Properties of a Recombinant Human Uracil-DNA Glycosylase from the *UNG* Gene and Evidence that *UNG* Encodes the Major Uracil-DNA Glycosylase†

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Received August 1, 1994; Revised Manuscript Received October 11, 1994⊗

ABSTRACT: We have expressed a human recombinant uracil-DNA glycosylase (UNGΔ84) closely resembling the mature form of the human enzyme (UNG, from the *UNG* gene) in *Escherichia coli* and purified the protein to apparent homogeneity. This form, which lacks the first seven nonconserved amino acids at the amino terminus, has properties similar to a 50% homogeneous UDG purified from human placenta except for a lower salt optimum and a slightly lower specific activity. The recombinant enzyme removed U from ssDNA approximately 3-fold more rapidly than from dsDNA. In the presence of 10 mM NaCl, K_m values were 0.45 and 1.6 μM with ssDNA and dsDNA, respectively, but K_m values increased significantly with higher NaCl concentrations. The pH optimum for UNGΔ84 was 7.7−8.0; the activation energy, 50.6 kJ/mol; and the p*I* between 10.4 and 10.8. The enzyme displays a striking sequence specificity in removal of U from UA base pairs in M13 dsDNA. The sequence specificity for removal of U from UG mismatches (simulating the situation after deamination of C) was essentially similar to removal from UA matches when examined in oligonucleotides. However, removal of U from UG mismatches was in general slightly faster, and in some cases significantly faster, than removal from UA base pairs. Immunofluorescence studies using polyclonal antibodies against UNGΔ84 demonstrated that the major fraction of UNG was located in the nucleus. Furthermore, >98% of the total uracil-DNA glycosylase activity from HeLa cell extracts was inhibited by the antibodies, indicating that the UNG protein represents the major uracil-DNA glycosylase in the cells.

Uracil in DNA may result from incorporation of dUTP instead of dTTP during replication (Tye et al., 1977; Brynolf et al., 1978; Wist et al., 1978) or from deamination of cytosine in DNA (Lindahl & Nyberg, 1974; Shapiro, 1980). The latter process results in a premutagenic UG mismatch that, unless repaired before the next round of replication, will result in a GC to AT transition mutation. The DNA repair enzyme uracil-DNA glycosylase (EC 3.2.2.3), which hydrolyzes the bond between uracil and deoxyribose in DNA, was first detected in *Escherichia coli* and subsequently purified to homogeneity from the same source (Lindahl, 1974; Lindahl et al., 1977). An analogous enzyme activity was later detected in a number of organisms, including mammalian cells and tissues (Sekiguchi et al., 1976; Wist et al., 1978; Caradonna & Cheng, 1980; Talpaert-Borlè et al., 1982; Arenaz & Sirover, 1983; Wittwer et al., 1989). In human tissues a large interindividual and interorgan variation in activity has been reported (Myrnes et al., 1983; Krokan et al., 1983; Slupphaug et al., 1992). *Escherichia coli* mutants in the *ung* gene show a 5-fold increased spontaneous mutation rate (Duncan & Miller, 1980) rising to 30-fold at certain bases (Duncan & Weiss, 1982), whereas a 20-fold increase in spontaneous mutations has been found in similar yeast mutants (Impellizzeri et al., 1991). Similar mutants in mammalian cells are not available. Cloning of uracil-DNA glycosylase from *E. coli* (Varshney et al., 1988), *Streptococcus pneumoniae* (Mèjean et al., 1990), *Saccharomyces cerevisiae* (Percival et al., 1989), different herpes viruses (Baer et al., 1984; Davison & Scott, 1986; McGeoch et al., 1988; Worrad & Caradonna, 1988), and human placenta (Olsen et al., 1989) has demonstrated that the enzyme is highly conserved among different species. Two other human cDNAs possibly encoding uracil-DNA glycosylase activities have been reported (Meyer-Siegler et al., 1991; Muller & Caradonna, 1991), which are unrelated to the group of homologous uracil-DNA glycosylases, as well as to each other. There has, however, been considerable controversy as to which of the human cDNAs encodes the major and nuclear uracil-DNA glycosylase.

In eukaryotic cells the major fraction of uracil-DNA glycosylase activity is located in the nuclei with a minor fraction in the mitochondria (Anderson & Friedberg, 1980; Gupta & Sirover, 1981; Colson & Verly, 1983; Wittwer & Krokan, 1985). cDNA for human conserved uracil-DNA glycosylase (*UNG15*) (Olsen et al., 1989) encodes a major human uracil-DNA glycosylase (Slupphaug et al., 1991). Recent results have demonstrated that the gene corresponding to this cDNA encodes both the nuclear and the mitochondrial form (Slupphaug et al., 1993), a finding that also explains the similar biochemical characteristics of the two forms in HeLa cells (Wittwer & Krokan, 1985). The primary transla-

† This study was financially supported by the Norwegian Research Council, the Norwegian Cancer Society, the Blix family fund, Rakel and Otto Bruun's fund, Grete Harbitz' fund, and the Nansen fund.

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1994.

0006-2960/95/0434-128$09.00/0

tion product of human uracil-DNA glycosylase based on the open reading frame in *UNG15* was predicted to have a size of 304 amino acids. A species of a size in agreement with this was detected as a minor activity in the cytosol (Slupphaug et al., 1993). Prior to translocation to nuclei and mitochondria, an amino-terminal presequence of 77 amino acids is removed by a mechanism that has not been elucidated. This presequence appears to be necessary for import to the mitochondria, but not for nuclear import (Slupphaug et al., 1993).

In general, uracil-DNA glycosylases have a preference for single-stranded uracil-containing DNA as a substrate and are inhibited by uracil. Furthermore, the rate of removal of uracil is sequence specific for *E. coli,* calf thymus, and rat liver UDG (Delort et al., 1985; Domena et al., 1988; Eftedal et al., 1993), with similar specificities for the former two enzymes (Delort et al., 1985; Eftedal et al., 1993). In addition, uracil appears to be removed faster from UG mismatches than from UA matches in some sequence contexts (Verri et al., 1992), but not all (Eftedal et al., 1993). In the present paper, we have purified recombinant human uracil-DNA glycosylase in good yield and characterized the enzyme with respect to various kinetic parameters, as well as sequence specificity. We find that human uracil-DNA glycosylase displays a striking sequence specificity that in general is similar for removal of U from UA matches and UG mismatches. However, for most sequences removal of U is slightly faster from mismatches than from matches, and for some sequences the removal from mismatches is significantly faster. These data indicate that sequence-specific repair may be a determinant to be considered in mutagenesis. Furthermore, we demonstrate that >98% of the total uracil-DNA glycosylase activity in HeLa cell extracts is inhibited by antibodies raised against purified recombinant UNGΔ84, strongly indicating that UNG[1] represents the major uracil-DNA glycosylase in the cells.

EXPERIMENTAL PROCEDURES

Materials.[2] The expression vector pTrc99A was purchased from Pharmacia Biotechnology Inc., and pGEM7Zf+ was from Promega (Madison, WI). Restriction and DNA-modifying enzymes were from New England Biolabs (Beverly, MA). [α-^{35}S]dATP, [γ-[^{33}P]ATP, and [^{35}S]methionine were from DuPont (Germany), and [^{3}H]dUTP was from Amersham (U.K). Antibiotics, IPTG, and protamine sulfate were from Sigma. Oligonucleotides were from Genosys Biotechnologies Inc., (TX), and linkers were from Promega. *Escherichia coli* NR8052 [Δ(*pro-lac*), *thi*$^-$, *ara*, *trpE9777*, *ung1*] (Kunkel, 1985; Varshney & van de Sande, 1989) was provided by Dr. Thomas A. Kunkel, National Institute of Environmental Health Sciences (NC). Media for bacterial culture were from DIFCO (Detroit, MI), and media for human cell culture were from GIBCO (Gaithersburg, MD). Antibodies and streptavidin−FITC were from DAKO (Denmark). M13mp18 DNA, M13 primer, and Sequenase sequencing kit were from United States Biochemical (Cleveland, OH).

Plasmid Constructions. The *E. coli* expression vector pTrc99A has a strong *trc* promoter upstream of the multiple cloning site and a strong transcription termination signal (*rrnB*) downstream. It also contains the *lac* I^q gene, allowing the vector to be used in hosts lacking the lactose repressor gene (Amann et al., 1988). The plasmid pUNG15 contains a full-length cDNA encoding human uracil-DNA glycosylase (Olsen et al., 1989). To facilitate subcloning of *UNG15* in pTrc99A, *Sal*I linkers were introduced in *UNG15* at positions 57 (*Rsr*II) and 1064 (*Hpa*I), and the resultant fragment was ligated into pTrc99A after *Sal*I digestion. The ligation mixture was used to transform *E. coli* DH5α, and transformants having correct orientation of the insert (pTUNG) were used in deletion experiments. pTUNG was isolated by CsCl-gradient centrifugation and extracted with acidic phenol (pH 4.8) to remove nicked plasmids. After *Sac*I/*Xba*I digestion of pTUNG, progressive deletions were introduced in the 5′-region of *UNG15* using the Erase-A-Base system (Promega). The exonuclease III digestion step was performed at 20 °C, and samples were withdrawn at 1-min intervals. After S1 nuclease treatment and religation, deletion mutants were used to transform the *ung*$^-$ *E. coli* strain NR8052. Transformants (approximately 200) were screened by restriction analysis, and selected clones were assayed for UDG activity after induction with IPTG. The 5′-region of interesting *UNG* inserts was analyzed by DNA sequencing to determine the structure of the construct.

Growth Conditions and Induction of the trc Promoter. For routine screening of deletion mutants, transformed *E. coli* NR8052 was grown in 5 mL of LB medium with 100 μg/mL ampicillin (5% inoculum) at 37 °C. Induction of the *trc* promoter was carried out after 2 h by addition of 1 mM IPTG (final), and the culture was continued for 2 h. One-milliliter samples were centrifuged at 2000*g* for 5 min (4 °C) and resuspended in 2 mL of 20 mM Tris-HCl (pH 7.5), 60 mM NaCl, 1 mM EDTA, and 1 mM DTT. Cells were disintegrated for 5 min on ice in a Branson Sonifer sonicator at output control 3.5 and 20% duty cycle. Extracts were centrifuged at 11000*g* for 10 min (4 °C) and assayed for UDG activity. On the preparative scale, UDG expression was studied using 14-L fermenters (Chemap). Bacteria were grown in 7 L of medium containing 3× LB, 15 g/L glycerol, and 100 μg/mL ampicillin at 37 °C and 130−913 rpm to a cell density corresponding to OD_{660} = 6.0. Induction of the *trc* promoter was carried out by addition of 1 mM IPTG, and the culture was continued for 2 h before cell harvesting.

To analyze possible plasmid loss during fermention, 0.1 mL of bacterial suspension was withdrawn at the time of harvest. Serial dilutions were plated on LB medium without ampicillin, and the bacteria were incubated at 37° overnight. One hundred colonies were transferred to plates containing 100 μg/mL ampicillin. After an overnight incubation, viable colonies were counted to determine the percentage of plasmid loss.

[1] Abbreviations: BSA, bovine serum albumin; CM, carboxymethyl; DEAE, diethylaminoethyl; ds, double stranded; DTT, dithiothreitol; EDTA, ethylenedinitrilotetraacetic acid (disodium salt); ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; ss, single stranded; UDG, uracil-DNA glycosylase; UNG, human uracil-DNA glycosylase encoded by *UNG15* (Olsen et al., 1989).

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by our laboratory, nor does it imply that the materials and equipment identified are necessarily the best available for the purpose.

Purification of Uracil-DNA Glycosylase. All operations were carried out at 4 °C if not otherwise stated. All buffers were thoroughly degassed, and PMSF and DTT were added just prior to usage. The frozen bacterial cell pellet (75 g) was thawed and resuspended in 190 mL of 50 mM Tris−acetate (pH 7), 10 mM NaCl, 0.01% NaN_3, 0.5 mM PMSF, and 1 mM DDT (buffer A). Cells were disintegrated in a Dyno-Mill type KDL (Willy A. Bachofen AG, Basel) with 0.1-mm glass beads for 15 × 3 min with a 1-min pause between cycles. Cell disintegration was verified by phase-contrast microscopy. The cell homogenate was centrifuged at 5900*g* for 10 min, and the pellet was washed with 100 mL of buffer A and recentrifuged as described. To the combined supernatants (crude extract, 325 mL) was added 50 mL of 4% protamine sulfate in buffer A, and the extract was thoroughly mixed for 5 min. Precipitates were pelleted at 5900*g* for 10 min, and the supernatant was treated with 25 mL of 4% protamine sulfate and centrifuged as before.

The protamine sulfate fraction (350 mL) was loaded onto a DEAE-cellulose column (DE-52, Whatman, 5 × 9 cm) (diameter x height values are given throughout) coupled in series with a CM Sephadex C-50 column (Pharmacia, 2.5 × 13 cm) and preequilibrated with buffer A. Flow was maintained at 0.8 mL/min. After loading of the extract and a 40-mL wash with buffer A, the DE-52 column was bypassed and the CM Sephadex column was washed with the same buffer until zero absorbance at 280 nm was reached. The adsorbed proteins were eluted in a 320-mL linear gradient of NaCl (0−0.4 M) in buffer A. Fractions containing uracil-DNA glycosylase activity were pooled (peak activity eluted at approximately 0.15 M NaCl).

The CM Sephadex fraction (48 mL) was concentrated by ultrafiltration to 3.9 mL and applied to a HiLoad 16/60 Superdex 75 prep grade column (Pharmacia), preequilibrated with buffer A containing 150 mM NaCl to avoid nonspecific adsorbtion of UDG and eluted with the same buffer at a flow rate of 0.8 mL/min. Fractions containing uracil-DNA glycosylase activity were pooled (peak activity eluted at 77 mL).

One-third of the Superdex 75 fraction was diluted with 2 vol of 20 mM Tris−acetate (pH 7) and 1 mM DTT (buffer B) and applied to a MonoS HR 5/5 column (Pharmacia) at room temperature. Flow was maintained at 0.2 mL/min. After a thorough wash with the same buffer, adsorbed proteins were eluted in a 20-mL linear gradient of NaCl (0−0.4 M) in buffer B. Fractions were collected on ice, and fractions containing uracil-DNA glycosylase activity were pooled (peak activity eluted at 0.12 M NaCl).

In Vitro Transcription/Translation. To obtain quantification of the specific activity of the N-terminally deleted UDG products, deletion mutants were subcloned in pGEM7Zf+ (Promega), and coupled *in vitro* transcription/translation in rabbit reticulolysates was performed using the TNT system (Promega). The UDG activity was assayed and compared to the level of [^{35}S]Met-labeled UDG produced in parallel reactions as determined by laser densitometry on an LKB Ultroscan XL laser densitometer (Pharmacia) after SDS−PAGE and fluorography.

Assays. Unless otherwise stated, UDG activity was measured in 20 μL of assay mixture containing 10 μL of assay buffer (60 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT), 5 μL (10.4 μM) of [^{3}H]dUMP-containing DNA (sp act. 0.5 mCi/mmol) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaCl, and 5 μL of UDG extract diluted in assay buffer (referred to as standard conditions). The mixture was incubated for 10 min at 30 °C, and the amount of uracil released was measured as described (Krokan & Wittwer, 1981). UDG activity after *in vitro* transcription/translation was measured after dilution of the reticulolysate reactions with equal amounts of assay buffer. One unit of UDG was defined as the amount of enzyme that released 1 nmol of uracil from the substrate per minute at 30 °C. Protein concentration was measured using the Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as standard. Michaelis constants and reaction velocities were measured in the presence of 0.5−10 μM [^{3}H]-dUMP-containing DNA (specific activity as above). Single-stranded substrate was prepared by heating the dsDNA substrate at 100 °C for 10 min and then rapidly chilling it on ice. The UDG concentrations were 55 and 275 pg per 20-μL reaction mixure for ss- and dsDNA, respectively, and the NaCl concentrations were 10, 30, and 60 mM. The effect of uracil inhibition was investigated in the presence of 2 and 5 mM uracil at each of the above NaCl-concentrations.

Polyclonal Antisera and Immunocytochemical Analysis. Purified recombinant UDG was emulsified in Freund's complete adjuvant and injected into chinchilla rabbits (Dr. Carl Thomae, Germany). Five hundred micrograms of UDG was administered subcutaneously three times at about 4-week intervals (the last two injections were with Freund's incomplete adjuvant). The antisera were collected 14 days after the last injection, and the serum lipids were removed using trichlorotrifluoroethane (Frigen, Hoechst). The IgG fractions were purified by affinity chromatography on protein A HiTrap columns (Pharmacia) using the FPLC system. The IgG fraction giving the highest titer in ELISA was designated PU101 and used for immunocytochemical staining. $HeLaS_3$ cells were grown on glass coverslips as previously described (Slupphaug et al., 1993). After a brief wash in PBS, cells were fixed in freshly prepared 4% paraformaldehyde for 10 min at room temperature. Cells were then washed in PBS for 3 × 5 min, followed by 0.2% Triton X-100 in PBS for 2 min and a washing step as described. After blocking with 0.5% BSA and 2% FCS in PBS, cells were incubated in 8.7 μg/mL PU101 in PBS + 0.5% BSA for 1 h. Cells were also incubated in parallel with preimmune IgG from the same rabbit. After a 5 × 5 min wash in PBS, cells were incubated with biotinylated goat × anti-rabbit secondary antibodies (Dako, 1:100 in PBST) for 1 h. Cells were then washed for 5 × 5 min in PBS and incubated with fluorescein-conjugated streptavidin (Dako, 1:100 in PBST) for 30 min. After a final 3 × 5 min wash in PBS, coverslips were mounted in Mowiol (Heimer & Taylor, 1974) and photographed using a standard Nikon immunofluorescence microscope equipped for fluorescein. For analysis of specific UDG inhibition by P101, $HeLaS_3$ cells were cultured in Dulbecco's modified Eagle's medium and harvested as previously described (Slupphaug et al., 1993). Approximately 10^7 cells were resuspended in 2 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 60 mM NaCl, 1 mM EDTA, and 1 mM DTT) and sonicated on ice in a Branson Sonifer until organelles were completely disrupted, as judged by phase-contrast microscopy. To half of the cell homogenate were added Triton X-100 and NaCl to final concentrations of 0.5% and 0.5 M, respectively, and both of the homogenates were centrifuged in a microfuge at 25000*g* for 30 min at 4 °C. Both supernatants were then

thoroughly dialyzed against extraction buffer (4 °C), and the dialyzed homogenates were diluted in extraction buffer to a final UDG concentration of 0.2 unit/mL. To 4 μL of HeLa extract was added 11 μL of various dilutions of PU101 or preimmune PU101 in extraction buffer, and the mixtures were incubated on ice for 10 min. Five microliters of UDG substrate was then added, and the UDG assay was performed as described.

Substrates for Analysis of Sequence Specificity. Double-stranded M13 DNA containing on average one dUMP per 300−400 base pairs (matched to A residues) was prepared as described (Eftedal et al., 1993). Single-stranded PAGE-purified synthetic oligodeoxyribonucleotides containing dUMP at fixed positions were 5′-end-labeled using [γ-^{33}P]ATP and T4 polynucleotide kinase and annealed to their complementary strands containing either adenine or guanine opposite uracil as described (Eftedal et al., 1993). The same procedure was employed to generate a substrate to test for excisional activity of human UDG on thymine in GT mispairs. For analysis of uracil excision, 0.5 pmol of M13 double-stranded dUMP-DNA or 0.9 pmol of dUMP oligonucleotides was mixed with 5×10^{-3} unit (M13 DNA) or 2×10^{-3} unit (oligonucleotides) of recombinant human UDG in a buffer containing 60 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.1 mg/mL BSA, and 10 mM EDTA in a total volume of 20 μL and incubated at 37 °C for 5−60 min. The reaction was stopped by addition of 100 μL of 1.2 M piperidine, and the mixture was immediately incubated at 90 °C for 30 min to cleave at apyrimidinic sites as described (Brash, 1981). The samples were resuspended in loading buffer containing 57% formamide, 12 mM EDTA, and 0.03% each of bromophenol blue and xylene cyanol prior to electrophoresis. Electrophoresis was performed in 7% 0.45 mm thick (M13 DNA) or 8% 0.75 mm thick (oligonucleotides) polyacrylamide gels containing 7 M urea in 50 mM Tris−borate buffer. After fixation and drying, gels were exposed on β-max film (Amersham, U.K.) without intensifying screens. Band intensities on the films were detected and analyzed using laser densitometry as described.

DNA Sequencing. DNA sequencing for the screening of deletion mutants was done with a Macrophor apparatus (Pharmacia). Sequenase version 2.0 (U.S. Biochemicals) was used, according to the manufacturer's protocol. Template DNA was denatured by alkaline pH (Chen & Seeburg, 1985).

Protein Amino-Terminal Sequence Analysis. Purified recombinant UDG was thoroughly dialyzed against 50 mM NH_4HCO_3 and subjected to 7 cycles of Edman degradation using an Applied Biosystems 477A Sequenator.

SDS−Polyacrylamide Electrophoresis and Isoelectric Focusing. SDS−PAGE was performed on the Pharmacia Phast system in 10−15% gradient gels and the gels were silver stained according to the manufacturer's protocol. Isoelectric focusing was performed in a Rotofor preparative isoelectric focusing cell (Bio-Rad) using 2% Pharmalyte 8−10.5 carrier ampholytes (Pharmacia). The ampholytes were prefocused for 2 h at 12 W prior to addition of 500 μg of UNGΔ84, and the focusing was continued for 3 h at 12 W before harvesting. The pH in each compartment was measured with a Radiometer PHM92 pH meter equipped with a GK 2421C electrode, and samples from each focusing compartment were adjusted to pH 7.5 and assayed for UDG activity. Isoelectric focusing was also performed in Pharmacia dry IEF gels rehydrated in 6.25% Pharmalyte 8−10.5 and stained with coomassie brilliant blue. For densitometric scanning of [^{35}S]-Met-labeled mutants, SDS−PAGE was performed in 12% (separating gel) and 4% (stacking gel) polyacrylamide gels on the Mini-Protean II system (Bio-Rad) according to the manufacturer's protocol. Gels were immersed in Amplify (Amersham, U.K.) for 30 min before drying.

RESULTS

Expression of Human Uracil-DNA Glycosylase in E. coli. Using a standard exonuclease digestion technique, we have prepared a series of constructs lacking increasing portions of the 5′-part of human UNG cDNA (Figure 1). The purpose of this was threefold: (a) to investigate the possible significance of the 77 amino acid presequence for enzymatic activity, (b) to obtain a construct that permitted good expression of human UDG in *E. coli*, and (c) to examine how much of the amino-terminal portion of the mature form, if any, could be removed before activity was lost. The truncated inserts obtained after nested deletions gave rise to proteins lacking between 28 and 107 residues of the N-terminal part of *UNG15* and in addition containing Met-Glu-Phe encoded by the vector. Although the starting construct for the deletion analysis encoded 33 additional N-terminal amino acids as compared to *UNG15*, UDG activity was readily observed after expression of this construct in *E. coli* (Table 1). However, a construct encoding full-length UNG lacking the additional residues (obtained by construction of an *Nco*I site at the ATG start codon by site-directed mutagenesis and subcloning of the resultant *UNG15* fragment in pTRc99A) did not yield UDG expression in *E. coli*, although the sequence context upstream of the start ATG codon was identical to that of all clones in Table 1 as verified by sequencing. Western analysis demonstrated that this was because no UNG-related protein was expressed in *E. coli*. Expression of the full-length cDNA in reticulolysates with the *Nco*I mutant, however, yielded readily detectable UDG activity (data not shown).

Considerable variation in UDG activity was observed in the deletion clones. The highest UDG expression was obtained with pTUNGΔ84, which lacked the 77-residue N-terminal presequence and the first seven nonconserved residues of the presumed mature region of UDG (Olsen et al., 1989). Extending the deletions beyond this point gave a sharp reduction in expressed activity, and when 104 or more amino acids were deleted, UDG activity was completely lost (Table 1). To find whether the observed differences in expressed UDG activity in *E. coli* were due to large changes in catalytic activity of the enzyme or rather reflected variations in translational efficiency, the deletion mutants were subcloned in pGEM7Zf+ containing a T7 promoter upstream of the *UNG* insert and expressed in an *in vitro* transcription/translation rabbit reticulolysate system. Uracil-DNA glycosylase activity was expressed in all mutants lacking up to 93 N-terminal residues (no rabbit UDG activity was observed in the transcription/translation system). Each of the mutants gave rise to a single new [^{35}S]Met-labeled protein band (as compared to the background labeling generated from lysate only) of the expected size after SDS−PAGE and fluorometry (Figure 2), excluding translation initiation from alternative AUG codons in *UNG* mRNA. The results furthermore demonstrated that the first 93 residues of the preprotein (including 16 residues of the mature protein)

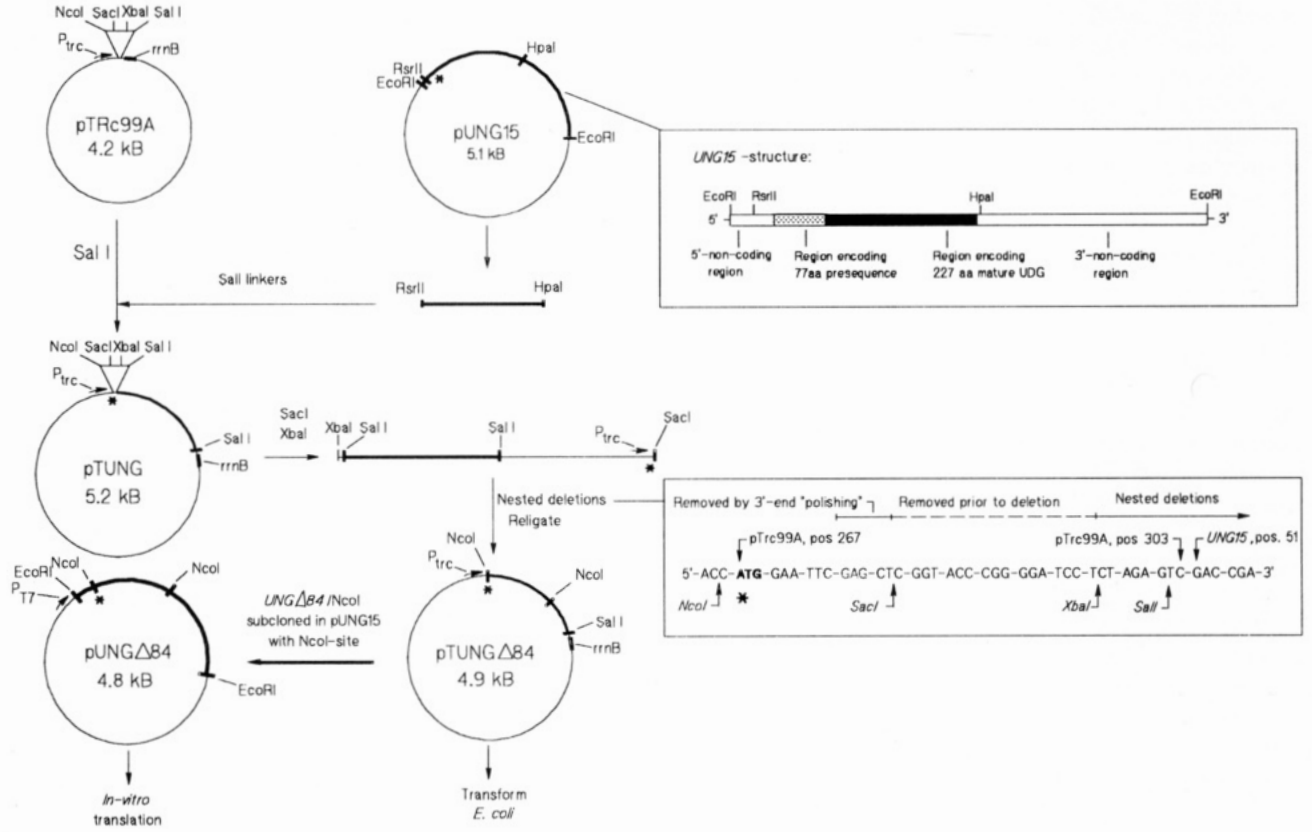

FIGURE 1: Structure of *UNG15* and construction of plasmids pTUNGΔ84 and pUNGΔ84. Thick dark lines indicate cloned DNA. Asterisks indicate the positions of start codons. The gene structure and coding sequence of *UNG15* are given in the upper box. Sequences removed during nested deletions are indicated in the lower box. The AUG codon originating from pTRc99A is in bold.

Table 1: Uracil-DNA Glycosylase Activity in Cell-Free Extracts from Deletion Mutants after Expression in *E. coli* NR 8052[a]

no. of N-terminally deleted amino acids	UDG activity (units/mg)
0[b]	0.6 ± 0.1
28	1.0 ± 0.4
35	1.0 ± 0.4
39	4.8 ± 0.5
53	3.2 ± 1.0
57	9.3 ± 3.8
73	1.8 ± 0.1
75	2.2 ± 0.1
84	92.5 ± 18.0
93	0.8 ± 0.2
104	0.0 ± 0.0
106	0.0 ± 0.0
107	0.0 ± 0.0

[a] Values are the mean of 2 or 3 independent experiments each assayed in duplicate, with standard deviations as indicated. [b] The start construction encoded 33 amino acid residues in addition to UNG15 encoded by the polylinker and codons upstream of the *UNG15* start codon.

were not essential for enzyme activity. When the expressed UDG activities after *in vitro* translation were related to the respective amounts of [^{35}S]Met-labeled UDG produced, all deletion mutants lacking segments of various length N-terminal to residue 93 showed higher relative activity as compared to nondeleted UNG15 (ranging from 1.6- to 2.2-fold). The highest relative activity was observed with UNGΔ84, which also gave the highest expression in *E. coli*. This clone was therefore chosen for preparative-scale expression. When more than 104 residues were deleted, UDG activity was completely lost, although *in vitro* expression of UDG remained essentially unchanged.

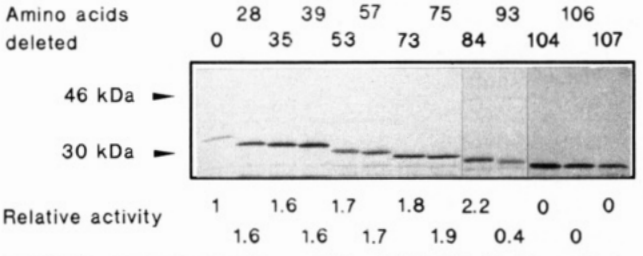

FIGURE 2: Analysis of relative activity of UNG15 deletion mutants. SDS−PAGE, fluorography, and densitometric scanning were as described in Experimental Procedures. The activity of each mutant is given relative to the specific activity of nondeleted UNG15. Additional minor bands observed at 45 and 27 kDa were background bands originating from the lysate itself.

In fermenter experiments several parameters such as medium composition, carbon source, cell density during induction, and induction time were investigated (data not shown). The optimal total UDG activity was obtained in 3× LB medium supplemented with 15 g/L glycerol and 100 μg/mL ampicillin and addition of 1 mM IPTG at OD_{660} = 6.0. Under these conditions a yield of 5.3 g of biomass/L (dry weight) was obtained 2 h after induction (OD_{660} = 12.3). The specific activity in the cell-free extract (sonicated) was 108 units/mg. No plasmid loss from the cells was observed at the end of the fermentation, indicating that pTUNGΔ84 was stable in the host cells under these conditions (data not shown).

Purification of Uracil-DNA Glycosylase. Recombinant UNGΔ84 was purified from a 7-L batch culture of *E. coli* NR8052 transformants as described (Table 2). A sample from each purification step was analyzed by SDS−PAGE and silver staining (Figure 3), and UNGΔ84 migrated as one

Table 2: Purification of Recombinant Human Uracil-DNA Glycosylase[a]

fraction	total act. (units)	total protein (mg)	sp act. (units/mg)	yield (%)	purification (x-fold)
crude extract	465 000	3820	122	100	
protamine sulfate	466 000	1760	265	100	2.2
DE 52/CM Sephadex	303 000	108.4	2800	65	23
Superdex 75	277 000	65.2	4200	60	34
MonoS[b]	78 200	18.2	4300	35	

[a] Uracil-DNA glycosylase was purified from transformed *E. coli* NR8052 as described under Experimental Procedures. Seven-liter cultures were grown at 37 °C in modified LB medium and harvested 2 h after IPTG induction. Standard conditions (60 mM NaCl) were used in UDG assays throughout the purification, and the apparent specific activities are consequently approximately 50% of the activity measured at 10 mM NaCl. [b] Approximately 1/3 of the Superdex 75 fraction was applied on the MonoS column.

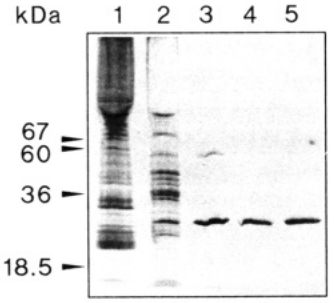

FIGURE 3: SDS–PAGE analysis of purification of recombinant human UDG. Details of the purification are described under Experimental Procedures. Lane 1, 2 μg of protein of crude extract; lane 2, 2 μg of protein of supernatant after protamine sulfate precipitation; lane 3, 0.3 μg of protein after DE52/CM Sephadex chromatography; lane 4, 0.3 μg of protein after Superdex 75 gel filtration; lane 5, 0.3 μg of protein after MonoS chromatography. The molecular mass standards were bovine serum albumin (67 kDa), beef liver catalase (60 kDa), beef heart lactate dehydrogenase (36 kDa), and horse spleen ferritin (18.5 kDa). Proteins were silver stained.

band corresponding to a molecular mass of 27 kDa. The homogeneity after MonoS purification was confirmed by heavy overloading of SDS gels, as no additional bands were visible after silver staining (data not shown). The identities of the first seven amino-terminal amino acid residues of the purified enzyme were determined by automated Edman degradation, and the sequence MEFFGES agreed with the sequence predicted from the corresponding DNA sequence.

Enzyme Activity. Purified UNGΔ84 had a specific activity of 4300 units/mg when assayed under standard conditions with 60 mM NaCl. However, at 10 mM NaCl, the activity increased to 8500 units/mg, which is approximately 50% of the activity reported for UDG purified from human sources (Myrnes & Wittwer, 1988; Wittwer et al., 1989). Using a three-factorial experimental setup (data not shown), optimum enzymatic activity was observed at 45 °C, pH 7.9, and in the presence of 10 mM NaCl. Under these conditions, the specific activity of the recombinant enzyme was 19 800 units/mg. Increasing the salt concentration beyond 10 mM resulted in a gradual decrease in activity, and at concentrations above 200 mM, UDG activity was essentially abolished. The specific activity using ssDNA as substrate was 3.4-fold higher than with dsDNA, which is in agreement with previous reports (Myrnes & Wittwer, 1988; Wittwer et al., 1989).

Isoelectric Point Determination. The UDG activity and protein profiles after isoelectric focusing of purified UNGΔ84 are shown in Figure 4. More than 95% of the activity focused above the theoretical p*I* of the protein (9.55), with a peak at pH 10.4. The relatively broad focusing range may

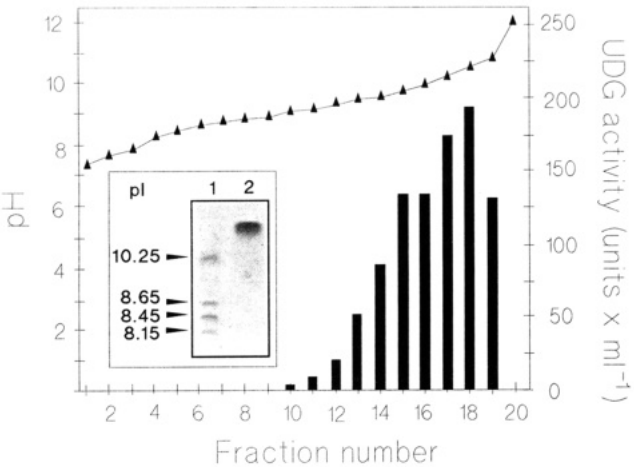

FIGURE 4: Isoelectric point analysis of recombinant human UDG. Isoelectric focusing of 500 μg of purified UNGΔ84 in the Rotofor preparative IEF cell was performed as described under Experimental Procedures. Triangles, pH of individual compartments in the focusing cell; bars, uracil-DNA glycosylase activity in the corresponding compartments. Inset: Isoelectric focusing of UNGΔ84 in polyacrylamide gel. Lane 1, p*I* standards cytochrome *c* (p*I* = 10.25) and lentil lectin, giving rise to three bands of p*I* = 8.65, 8.45 and 8.15, respectively; lane 2, 0.5 μg of purified UNGΔ84. Proteins were stained with coomassie brilliant blue.

Table 3: Kinetic Analysis of Recombinant Uracil-DNA Glycosylase[a]

	K_m (μM)			V_{max} (nmol/min/mg × 10^3)		
NaCl concn (mM)	10	30	60	10	30	60
dsDNA	1.6	2.9	4.5	10.5	10.6	13.2
ssDNA	0.4	0.8	2.3	21.0	24.6	26.6

[a] Kinetic parameters were analyzed as described under Experimental Procedures. The kinetic constants are calculated from Eisenthal–Cornish–Bowden plots.

indicate a microheterogeneity of the enzyme. This is most likely a result of the basic conditions near the p*I*, as refocusing of the pooled fractions 18 and 19 resulted in a shift of the activity profile toward a lower pH combined with a marked loss of enzyme activity (data not shown). The p*I* of the native enzyme was thus considered to be between 10.4 and 10.8, which also is in accordance with the results obtained from isoelectric focusing in polyacrylamide gels (Figure 4, inset).

Kinetic Parameters. Apparent Michaelis constants for UNGΔ84 against ss- and dsDNA substrates are given in Table 3. The K_m values showed a marked dependence on the NaCl concentration, with the lowest K_m observed at 10 mM NaCl (1.6 and 0.45 μM for ds- and ssDNA, respectively). An inverse effect of NaCl was observed on the

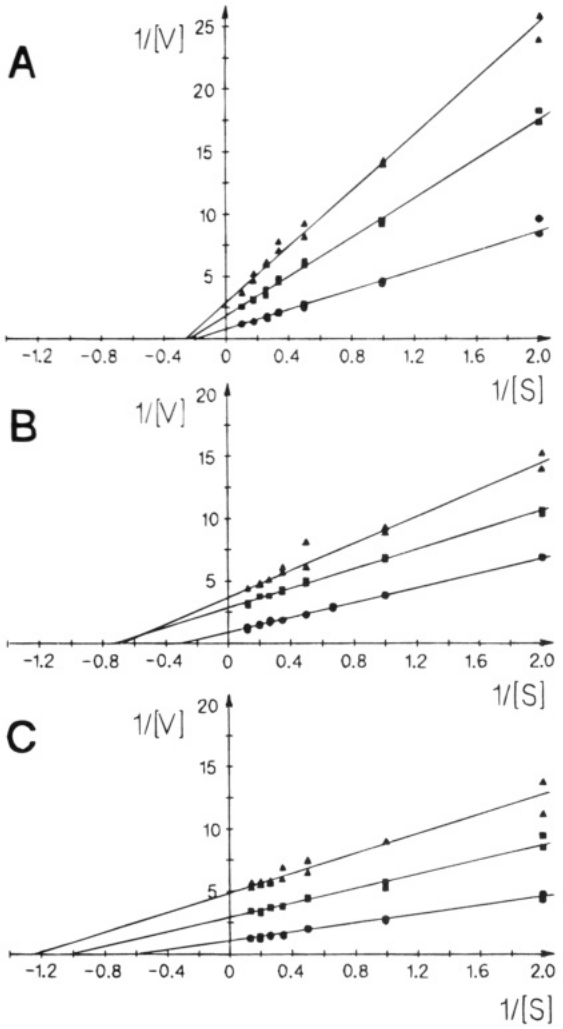

FIGURE 5: Effects of inhibition by uracil. Lineweaver–Burk plots of the effect of various concentrations of uracil at different NaCl concentrations. Panel A, 60 mM NaCl; panel B, 30 mM NaCl; panel C, 10 mM NaCl. Circles, no uracil; squares, 2 mM uracil; triangles, 5 mM uracil.

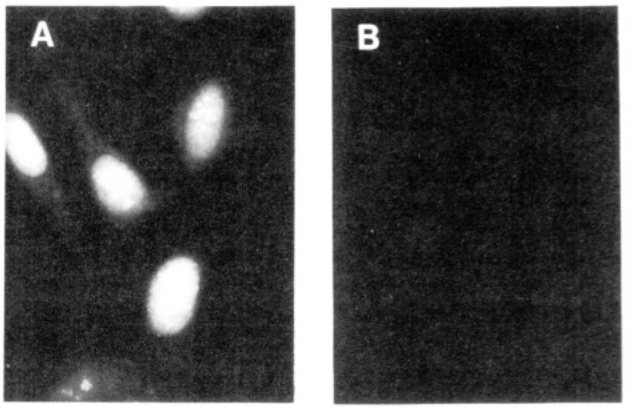

FIGURE 6: Immunocytochemical localization of UDG in $HeLaS_3$ cells. Cells were grown and treated for immunochemistry as described under Experimental Procedures. Panel A, cells labeled with PU101 IgG; panel B, control cells labeled with preimmune IgG from the same rabbit.

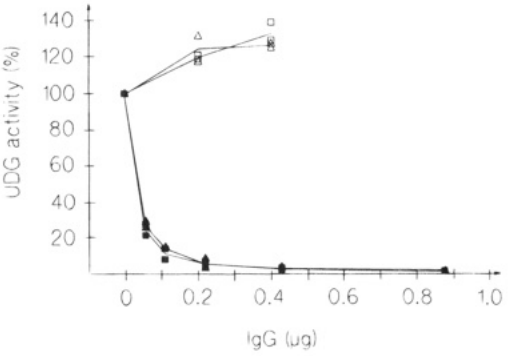

FIGURE 7: Inhibition of UDG activity in HeLa extracts by polyclonal IgGs against UNGΔ84. HeLa extracts were incubated with various amounts of PU101 IgG prior to analysis of UDG activity. Shaded symbols, incubated with PU101 IgG; open symbols, controls incubated with preimmune IgG from the same rabbit; squares, cells extracted with buffer containing 60 mM NaCl; triangles, cells extracted with buffer containing 0.5 M NaCl and 0.5% Triton X-100. The abscissa indicates the total amount of IgG added in each reaction.

maximum reaction velocities obtained, although the effect was not as marked as for the K_m (Table 3). To measure the energy of activation, UNGΔ84 was incubated at eight different temperatures (18–43 °C) and the energy was calculated from an Arrhenius plot. The activation energy was 50.6 kJ/mol, which is close to the value previously reported for HeLa UDG (Wittwer & Krokan, 1985).

Inhibition by Uracil. Human uracil-DNA glycosylase is subject to product inhibition by uracil, with a 50% inhibition occurring at 2 mM uracil (Krokan & Wittwer, 1981). We found a similar degree of inhibition of the recombinant enzyme (58% inhibition at 2 mM uracil using standard conditions and dsDNA as substrate). Although little variation in inhibition was observed using NaCl concentrations between 10 and 60 mM, a shift in the mechanism of inhibition occurred. At 60 mM NaCl inhibition was essentially noncompetitive as determined from Lineweaver–Burk plots, while a gradual shift toward uncompetitive inhibition was observed at lower salt concentrations (Figure 5).

Immunocytochemical Localization and Inhibition of UDG by PU101. $HeLaS_3$ cells were fixed in paraformaldehyde and stained with PU101 IgG raised against UNGΔ84 as described under Experimental Procedures. A strong fluorescence was observed in the nuclei, while a weaker staining was observed in the cytoplasm (Figure 6A), which is in accordance with results using peptide-specific antisera (Slupphaug et al., 1993). The specificity of the staining was confirmed using purified preimmune IgG from the same rabbit (Figure 6B). An identical staining pattern, although less intense, was observed with methanol-fixed cells (data not shown).

When HeLa cell extracts were preincubated with PU101 IgG on ice and subsequently assayed for UDG activity, more than 98% of the activity was lost as compared to extracts without added PU101 (Figure 7). In parallel experiments using preimmune IgG from the same rabbit, no UDG activity was lost; in fact the activity was reproducibly stimulated slightly. No endogeneous UDG activity was observed in the IgG fractions (data not shown). These results strongly indicate that the *UNG* gene encodes the major uracil-DNA glycosylase in human cells.

Sequence Specificity for Removal of Uracil. When partially replicated double-stranded M13 DNA containing U opposite A at random positions at an average frequency of 1 residue per 300–400 base pairs was employed as a substrate, a pronounced sequence specificity in uracil excision was observed when the amount of UNGΔ84 in a

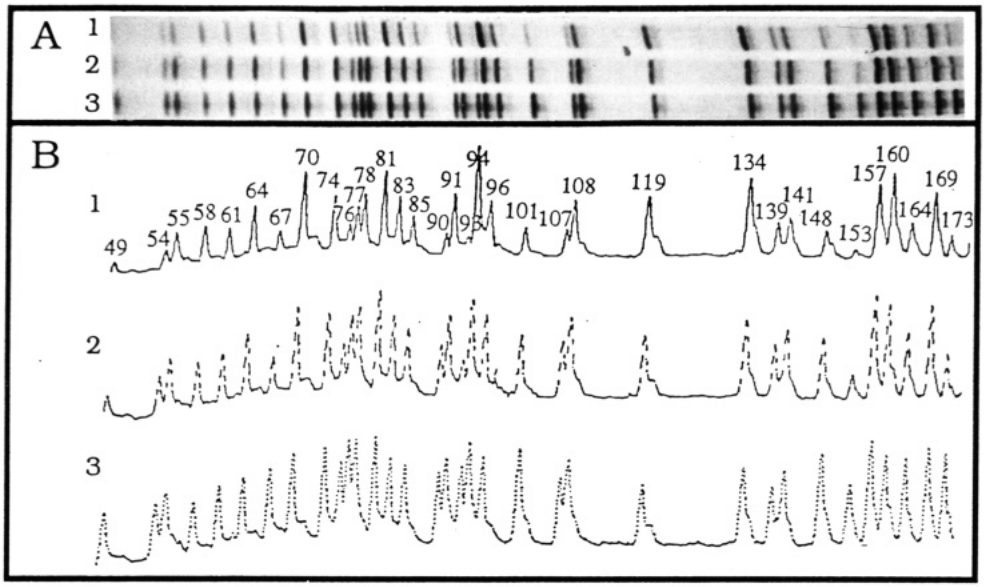

FIGURE 8: Analysis of uracil excision from defined positions in double-stranded M13 DNA by recombinant human UDG. Panel A shows an autoradiogram of dUMP DNA after incubation of 0.5 pmol of DNA with 5×10^{-3} unit of UDG for 5, 15, and 60 min, with subsequent strand cleavage at AP sites and electrophoresis as described. Panel B shows the intensities of the bands from panel A as they appear after laser scanning densitometric analysis of the autoradiogram. Peak areas represent a measure of strand cleavage and thus indirectly uracil excision at any particular site.

reaction was rate limiting (Figure 8). In Table 4 the nucleotide sequences surrounding individual uracil residues are given, together with the respective efficiencies of uracil excision. Under conditions where the amounts of UNGΔ84 were not rate limiting, uracils in all positions were equally well excised, giving bands of even intensity on autoradiography after electrophoresis as shown in Figure 8. This indicates that the incorporation of dUMP into M13 DNA by the modified T7 DNA polymerase has no significant sequence-dependent variation which might contribute to the heterogeneous pattern of cleaved apyrimidinic sites. By inspection of the sequences from which U is rapidly or slowly removed by UNGΔ84, it appears evident that a dTMP residue 3′ to U always results in slow removal. This is in accordance with similar results obtained with the corresponding enzyme from calf thymus (Eftedal et al., 1993). In addition, it appears that a high GC content surrounding the dUMP residue also results in slow removal. We calculated ΔGs for melting of five base pairs (two flanking base pairs on each side of U) for the first 10 and the last 10 sequences in Table 4 (representing good and poor removal, respectively). The mean ΔGs for the best and the poorest sequences were −1.96 and −2.78 kcal/mol, respectively, and this difference was significant ($p < 0.05$) according to the Mann−Whitney test. When sequences containing dTMP directly 3′ to the dUMP were not included in the calculation, ΔGs were −1.96 and −2.73 kcal/mol, respectively.

On the basis of the sequence-dependent action of UNGΔ84 on dUMP-DNA, a set of six oligodeoxyribonucleotides of 19 to 22 bases were synthesized, each containing a central dUMP residue in a DNA context identical to a part of the M13 sequence. Complementary strands contained either adenine or guanine opposite uracil to mimic products from dUMP incorporation or cytosine deamination, respectively. The oligonucleotides in Figure 9 were named according to the distance from the M13 primer to the inserted dUMP in compliance with the entries in Table 4. They were chosen so as to cover sequences from the upper, middle, and lower parts of Table 4, representing repair frequencies ranging from 5% to 100%. The overall pattern of sequence specificity from dUMP DNA was retained in these double-stranded oligonucleotide substrates, as is shown in Figure 9. However, intersequence differences were somewhat diminished in the homogeneous oligonucleotide substrates as compared to the M13 DNA where uracil appeared in multiple positions. This may indicate that UNGΔ84 removes uracil in the most readily available positions in a segment of DNA before attacking less accessible sites, thus creating very large initial sequence-dependent variation in excisional efficiency on heterogeneous substrates.

UNGΔ84 generally displayed slightly higher activity on mismatched uracil (UG) as compared to uracil in UA base pairs in oligonucleotide substrates, mostly so at uracil positions flanked on one side by A or T and on the other by G or C as is the case for U91, U93, and U141. The mismatched uracil was also most efficiently excised in oligo U291 where uracil is situated in a run of five Gs. In positions U90 and U94, where uracil is flanked on both sides by A or T, no significant difference between UA and UG base pairs was observed. As shown in Figure 9, the sequence-dependent variation in repair rates by far outweighs the differences caused by the nature of the base opposing uracil under our assay conditions. These results suggest that the surrounding sequence is a more important determinant of UDG excisional activity than is the origin of the lesion.

To assess whether sequence specificity of UNGΔ84 prevails in the absence of double-stranded DNA structure, single-stranded oligonucleotides were subjected to the same treatment. The results of these experiments demonstrated that in ssDNA the sequence specificity was less prononunced, and the small differences observed were not clearly related to the GC or AT content of the substrate (data not shown).

Potential activity of UNGΔ84 on thymine in TG mismatched base pairs was assayed for a double-stranded oligonucleotide containing thymine instead of uracil but otherwise identical to the mismatched U90. Incubation was with 4 times the amount of enzyme used on dUMP substrates. After 30 min of incubation, no sites susceptible

Table 4: Sequence Specificity of Uracil Excision for Human UDG[a]

Uracil position	Sequence (5' to 3' direction)	% removal of uracil[b]
134	A A G C A **U** A A A G T	100
160	C C T A A **U** G A G T G	85
157	G T G C C **U** A A T G A	77
94	A T T G T **U** A T C C G	77
70	G G T C A **U** A G C T G	69
169	T G A G C **U** A A C T C	66
81	T T T C C **U** G T G T G	55
141	A A G T G **U** A A A G C	54
64	A A T C A **U** G G T C A	52
58	A T T C G **U** A A T C A	50
108	A C A A T **U** C C A C A	45
96	T G T T A **U** C C G C T	44
83	T C C T G **U** G T G A A	44
91	G A A A T **U** G T T A T	42
119	C A A C A **U** A C G A G	41
74	A T A G C **U** G T T T C	40
78	C T G T T **U** C C T G T	38
55	C G A A T **U** C G T A A	37
61	C G T A A **U** C A T G G	33
77	G C T G T **U** T C C T G	33
90	T G A A A **U** T G T T A	31
164	A T G A G **U** G A G C T	29
139	T A A A G **U** G T A A A	29
54	T C G A A **U** T C G T A	27
85	C T G T G **U** G A A A T	26
107	C A C A A **U** T C C A C	23
76	A G C T G **U** T T C C T	20
67	C A T G G **U** C A T A G	19
173	C T A A C **U** C A C A T	19
101	T C C G C **U** C A C A A	19
49	C G A G C **U** C G A A T	17
93	A A T T G **U** T A T C C	15
153	T G G G G **U** G C C T A	8
291	C A G G G **U** G G T T T	5

[a] Removal of uracil from double-stranded DNA after a 5-min incubation of 0.5 pmol dUMP DNA with 5×10^{-3} unit of human UDG. [b] The percentages of uracil excision are mean values based on three independent experiments like the one shown in Figure 8.

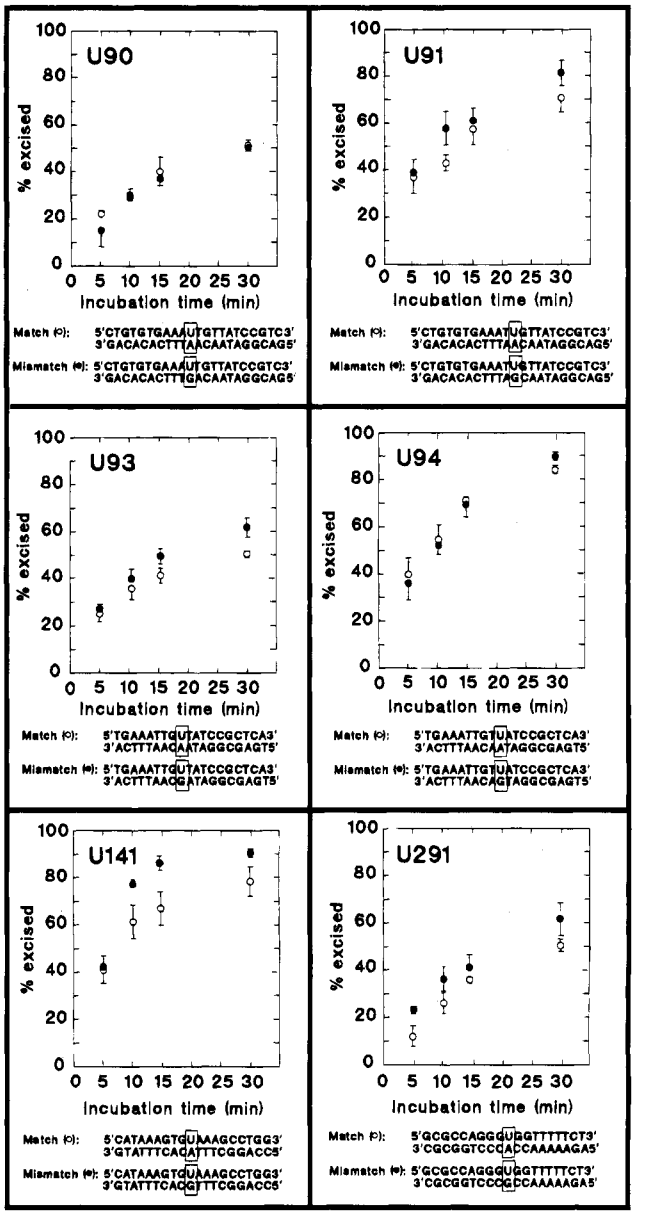

FIGURE 9: Analysis of uracil excision from double-stranded oligonucleotides containing uracil at defined positions by recombinant human UNGΔ84. Excision was monitored as processing of full-length 5'-end-labeled oligonucleotides to shorter fragments on denaturing gels after uracil excision and β-elimination cleavage at the induced apyrimidinic sites with hot piperidine. For each set of oligos containing matched (UA) or mismatched (UG) uracil, the reactions were performed with 2×10^{-3} unit of UDG and 0.9 pmol of double-stranded substrate. Reaction times were 0, 5, 10, 15, and 30 min. The curves show percentages of uracil removed in UA and UG base pairs as means of three independent experiments with SEM value error bars.

to β-elimination by piperidine were observed, indicating that thymine is not excised by the enzyme.

DISCUSSION

The present work describes expression of recombinant human uracil-DNA glycosylase (UNGΔ84) in good yield and biochemical characterization of the enzyme in pure form. In addition, we demonstrate that uracil-DNA glycosylase from the UNG gene is a major nuclear form. We have previously shown that the preform of the enzyme resides in the cytoplasm and that it is enzymatically active (Slupphaug et al., 1993). However, expression of the preform in *E. coli* was shown in the present paper to be inefficient, and when different 5'-terminally deleted *UNG15* cDNAs were expressed in *E. coli*, a striking variation in the UDG activity was observed. This is mainly due to variation in the amount of recombinant protein produced. The variation may rely on sequences in the UNG-coding region itself, as the sequences flanking the AUG initiation codon are identical in all the mutants. The markedly increased translational efficiency in *E. coli* observed for UNGΔ84 may rely on the removal of a potential stable hairpin structure in the 339–361 region of *UNG15* (corresponding to amino acids 78–84). As the effect of hairpins on translation appears to be less in higher eukaryotes (Shakin & Liebhaber, 1986), this may explain the lower variation in UDG expression in the reticulolysates.

The biochemical properties of recombinant UNGΔ84 appear to be very similar to those of UDG purified from

human sources, and the only clear difference observed is the change in the NaCl optimum from 30−60 mM for the human UDG to 10 mM for the recombinant enzyme. However, we cannot formally rule out the possibility that other properties not previously examined for the natural cellular form of UNG could be altered. A recent report from Higley and Lloyd (1993) demonstrated that *E. coli* UDG utilizes a limited processive mechanism for uracil search at NaCl concentrations up to 50 mM, while at higher salt concentrations a three-dimensional (distributive) search mechanism gradually becomes dominant. Such a mechanism may also explain the increase in K_m observed at increasing NaCl concentrations. Uracil inhibits UDG activity by an uncompetitive (at low salt) or a noncompetitive (at higher salt) mechanism, both types of inhibition indicating that uracil binds to a site different from the substrate-binding site.

We have found that the sequence specificity of the recombinant human enzyme resembles that of the analogous enzymes from *E. coli* and calf thymus, a finding consistent with their high degree of conservation (Olsen et al., 1989; Eftedal et al., 1993). Interestingly, the rate of U removal appears to depend on at least two independent variables. First, a dTMP residue directly 3′ to dUMP consistently results in poor U removal. Second, a high local GC content resulting in a high local melting temperature also is an obstacle to efficient uracil removal. The latter observation may reflect the increased affinity of the enzyme toward ssDNA as compared to dsDNA and could also, at least in part, explain the observed reduction in sequence specificity when ssDNA was used as substrate. This is also in compliance with an earlier report by Delort et al. (1985) who found little sequence-dependent variation in excisional activity of *E. coli* and *Micrococcus luteus* uracil-DNA glycosylases on ssDNA where uracil was induced via deamination of cytosine. However, since the rates of removal of U from a few sequences do not comply with the general rules as outlined above, other determinants not yet identified probably are involved.

The diminished intersequence variation in excisional activity observed when comparing M13 dUMP-DNA to homogeneous oligonucleotides may indicate that UDG, when presented with a choice, removes uracil in the most readily available positions in a segment of DNA before attacking less accessible sites, thus creating very large initial sequence-dependent variation in excisional efficiency on heterogeneous substrates. Regardless of this effect, the general pattern of sequence-specific excision prevails whether uracil appears in full-length DNA or in short oligonucleotides in UA or in mismatched base pairs with guanine. In general, we have found that the sequence surrounding the U residue is a more important determinant for the rate of removal of U than whether U is present in a UA match or a UG mismatch. In some positions, however, the rate of removal is considerably faster from UG mismatches, consistent with an observation from a study in which removal of U from UG or UA from otherwise identical oligonucleotides was examined (Verri et al., 1992). This might imply an increase in accessibility due to "wobble" formation when uracil forms a base pair with guanine as indeed is the case in TG base pairs (Hunter, 1992).

Two different cDNAs claimed to encode human uracil-DNA glycosylase have been isolated by other groups (Vollberg et al., 1989; Muller & Caradonna, 1991), and this has resulted in considerable confusion as to which of the cDNAs encodes the major (nuclear) form of UDG in human cells. We have recently demonstrated that *UNG15* encodes UDG which is mainly located in nuclei, but a minor fraction is transported to mitochondria (Slupphaug et al., 1993). In addition, translational hybrid arrest experiments have demonstrated that antisense *UNG* mRNA inhibits approximately 90% of the UDG activity produced after translation of total mRNA from human fibroblasts (Slupphaug et al., 1991), again indicating that *UNG15* encodes the major human UDG. The present immunolocalization of UDG in HeLa nuclei using polyclonal antibodies against purified UNGΔ84 supports previous studies using peptide-specific antibodies (Slupphaug et al., 1993) in that the main fraction of the *UNG15* gene product is localized in the nuclei. Furthermore, the fact that the polyclonal IgG PU101 antibodies inhibited UDG activity in HeLa cell extracts by more than 98% strongly suggests that the UNG protein is the main uracil-DNA glycosylase in the cells. Formally, a different UDG with antigenic properties similar to those of the UNG protein could be hypothesized, but this is judged as being very unlikely, especially since the other cDNAs claimed to encode UDG activity are unrelated to *UNG15*. The cDNA isolated by Vollberg et al. (1989) is identical to the gene encoding the 37-kDa subunit of glyceraldehyde-3-phosphate dehydrogenase (G3PD) (Meyer-Siegler et al., 1991). The specific activity of this gene product is several orders of magnitude lower than that of the UNG protein and may represent a cryptic activity. The functional significance of the cDNA isolated by Muller and Caradonna (1991) is also still not clear. This gene encodes a protein with a significant similarity to the conserved region of the cyclin protein family, and thus bears no obvious resemblance to the structurally conserved UDG family. *In vitro* transcription/translation of the cDNA did, however, result in functional uracil-DNA glycosylase activity, but the pattern of cell cycle variation of both the mRNA and its protein (Muller & Caradonna, 1993) is not consistent with this being a major uracil-DNA glycosylase.

A uracil-excising activity of the mismatch-specific thymine-DNA glycosylase of HeLa cells was recently reported (Neddermann & Jiricny, 1994). This enzyme has previously been demonstrated to remove thymine from any DNA duplex in which the thymine is not in a Watson−Crick base pair with adenine (Neddermann & Jiricny, 1993). It removes uracil from GU base pairs, but not from AU base pairs. The thymine-DNA glycosylase may thus constitute a backup system in the cells for the repair of GU mispairs in GC-rich regions since U is poorly removed from GC-rich sequences (Eftedal et al., 1993; this paper).

ACKNOWLEDGMENT

We greatly appreciate the assistance of the following graduate students: Lena Kristiansen for preparing the starting *UNG15* construct containing *Sal*I linkers, Gry Lindstad and Ragnhild Weie for optimizing fermenter conditions, and Wenche Brenne for preparing the photomicrographs. Thanks to Dr. Knut Sletten at the University of Oslo Biotechnology Center who performed the N-terminal amino acid sequencing.

REFERENCES

Amann, E., Ochs, B., & Abel, K.-J. (1988) *Gene 69*, 301−315.
Anderson, C. T. M., & Friedberg, E. C. (1980) *Nucleic Acids Res. 8*, 875−888.

Arenaz, P., & Sirover, P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5822−5826.
Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C., Tuffnell, P. S., & Barrell, B. G. (1984) *Nature 310*, 207−211.
Brash, D. E. (1981) in *DNA repair, a laboratory manual of research procedures* (Friedberg, E. C., & Hanawalt, P. C., Eds.) Vol. 3, pp 327−345, Marcel Dekker Inc., New York.
Brynolf, K., Eliasson, R., & Reichard, P. (1978) *Cell 13*, 573−580.
Caradonna, S. J., & Cheng, Y.-C. (1980) *J. Biol. Chem. 255*, 2293−2300.
Chen, E. Y., & Seeburg, P. H. (1985) *DNA 4*, 165−170.
Colson, P., & Verly, W. G. (1983) *Eur. J. Biochem. 134*, 415−420.
Davison, A. J., & Scott, J. E. (1986) *J. Gen. Virol. 67*, 1759−1816.
Delort, A.-M., Duplaa, A.-M., Molko, D., Teoule, R., Leblanc, J.-P., & Laval, J. (1985) *Nucleic Acids Res. 13*, 319−335.
Domena, J. D., Timmer, R. T., Dicharry, S. A., & Mosbaugh, D. W. (1988) *Biochemistry 27*, 6742−6751.
Duncan, B. K., & Miller, J. H. (1980) *Nature 287*, 560−561.
Duncan, B. K., & Weiss, B. (1982) *J. Bacteriol. 151*, 750−755.
Eftedal, I., Guddal, P. H., Slupphaug, G., Volden, G., & Krokan, H. E. (1993) *Nucleic Acids Res. 21*, 2095−2101.
Gupta, P. K., & Sirover, M. A. (1981) *Cancer Res. 41*, 3133−3136.
Heimer, G. V., & Taylor, C. E. D. (1974) *J. Clin. Pathol. 27*, 254−256.
Higley, M., & Lloyd, R. S. (1993) *Mutat. Res. 294*, 109−116.
Hunter, W. N. (1992) in *Methods in Enzymology* (Wyckoff, H., Hirs, C. H. W., & Timasheff, S. N., Eds.) Vol. 211, pp 221−231, Academic Press, Inc., New York.
Impellizzeri, K. J., Anderson, B., & Burgers, P. M. J. (1991) *J. Bacteriol 173*, 6807−6810.
Krokan, H. E., & Wittwer, C. U. (1981) *Nucleic Acids Res. 9*, 2599−2613.
Krokan, H., Haugen, Aa., Myrnes, B., & Guddal, P. H. (1983) *Carcinogenesis 4*, 1559−1564.
Kunkel, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Lindahl, T. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3649−3653.
Lindahl, T., & Nyberg, B. (1974) *Biochemistry 13*, 3405−3410.
Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., & Sperens, B. (1977) *J. Biol. Chem. 252*, 3286−3294.
McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E., & Taylor, P. (1988) *J. Gen. Virol. 69*, 1531−1574.
Méjean, V., Rives, I., & Claverys, J.-P. (1990) *Nucleic Acids Res. 18*, 6693.
Meyer-Siegler, K., Mauro, D. J., Seal, G., Wurzer, J., deRiel, J. K., & Sirover, M. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8460−8464.
Muller, S. J., & Caradonna, S. (1991) *Biochim. Biophys. Acta 1088*, 197−207.
Muller, S. J., & Caradonna, S. (1993) *J. Biol. Chem. 268*, 1310−1319.
Myrnes, B., & Wittwer, C. U. (1988) *Eur. J. Biochem. 173*, 383−387.
Myrnes, B., Giercksky, K.-E., & Krokan, H. (1983) *Carcinogenesis 4*, 1565−1568.
Neddermann, P., & Jiricny, J. (1993) *J. Biol. Chem. 268*, 21218−21224.
Neddermann, P., & Jiricny, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1642−1646.
Olsen, L. C., Aasland, R., Wittwer, C. U., Krokan, H. E., & Helland, D. E. (1989) *EMBO J. 8*, 3121−3125.
Percival, K. J., Klein, M. B., & Burgers, P. M. J. (1989) *J. Biol. Chem. 264*, 2593−2598.
Sekiguchi, M., Hayakawa, H., Makino, F., Tanaka, K., & Okada, Y. (1976) *Biochem. Biophys. Res. Commun. 73*, 293−299.
Shakin, S. H., & Liebhaber, S. A. (1986) *J. Biol. Chem. 261*, 16018−16025.
Shapiro, R. (1980) in *Chromosome Damage and Repair* (Seeberg, E., & Kleppe, K., Eds.) pp 3−18, Plenum Press, New York.
Slupphaug, G., Olsen, L. C., Helland, D., Aasland, R., & Krokan, H. E. (1991) *Nucleic Acids Res. 19*, 5131−5137.
Slupphaug, G., Lettrem, I., Myrnes, B., & Krokan, H. E. (1992) *Carcinogenesis 13*, 1769−1773.
Slupphaug, G., Markussen, F.-H., Olsen, L. C., Aasland, R., Aarsæther, N., Bakke, O., Krokan, H. E., & Helland, D. E. (1993) *Nucleic Acids Res. 21*, 2579−2584.
Talpaert-Borlè, M., Campagnari, F., & Creissen, D. M. (1982) *J. Biol. Chem. 257*, 1208−1214.
Tye, B. K., Nyman, P. O., Lehman, I. R., Hochhauser, S., & Weiss, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 154−157.
Varshney, U., & van de Sande, J. H. (1989) *Nucleic Acids Res. 17*, 813.
Varshney, U., Houtcheon, T., & van de Sande, J. H. (1988) *J. Biol. Chem. 263*, 7776−7784.
Verri, A., Mazzarello, P., Spadari, S., & Focher, F. (1992) *Biochem J. 287*, 1007−1010.
Vollberg, T. M., Siegler, K. M., Cool, B. L., & Sirover, M. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8693−8697.
Wist, E., Unhjem, O., & Krokan, H. (1978) *Biochim. Biophys. Acta 520*, 253−270.
Wittwer, C. U., & Krokan, H. E. (1985) *Biochim. Biophys. Acta 832*, 308−318.
Wittwer, C. U., Bauw, G., & Krokan, H. E. (1989) *Biochemistry 28*, 780−784.
Worrad, D. M., & Caradonna, S. (1988) *J. Virol. 62*, 4774−4777.

BI941761F