Reconstitution of Human Base Excision Repair with Purified Proteins†

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ABSTRACT: Base excision repair is a major mechanism for correcting aberrant DNA bases. We are using an *in vitro* base excision repair assay to fractionate and purify proteins from a human cell extract that are involved in this type of repair. Three fractions are required to reconstitute base excision repair synthesis using a uracil-containing DNA as a model substrate. We previously showed that one fraction corresponds to DNA polymerase β. A second fraction was extensively purified and found to possess uracil-DNA glycosylase activity and was identified as the product of the UNG gene. A neutralizing antibody to the human UNG protein inhibited base excision repair in crude extract by at least 90%. The third fraction was highly purified and exhibited apurinic/apyrimidinic (AP) endonuclease activity. Immunoblot analysis identified HAP1 as the major polypeptide in fractions possessing DNA repair activity. Recombinant versions of UNG, HAP1, and DNA polymerase β were able to substitute for the proteins purified from human cells. Addition of DNA ligase I led to ligated repair products. Thus, complete base excision repair of uracil-containing DNA was achieved by a combination of UNG, HAP1, DNA polymerase β, and DNA ligase I. This is the first complete reconstitution of base excision repair using entirely eukaryotic proteins.

Different types of DNA damage are corrected by distinct DNA repair pathways (Friedberg *et al.,* 1995). The base excision repair (BER)[1] pathway is generally initiated by a DNA glycosylase that recognizes and removes damaged bases. DNA glycosylases are specific for a particular type or a few related forms of base damage. The model of DNA damage used in this study is uracil. Deamination of cytosine to uracil in DNA genomes is one of the most common spontaneous premutagenic events and occurs hundreds of times a day in every human cell (Lindahl, 1993; Friedberg *et al.,* 1995). Uracil can also be introduced into the DNA as a dUMP residue during the course of DNA replication. Efficient repair of uracil-containing DNA is essential to prevent mutagenesis (Impellizzeri *et al.,* 1991; Duncan & Weiss, 1982).

A number of models for eukaryotic BER of uracil-containing DNA have been proposed (Matsumoto & Bogenhagen, 1991; Dianov & Lindahl, 1994; Friedberg *et al.,* 1995; Lindahl *et al.,* 1995). The essential features can be summarized as follows. The N-glycosylic bond between the uracil base and the deoxyribose sugar is cleaved by a uracil-DNA glycosylase (UDG) to produce an abasic site. The phosphodiester bond 5′ to the abasic site is then hydrolyzed by an apurinic/apyrimidinic (AP) endonuclease, and the deoxyribose phosphate residue is excised by a deoxyribophosphodiesterase (dRpase) or exonuclease. The resulting single-nucleotide gap is filled in by a DNA polymerase, and a DNA ligase completes the repair reaction. BER has been reconstituted with purified *Escherichia coli* proteins (Dianov & Lindahl, 1994). Although proteins with the appropriate enzymatic activities have been identified in mammalian cells, the precise identity of the enzymes involved in BER remains unclear due to limited genetics and the fact that BER has not been fully reconstituted with purified mammalian proteins.

There have been reports of four distinct UDGs in human cells, and their relative contribution to BER has become a subject of controversy (Mosbaugh & Bennett, 1994). The uracil-DNA glycosylase, designated UNG, identified by Krokan and colleagues has been extensively studied. Notably, the protein sequence is remarkably similar to UDGs from other organisms, and the recombinant protein exhibits UDG activity (Olsen *et al.,* 1989). Evidence from the Krokan laboratory suggests that the UNG protein may be the major UDG in both mitochondria and nuclei (Slupphaug *et al.,* 1995, 1993, 1991). Two other purported UDGs have been cloned and the genes were found to encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a protein with sequence similarity to cyclins (Meyer-Siegler *et al.,* 1991; Muller & Caradonna, 1991). A fourth human UDG was originally identified as a mismatch-specific thymine DNA glycosylase but has more recently been shown to efficiently excise uracil from U-G mispairs but not U-A base pairs (Wiebauer & Jiricny, 1990; Neddermann & Jiricny, 1993, 1994).

A single 5′-AP endonuclease has been purified to homogeneity and cloned from human cells (variously designated HAP1, APE, APEX, and Ref-1) (Robson & Hickson, 1991; Demple *et al.,* 1991; Seki *et al.,* 1992; Xanthoudakis *et al.,* 1992). However, the possibility remains that there may be

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[1] Abbreviations: AP, apurinic/apyrimidinic; bp, base pair(s); BER, base excision repair; BSA, bovine serum albumin; dRpase, deoxyribophosphodiesterase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen; Pol, DNA polymerase; ssDNA, single-stranded DNA; UDG, uracil-DNA glycosylase; Ugi, uracil-DNA glycosylase inhibitor of *Bacillus subtilis* bacteriophage PBS1.

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additional AP endonucleases involved in the repair of different types of DNA damage (Chen *et al.*, 1991). *E. coli*, for example, has two different AP endonucleases with distinct enzymatic activities and substrate specificities (Friedberg *et al.*, 1995).

Once the DNA has been incised, it is unclear how the abasic, deoxyribose phosphate moiety is excised. One report provided evidence that DNA synthesis may precede the excision step during the repair of a synthetic abasic site (Matsumoto & Bogenhagen, 1991). Some papers have reported BER patches up to 10 nucleotides in length, suggesting the involvement of a 5′ → 3′ exonuclease (Frosina *et al.*, 1994; Matsumoto *et al.*, 1994). Other reports indicated that the repair patch may be only a single nucleotide in length, suggesting the action of a dRpase that can release a free deoxyribose phosphate residue (Dianov *et al.*, 1992; Singhal *et al.*, 1995).

The identity of the DNA polymerase involved in BER has been the subject of numerous studies (Matsumoto *et al.*, 1994; Dianov *et al.*, 1992; Wiebauer & Jiricny, 1990; Singhal *et al.*, 1995; Sobol *et al.*, 1996). Recently, it has been suggested that DNA polymerase β (Polβ) catalyzes the majority of mammalian BER synthesis while a PCNA-dependent, aphidicolin-sensitive polymerase (Pol δ or ϵ) participates in a second BER pathway (Frosina *et al.*, 1996; Nealon *et al.*, 1996).

Three distinct DNA ligases (I, III, and IV) have been identified in humans (Tomkinson *et al.*, 1991; Lindahl & Barnes, 1992; Barnes *et al.*, 1990; Wei *et al.*, 1995). DNA ligase I was recently shown to interact with Polβ in a multiprotein BER complex (Prasad *et al.*, 1996). However, the ligase requirement for BER is unclear (Prigent *et al.*, 1994; Wei *et al.*, 1995; Caldecott *et al.*, 1994).

The purpose of this study was to identify the proteins required for BER of uracil-containing DNA in human cells. By fractionating and purifying BER proteins from a human cell extract, we have identified the UDG (the UNG protein) and AP endonuclease (the HAP1 protein) able to cooperate with Polβ in this repair pathway. Reconstitution of the complete BER reaction was accomplished by combining UNG, HAP1, Polβ, and DNA ligase I. These proteins led to the formation of a one nucleotide repair patch and did not require the addition of an extrinsic dRpase or exonuclease.

EXPERIMENTAL PROCEDURES

Reagents. The following materials were obtained from commercial sources: uracil glycosylase inhibitor (Ugi) of *Bacillus subtilis* bacteriophage PBS1 (New England Biolabs); *E. coli* UDG and T4 DNA ligase (Gibco BRL); and human erythrocyte GAPDH (Sigma). Anti-GAPDH (monoclonal 6C5) and anti-HAP1/Ref-1 (C-20, rabbit polyclonal IgG) antibodies were purchased from Biodesign International and Santa Cruz Biotechnology, respectively. Anti-UNG antibody (PU101, rabbit polyclonal IgG) and recombinant UNGΔ84 expression clone were provided by Dr. H. Krokan (University of Trondheim, Norway). Anti-cyclin-like UDG monoclonal antibody [mAb-u(91−243)] and recombinant MBP-cyclin-like UDG fusion protein were supplied by Dr. M. Walsh (Mount Sinai School of Medicine, New York). The HAP1 expression plasmid was a gift of Dr. I. Hickson (University of Oxford, U.K.). Recombinant human DNA ligase I (purified from baculovirus-infected cells) was provided by Dr. A. Tomkinson (University of Texas at San Antonio). Recombinant rat Polβ was purified as previously described (Nealon *et al.*, 1996). Other reagents were obtained as previously reported (Nealon *et al.*, 1996). Protein concentrations were determined using Bio-Rad protein dye reagent and bovine serum albumin (BSA) as a standard.

BER Synthesis Assay. This assay measures DNA repair synthesis using a radioactive nucleotide, a uracil-containing duplex oligonucleotide, and a human cell extract or fractions derived therefrom. The assay does not measure the ligation step of the repair reaction. The experiments shown in this paper used the following 30 bp UG oligonucleotide unless otherwise indicated:

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5' GAGCCGGCACTGGUGCCCAGCTGATATCGC 3'
3' CTCGGCCGTGACCGCGGGTCGACTATAGCG 5'
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Reactions (25 μL) contained 40 mM creatine phosphate-diTris salt (pH 7.7), 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 20 μM each dATP, dGTP, dTTP, and dCTP, 1 μCi of [α-^{32}P]dCTP, 2.5 μg of creatine phosphokinase, 50 mM NaCl, 0.5 μg (25 pmol) of 30 bp UG oligo, and protein fractions as indicated. Reaction mixtures were incubated at 37 °C for 30 min. Reactions were stopped by addition of 5 μL of 6× gel loading dye (20% Ficoll, 100 mM EDTA, 2% SDS, 0.2% bromophenol blue, and 0.2% xylene cyanol). Samples were directly loaded onto a 15% polyacrylamide gel (29:1 acrylamide:bis) and electrophoresed at 150 V for ∼90 min. The dried gel was exposed to film and the radioactive bands were excised from the gel for quantitation in a scintillation counter.

UDG Assay. The substrate for UDG assays was [^{3}H]-dUMP-containing DNA (specific activity 3×10^4 cpm/μg of DNA), prepared by nick-translation of calf thymus DNA as previously described (Krokan & Wittwer, 1981). UDG activity was measured as previously reported (Krokan & Wittwer, 1981). Briefly, assay mixtures (20 μL) contained 20 mM Tris-HCl, pH 7.5, 10 mM NaCl (unless otherwise stated), 5 μM [^{3}H]dUMP-DNA (the concentration refers to uracil), 0.5 mg/mL BSA, 1 mM EDTA, 1 mM DTT, and protein fractions as indicated. Assay mixes were incubated for 10 min at 30 °C. Reactions were stopped with the addition of 0.1 mL of 0.15 M sodium pyrophosphate, 0.1 mL of ssDNA (1 mg/mL), and 0.9 mL of 10% trichloroacetic acid, and mixtures were incubated for 10 min on ice. Samples were centrifuged and the acid-soluble radioactivity was measured by liquid scintillation counting. One unit of UDG is defined as the amount of enzyme that releases 1 nmol of uracil/min at 30 °C as measured under the above conditions (Krokan & Wittwer, 1981). A different definition is used to describe the activity of commercially available *E. coli* UDG: one unit catalyzes the release of 1 nmol of free uracil in 1 h at 37 °C from ^{3}H-poly(dU) (Gibco product description).

When poly(U)-Sepharose fractions were tested in the UDG assay, the identity of the soluble radioactivity was confirmed as uracil by thin-layer chromatography. Briefly, radioactivity migrated with nonradioactive uracil when polyethylenimine-cellulose plates were developed with water (Krokan & Wittwer, 1981).

AP Endonuclease Assay. The substrate for AP endonuclease activity was plasmid DNA containing abasic sites (AP-DNA). Plasmid pSV01ΔEP (100 μg) was incubated in 200 μL of 100 mM sodium acetate, pH 5.2, at 70 °C for

15 min to produce AP sites. The DNA solution was transferred to 4 °C and neutralized by the addition of 20 μL of 1 M Tris-HCl, pH 8.0. AP-DNA was extensively dialyzed against TE buffer at 4 °C. AP endonuclease activity was measured as previously described (Robson *et al.*, 1991). Briefly, assay mixtures (10 μL) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.5 μg of AP-DNA, 2.5 μg of BSA, and protein fractions as indicated. Reactions mixtures were incubated for 5 min at 37 °C. Reactions were transferred to 4 °C and stopped by the addition of 6× gel loading dye. Samples were electrophoresed in a 0.8% agarose gel (containing 0.5 μg/mL ethidium bromide).

Analysis of Repair Products. For analysis of the repair intermediates and the ligation step, the 46 bp UG oligonucleotide was used. Prior to their use in repair reactions, the individual oligonucleotides were first gel-purified by urea−PAGE (12%). The uracil-containing strand was 5′-^{32}P-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP and then annealed to the unlabeled complementary strand. After incubation in standard repair reactions, with proteins as indicated, the DNA products were subjected to urea−PAGE (12%) and the gel was exposed to film.

SDS−PAGE and Immunoblotting. Aliquots of chromatographic fractions were precipitated (Wessel & Flügge, 1984) prior to analysis by SDS−PAGE. Immunoblot analysis was performed using standard procedures (Harlow & Lane, 1988) and enhanced chemiluminescence detection (ECL, Amersham).

Preparation of HeLa Cell Extracts. HeLa S3 cells were extracted as previously described (Wobbe *et al.*, 1985; Kenny, 1993). Briefly, the cell pellet from a 50 L culture (6.2×10^5 cells/mL) was washed with PBS and resuspended in 125 mL of hypotonic buffer (20 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM PMSF, 0.5 μg/mL pepstatin, 0.5 μg/mL leupeptin, and 1 mM DTT). All procedures were performed at 4 °C unless otherwise stated. Cells were lysed by Dounce homogenization (30 strokes, B pestle) and to the extract was added 5 M NaCl to a concentration of 200 mM. The extract was centrifuged at 35000*g* for 30 min. The resulting pellet was frozen at −80 °C, and the decanted supernatant was centrifuged at 100000*g* for 1 h. The resulting supernatant was decanted and dialyzed 2 times against 2 L of buffer A [20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10% glycerol (v/v), 0.1 mM EDTA, 0.1 mM PMSF, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, and 1 mM DTT]. The dialysate was centrifuged at 100000*g* for 1 h, and the supernatant (denoted S-100 extract) was decanted and stored at −80 °C.

The 35000*g* pellet was further treated with high salt to extract additional proteins remaining in the cell nuclei after the initial extraction. The 35000*g* pellet was thawed, resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 2 M NaCl, and 2 mM DTT and Dounce homogenized on ice (5 strokes, B pestle) (Caradonna & Cheng, 1980). The mixture was stirred for 3 h on ice and centrifuged at 27000*g* for 45 min. The supernatant was decanted and filtered through sterile gauze. The filtered supernatant was extensively dialyzed against buffer A (minus NaCl, with 2 mM DTT), and the resulting precipitate was removed by centrifugation at 100000*g* for 1 h at 2 °C. The supernatant was decanted and stored at −80 °C prior to further analysis (designated HS-100 extract, 196 mg).

Fractionation of the S-100 Extract. The HeLa S-100 extract (2800 mg of protein) was fractionated employing DEAE-cellulose and SP-Sepharose essentially as described previously (Nealon *et al.*, 1996). Briefly, protein flowing through the DEAE-cellulose column was loaded directly onto an SP-Sepharose column connected in series. Bound protein was eluted off the SP-Sepharose column with increasing salt concentrations: protein peaks were pooled for the flowthrough (DSP1, 1008 mg), 250 mM NaCl elution (DSP2, 146 mg), and 1M NaCl elution (DSP3, 74 mg). DNA repair activity was recovered by combining fractions DSP2 and DSP3. Recombinant rat Polβ was capable of substituting for fraction DSP3 (Nealon *et al.*, 1996).

Fraction DSP2 was dialyzed overnight against 50 mM sodium phosphate buffer, pH 7.4, containing 250 mM NaCl, 10% glycerol (v/v), 0.2 mM EDTA, and 0.5 mM DTT, to which saturated ammonium sulfate solution was added to a final concentration of 30% (v/v). The dialysate was diluted 3-fold with buffer 30P [50 mM sodium phosphate, pH 7.4, 30% ammonium sulfate (v/v), 10% glycerol, 0.2 mM EDTA, and 1 mM DTT] and loaded onto a phenyl-Sepharose column (50 cm^3) which had been equilibrated with buffer 30P. The column was washed sequentially with (1) buffer 30P, (2) buffer 15P [50 mM sodium phosphate, pH 7.4, 15% ammonium sulfate (v/v), 10% glycerol (v/v), 0.2 mM EDTA, and 1 mM DTT], and (3) buffer NP-40 [50 mM sodium phosphate, pH 7.4, 10% glycerol (v/v), 0.2 mM EDTA, 0.1% NP-40 (v/v), and 1 mM DTT]. Fractions resulting from buffer 30P elution (flowthrough and wash) were pooled and concentrated by ultrafiltration (YM10 filter) using an Amicon 8400 stirred cell. The concentrate was dialyzed against buffer AN [20 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), 0.1 mM EDTA, 0.01% NP-40 (v/v), and 1 mM DTT] containing 100 mM NaCl (fraction PS1, 230 μg of protein). Protein peaks were pooled from the buffer 15P elution (fraction PS2) and buffer NP-40 elution (fraction PS3, 46 mg of protein). Fraction PS3 was dialyzed against buffer AN−100 mM NaCl. Fraction PS2 was concentrated by ultrafiltration and dialyzed against buffer AN−50 mM NaCl (fraction PS2, 2.8 mg of protein). DNA repair activity was recovered by combining fractions PS2 and PS3 with recombinant rat Polβ.

Purification of Repair Factor in Fraction PS3. Fraction PS3 (46 mg) was loaded onto a ssDNA−cellulose column (25 cm^3) equilibrated in buffer AN−100 mM NaCl. The column was washed with 140 mL of buffer AN−100 mM NaCl. A linear gradient elution of 125 mL from 0.1 to 0.4 M NaCl in buffer AN was employed. Finally, a step elution was performed with buffer AN−1 M NaCl. Aliquots of ssDNA−cellulose fractions were assayed to identify those that supported DNA repair synthesis in combination with fraction PS2 and Polβ. Fractions were additionally tested for UDG activity. Fractions supporting DNA repair synthesis were pooled.

The ssDNA−cellulose pool (major peak, 92 mL) was concentrated by ultrafiltration (to 2.3 mL) and dialyzed against buffer AN−25 mM NaCl. The dialysate (1.25 mg) was loaded onto a Mono S column (1 cm^3) equilibrated with buffer AN−25 mM NaCl. The column was washed with 12 mL of buffer AN−25 mM NaCl. A linear gradient elution of 20 mL from 25 to 400 mM NaCl in buffer AN and a 1 M NaCl step elution were employed. Fractions were assayed as described above, to identify those that supported DNA repair synthesis and exhibited UDG activity. To facilitate purification, only those fractions which supported

DNA repair synthesis with the highest specific activity were pooled.

Pooled Mono S fractions were dialyzed against buffer AN−100 mM NaCl and loaded onto a poly(U)-Sepharose column (14 cm^3) that had been equilibrated with buffer AN−100 mM NaCl. The column was washed with 45 mL of buffer AN−100 mM NaCl. A linear gradient elution of 140 mL from 100 to 400 mM NaCl in buffer AN and a 1 M NaCl step elution were used. Fractions (52−60) that supported DNA repair synthesis were pooled and concentrated by ultrafiltration (Centriprep 10). Glycerol was added to a concentration of 50% (v/v), and the Mono S pool was stored at −20 °C. UDG activity was ∼3.5 units/mL.

Purification of Repair Factor in Fraction PS2. Fraction PS2 (2.8 mg) was loaded onto a ssDNA−cellulose column (7 cm^3) equilibrated in buffer AN−50 mM NaCl. The column was washed with 43 mL of buffer AN−50 mM NaCl. A linear gradient elution of 70 mL from 0.05 to 1.0 M NaCl in buffer AN was employed. Fractions were assayed to identify those that supported DNA repair synthesis in combination with HeLa UNG [poly(U)-Sepharose pool] and recombinant Polβ. Fractions supporting DNA repair synthesis (eluting at ∼450 mM NaCl) were pooled and dialyzed against buffer AN−100 mM NaCl. The dialysate was loaded onto a HiTrap heparin−Sepharose column (1 cm^3) equilibrated in buffer AN−100 mM NaCl. The column was washed with 19 mL of buffer AN−100 mM NaCl. A linear gradient elution of 20 mL from 0.1 to 1.0 M NaCl in buffer AN was employed. Fractions supporting DNA repair synthesis (eluting at ∼400 mM NaCl) were pooled and dialyzed against buffer AN−100 mM NaCl. The dialysate was loaded onto a Mono S column equilibrated in buffer AN−100 mM NaCl. The column was washed with 10 mL of buffer AN−100 mM NaCl. A linear gradient elution of 20 mL from 0.1 to 1.0 M NaCl in buffer AN was employed. Fractions were assayed to identify those that supported DNA repair synthesis in combination with human UNG and Polβ.

RESULTS

Fractionation of Repair Factors. We previously reported the development of a rapid, sensitive, and quantitative *in vitro* assay to measure the BER of uracil-containing DNA by extracts from HeLa cells (Nealon *et al.*, 1996). The assay measures DNA repair synthesis using as a substrate a 30 bp duplex oligonucleotide containing a centrally located uracil on one strand (see Experimental Procedures). The uracil-containing oligonucleotide mimics the result of cytosine deamination in normal duplex DNA. Extracts from HeLa cells were fractionated using DEAE-cellulose and SP-Sepharose chromatography (Figure 1). DNA repair synthesis was reconstituted when two fractions, DSP2 and DSP3, were combined. The required component in fraction DSP3 was previously identified as Polβ (Nealon *et al.*, 1996). Figure 2 (lane 3) shows that repair synthesis occurred when the uracil-containing (UG) oligonucleotide was incubated with Polβ and fraction DSP2 in the BER assay. Note that reaction products from the BER assay were analyzed by polyacrylamide gel electrophoresis (PAGE) under native conditions.

Preliminary analyses indicated that fraction DSP2 contained multiple components (data not shown). We set out to characterize the components in fraction DSP2, which, in conjunction with Polβ, were responsible for repair of the UG oligonucleotide. DSP2 was fractionated using phenyl-Sepharose chromatography. This procedure yielded three fractions (PS 1, 2, and 3) which were tested for their repair capacity in the BER assay in combination with Polβ. As shown in Figure 2, the individual fractions were incapable of supporting significant repair synthesis. However, combining fractions PS2 and PS3 led to high levels of repair synthesis. Fraction PS1, when included with fractions PS2 and PS3, had no effect on repair synthesis. As uracil-DNA glycosylase (UDG) activity is believed to be required in the initial stages of BER of uracil-containing DNA, fractions were tested for UDG activity employing a previously described assay using [^{3}H]dUMP-containing calf thymus DNA as substrate (Krokan & Wittwer, 1981). Approximately 70% of UDG activity was recovered in fraction PS3 after phenyl-Sepharose chromatography of fraction DSP2.

Purification of the Repair Factor in Fraction PS3. Fraction PS3 was loaded onto a ssDNA−cellulose column and developed with a linear salt gradient from 0.1 to 0.4 M NaCl. Fractions were assayed for repair synthesis activity when combined with fraction PS2 and Polβ, and for UDG activity. Repair complementation and UDG activities co-eluted in two peaks: as a minor peak in the flowthrough and as a major peak at ∼0.3 M NaCl (data not shown). Approximately 80% of UDG activity was calculated to reside

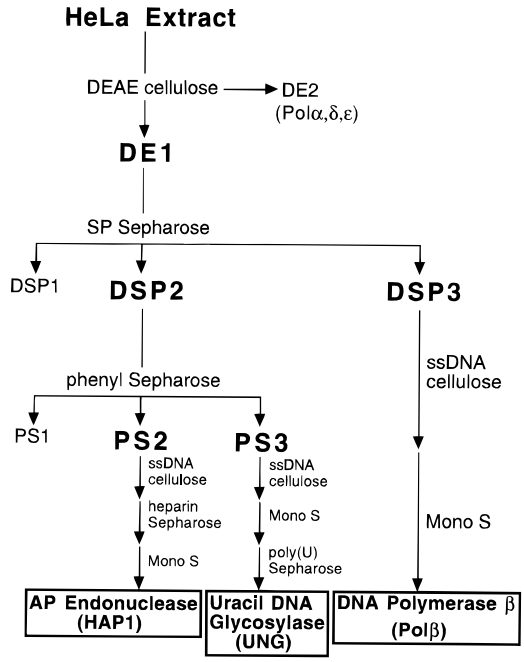

FIGURE 1: Fractionation of HeLa cell extract.

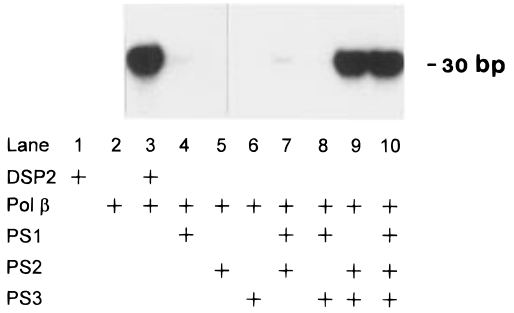

FIGURE 2: Reconstitution of repair synthesis with phenyl-Sepharose fractions. Repair synthesis reactions contained the 30 bp UG oligonucleotide, [α-^{32}P]dCTP, and the following fractions as indicated: DSP2 (2.4 μg), PS1 (150 ng), PS2 (27 ng), PS3 (460 ng), and recombinant Polβ (5.2 ng). Products were analyzed by native PAGE and autoradiography.

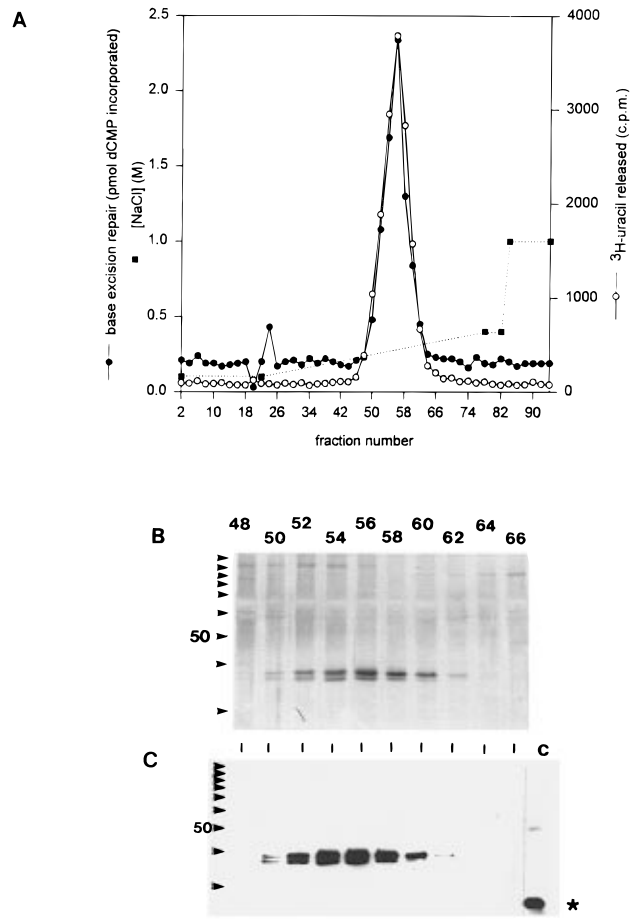

FIGURE 3: Copurification of PS3 repair activity and the UNG uracil-DNA glycosylase using poly(U)-Sepharose chromatography. The peak of repair activity from the Mono S column was pooled and chromatographed on a poly(U)-Sepharose column and fractions were analyzed as follows: (A) Repair and UDG activities. Fractions were assayed for repair activity in combination with fraction PS2 (27 ng) and Polβ (5.2 ng) and for UDG activity. (B) SDS−PAGE. Fractions of peak activity (48−66) were analyzed by SDS−PAGE using a 10% resolving gel and silver staining. (C) Western blot. Peak fractions (48−66) were analyzed by immunoblotting using an anti-UNG polyclonal antibody PU101 (IgG fraction, 1:1000 dilution). Lane C contained truncated, recombinant UNG protein (UNGΔ84, asterisk). Arrowheads at the left indicate migration of 10 kDa molecular mass markers: for orientation, the 50 kDa marker is indicated.

with the second peak. This major peak of activity was pooled and loaded onto a Mono S column. Bound protein was eluted with a linear salt gradient from 0.1 to 0.4 M NaCl. Repair and UDG activities bound to the column and coeluted over a broad NaCl concentration range (data not shown). To facilitate purification of the repair activity, Mono S fractions that supported DNA repair synthesis with the highest specific activity were pooled and loaded onto a poly-(U)-Sepharose column. The poly(U)-Sepharose matrix was previously reported to be highly effective in the purification of a UDG from human placenta (Wittwer *et al.*, 1989). DNA repair and UDG activities bound to the column and were eluted with a linear salt gradient of 0.1−0.4 M NaCl. Figure 3A shows the coelution of DNA repair and UDG activities with peak activity eluting at ~0.25 M NaCl. The purification of UDG activity from the HeLa S-100 extract, using the fractionation procedures described above, was estimated at ~5000-fold with a yield of ~5%. Specific activity was estimated at ~500 units/mg of protein. The product resulting from testing poly(U)-Sepharose fractions in the UDG assay

Table 1: Effect of Various Compounds on UDG Activity[a]

compound	inhibition	IC_{50}
uracil	+	0.4 mM
adenine	−	
cytosine	−	
thymine	−	
3-deazauracil	−	
5-aminouracil	−	
5-chlorouracil	−	
5-fluorouracil	−	
6-aminouracil	+	0.2 mM
6-chlorouracil	+	0.1 mM
6-azauracil	−	
deoxyuridine	−	
dUMP	−	
dNTPs	−	
ATP	−	
p-hydroxymercuribenzoate	+	3 mM
N-ethylmaleimide	−	
EDTA	−	
Ugi protein	+	0.1 unit

[a] Various compounds and the Ugi protein were added to a UDG assay performed at 10 mM NaCl and containing the poly(U)-Sepharose pool (fractions 52−60, ~1.4×10^{-3} unit of UDG activity). The effect of bases and nucleotides were examined up to a concentration of 2 mM. The Ugi protein was tested up to 0.25 unit, and EDTA, *N*-ethylmaleimide, and *p*-hydroxymercuribenzoate were tested up to 5 mM. For *p*-hydroxymercuribenzoate and NEM, DTT was omitted from the UDG assay. One unit of Ugi is defined as the amount of protein required to inhibit 1 unit of *E. coli* UDG (New England Biolabs product description). IC_{50} values were calculated using Enzfitter software (Cambridge Biosoft, Cambridge, U.K.) programmed with the median-effect equation (Chou, 1976).

was confirmed as ^{3}H-uracil by thin-layer chromatography (data not shown).

Characterization of the Purified UDG. Polypeptides in peak poly(U)-Sepharose fractions were analyzed by SDS−PAGE (Figure 3B). Two major bands with molecular masses of approximately 37 and 35 kDa were apparent upon silver staining, corresponding to those fractions possessing UDG and DNA repair activities. Three proteins of ~37 kDa have been reported to exhibit UDG activity: the UNG protein, GAPDH, and a cyclin-like UDG (Olsen *et al.*, 1989; Meyer-Siegler *et al.*, 1991; Muller & Caradonna, 1991). Peak poly-(U)-Sepharose fractions were tested for these proteins by immunoblot analysis. An antibody against the UNG protein (PU101) clearly recognized the 35/37 kDa protein doublet (Figure 3C). PU101 did not cross-react with GAPDH or the cyclin-like UDG (data not shown). Furthermore, antibodies against GAPDH (monoclonal 6C5) and cyclin-like UDG [monoclonal u(91−243)] failed to react with any proteins in the poly(U)-Sepharose fractions (data not shown).

The effect of various bases, nucleotides, uracil analogues, and other compounds on the UDG activity present in peak poly(U)-Sepharose fractions (52−60) was examined to further characterize the purified protein (Table 1). Uracil, 6-aminouracil, 6-chlorouracil, and *p*-hydroxymercuribenzoate clearly inhibited enzymatic activity. Moreover, dose-dependent inhibition was also noted with uracil-DNA glycosylase inhibitor (Ugi), a 9.5 kDa protein produced by the bacteriophage PBS1 (Mosbaugh & Bennett, 1994). Inhibition by uracil, 6-aminouracil, and *p*-hydroxymercuribenzoate is consistent with that previously noted for the UNG protein (Krokan & Wittwer, 1981). As far as we are aware, this is the first time that 6-chlorouracil has been examined and shown to be an inhibitor of UDG activity. The NaCl sensitivity of the UDG present in the poly(U)-Sepharose pool

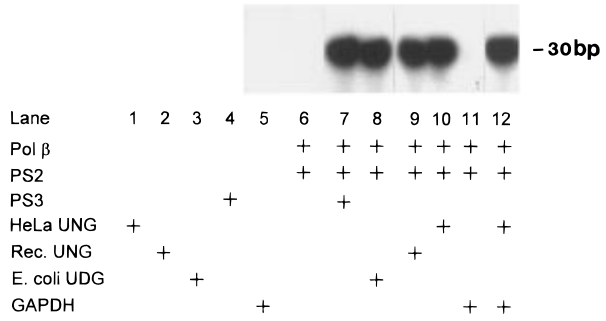

FIGURE 4: Reconstitution of repair activity with purified UDGs. The ability of various purified proteins to complement fraction PS2 and Polβ in the repair synthesis assay was tested. Repair reactions contained (as indicated) recombinant Polβ (5.2 ng), fraction PS2 (28 ng), fraction PS3 (460 ng), HeLa UNG protein [pooled poly(U)-Sepharose fractions 52−60 (see Figure 3), 1.8 ng], recombinant UNG protein (UNGΔ84, 2.3 ng), *E. coli* UDG (1 unit), and GAPDH (6 μg).

was also examined in light of the substantial differences observed with previously purified UDGs (Domena & Mosbaugh, 1985). UDG activity was maximal at approximately 60 mM NaCl. Half-maximal activity was seen without salt and ~70% maximal activity was found at physiological (150 mM) monovalent salt concentrations. The UDG activity examined here is clearly distinguished from the mismatch-specific thymine-DNA glycosylase, which is unaffected by Ugi, is considerably larger (55 kDa), and acts upon U-G mispairs but not U-A base pairs (Neddermann & Jiricny, 1994).

As mentioned above, additional fractions from the ssDNA−cellulose and Mono S columns contained both repair and UDG activities. These fractions were further analyzed to determine the identity of the UDG in these fractions. Immunoblot analysis using PU101 revealed the presence of the UNG protein in fractions containing UDG and DNA repair activities (data not shown). Taken together, the data indicate that the required repair factor in fraction PS3 is the UNG protein.

Reconstitution of DNA Repair Synthesis Using Purified UDGs. The ability of various UDGs to support BER synthesis in combination with fraction PS2 and Polβ was examined (Figure 4). The purified HeLa UNG protein [poly(U)-Sepharose pool] was as effective as fraction PS3 in reconstituting repair synthesis. A recombinant, truncated form of the human UNG protein (UNGΔ84) and *E. coli* UDG also supported high levels of repair in this complementation assay. Repair synthesis was dependent on Polβ, fraction PS2, and one of the aforementioned UDGs. However, human GAPDH was incapable of complementing fraction PS2 and Polβ, even when ~3000-fold more GAPDH than UNG protein was tested in the assay (Figure 4, lane 11). Moreover, addition of GAPDH did not stimulate or inhibit repair synthesis seen with HeLa UNG protein, fraction PS2, and Polβ (Figure 4, lane 12).

Inhibition of BER Synthesis in HeLa Extracts by Anti-UNG Antibodies. The UNG gene product is clearly implicated in BER of uracil-containing DNA, based on its enzymatic activity and the data presented above. However, because of the controversy regarding the possibility of multiple cellular UDGs and the identity of the nuclear UDG, we investigated to what extent the UNG protein is required for BER in HeLa extracts. The anti-UNG polyclonal antibody (PU101, IgG fraction) has recently been shown to be extremely effective in neutralizing UDG activity in HeLa extracts (Slupphaug *et al.*, 1995). Therefore, the capacity of PU101 to inhibit DNA repair synthesis in the HeLa S-100 extract was tested (Figure 5A). Repair synthesis was inhibited in a dose-dependent fashion by up to 90%. The remaining nucleotide incorporation is predominantly background synthesis that is not dependent on uracil-containing DNA (data not shown). In contrast, the addition of similar amounts of an affinity-purified anti-PCNA polyclonal antibody to the HeLa S-100 extract did not inhibit repair (Figure 5A).

In order to confirm that PU101 inhibition of repair synthesis was specific, purified HeLa UNG, recombinant human UNG, and *E. coli* UDG were added to the HeLa S-100 extract along with the neutralizing antibody (Figure 5B). Inhibition of repair synthesis by PU101 was clearly overridden, in a dose-dependent fashion, by HeLa UNG, recombinant UNG, and *E. coli* UDG. This indicates that the inhibition observed by PU101 was due to the specific neutralization of UDG activity in the extract.

As mentioned previously, a 37 kDa cyclin-like protein purified from HeLa cells was reported to possess UDG activity (Muller & Caradonna, 1991). Since high salt (2 M NaCl) was used in the extraction of this cyclin-like protein from HeLa cells and our extraction protocol used only 200 mM NaCl, we were concerned that this putative UDG would not be present in our S-100 extract. Therefore, the nuclear pellet resulting after the initial extraction of HeLa cells was reextracted with high salt (2 M NaCl), essentially as described previously (Caradonna & Cheng, 1980). Total UDG activity in the resulting HS-100 extract was estimated at approximately 25% of that found in the S-100 supernatant. The capacity of PU101 to inhibit the UDG activity in HS-100 extract was examined in order to determine how much of this activity was due to the UNG protein (Figure 5C). UDG activity was specifically inhibited >95% by PU101.

Purification of the Repair Factor in Fraction PS2. The remaining uncharacterized fraction, PS2, was loaded onto a ssDNA−cellulose column and developed with a linear salt gradient from 0.05 to 1 M NaCl. Fractions were assayed for repair synthesis activity in conjunction with HeLa UNG protein and recombinant Polβ. Repair complementing activity eluted as a single peak at ~0.45 M NaCl (data not shown). Peak fractions were pooled and loaded onto a heparin−Sepharose column. Bound protein was eluted with a linear salt gradient from 0.1 to 1 M NaCl. Repair complementation activity eluted as a single peak at ~0.4 M NaCl (data not shown).

Heparin−Sepharose fractions containing the peak of repair activity were pooled and loaded onto a Mono S column. Bound protein was eluted with a linear salt gradient from 0.1 to 0.8 M NaCl. Fractions were assayed for repair synthesis activity in combination with the UNG protein and Polβ. Repair activity eluted as a single peak at ~0.4 M NaCl (Figure 6A). Fractions possessing repair activity were also tested for AP endonuclease activity (Figure 6B). A clear correlation between fractions exhibiting repair activity and AP endonuclease activity is seen. Polypeptides in peak Mono S fractions were analyzed by SDS−PAGE (Figure 6C). Fractions possessing the highest repair activity all contained polypeptides of ~35 kDa. Since the human AP endonuclease HAP1 (also known as redox factor Ref-1) has been postulated to play a role in BER, Mono S peak fractions were subjected to immunoblot analysis using an antibody against HAP1 (Figure 6D). The antibody recognized polypep-

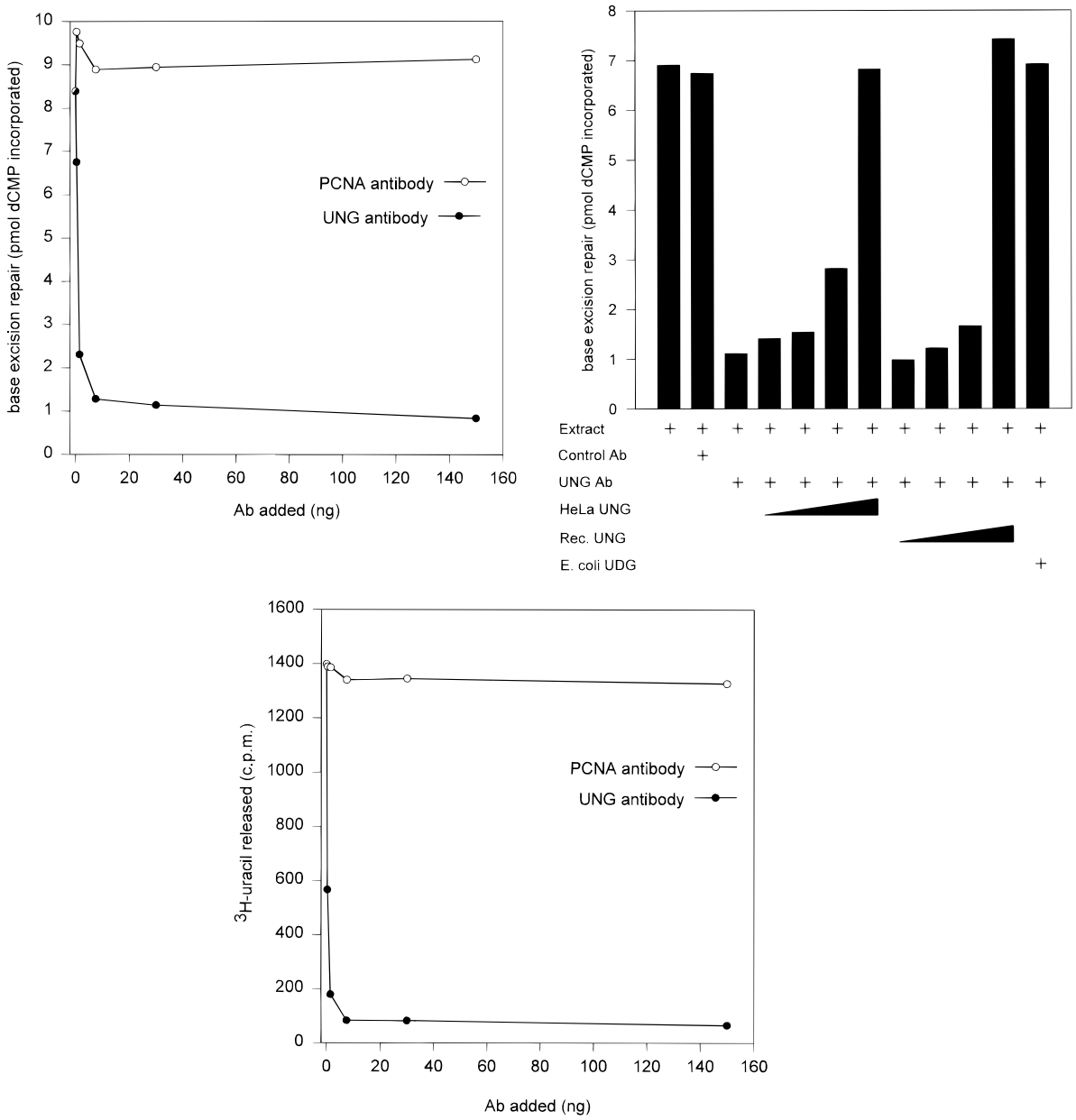

FIGURE 5: Effect of anti-UNG antibodies on BER and UDG activity in HeLa extracts. (A, upper left panel) The effect of affinity-purified, anti-UNG (PU101) or control, anti-PCNA antibodies on repair synthesis in HeLa S-100 extract (13 μg of protein, $\sim 1.4 \times 10^{-3}$ unit of UDG activity) was examined. (B, upper right panel) The ability of exogenously added, purified UDGs to reverse the inhibition of repair synthesis by anti-UNG antibodies was tested. Repair reactions contained HeLa UNG [poly(U)-Sepharose peak fractions 52−60 (see Figure 3); 0.35, 1.0, 3.5, and 10.4×10^{-3} unit], recombinant UNG protein (UNGΔ84; 0.3, 1.0, 3.0, and 10 ng), *E. coli* UDG (3 units), S-100 extract (13 μg), and anti-UNG (PU101) or control, anti-PCNA antibody (6 ng), as indicated. (C, lower panel) The effect of anti-UNG (PU101) or control, anti-PCNA antibody on UDG activity was examined using HeLa HS-100 extract (3.7 μg of protein, 1.4×10^{-3} unit of UDG activity). (Note that antibodies were preincubated with extracts and proteins in reaction mixtures for 10 min at 4 °C prior to incubation at normal reaction temperatures.) Ab = antibody.

tides of ~35 kDa in the Mono S fractions. The reactivity of the antibody with multiple bands may reflect proteolytic action during fractionation and purification steps. On the basis of these data, the required repair component in fraction PS2 appears to be the HAP1 protein.

Reconstitution of BER Synthesis with Purified Human Proteins. The ability of highly purified proteins to reconstitute BER was examined using a 46 bp duplex oligonucleotide containing a UG base pair. A combination of UNG and HAP1 proteins, purified from HeLa cells or from a recombinant source, were able to support repair synthesis in the presence of recombinant Polβ (Figure 7A). All three proteins were required since omission of any one protein abolished synthesis. The level of synthesis observed was comparable to that seen in crude S-100 extract (data not shown). Repair synthesis with purified UNG protein, HAP1 protein, and Polβ was also detected using the 30 bp UG oligonucleotide, but repair was not observed with substrates lacking a uracil (data not shown).

Product Analysis and the Ligation Step. In order to investigate the roles of the individual proteins in BER, a product analysis was performed. In addition to UNG, HAP1, and Polβ, repair reactions were supplemented with recombinant human DNA ligase I or bacteriophage T4 DNA ligase. The 46 bp UG oligonucleotide, which was prelabeled at the 5′-terminus of the uracil-containing strand, was used as a substrate (see Figure 7 legend). Products of the repair reaction were analyzed by denaturing PAGE (Figure 7B). The 46 nucleotide (nt) uracil-containing strand was unaltered in a reaction lacking the UNG protein (Figure 7B, lane 1).

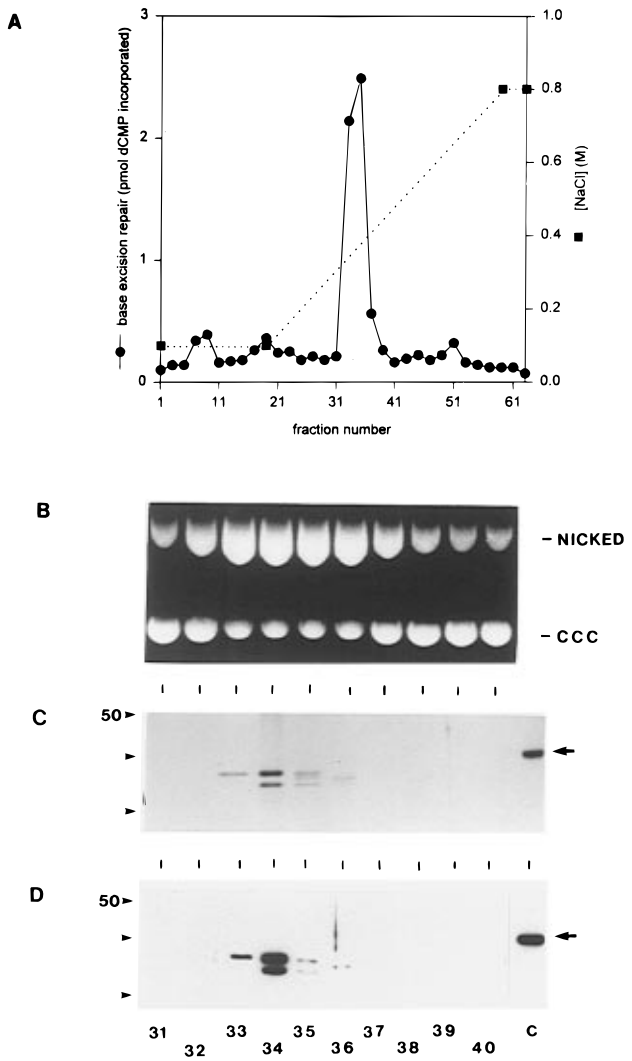

FIGURE 6: Copurification of PS2 repair activity and the HAP1 AP endonuclease using Mono S chromatography. The peak of repair activity from the heparin−Sepharose column was pooled and chromatographed on a Mono S column. Fractions were analyzed as follows: (A) Repair. Fractions were assayed for BER activity in combination with HeLa UNG [poly(U)-Sepharose pool, 3.5×10^{-3} unit] and recombinant Polβ (5.2 ng). (B) AP endonuclease. The peak of repair activity (fractions 31−40) was assayed for AP endonuclease activity by monitoring the conversion of covalently closed circular (ccc) AP-DNA to nicked DNA. (C) SDS−PAGE. Peak fractions were analyzed by SDS−PAGE using a 10% resolving gel and silver staining. (D) Western blot. Immunoblot analysis was performed using anti-HAP1/Ref-1 antibody (C-20, rabbit polyclonal IgG, 50 ng/mL). Lane C contains recombinant HAP1 (6× His-tagged, arrow). Note that the recombinant, His-tagged protein migrates more slowly than expected for the unmodified HAP1. Arrowheads at the left indicate migration of 10 kDa incremental molecular mass markers: for orientation, the 50 kDa marker is indicated.

A reaction containing UNG but missing HAP1 produced no apparent change in the substrate (Figure 7B, lane 2). The uracil has presumably been excised to yield a 46 nt strand containing an AP site, but this product is not resolved from the substrate. In the absence of Polβ, the UNG and HAP1 proteins combined to produce a 19 nt product consistent with uracil excision and cleavage to the 5′-side of the resultant AP site (Figure 7B, lane 3). Addition of Polβ to UNG and HAP1 resulted in a 20 nt product (Figure 7B, lane 4). This is in agreement with the reported ability of Polβ to remove the 5′-deoxyribose phosphate moiety and fill in the resultant one-nucleotide gap (Matsumoto & Kim, 1995). Further addition of human ligase I to UNG, HAP1, and Polβ converted the 20 nt product to a ligated, fully-repaired 46 nt product (Figure 7B, lane 5). T4 DNA ligase was able to ligate at least a fraction of the repair intermediates (Figure 7B, lane 6).

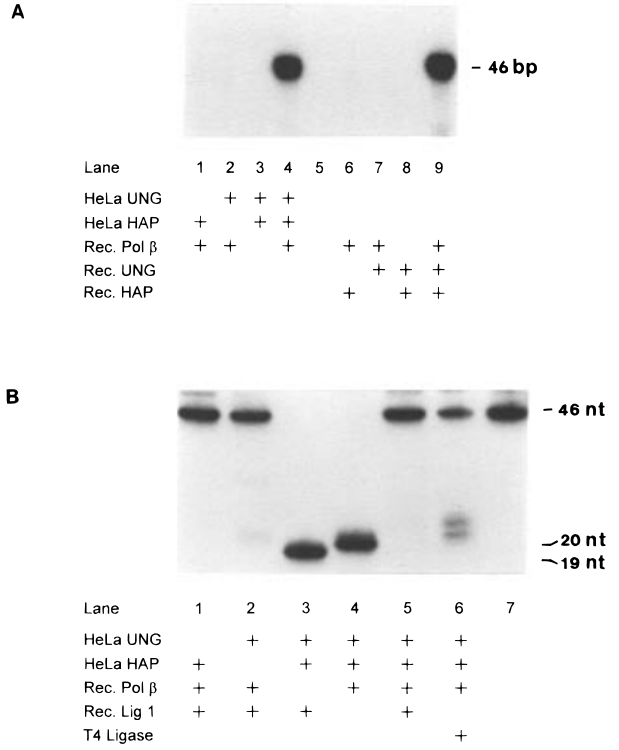

FIGURE 7: BER with purified proteins. (A) Reconstitution of repair synthesis. Repair synthesis was assayed using the 46 bp UG oligonucleotide. Reactions contained HeLa UNG protein [poly(U)-Sepharose pool; 1.7×10^{-3} unit], HeLa HAP1 protein (Mono S peak fraction 34; 0.5 μL), recombinant rat Polβ (5.2 ng), recombinant human UNG protein (UNGΔ84; 2.0 ng), and recombinant human HAP1 protein (0.27 ng), as indicated. Repair products were analyzed by native PAGE. (B) Product analysis of the complete repair reaction including ligation. BER reactions were carried out using as a substrate the 46 bp UG oligonucleotide, prelabeled with ^{32}P at the 5′-end of the uracil-containing strand. In addition to the proteins listed above, the indicated reactions contained recombinant human ligase I (Rec. Lig 1; 30 ng) or bacteriophage T4 DNA ligase (1.0 unit). Products were denatured by heating in formamide loading buffer and analyzed by 8 M urea−12% PAGE. The migration of size markers are indicated on the left. The 46 bp UG oligonucleotide used in this figure contained the following sequence:

```
    1                   19 20                  46
5' ACTCTAGAGGATCCCCGGGUACCGAGCTCGAATTCGCCCTATAGTG 3'
3' TGAGATCTCCTAGGGGCCCGTGGCTCGAGCTTAAGCGGGATATCAC 5'
```

DISCUSSION

We have undertaken the systematic fractionation of extracts from HeLa cells to identify the enzymes required for BER of uracil-containing DNA. This method makes no assumptions based on preconceived ideas of what factors are thought to be involved. We previously reported that Polβ and fraction DSP2 are required to reconstitute repair synthesis using uracil-containing DNA in an *in vitro* assay (Nealon *et al.*, 1996). In this study, DSP2 was separated into two required fractions, PS2 and PS3, which along with Polβ are required for efficient repair synthesis. The PS3 repair complementing activity was extensively purified and identified as a UDG, identical to the product of the UNG gene, on the basis of several criteria: (i) copurification of repair

and UDG activities, (ii) molecular weights of the major, copurifying protein species are consistent with that previously reported for the product of the UNG gene (Slupphaug *et al.*, 1993), (iii) immunological relatedness, (iv) similar sensitivity to inhibitors, and (v) purified, recombinant UNG can substitute for fraction PS3.

A neutralizing antibody against UNG inhibited repair synthesis in the S-100 extract by at least ~90%. Additionally, employing the same antibody, UDG activity remaining in the high-salt nuclear extract (HS-100) was inhibited by >95%. The specificity of the antibody inhibition was confirmed by the ability of purified UNG to block the inhibition and by the lack of antibody cross-reaction with GAPDH or cyclin-like UDG. These results are consistent with previous findings from the Krokan laboratory, which suggested that UNG is the major UDG in human cells (Slupphaug *et al.*, 1995; Nagelhus *et al.*, 1995). Our results further indicate that UNG is responsible for the vast majority of uracil excision during the multienzyme BER reaction in HeLa cell extracts.

The inability of purified GAPDH to function in the repair complementation assay supports the assertion that the UDG activity previously attributed to GAPDH is of limited activity and significance (Slupphaug *et al.*, 1991, 1995; Caradonna *et al.*, 1996). In light of the similar molecular weights of the GAPDH and UNG proteins, it would appear prudent to rule out cross-contamination of these proteins even when they are isolated by SDS−PAGE.

It has been suggested that the UNG protein is the mitochondrial UDG and that the 37 kDa cyclin-like UDG is the nuclear UDG (Caradonna *et al.*, 1996). The results of our antibody inhibition experiments do not support this view. *In vitro* transcription and translation of the cyclin-like UDG cDNA was reported to yield UDG activity (Muller & Caradonna, 1991). In view of the present controversy over the identity of the major and nuclear UDG, it would seem important to demonstrate UDG activity in purified or recombinant cyclin-like UDG that is free of contaminating UNG protein.

The work described herein additionally limits the extent to which the 55 kDa mismatch-specific thymine DNA glycosylase is involved in BER of uracil-containing DNA. However, like GAPDH and cyclin-like UDG, a backup role for this enzyme cannot be excluded at this time. Analysis of human cell lines lacking the aforementioned UDG genes is necessary to unequivocally determine the relative roles of the above proteins in uracil-initiated BER.

During the course of characterizing the UDG activity of the HeLa UNG protein, a novel inhibitor was identified. 6-Chlorouracil inhibited UDG activity with an apparent IC_{50} of 100 μM, which is somewhat lower than those of previously described inhibitors (Krokan & Wittwer, 1981; Focher *et al.*, 1993). This finding is of particular interest since cellular and viral UDGs are potential chemotherapeutic targets for cancer and viral diseases, respectively.

The effect of two sulfhydryl-modifying reagents on the UDG activity of the HeLa UNG protein was examined. *p*-Hydroxymercuribenzoate inhibited the enzymatic activity while *N*-ethylmaleimide did not (Table 1). This same curious pattern of inhibition was previously observed with the human UNG protein (Krokan & Wittwer, 1981) as well as with *E. coli* UDG (Lindahl *et al*., 1977). It is unclear why these two reagents differ in their ability to inhibit UDGs, but it raises the possibility that *p*-hydroxymercuribenzoate may be exerting its effect by some mechanism other than modification of sulfhydryl groups.

The repair-complementing activity in fraction PS2 was purified and identified as HAP1 on the basis of several observations: (i) copurification of repair and AP endonuclease activities, (ii) molecular weight of the major polypeptide species in peak fractions is consistent with that of HAP1 (Robson & Hickson, 1991), (iii) immunological relatedness, and (iv) purified, recombinant HAP1 can substitute for fraction PS2. Our findings are consistent with HAP1 being the major AP endonuclease involved in BER of uracil-containing DNA. There is evidence that human cells may contain a second minor AP endonuclease, as is the case in *E. coli* (Chen *et al.*, 1991). This secondary AP endonuclease may provide redundancy or a different substrate specificity.

The repair of uracil-containing DNA demonstrated here with UNG, HAP1, Polβ, and DNA ligase I represents the first time that BER has been reconstituted with purified mammalian proteins. A dRpase activity that catalyzes the release of 5′-terminal deoxyribose phosphate residues by hydrolysis has been identified in human cell extracts but it has not been extensively purified or cloned (Price & Lindahl, 1991). Recently, it was shown that DNA polymerase β (Polβ) can excise deoxyribose phosphate residues, thus raising the possibility that a separate dRpase might not be necessary (Matsumoto & Kim, 1995). The deoxyribose phosphate residues are removed by Polβ using a β-elimination mechanism as opposed to hydrolysis. Polyamines and basic proteins have also been shown to excise deoxyribose phosphate residues by relatively slow, nonenzymatic β-elimination. It has been suggested that the removal of deoxyribose phosphate residues by nonenzymatic β-elimination may not be physiologically relevant and thus the importance of the dRpase activity in Polβ has remained uncertain (Price & Lindahl, 1991). The absence of a separate dRpase or exonuclease protein from the reconstituted system described here suggests that the dRpase activity intrinsic to the Polβ protein is relevant to BER. In accordance with our observations in crude extracts, BER with purified proteins produced a one-nucleotide repair patch. Although we demonstrate that DNA ligase I is capable of completing the final stage of the reaction *in vitro*, it is unclear whether other human DNA ligases may serve in this role *in vitro* or *in vivo*.

Previous studies indicate that there may be more than one BER pathway which utilizing different DNA polymerases (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996; Nealon *et al.*, 1996). The Polδ/ϵ pathway may have somewhat different protein requirements than the Polβ-dependent reaction described here. A distinct dRpase or exonuclease would presumably be required in conjunction with Polδ/ϵ. There are indications that some BER may be coupled to transcription, and repair of chromatin may also necessitate additional factors (Leadon & Cooper, 1993; Cooper & Leadon, 1994; Ishiwata & Oikawa, 1979).

NOTE ADDED IN PROOF

Kubota *et al*. have recently reconstituted BER using purified UNG, HAP1, Polβ, XRCC1, and DNA ligase III (Kubota *et al*., 1996).

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