides derived from RecA protein (*27*), implying an alternative toxic mechanism of fU in DNA. Accordingly, fU is a potentially genotoxic and cytotoxic lesion formed by reactive oxygen species.

In *E. coli*, fU in an fU·A pair is repaired by the BER pathway where fU is excised from DNA by AlkA (3-methyladenine−DNA glycosylase II) (*28*), whereas fU in an fU·G mispair generated by misincorporation of a dNTP form of fU is likely repaired by the mismatch repair pathway (*21*). There is also an activity that excises fU from DNA in rat liver and HeLa cells (*29*, *30*), but the activity has been neither purified nor characterized to date. It has been recently reported that *E. coli* Endo III, Endo VIII, Fpg, and human NTH1 (hNTH1) excise fU from DNA when used in large excess over the substrate (*31*, *32*). However, it remains unclear whether the activity of the enzymes for fU is physiologically significant. In this study, the kinetic parameters of the above enzymes for fU have been determined and compared to those for intrinsic substrates to elucidate whether their activity for fU is physiologically significant. Then, 5-formyluracil−DNA glycosylase (FDG) activity has been partially purified from rat liver and characterized. We report here that the excision activity of Endo III, Endo VIII, Fpg, and mammalian NTH1 for fU is very low relative to that for their intrinsic substrates (Tg and 8oxoG) and, therefore, physiologically insignificant. Consistent with this, the FDG activity in the mouse liver cell extract is chromatographically separated from the NTH1 activity. More importantly, purified rat FDG acts as a monofunctional DNA glycosylase for fU in single-stranded (ss) and double-stranded (ds) DNA and exhibits additional activities for uracil (U), 5-hydroxyuracil (hoU), and 5-hydroxymethyluracil (hmU). These and immunological data combined together indicate that FDG is a rat homologue of single-strand-selective monofunctional uracil−DNA glycosylase (SMUG1).

EXPERIMENTAL PROCEDURES

Oligonucleotide Substrates and Enzymes. The substrates used in this study were summarized in Table 1. 19TG, 25FU, and 25MG containing Tg, fU, and 7-methylguanine (7mG), respectively, were synthesized as described previously (*20*, *33*, *34*). 19HOU, 25HMU, and 25OG containing hoU, hmU, and 8oxoG, respectively, were synthesized by the phosphoramidite method using the corresponding phosphoramidite monomers (Glen Research) and deprotected following the manufacturer's instructions. Oligonucleotides containing normal bases and U were synthesized by the standard phosphoramide method. The oligonucleotides containing the base lesions were 5′-end labeled with [γ-^{32}P]ATP (110 TBq/mmol, Amersham Biosciences) and T4 polynucleotide kinase (New England BioLabs) and purified by a Sep-Pak cartridge (Waters). The labeled oligonucleotides were used as single-stranded (ss) substrates. Alternatively, they were annealed to appropriate complementary strands (Table 1) and used as double-stranded (ds) substrates. Purification of Endo III, Endo VIII, Fpg, mouse NTH1 (mNTH1), and human OGG1 (hOGG1) were reported previously (*34*−*37*). *E. coli* uracil−DNA glycosylase (Ung) and a uracil−DNA glycosylase inhibitor (Ugi) were obtained from USB and New England BioLabs, respectively. hNTH1 was expressed as a glutathione *S*-transferase (GST) fusion protein in *E. coli* BL21(DE3)-pLysS carrying pGEX-hNTHmet1 [gift from S. Ikeda (*38*)]. The fusion protein was digested with thrombin, and hNTH1 was purified as reported (*38*). Human and mouse methylpurine glycosylases (hMPG and mMPG) were gifts from T. R. O'Connor (39) and R. Roy (*40*), respectively. Purification of rat and human SMUG1(rSMUG1 and hSMUG1) is described in the accompanying paper (*41*).

Table 1: Oligonucleotide Substrates Used in This Study

substrate	damage (X)[a]	sequence (5′ → 3′)	paired base[b]
19U	U	ACAGACGCCAXCAACCAGG	ss, G
19HOU	hoU	ACAGACGCCAXCAACCAGG	ss, G
19TG	Tg	ACAGACGCCAXCAACCAGG	A
19T	T	ACAGACGCCAXCAACCAGG	G, A
25FU	fU	CATCGATAGCATCCGXCACAGGCAG	ss, A
25HMU	hmU	CATCGATAGCATCCGXCACAGGCAG	ss, A
25TpG	T	CATCGATAGCATCCGXGACAGGCAG	G
25OG	8oxoG	CATCGATAGCATCCTXCCTTCTCTC	C
25MG	7mG	CATCGATAGCATCCTXCCTTCTCTC	C

[a] Abbreviations: U, uracil; hoU, 5-hydroxyuracil; Tg, thymine glycol; fU, 5-formyluracil; hmU, 5-hydroxymethyluracil; 8oxoG, 7,8-dihydro-8-oxoguanine; 7mG, 7-methylguanine. Note that T is not damage but is present as a T·G mispair in a CpG sequence (25TpG) or non-CpG sequence (19T) context. [b] ss indicates single-stranded substrates without complementary strands. G, A, and C indicate the base opposite the damage (X) in double-stranded substrates. The length of the complementary strand was the same as the lesion strand for each substrate.

Comparison of the Activities of Purified Enzymes for fU and Intrinsic Substrates. For parameter analysis, dsDNA containing a Tg·A pair was used as an intrinsic substrate for Endo III, Endo VIII, mNTH1, hNTH1, and dsDNA containing an 8oxoG·C pair for Fpg (Table 1). The intrinsic substrates [0.01−0.6 pmol (i.e., 1−60 nM)] were incubated with 1 ng of Endo III, Fpg, mNTH1, and hNTH1 or 5 ng of Endo VIII in the appropriate buffer (10 μL) at 37 °C for 3 min. The substrate containing an fU·A pair [0.1−4 pmol (i.e., 10−400 nM)] was similarly incubated with 20 ng of Endo III, Endo VIII, and Fpg or 10 ng of mNTH1 and hNTH1 at 37 °C for 5 min. The reaction buffer for Endo III, Endo VIII, and Fpg consisted of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA, and that for mNTH1 and hNTH1 consisted of 50 mM Tris-HCl (pH 8.0), 75 mM NaCl, 1 mM DTT, and 0.1 mg/mL BSA. The reaction was terminated by addition of PAGE loading buffer (0.05% xylene cyanol, 0.05% bromphenol blue, 20 mM EDTA, and 98% formamide). The products were separated by 16% denaturing PAGE and quantitated on a phosphorimaging analyzer, Fuji BAS2000. Parameters (K_m and k_{cat}) were evaluated from S−V plots using a hyperbolic curve-fitting program. The activity of hOGG1, hMPG, and mMPG was assayed using intrinsic substrates (8oxoG·C for hOGG1 and 7mG·C for mMPG and hMPG) and that containing an fU·A pair (Table 1). The substrates [0.05 pmol (i.e., 5 nM)] were incubated in the appropriate reaction buffer (10 μL) with the enzymes

(100 ng) at 37 °C for 10 min. The composition of reaction buffer for hOGG1 was 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM EDTA, and 0.1 mg/mL BSA, and that for mMPG and hMPG was 50 mM Hepes−KOH (pH 7.8), 100 mM KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 mg/mL BSA. The sample for mMPG and hMPG was further treated with 0.1 M NaOH at 70 °C for 5 min to cleave AP sites and was neutralized. Products were analyzed by denaturing PAGE as described above.

$NaBH_4$ Trapping Assays. dsDNA substrates (0.1 pmol) containing Tg, 8oxoG, or fU were incubated with Endo III, Endo VIII, Fpg (10 ng each), hNTH 1 (10−60 ng), or mNTH1 (10−60 ng) in the presence of 50 mM $NaBH_4$ at 37 °C for 30 min. The volume (10 μL) and compositions of reaction buffer were the same as those for the nicking assay described above except that the concentration of NaCl was 50 mM. After incubation, the sample was mixed with SDS loading buffer [100 mM Tris-HCl (pH 6.8), 8% SDS, 24% glycerol, 4% 2-mercaptoethanol, and 0.02% SERVA Blue G], heated, and separated by 10% SDS−PAGE.

Purification of fU-Excising Activity from Rat Liver. All procedures were performed at 4 °C or on ice. Fresh liver (ca. 100 g) from male rats (8−10 weeks old) was cut into small pieces with scissors and was suspended in 350 mL of homogenation buffer [50 mM Hepes−KOH (pH 7.5), 300 mM KCl, 3 mM EDTA, 5 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin]. The liver was homogenized by a Potter−Elvehjem homogenizer (1500 rpm). The homogenized solution was centrifuged at 100000*g* for 35 min, and the supernatant was recovered (fraction I). Fraction I was first precipitated with solid $(NH_4)_2SO_4$ to a concentration of 20% saturation. The resulting pellets were removed by centrifugation (7500*g* for 25 min). To the supernatant was further added solid ammonium sulfate to the final concentration of 50% saturation. The pellets were recovered by centrifugation and were suspended in 40 mL of buffer A [50 mM KP_i (pH 7.1), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol] containing 500 mM $(NH_4)_2SO_4$, yielding fraction II. Fraction II was diluted to 315 mL with buffer A containing 500 mM $(NH_4)_2SO_4$ and was applied to a phenyl-Sepharose CL-4B column (Amersham Biosciences, 250 mL). Protein was eluted with a stepwise gradient of $(NH_4)_2SO_4$ in buffer A at a flow rate of 7.5 mL/min. The concentration of $(NH_4)_2SO_4$ was 500 mM (elution volume 500 mL), 250 mM (750 mL), and 0 mM (1000 mL). Fractions were collected as 40 mL aliquots and were assayed for fU-excising activity as described below. The fractions containing relatively high activity (total 480 mL) were pooled as fraction III. Fraction III was dialyzed overnight against buffer B [20 mM Hepes−KOH (pH 7.3), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol] containing 20 mM NaCl. A half-volume of dialyzed fraction III was applied to a Macro-Prep High S Support column (Bio-Rad, 30 mL). The column was washed with 45 mL of buffer B containing 20 mM NaCl and was eluted with a linear gradient of NaCl (20−600 mM) in buffer B (total 150 mL) at a flow rate of 1 mL/min. Fractions were collected as 5 mL aliquots and were assayed for fU-excising activity. The same procedure was repeated twice. The fractions containing the activity (total 50 mL) were pooled as fraction IV. One-fourth volume of fraction IV was desalted by a HiPrep 26/10 desalting column (Amersham Biosciences, 53 mL). Elution was carried out with buffer B containing 20 mM NaCl at a flow rate of 10 mL/min. The same procedure was repeated four times. The fractions containing protein (detected by absorbance at 280 nm) were pooled (50 mL). A half-volume of the pooled fraction was loaded onto a DNA (denatured)−cellulose column (Amersham Biosciences, 5 mL). The column was washed with 8 mL of buffer B containing 20 mM NaCl and was eluted with a linear gradient of NaCl (20−600 mM) in buffer B (total 25 mL) at a flow rate of 0.8 mL/min. Fractions were collected as 1 mL aliquots and were assayed for the activities to fU and other substrates. The same procedure was repeated twice. The fractions containing fU-excising activity (total 13 mL) were pooled as fraction V. To reduce the volume, fraction V was desalted by a HiPrep 26/10 desalting column and applied to a DNA−cellulose column (5 mL). The column was washed with 8 mL of buffer B containing 20 mM NaCl and was eluted with 15 mL of buffer B containing 600 mM NaCl at a flow rate of 0.5 mL/min. One milliliter aliquots were collected. The pooled fraction containing protein (detected by absorbance at 280 nm, 4.5 mL) was applied to a Superdex 75 XK16/60 column (Amersham Biosciences, 120 mL). Protein was eluted with 180 mL of buffer C [20 mM Hepes−KOH (pH 7.3), 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol] at a flow rate of 0.1 mL/min. Fractions were collected as 2.5 mL aliquots and were assayed for fU-excising activity. The active fractions (total 10 mL) were pooled as fraction VI. The protein concentration was determined by a BCA protein assay kit (Pierce) using BSA as a standard.

Activity Assays of Column Fractions. Aliquots of the column fractions were dialyzed against buffer D [20 mM Hepes−KOH (pH 7.3), 20 mM NaCl, 1 mM EDTA] at 4 °C for 1 h using microdialysis cups (molecular weight cutoff 12000). dsDNA substrates containing an fU·A pair or other base lesions (when necessary) (0.1 pmol) were incubated with the dialyzed column fraction (25 μL) in a total volume of 100 μL at 37 °C for 30 min. The final composition of the reaction buffer was 20 mM Hepes−KOH (pH 7.3), 20 mM NaCl, 1 mM EDTA, and 0.1 mg/mL BSA (buffer E). The reaction was terminated by the addition of 1 M NaOH (final concentration 0.1 M). The reaction mixture was heated at 70 °C for 5 min to cleave AP sites and neutralized with 1 M acetic acid. The sample was extracted with an equal volume of phenol. DNA was precipitated by ethanol and suspended in PAGE loading buffer. Products were separated by 16% denaturing PAGE and quantitated on a phosphorimaging analyzer, Fuji BAS2000.

Characterization of Enzymatic Activity of FDG. Unless otherwise noted, all activity assays for FDG were performed after fraction VI was dialyzed against buffer D at 4 °C for 1.5 h. For analysis of substrate specificity, ssDNA and dsDNA substrates containing various base lesions (0.1 pmol) shown in Table 1 were incubated with FDG (fraction VI, 50 μL) in a total volume of 100 μL at 37 °C for 30 min. The final buffer composition of the reaction mixture was the same as buffer E. The sample was further treated and analyzed as described for the activity assay with column fractions. For competitive inhibition experiments, the activity of FDG for dsDNA containing an fU·A pair was assayed in a similar manner except that unlabeled competitor dsDNA (U·G, hoU·G, hmU·A, T·A) or ssDNA (ssU) was present

in the reaction mixture (5- or 10-fold molar excess). For antibody inhibition experiments, FDG (fraction VI, 50 μL) was incubated without or with hSMUG1 antibodies (1 μL of antiserum) for 10 min at 20 °C. After incubation, the activities of FDG for ssDNA and dsDNA substrates were assayed as described above. Antibody inhibition experiments for the cell extracts were performed in an essentially similar manner using fraction II (20 μL) and hSMUG1 antibodies or control rabbit IgG (1 μg, Sigma). To elucidate the effect of a uracil−DNA glycosylase inhibitor (Ugi), FDG (fraction VI, 50 μL) was preincubated with or without 1 unit of Ugi at 37 °C for 10 min. Then, FDG was incubated with dsDNA (fU·A, U·G) or ssDNA (ssU) substrates (0.1 pmol) as described above. The reaction with *E. coli* Ung was performed in an essentially similar manner except that the amount of Ung was 0.1 unit and the reaction was performed in 50 mM Hepes−KOH (pH 7.3), 10 mM NaCl, 1 mM EDTA, and 1 mM DTT. For the analysis of salt concentration dependence, fraction VI was dialyzed against buffer D (NaCl omitted) at 4 °C for 2.5 h. ssDNA and dsDNA substrates containing fU, hmU, hoU, and U (0.1 pmol) were incubated with the dialyzed fraction (50 μL) in a total volume of 100 μL at 37 °C for 30 min. The final composition of the reaction buffer was the same as buffer E except that the NaCl concentration was varied (5, 10, 25, 50, 75, 100, 125, 150 mM).

Preparation of Antibody and Western Blotting. Two New Zealand White rabbits were immunized with purified recombinant hSMUG1 protein (*41*). hSMUG1 protein (100 μg) in phosphate-buffered saline was mixed with complete (first immunization) or incomplete (subsequent immunization) Freund's adjuvant and was subcutaneously injected in rabbits at 2 week intervals for a total of five injections. After each boost, the serum was analyzed by an enzyme-linked immunosorbent assay for the development of antibodies. After the final boost, the sera of the two rabbits contained a significant titer of antibodies specific for hSMUG1. The antiserum from one rabbit was stored and used for experiments. Western blot analysis was carried out with hSMUG1 antibodies as follows. Proteins in the final column fraction (fraction VI) were precipitated by trichloroacetic acid. The samples [hSMUG1, rSMUG1, hNTH1 (50 ng each), and fraction VI (ca. 1 μg protein)] and prestained molecular weight markers (Bio-Rad) were separated by 10% SDS−PAGE and electrophoretically transferred to a nitrocellulose membrane. After being blocked with TBS [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl] containing 20 mg/mL BSA and 5 mM sodium azide, the membrane was incubated with hSMUG1 antibodies (1:50 dilution) in TBS containing 0.1 mg/mL BSA at 37 °C for 1 h and washed with TBS containing 0.05% (w/v) Tween 20. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000 dilution; Amersham Biosciences) in TBS containing 0.1 mg/mL BSA at 37 °C for 1 h and washed with TBS containing 0.05% (w/v) Tween 20. The membrane-bound antibody was detected using hydrogen peroxide and 3,3′-diaminobenzidine (Aldrich) as a chromogenic reagent.

Chromatographic Separation of FDG and NTH1 Activities in the Mouse Liver Cell Extract. The cell extract from mouse liver was prepared as described for that of rat liver. The fraction obtained by $(NH_4)_2SO_4$ precipitation (60% saturation) was dialyzed against buffer B containing 20 mM NaCl and applied to a Macro-Prep High S Support column (15 mL). The column was eluted with a linear gradient of NaCl (20 mM−1 M) in buffer B. An aliquot of the column fractions was assayed for the activity using dsDNA substrates containing fU·A or Tg·A pairs as described for the rat liver samples except that the concentration of NaCl of the reaction buffer was 50 mM. $NaBH_4$ trapping assays were performed using column fractions (4 μL), purified mNTH1 and hNTH1 as standards, and a dsDNA substrate containing Tg·A as described above.

Table 2: Kinetic Parameters of Tg- and 8oxoG−DNA Glycosylases for fU and Intrinsic Substrates

enzyme	substrate	damage	K_m[a] (nM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m[b] ($min^{-1}\,nM^{-1}$)
Endo III	19TG	Tg·A	16	0.83	5.2×10^{-2} (430)
	25FU	fU·A	370	0.043	1.2×10^{-4} (1)
Endo VIII	19TG	Tg·A	9.8	0.064	6.5×10^{-3} (50)
	25FU	fU·A	250	0.033	1.3×10^{-4} (1)
Fpg	25OG	8oxoG·C	2.1	0.19	9.0×10^{-2} (1200)
	25FU	fU·A	300	0.023	7.7×10^{-5} (1)
hNTH1	19TG	Tg·A	6.9	0.14	2.0×10^{-2} (50)
	25FU	fU·A	220	0.088	4.0×10^{-4} (1)
mNTH1	19TG	Tg·A	2.5	0.050	2.0×10^{-2} (74)
	25FU	fU·A	170	0.046	2.7×10^{-4} (1)

[a] Average of two independent experiments. [b] The value in parentheses indicates the relative k_{cat}/K_m for each enzyme.

RESULTS

Activity of Purified DNA Glycosylases for fU. To quantitatively compare the activity of Endo III, Endo VIII, Fpg, hNTH1, and mNTH1 for fU and their intrinsic substrates, enzymatic parameters for the base lesions were determined (Table 2). The k_{cat}/K_m values of Endo III, Endo VIII, hNTH1, and mNTH1 for fU were 50−430-fold lower than those for Tg. Similarly, the k_{cat}/K_m value of Fpg for fU was 1200-fold lower than that for 8oxoG. The low activity for fU was primarily due to a large decrease (23−143-fold) in the affinity of the enzymes for fU-containing DNA (i.e., an increase in K_m). An additional decrease in k_{cat} for fU relative to Tg or 8oxoG made fU a further unfavorable substrate for the enzymes, which was particularly the case for Endo III and Fpg. hOGG1, hMPG, and mMPG exhibited no detectable activity for fU, although they excised 8oxoG (hOGG1) and 7mG (hMPG and mMPG) from DNA very efficiently (data not shown).

The weak activity of the enzymes for fU was further confirmed by $NaBH_4$ trapping assays of a Schiff base intermediate formed between the substrate and enzymes (*34*−*36*, *42*, *43*). Figure 1A shows the results of the $NaBH_4$ trapping assay when the same amount of enzyme (10 ng) was incubated with dsDNA substrates containing Tg (Endo III and Endo VIII), 8oxoG (Fpg), and fU (Endo III, Endo VIII, and Fpg). Although trapped complexes were observed for all the substrates in SDS−PAGE analysis, the amount of the complex formed between fU-containing DNA and the enzyme (lanes 7−9) was by far lower than that formed for Tg-containing DNA (Endo III and Endo VIII, lanes 2 and 3) or 8oxoG-containing DNA (Fpg, lane 5). Incubation of hNTH1 with Tg-containing DNA resulted in a slow migrating band indicative of a trapped complex, and the amount of the complex increased with the increasing amount of hNTH1 protein (Figure 1B, lanes 2−5). A band of a trapped

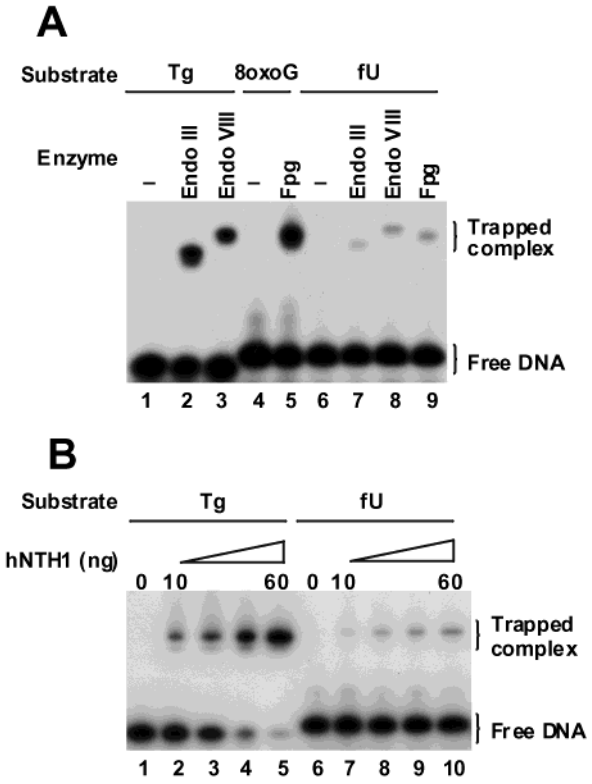

FIGURE 1: $NaBH_4$ trapping assays of reaction intermediates formed with intrinsic (Tg and 8oxoG) and fU substrates. (A) dsDNA substrates (0.1 pmol) containing Tg·A, 8oxoG·C, or fU·A were incubated with the indicated base excision repair enzymes (10 ng) and $NaBH_4$ (50 mM) at 37 °C for 30 min. The sample was separated by 10% SDS−PAGE, and the gel was autoradiographed. Substrates and enzymes used were indicated on the top of the gel. (B) Reactions were performed with hNTH1 in a manner similar to that of panel A except that the amount of hNTH1 was varied (10, 20, 40, and 60 ng).

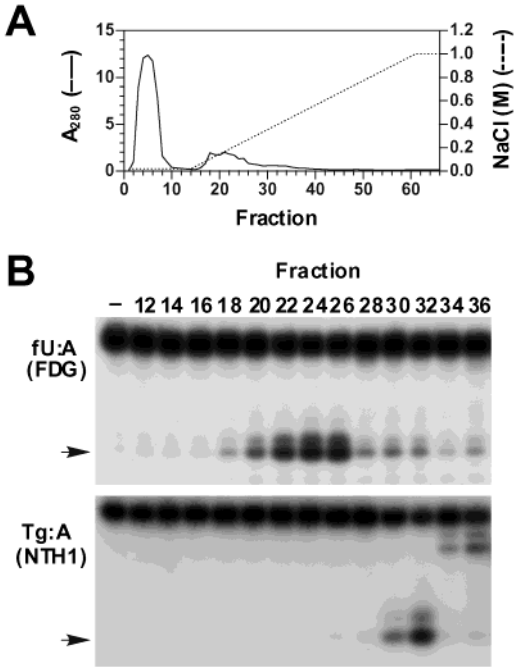

FIGURE 2: Chromatographic separation of mouse FDG and NTH1 activities. (A) The cell extract from mouse liver was applied to a High S Support column, and the column was eluted with a linear gradient of NaCl (20 mM−1 M). Key: solid line, protein elution profile monitored by A_{280}; dotted line, NaCl gradient. (B) PAGE analysis of FDG and NTH1 activities. An aliquot of the column fraction (25 μL) was incubated with dsDNA (0.1 pmol) containing fU·A (top gel) or Tg·A (bottom gel) in buffer E containing 50 mM NaCl (total volume 100 μL) at 37 °C for 30 min. The sample was treated with NaOH and separated by denaturing PAGE. Nicked products (β- and δ-elimination products) were indicated by arrows.

complex was also observed for fU-containing DNA, but the amount was very low relative to that for Tg-containing DNA (lanes 7−10). Tg-containing DNA was mostly converted to a trapped complex in the presence of an excessive amount of hNTH1 (e.g., hNTH1:DNA = 17:1, lane 5), whereas a very small fraction of fU-containing DNA was trapped under the same conditions (lane 10). Similar results were obtained with mNTH1 (data not shown). Therefore, the results of the parameter analysis and $NaBH_4$ trapping assays clearly indicate that Endo III, Endo VIII, Fpg, hNTH1, and mNTH1 excise fU from DNA very poorly.

Chromatographic Separation of FDG and NTH1 Activities. To analyze the contribution of NTH1 and other enzyme(s) to cellular fU-excising activity, the cell extract was prepared from mouse liver and separated by a High S Support cation-exchange column (Figure 2A). Analysis of repair activity in the column fractions revealed that the fU-excising and Tg-excising activities attributable to FDG and mNTH1, respectively, were eluted in separate fractions: FDG in fractions 20−26 and mNTH1 in fractions 30−32 (Figure 2B). The Tg-excising activity in the FDG fractions was virtually negligible. The presence of mNTH1 in the latter fractions was further confirmed by the SDS−PAGE analysis of a $NaBH_4$-trapped complex. The size of the trapped complex was comparable to those obtained with purified mNTH1 and hNTH1 (data not shown). These results clearly demonstrated that the cellular fU-excising activity was exclusively attributable to protein other than mNTH1.

Table 3: Purification of fU-Excising Activity (FDG) from Rat Liver

no.	fraction	vol (mL)	total protein (mg)	specific activity (pmol h^{-1} mg^{-1})	total activity (pmol/h)	purification (*x*-fold)
I	cell extract	380	8700	0.14	1200	1
II	ammonium sulfate	40	2600	0.24	620	1.7
III	phenyl-Sepharose	480	180	1.5	270	11
IV	High S Support	50	15	1.6	24	11
V	DNA−cellulose	13	0.72	7.1	5.1	51
VI	Superdex 75	10	0.13	23	3.0	160

Purification of FDG from Rat Liver. The fU-excising activity in the rat liver cell extract (fraction I) was purified by $(NH_4)_2SO_4$ precipitation followed by four chromatography steps (Table 3). In the first hydrophobic chromatography (phenyl-Sepharose), the fU-excising activity was eluted as a broad peak in 0 mM $(NH_4)_2SO_4$ elution. The activity was then purified by cation-exchange chromatography (High S Support) and affinity chromatography (DNA−cellulose), where the activity was eluted around 300 and 240 mM NaCl, respectively. In DNA−cellulose chromatography, the fU-excising activity was separated from the major protein peak and comigrated with the excision activities for hmU, hoU, and U in dsDNA substrates (Figure 3) (see also below). These activities were further copurified in the next gel filtration chromatography. In gel filtration chromatography (Superdex 75), the fU-excising activity was eluted in fractions

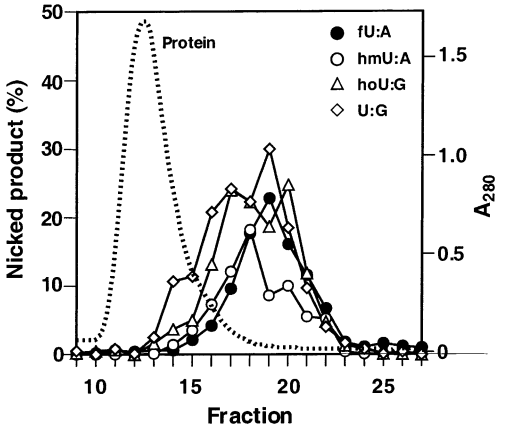

FIGURE 3: Elution profiles of damage-excising activities and protein in a DNA cellulose column. Fraction IV was separated on a DNA−cellulose column as described in Experimental Procedures. After dialysis against buffer D, an aliquot of the column fraction (25 μL) was incubated with dsDNA (0.1 pmol) containing various base lesions at 37 °C for 30 min. The sample was treated with NaOH, and the amount of nicked products was quantitated by PAGE analysis. Substrates: dsDNA containing fU·A (●), hmU·A (○), hoU·G (△), or U·G (◇). The elution profile of proteins (measured by A_{280}, right ordinate) is shown with a dotted line.

corresponding to a molecular weight around 35000 [calibrated with BSA (67000), ovalbumin (43000), and chymotrypsinogen (25000)]. In the final preparation (fraction VI), the fU-excising activity was purified 160-fold from the cell extract. The fU-excising activity tended to die in all chromatographic steps used (Table 3) so that the recovery of the activity from the cell extract was 0.25%. This also resulted in moderate purification of the activity (160-fold). SDS−PAGE analysis of fraction VI revealed several protein bands with molecular weights between 22000 and 51000 (data not shown). Therefore, the fU-excising activity could not be attributed to a particular protein band. The fU-excising activity in fraction VI was designated 5-formyluracil−DNA glycosylase (FDG).

Enzymatic Properties of FDG. To clarify whether the fU-excising activity is a monofunctional or bifunctional DNA glycosylase, a dsDNA substrate containing an fU·A pair was incubated with FDG (fraction VI), and products were analyzed by PAGE. A part of the FDG-treated sample was further treated with NaOH and was similarly analyzed. The band of a nicked product was very weak without NaOH treatment but was markedly enhanced by NaOH treatment (Figure 4A, lanes 2 and 4). These results indicate that FDG

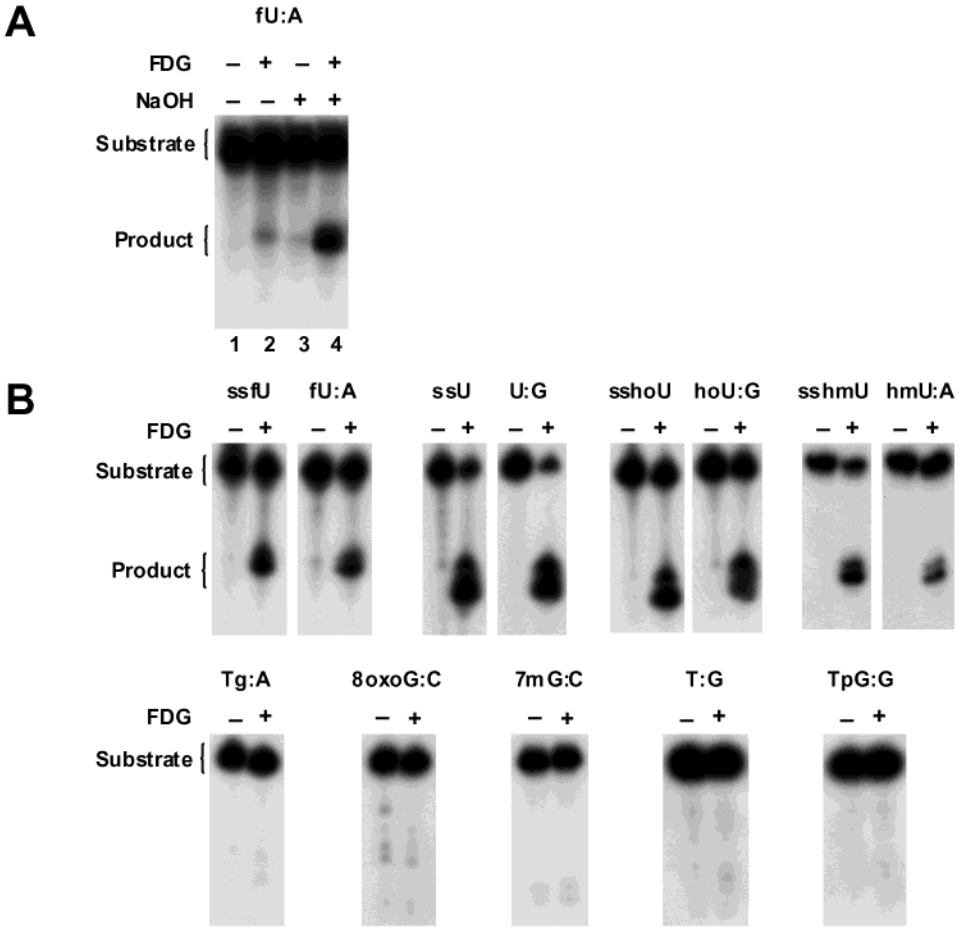

FIGURE 4: Analysis of substrate specificity of FDG. (A) The dsDNA substrate containing an fU·A pair (0.1 pmol) was incubated with FDG (fraction VI, 50 μL) at 37 °C for 30 min. A part of the sample was further treated with NaOH (0.1 M) at 70 °C for 5 min, neutralized with acetic acid (1 M), and analyzed by denaturing PAGE (lane 4). The rest of the sample was analyzed without NaOH treatment (lane 2). Treatment without (−) or with (+) FDG and NaOH was indicated on the top of the gel. (B) The ssDNA and dsDNA substrates containing various base lesions (0.1 pmol) were incubated with FDG (fraction VI, 50 μL) at 37 °C for 30 min. The sample was further treated with NaOH and analyzed by denaturing PAGE. Substrates and treatment without (−) or with (+) FDG were indicated on the top of the gel. Single-stranded substrates were indicated with a prefix ss (e.g., ssfU), and double-stranded substrates were indicated by base pairs (e.g., fU·A) present in DNA.

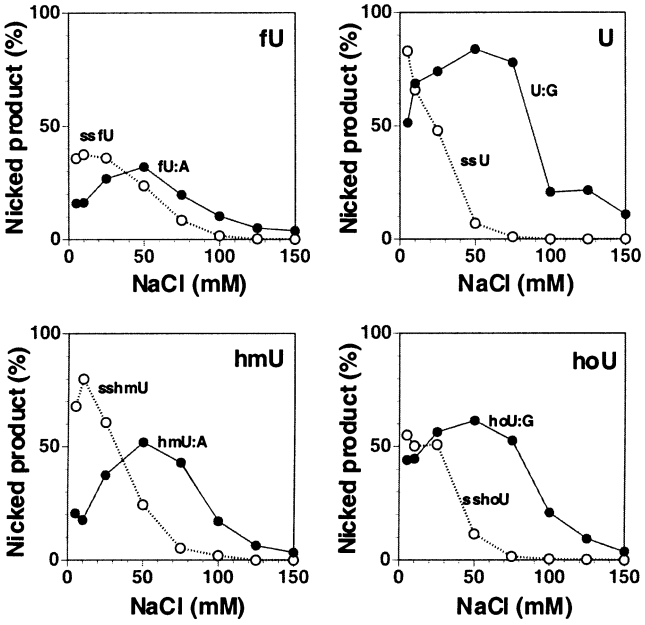

FIGURE 5: Salt concentration dependences of FDG for various substrates. The ssDNA and dsDNA substrates containing various base lesions (0.1 pmol) were incubated with FDG (fraction VI, 50 μL) in the presence of varying concentrations of NaCl (5, 10, 25, 50, 75, 100, 125, 150 mM) at 37 °C for 30 min. The sample was treated with NaOH, and nicked products were quantitated by PAGE analysis. The base lesion present in the substrate was indicated on the graph. Key: single-stranded substrates, ○; double-stranded substrates, ●.

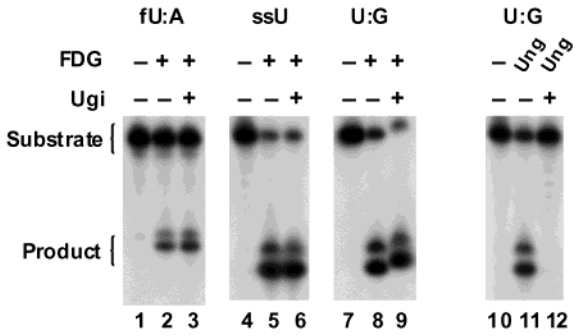

FIGURE 6: Effects of Ugi on the activity of FDG. FDG (fraction VI, 50 μL) was preincubated without or with Ugi (1 unit) at 37 °C for 10 min. Then, FDG was incubated with a ssDNA substrate containing U (ssU) or dsDNA substrates containing fU·A and U·G pairs (all 0.1 pmol) at 37 °C for 30 min. The sample was treated with NaOH, and products were analyzed by denaturing PAGE. Substrates and treatment without (−) or with (+) FDG and Ugi were indicated on the top of the gel (lanes 1−9). The rightmost panel shows the results of control reactions where dsDNA containing a U·G pair (0.1 pmol) was treated with *E. coli* Ung (0.1 unit) that was preincubated without (−) or with (+) Ugi (1 unit) (lanes 11 and 12).

is a monofunctional DNA glycosylase, leaving an intact AP site in DNA.

Damage specificity of FDG was systematically analyzed using ssDNA and dsDNA substrates shown in Table 1. FDG exhibited activities not only for fU·A but also for U·G, hoU·G, and hmU·A in dsDNA (Figure 4B, top panels). Furthermore, fU, U, hoU, and hmU in ssDNA were also the substrates of FDG (Figure 4B, ssfU, ssU, sshoU, and sshmU in the top panels). In PAGE analysis, nicked products for fU, U, hoU, and hmU were separated into two bands corresponding to β-elimination (slow migrating band) and δ-elimination (fast migrating band) products. Note that mild alkaline treatment of AP sites gave rise to these bands in a control reaction with Ung (see Figure 6). These bands were barely detected without NaOH treatment. Therefore, the mode of action of FDG was a simple DNA glycosylase and was not bifunctional ones such as NTH1, OGG1, and recently identified mammalian Endo VIII homologues (NEH1/NEI1 and NEH2/NEI2) (*6*−*10*). FDG did not exhibit any detectable activities for the substrates containing Tg·A, 8oxoG·C, and 7mG·C pairs and T·G and TpG·G mispairs (Figure 4B, bottom panels), which were the hallmarks of mammalian NTH1 (*35*, *37*, *38*), OGG1 (*34*), MPG (*39*), thymine−DNA glycosylase (TDG) (*44*), and methyl-CpG binding domain (MBD4) (*45*), respectively. FDG is also unlikely NEH1/NEI1 or NEH2/NEI2 since they recognize Tg (*8*−*10*).

The salt concentration dependence of FDG was notably different for ssDNA and dsDNA substrates (Figure 5). The activity for ssDNA (ssfU) favored low salt concentrations and showed an optimum around 10 mM NaCl, whereas that for dsDNA (fU·A) preferred relatively high salt concentrations and showed an optimum around 50 mM NaCl. Distinct salt concentration dependences for ssDNA and dsDNA were also observed for the substrates containing U (ssU and U·G), hmU (sshmU and hmU·A), and hoU (sshoU and hoU·G) (Figure 5). The overall features of the salt concentration versus activity profiles resembled those of fU. The final preparation of FDG (fraction VI) contained a uracil-excising activity for ssDNA and dsDNA (Figure 4B), implying that rat Ung might be present in the preparation. However, the activity for ssU and U·G was not inhibited by a uracil−DNA glycosylase inhibitor (Ugi) (Figure 6, lanes 6 and 9). The activity for fU·A was also insensitive to Ugi (lane 3). These results were in contrast to the fact that the activity of Ung (*E. coli*) was completely abolished by Ugi in a control reaction (lane 12). Considering that Ugi can efficiently inhibit both bacterial and mammalian uracil−DNA glycosylases (*46*), the uracil-excising activity in the FDG preparation was not rat UNG.

To elucidate whether the activity for fU and those for U, hoU, and hmU in the FDG preparation were attributable to a single enzyme or distinct enzymes, competition experiments were performed. For this purpose, labeled dsDNA containing an fU·A pair was incubated with FDG in the presence of a 5- or 10-fold excessive amount of cold competitor substrates (Figure 7). The amount of nicked products for fU·A was decreased by the competitors containing U·G, hoU·G, and hmU·A pairs in a concentration-dependent manner but not by the competitor containing no damage (T·A). The fU-excising activity was abolished almost completely by the addition of a 10-fold excessive amount of competitors containing U·G, hoU·G, and hmU·A pairs. These results indicate that the inhibition was damage-dependent and further suggest that the activity for fU and those for U, hoU, and hmU in the FDG preparation are attributable to the same enzyme. Interestingly, the fU-excising activity was not inhibited by a 10-fold excessive amount of ssDNA containing U that was also a substrate of FDG (see Discussion).

Inhibition of FDG Activity by hSMUG1 Antibodies. Polyclonal antibodies were raised against hSMUG1 and used in Western blot analysis of the FDG preparation (fraction

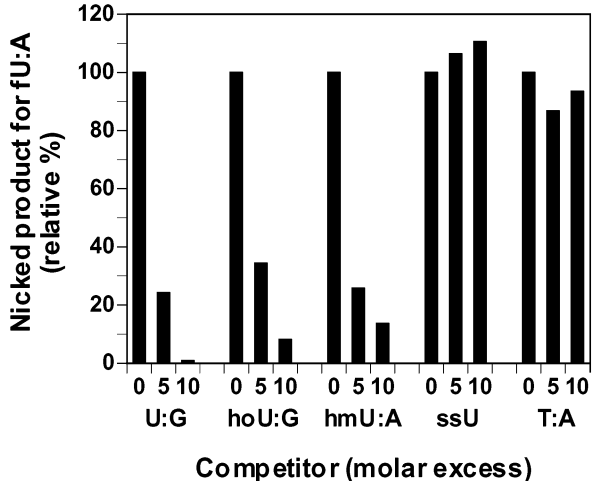

FIGURE 7: Inhibition of the fU-excising activity of FDG by competitor DNA. The dsDNA substrate containing an fU·A pair (0.1 pmol, ^{32}P-labeled) was incubated with FDG (fraction VI, 50 μL) in the absence and presence of competitor DNA (5- or 10-fold molar excess over the fU·A substrate, unlabeled) at 37 °C for 30 min. The competitor DNA was ssDNA containing U (ssU) or dsDNA containing U·G, hoU·G, hmU·A, and T·A pairs. The sample was treated with NaOH, and products were analyzed by denaturing PAGE. The amount of the nicked fU·A substrate (standardized to that obtained in the absence of competitors) was plotted against the amount of the competitors (molar excess over fU·A DNA).

VI). hSMUG1 antibodies cross-reacted with purified hSMUG1 and rSMUG1 but not with hNTH1 (Figure 8A, lanes 2, 3, and 5). A single band with a molecular weight comparable to that of rSMUG1 (=30000) was detected for FDG (lane 4), showing the presence of rSMUG1 in the FDG preparation. When FDG was preincubated with hSMUG1 antibodies, all of the activities for fU, hmU, hoU, and U in ssDNA and dsDNA were neutralized. Figure 8B shows typical gel data in the analysis of fU and hmU activities using hSMUG1 antibodies. Similarly, an inhibition effect of hSMUG1 antibodies was assessed using crude cell extracts obtained by ammonium sulfate precipitation (fraction II). hSMUG1 antibodies but not control IgG effectively neutralized the activities for fU·A and hmU·A in fraction II (Figure 8C).

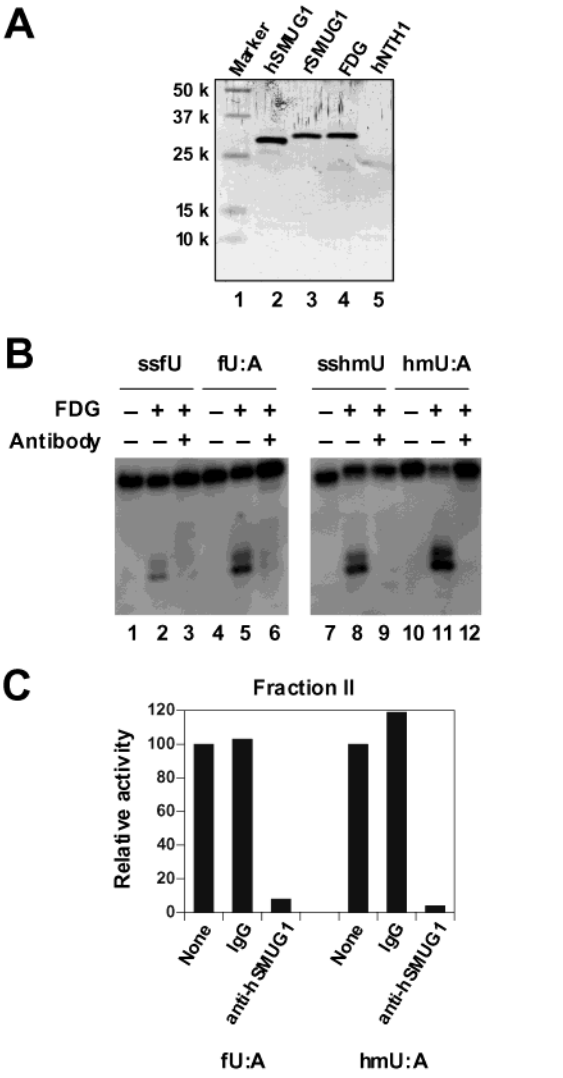

FIGURE 8: Characterization of FDG with hSMUG1 antibodies. (A) Western blotting analysis of the FDG preparation (fraction VI). hSMUG1 (50 ng, lane 2), rSMUG1 (50 ng, lane 3), hNTH1 (50 ng, lane 5), and the FDG fraction (ca. 1 μg of protein, lane 4) were separated by 10% SDS−PAGE. Proteins were transferred to a membrane and detected by polyclonal hSMUG1 antibodies. Lane 1 shows prestained molecular weight markers whose sizes were indicated on the left. (B) Neutralization of the activity of FDG for fU and hmU by hSMUG1 antibodies. FDG (fraction VI, 50 μL) was incubated without or with hSMUG1 antibodies (1 μL of antiserum) for 10 min at 20 °C. After incubation, FDG was incubated with ssDNA (ssfU, sshmU) or dsDNA (fU·A, hmU·A) containing fU and hmU, and products were separated by denaturing PAGE as described in Figure 4. The substrates and treatment without (−) with (+) FDG or hSMUG1 antibodies were indicated on the top of the gel. (C) Neutralization of the activity of cell extracts for fU and hmU by hSMUG1 antibodies. Crude cell extracts obtained by ammonium sulfate precipitation (fraction II, 20 μL) were incubated without or with control rabbit IgG or hSMUG1 antibodies (1 μL of antiserum) for 10 min at 20 °C. After incubation, the activities for dsDNA containing fU·A or hmU·A were assayed as described in Figure 4B. The activities were standardized to that obtained without IgG and hSMUG1 antibodies and plotted against the type of pretreatment.

DISCUSSION

In the present study, we have characterized mammalian 5-formyluracil−DNA glycosylase using two distinct approaches. In the first approach, the activity for fU was examined using purified DNA glycosylases. hNTH1 and mNTH1 showed a very weak activity for fU, but the parameter analysis and $NaBH_4$ trapping assays revealed that they were kinetically incompetent for repair of fU (Table 2 and Figure 1B). On the basis of the enzymatic parameters (k_{cat}/K_m) in Table 2 and those reported in the accompanying paper (*41*), hNTH1 excises fU approximately 20-fold less efficiently than the major cellular enzyme (hSMUG1; see below). Consistent with these data, the FDG activity of the mouse liver cell extract was chromatographically separated from mNTH1, a major repair enzyme for Tg (Figure 2B). hOGG1 and h(m)MPG did not exhibit any detectable activity for fU. Thus, NTH1, OGG1, and MPG are not involved in repair of fU in mammalian cells. This conclusion was further supported by the fact that the purified fraction of FDG from rat liver (fraction VI) did not contain the activities indicative of NTH1, OGG1, and MPG (Figure 4B) (see also below). In parallel experiments, it was also shown that Endo III, Endo VIII, and Fpg that were prokaryotic counterparts of NTH1,

NEH1/NEH2, and OGG1, respectively, were also kinetically incompetent for repair of fU.

In the second approach, FDG was purified from rat liver. Although the extent of purification was moderate (160-fold), the final fraction (fraction VI) allowed us to investigate several aspects of the enzymatic properties of FDG. FDG was a monofunctional DNA glycosylase (Figure 4A) and recognized fU in ssDNA and dsDNA (Figure 4B). These features are unusual for a DNA glycosylase that recognizes oxidized base lesions in light of the fact that such enzymes from *E. coli* (Endo III, Endo VIII, Fpg) and mammal (NTH1, NEH1/NEI1, NEH2/NEI2, OGG1) are bifunctional DNA glycosylases (*4*−*10*) and require dsDNA as a substrate (*4*−*9*; see also ref 10 for an exception). Fraction VI showed additional activities for U, hoU, and hmU in ssDNA and dsDNA but not for Tg, 8oxoG, 7mG, and mispaired T·G (both in CpG and non-CpG sequence contexts) (Figure 4B). Like fU, the activities for U, hoU, and hmU were all monofunctional DNA glycosylases. The lack of activities for Tg, 8oxoG, 7mG, and mispaired T·G ruled out the presence of rat homologues of NTH1 (and probably NEH1/NEI1 and NEH2/NEI2), OGG1, MPG, and TDG/MBD4 in fraction VI, respectively. The FDG activity was copurified with those for U, hoU, and hmU (Figure 3) and exhibited distinct salt concentration dependences for ssDNA and dsDNA (Figure 5). The profiles of the salt concentration dependence for fU were similar to those for U, hoU, and hmU (Figure 5). More importantly, the activity of FDG for fU was competitively inhibited by dsDNA containing U·G, hoU·G, and hmU·A pairs but not by dsDNA containing a T·A pair (Figure 7). From these results, we have concluded that FDG is a damage-specific monofunctional DNA glycosylase capable of excising not only fU but also U, hoU, and hmU in ssDNA and dsDNA.

So far, four mammalian DNA glycosylases have been identified that excise U from DNA. These include UNG, TDG, MBD4, and SMUG1 (*4*). TDG and MBD4 are mismatch-specific DNA glycosylases that recognize U·G and T·G mispairs and require dsDNA as a substrate (*44*, *45*). The latter feature clearly differentiates FDG and TDG/MBD4. Moreover, FDG did not show any detectable activity for T·G mismatches, the hallmark of TDG and MBD4. FDG and UNG were also differentiated since the uracil-excising activity of fraction VI was not inhibited by Ugi, a specific inhibitor of UNG (*46*). In contrast, the properties of FDG are consistent with those reported for SMUG1. First, although SMUG1 was originally identified as a monofunctional uracil−DNA glycosylase selective for ssDNA (*47*), it was shown active for U in dsDNA by the subsequent studies (*48*, *49*). Thus, like SMUG1, FDG recognizes base damage both in ssDNA and in dsDNA substrates. Second, there are conflicting reports on mammalian hmU−DNA glycosylase. Two reports showed that hmU−DNA glycosylase and SMUG1 were the same protein (*49*, *50*), whereas another one concluded that the enzyme was distinct from SMUG1 (also UNG and TDG) (*51*). To clarify whether FDG was a rat homologue of SMUG1 (rSMUG1), we raised polyclonal antibodies against hSMUG1. The antibodies cross-reacted with hSMUG1 and rSMUG1 (Figure 8). Western blotting analysis revealed the presence of rSMUG1 in the FDG preparation. Furthermore, the antibodies effectively neutralized all of the activities of FDG for fU, hmU, hoU, and U in ssDNA and dsDNA. Thus, we have concluded that FDG is rSMUG1.

It has been suggested that the primary role of SMUG1 in vivo is excision of U from a U·G mispair resulting from deamination of C in DNA, thereby serving as a backup enzyme for UNG (*48*, *52*). Since FDG and SMUG1 are the same protein, SMUG1 turns out to be a repair enzyme not only for U, a deamination product of C, but also for certain types of oxidized pyrimidine bases such as fU, hmU, and hoU that are not removed (or removed poorly) by NTH1 and OGG1. fU and hmU are produced by oxidation of the C5-methyl group of thymine, and hoU is formed by deamination and subsequent dehydration of unstable cytosine glycol. Unlike Tg, the base lesions (fU, hmU, and hoU) retain aromaticity and have an oxidized substituent group at C5 in common. Thus, FDG (SMUG1) may sense and use these features in damage recognition and catalysis. The size of rat FDG was roughly 35000 when estimated from gel filtration chromatography. This size is somewhat larger than those of human, mouse, and rat SMUG1 (ca. 30000) calculated from the deduced amino acid composition (*41*, *47*, *48*). It is not clear whether the discrepancy is due to a unique hydrodynamic property of SMUG1 protein, e.g., a deviation from globular protein. The activity of FDG for fU was inhibited by the dsDNA competitors containing U·G, hoU·G, and hmU·A pairs but not by the ssDNA competitor containing U (ssU) (Figure 7), though the latter was a substrate of FDG (Figure 4). The affinity of *Xenopus* SMUG1 for U in ssDNA (K_m = 1090 nM) is 31-fold lower than that for U·G in dsDNA (K_m = 35 nM) (*47*). Accordingly, it is reasonably expected that SMUG1 preferentially binds to a dsDNA substrate over a ssDNA substrate when both are present. Thus, the contrasting effects of dsDNA (U·G, hoU·G, hmU·A) and ssDNA (ssU) as competitors on the fU-excising activity further verify that FDG and SMUG1 are identical proteins.

In the accompanying paper (*41*), we will show that human and rat SMUG1 expressed from the corresponding cDNAs indeed excise a subset of oxidized pyrimidines (fU, hmU, and hoU) and U from ssDNA and dsDNA.

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