

Characterization of the DNA polymerase requirement of human base excision repair

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ABSTRACT

Base excision repair is one of the major mechanisms by which cells correct damaged DNA. We have developed an *in vitro* assay for base excision repair which is dependent on a uracil-containing DNA template. In this report, we demonstrate the fractionation of a human cell extract into two required components. One fraction was extensively purified and by several criteria shown to be identical to DNA polymerase β (Pol β). Purified, recombinant Pol β efficiently substituted for this fraction. *Escherichia coli* PolI, mammalian Pol δ and to a lesser extent Pol α and ϵ also functioned in this assay. We provide evidence that multiple polymerases function in base excision repair in human cell extracts. A neutralizing antibody to Pol β , which inhibited repair synthesis catalyzed by pure Pol β by ~90%, only suppressed repair in crude extracts by a maximum of ~70%. An inhibitor of Pol β , ddCTP, decreased base excision repair in crude extracts by ~50%, whereas the Pol $\alpha/\delta/\epsilon$ inhibitor, aphidicolin, reduced the reaction by ~20%. A combination of these chemical inhibitors almost completely abolished repair synthesis. These data suggest that Pol β is the major base excision repair polymerase in human cells, but that other polymerases also contribute to a significant extent.

INTRODUCTION

Cells have multiple strategies for repairing the various types of damage that constantly alter their DNA. These DNA repair mechanisms are crucial in preventing mutagenesis and carcinogenesis. Certain modified or improper bases are repaired by a base excision repair mechanism in which the offending base is removed, the sugar-phosphate backbone is nicked, and a short repair patch is formed (1–4). For example, the deamination of a dCMP residue in the DNA leads to a U–G mispair which, if not corrected, can result in a C to T mutation (4). Additionally, DNA polymerases can incorporate dUMP into DNA during replication, repair and recombination (2). Base excision repair of uracil-containing DNA is believed to be initiated by a uracil–DNA glycosylase which cleaves the bond between the uracil base and the deoxyribose sugar to yield an abasic site with the sugar-phosphate backbone intact. The phosphodiester bond 5' to the abasic site is

then nicked by an AP (apurinic/apyrimidinic) endonuclease, and the abasic sugar-phosphate residue is removed by the action of a deoxyribosephosphodiesterase (or a 5'→3' exonuclease). A DNA polymerase fills in the resulting single nucleotide gap and a DNA ligase seals the repair patch. Some reports suggest that this model for base excision repair may be overly simplistic or that alternative pathways may exist (5–9). Furthermore, the identity of the enzymes involved in base excision repair remains speculative.

For example, previous studies aimed at determining the identity of the DNA polymerase involved have been inconclusive or conflicting. Three reports have used polymerase inhibitors to conclude that base excision repair in mammalian cell extracts is carried out exclusively by Pol β (3,10,11). Singhal *et al.* (11) also show that reconstitution of the repair reaction with partially purified components can be accomplished using Pol β but not Pol α , δ or ϵ . Additionally, Sobol *et al.* (12) provide genetic evidence for the involvement of Pol β in base excision repair. A cell line lacking the Pol β gene was found to be hypersensitive to alkylating agents, and extracts from these cells did not support base excision repair unless Pol β was provided exogenously. This body of evidence has led to the conclusion that Pol β is essential for base excision repair in mammalian cells (3,10–12).

In contrast, experiments in other eukaryotic systems have suggested that Pol δ and/or ϵ play an important role in base excision repair. Using fractions derived from a *Xenopus* ovarian extract, Matsumoto *et al.* (13) demonstrated that repair of a synthetic tetrahydrofuran AP site was dependent on Pol δ and its accessory protein, proliferating cell nuclear antigen (PCNA). Henderson *et al.* (14,15) have shown that mutations in the *Drosophila* gene encoding the Pol δ/ϵ auxiliary factor, PCNA, cause a hypersensitivity to alkylating agents and ionizing radiation.

Inhibitor studies using intact or permeable mammalian cells have not conclusively determined the polymerase requirement for base excision repair. Early experiments indicated that Pol β is involved in repair of bleomycin-induced DNA damage while an aphidicolin-sensitive polymerase (probably Pol δ or ϵ) carries out repair of alkylated DNA (16,17). More recent reports suggest that both Pol β and Pol δ/ϵ may play a role in DNA repair induced by both bleomycin and alkylating agents (18–21). One limitation of these studies is their reliance on inhibitors with limited specificity (22). Another problem is that bleomycin and alkylating agents produce DNA damage that is repaired not only by base excision repair but by other mechanisms as well.

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Thus, uncertainty clearly remains over which DNA polymerase(s) and other proteins are involved in base excision repair. To address this question, we are using a DNA molecule containing a single, defined uracil residue as a substrate for base excision repair in human cell extracts. In this report we initiate the fractionation and purification of the components required for human base excision repair. We demonstrate that although Pol β is responsible for the majority of uracil-dependent repair synthesis in HeLa cell extracts, other DNA polymerases contribute to a significant degree. We suggest reasons why previous studies using mammalian cell extracts led to the conclusion that only Pol β could fulfill this role.

MATERIALS AND METHODS

Reagents

The following materials were obtained from commercial sources: HeLa S3 cells (National Cell Culture Center); [^3H]dTTP (Amersham); [^{32}P]dNTPs (DuPont-NEN); ATP, creatine phosphate, creatine phosphokinase, β -lactoglobulin A, aphidicolin, bovine serum albumin and single-stranded (ss) DNA-cellulose (Sigma); dideoxyTTP (ddTTP), SP Sepharose and Mono S (Pharmacia LKB); unlabelled dNTPs, phenylmethyl sulfonylfluoride (PMSF) and *E.coli* PolI-large fragment (Gibco-BRL); leupeptin and pepstatin (Boehringer Mannheim Biochemicals); DEAE-cellulose DE52 and phosphocellulose P-11 (Whatman); chromatography columns (Kontes). Pol α was immunoaffinity purified from HeLa cell extract (23). PCNA was purified from *E.coli* which contained a plasmid encoding the human PCNA cDNA under the control of an inducible bacteriophage T7 promoter (24). Pole and RF-C were purified from calf thymus and were generously provided by Vladimir Podust and Ulrich Hübscher. Purified Pol δ from HeLa cells was a gift from Hernan Flores-Rozas and Jerard Hurwitz.

Oligonucleotides

Lyophilized oligonucleotides (Oligos Etc. Inc.) were resuspended in TE (pH 8.0) and concentrations were adjusted according to spectrophotometric readings at OD₂₆₀. Equal concentrations (500 $\mu\text{g}/\text{ml}$ each) of complementary stands were annealed in the presence of 200 mM NaCl by heating at 90°C for 5 min and cooling slowly to room temperature. Annealing was checked by native polyacrylamide gel electrophoresis. The experiments shown in this paper used a 30 base pair (bp) UG oligonucleotide of the following sequence:



We have also used a 30 bp UA oligonucleotide containing a single UA base pair. As controls, the corresponding normal homoduplexes containing CG or TA base pairs were utilized.

Base excision repair assay

This assay measures DNA synthesis using radioactive nucleotides, a uracil-containing duplex oligonucleotide, and a human cell extract or fractions derived therefrom. Reactions (25 μl) contained 40 mM creatine phosphate-diTris salt (pH 7.7), 5 mM MgCl₂, 1 mM dithiothreitol, 2 mM ATP, 20 μM each of dATP, dGTP, dTTP and dCTP, 1 μCi [α - ^{32}P]dCTP (for UG and CG substrates) or dTTP (for UA and TA substrates), 2.5 μg creatine phosphokinase, 50 mM NaCl, 0.5 μg (25 pmol) 30 bp duplex oligonucleotide, and HeLa

crude extract (~10 μg), fractions or purified proteins as indicated. The experiments shown in this paper used the UG oligonucleotide. Reaction mixtures were incubated at 37°C for 30 min. Reactions were stopped by addition of 5 μl of 6 \times gel loading dye (20% Ficoll, 100 mM EDTA, 2% SDS, 0.2% bromophenol blue, 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.

Fractionation and purification of DSP3/Pol β from HeLa extract

HeLa cell S-100 extract. An S-100 extract from 50 l of HeLa S3 cells (5.1×10^5 cells/ml) was prepared as previously described (25,26).

DEAE-cellulose and SP Sepharose chromatography. DEAE-cellulose and SP Sepharose columns (120 c.c. each) were equilibrated with Buffer AN (20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM dithiothreitol) containing 50 mM NaCl and connected in series. HeLa cell extract (1024 mg of protein) was loaded onto the DEAE-cellulose column and flowed through onto the SP Sepharose column. While connected in series, the columns were washed with 600 ml of Buffer AN-50 mM NaCl. The columns were disconnected and the SP Sepharose column was eluted with Buffer AN-250 mM NaCl (360 ml) followed by Buffer AN-1.0 M NaCl (360 ml). Protein peaks were pooled for the flow through (DSP1, 585 mg), 250 mM elution (DSP2, 49 mg) and 1 M elution (DSP3, 14 mg). DNA repair activity was recovered by combining the DSP2 and DSP3 fractions.

In the experiment presented in Table 1, the DEAE-cellulose column was not connected to the SP Sepharose column. The extract was loaded onto the DEAE-cellulose column and the column was washed as above. Bound proteins were eluted with Buffer AN-1.0 M NaCl. The fractions containing the peak protein concentrations were pooled and designated fractions DE1 (flow through) and DE2 (bound).

Single stranded DNA-cellulose chromatography. DSP3 (14 mg) was dialyzed overnight against Buffer AN-250 mM NaCl and loaded onto a ssDNA-cellulose column (9 c.c.) which had been equilibrated in Buffer AN-250 mM NaCl. The column was washed with 50 ml of Buffer AN-250 mM NaCl. A linear gradient elution of 80 ml from 0.25 to 1.0 M NaCl in Buffer AN was performed. Fractions supporting repair synthesis when combined with the DSP2 fraction eluted at ~700 mM NaCl and were pooled.

Mono S chromatography. The ssDNA-cellulose pool (192 μg) was dialyzed against Buffer AN-250 mM NaCl, and lactoglobulin was added to 100 $\mu\text{g}/\text{ml}$ to the dialysate as a carrier. A Mono S FPLC column (8 c.c.) was equilibrated with Buffer AN-250 mM NaCl. The sample was loaded and the column was washed with 20 ml of Buffer AN-250 mM NaCl containing 30 $\mu\text{g}/\text{ml}$ of lactoglobulin. A 20 ml linear gradient to 1.0 M NaCl in Buffer AN containing 30 $\mu\text{g}/\text{ml}$ lactoglobulin was used for elution. Repair complementation activity and DNA polymerase activity co-eluted at ~750 mM NaCl and peak fractions were pooled.

DNA polymerase assay

Standard DNA polymerase reactions (50 μl) contained 50 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 4 mM dithiothreitol, 10 μg

bovine serum albumin, 40 μ M each of dATP, dGTP and dCTP, 10 μ M [³H]dTTP (~500 c.p.m./pmol), and 10 μ g activated DNA (DNase I-treated calf thymus DNA). Where indicated, polymerase activity was measured under repair assay conditions. These conditions were identical to those of base excision repair reactions except for the addition of 10 μ g of bovine serum albumin, the use of 10 μ g of activated DNA as the DNA template, and the use of 10 μ M [³H]dTTP as the radioactive nucleotide. All DNA polymerase reactions were incubated at 37°C for 60 min and acid-insoluble radioactivity was determined.

***In situ*, DNA polymerase activity gel assay**

This method was performed essentially as described by Karawya *et al.* (27). Briefly, protein samples were heated to only 37°C prior to electrophoresis on a 10% SDS–polyacrylamide gel containing 100 μ g/ml activated DNA. Following electrophoresis, the gel was incubated in several changes of wash buffer to ensure removal of the SDS and renaturation of proteins. The gel was then incubated in buffer containing radioactive dNTPs overnight at room temperature. This allows incorporation of labelled nucleotides at the site of any DNA polymerase within the gel. Unincorporated nucleotides were then removed by several washes in 5% trichloroacetic acid, 1% sodium pyrophosphate. The gel was dried and autoradiographed.

Immunoblot assay

Immunoblot (Western blot) assays were performed by standard methods (28). Protein samples were subjected to SDS–PAGE and then transferred to nitrocellulose. Pol β was detected using rabbit polyclonal serum (1:500 dilution), horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin and enhanced chemiluminescence detection (ECL, Amersham). For quantitative immunoblotting, several amounts of each protein fraction were analyzed and the relative band strengths were compared.

Expression and purification of recombinant Pol β

Escherichia coli strain BL21 (DE3) pLysE (Novagen, Inc.), transformed with the rat Pol β expression plasmid pRSET (generously supplied by Dr Samuel Wilson), was cultured in LB broth (800 ml) at 37°C until the OD₆₀₀ reached 0.6. IPTG was added to a concentration of 1 mM, and incubation was continued at 37°C for 4 h. The cells were pelleted and frozen at –20°C. The bacterial pellet was thawed and resuspended in 80 ml of 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 mM PMSF, 0.5 μ g/ml leupeptin and 0.5 μ g/ml pepstatin A. The suspension was incubated for 15 min at room temperature allowing endogenous lysozyme to act. The suspension was vortexed and disrupted by sonication (while cooling on ice) until the viscosity was minimal. All subsequent procedures were carried out at 4°C. The mixture was then centrifuged at 12 000 g for 20 min, and the supernatant decanted. To the supernatant, 50 mM Tris–HCl pH 7.5, 1 mM EDTA and 4 M NaCl was added to give a final NaCl concentration of 200 mM. The supernatant was dialyzed overnight against Buffer A (20 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol) containing 200 mM NaCl. The dialysate was clarified by centrifugation at 12 000 g for 20 min. Pol β was purified by sequential chromatography on DEAE–cellulose, SP Sepharose, ssDNA–cellulose and phosphocellulose, essentially as described above for purification from HeLa extract. Approxi-

mately 6 mg of purified Pol β were obtained starting from an 800 ml culture.

Pol β antibodies

Rabbits were immunized with purified Pol β (Cocalico Biologicals). Polyclonal antibodies against Pol β were purified from rabbit antisera using a Pol β affinity column (28). The affinity resin was prepared by coupling purified recombinant rat Pol β (1 mg in PBS) to cyanogen bromide-activated Sepharose 4B (Sigma). Pol β -specific antibodies were eluted from this column with 100 mM glycine–HCl pH 2.5 or 100 mM triethylamine pH 11.5. Purified antibodies were dialyzed against PBS, concentrated using a Centriprep 10 apparatus (Amicon), and stored in aliquots at –80°C. Affinity-purified anti-PCNA antibodies were produced in a similar manner using purified recombinant human PCNA as the immunogen and in preparation of the affinity resin.

RESULTS

The base excision repair assay

We established a base excision repair assay based on those reported by Dianov *et al.* (3) and Wang *et al.* (29). Our *in vitro* assay measures uracil-dependent repair synthesis using a human HeLa cell extract and a duplex oligonucleotide containing a single uracil residue. A 30 bp UG oligonucleotide was identical to a CG oligonucleotide except for a single dUMP in place of a dCMP at position 14 (see Materials and Methods). Likewise, a 30 bp UA oligonucleotide differed from a TA oligonucleotide by virtue of a single uracil residue in place of a thymine. A UG base pair represents the result of cytosine deamination in normal duplex DNA, whereas a UA base pair would result from the incorporation of a dUMP during the course of DNA replication. The oligonucleotides were incubated in HeLa cell extract with radiolabelled nucleotides and the reaction products were electrophoresed on a polyacrylamide gel. We typically observed \geq 10-fold more synthesis on the uracil-containing oligonucleotides compared with the normal duplex oligonucleotides. A duplex oligonucleotide containing a GT mismatch (which would result from deamination of a 5-methyl cytosine residue) did not support significant levels of repair synthesis. Likewise, single-stranded oligonucleotides were not efficient substrates in this reaction. The vast majority of repair events consisted of a single nucleotide repair patch (3,11; K.N. and M.K.K., unpublished observations).

Because we intended to use this assay for fractionation and purification, it was essential for it to be rapid, sensitive and quantitative. Importantly, we found that purification of the reaction products was neither necessary nor desirable. Addition of gel loading dye (containing SDS and EDTA) to stop the reaction followed by direct loading of the mixture onto the gel led to more rapid and quantitatively more reproducible results. Quantitation of repair synthesis was achieved by excising radioactive bands from the dried gel and measuring radioactivity in a scintillation counter. Using ~10 μ g of extract, the reaction remained linear for at least 30 min at which point ~20% of the template molecules are typically repaired. This compares favorably with other complex mammalian *in vitro* DNA synthesis assays such as SV40 DNA replication and nucleotide excision repair.

A potential role for poly ADP-ribose polymerase was investigated because of previous evidence suggesting the involvement of this enzyme in DNA repair processes (2). The addition of the poly

ADP-ribose polymerase substrate (NAD) or inhibitor (3-amino-benzamide) had no effect on repair synthesis. Surprisingly, a 1.5–3-fold stimulation of repair synthesis by ATP was consistently observed, and thus ATP and an ATP-regenerating system were included in all assays. Neutralizing antibodies to human SSB (RP-A) and Pol α had no significant effect on the repair reaction.

Fractionation and reconstitution of repair synthesis

To investigate the protein requirements for base excision repair of a uracil-containing template further, we have begun fractionating the crude extract. Preliminary experimentation with various fractionation steps indicated that base excision repair activity flowed through a DEAE-cellulose column when loaded at 50 mM NaCl. This procedure removed ~50% of the protein and the majority of the nucleic acids in the crude extract which contributed to some non-specific DNA synthesis. It was also noted that the vast majority of DNA polymerase activity bound to the DEAE-cellulose.

The material that flowed through the DEAE-cellulose column was directly loaded onto an SP Sepharose column. The column was washed with buffer containing 50 mM NaCl and protein was eluted with two steps of 250 mM and 1 M NaCl. The protein peaks of the flow through and the step elutions were collected and these fractions were designated DSP 1, 2 and 3, respectively (Fig. 1A). These fractions were assayed individually and in combination for repair synthesis on the UG oligonucleotide. As shown in Figure 1B the fractions were individually incapable of supporting repair synthesis. However, the combination of the fractions DSP 2 and 3 led to high levels of repair. DSP 1 had no effect on the synthesis by the other two fractions. Repair synthesis by DSP 2 and 3 exhibited even greater dependence on a uracil-containing DNA template than the crude extract presumably due to removal of non-specific nucleases. The reconstituted system was able to utilize both the UG and UA oligonucleotides, and the size of the repair patch synthesized was a single nucleotide (data not shown).

Co-purification of DSP3 and Pol β

Because DSP3 contained $\leq 2\%$ of the protein of the crude extract, and because initial fractionation attempts suggested that it may contain a single required component, attention was focused on purifying that component from DSP3. Since virtually all of the DNA polymerase activity that flowed through the DEAE-cellulose column was found in fraction DSP3, fractions were assayed for polymerase activity as well as for the ability to perform repair synthesis in combination with DSP2. DSP3 was loaded onto a ssDNA-cellulose column and eluted with buffer containing a linear gradient from 0.25 to 1.0 M NaCl. The majority of the repair synthesis and DNA polymerase activity bound to the resin and co-eluted in a peak at ~0.7 M NaCl, resulting in a 26-fold purification (data not shown).

This peak was pooled and loaded onto a Mono S FPLC column. Both repair and polymerase activities bound to this column and were eluted with a linear salt gradient from 0.25 to 1.0 M NaCl. Figure 2A shows the co-elution of repair and polymerase activities around the peak fractions at ~0.75 M NaCl. Initial characterization of this co-eluting polymerase suggested that it was Pol β . To test this possibility further, a DNA polymerase activity gel assay was performed. This assay measures DNA polymerase activity *in situ* in an SDS-polyacrylamide gel and thus provides information on the molecular weight of the polymerase.

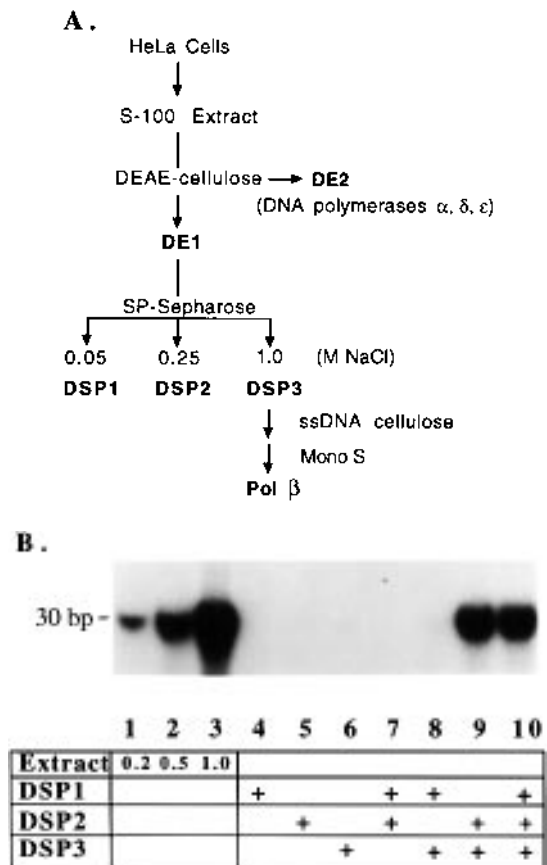


Figure 1. Fractionation of HeLa cell extract. (A) Fractionation scheme. (B) Reconstitution of repair synthesis with fractions. Indicated below each lane is the volume (in μ l) of extract (17 μ g/ μ l) added or which fraction(s) was added (DSP1, 0.60 μ g; DSP2, 0.36 μ g; DSP3, 0.22 μ g).

Figure 2B shows an activity gel analysis of the Mono S fractions containing the peak of repair activity. A DNA polymerase activity which migrated identically to purified Pol β at 39 kDa, co-eluted with repair activity. Figure 2C shows the result of an immunoblot analysis of these same peak fractions using antibodies against Pol β . A 39 kDa band, immunologically related to Pol β , also co-eluted with repair activity. Although it is difficult to determine the precise degree of purification achieved by Mono S chromatography due to the presence of carrier protein in the elution buffer, we estimate that the overall purification of this factor from crude extract is >1000-fold.

To clarify further the identity of the polymerase in DSP3, the pooled fractions from the Mono S column were compared with the known mammalian nuclear DNA polymerases with regard to their sensitivity to polymerase inhibitors (data not shown). The polymerase activity of the purified DSP3 fraction was resistant to aphidicolin (at 100 μ g/ml) and sensitive to ddTTP (at 100 μ M; 5:1 of ddTTP:dTTP). Purified, recombinant Pol β exhibited the same sensitivity to these inhibitors while purified Pol α , δ and ϵ showed the opposite sensitivity. Taken together, these data indicate that the factor in DSP3, required for DNA repair, is Pol β .

Specificity of DNA polymerases in base excision repair

Although Pol β was purified as a factor required for base excision repair, it is possible that other polymerases might also function in

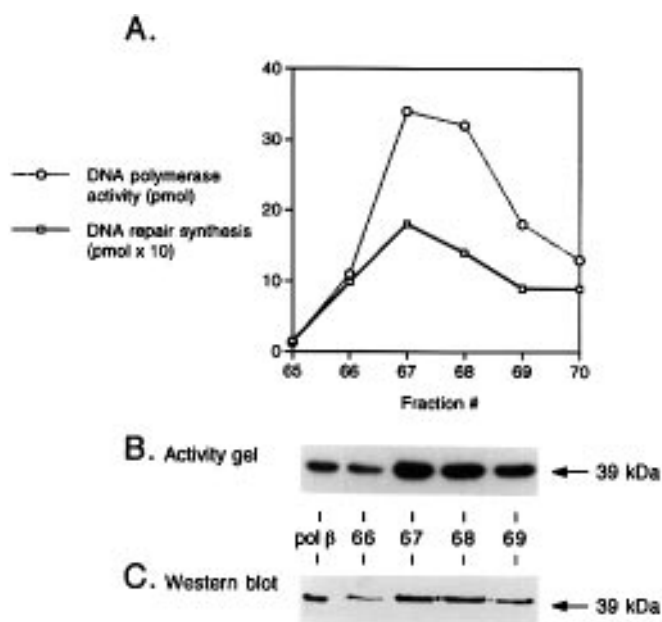


Figure 2. Co-purification of DSP3 repair activity and Pol β using Mono S chromatography. The ssDNA-cellulose pool was chromatographed on a Mono S FPLC column and fractions surrounding the peak of repair activity were analyzed by the following assays. Lanes labelled Pol β contained purified recombinant rat Pol β . (A) Repair synthesis (in the presence of DSP2, 1.3 μ g) and DNA polymerase activity. (B) *In situ*, DNA polymerase activity gel analysis. (C) Immunoblot analysis.

this type of DNA repair. Various DNA polymerases were tested for their ability to complement DSP2 in the DNA repair assay (Fig. 3). Purified recombinant Pol β was able to fully substitute for DSP3 in its ability to complement DSP2 in the repair reaction. This further confirms that there is only one essential factor in DSP3 and that this factor is Pol β . *Escherichia coli* PolI also supported efficient repair synthesis in combination with DSP2. Of the other mammalian DNA polymerases tested, Pol δ yielded the highest level of repair synthesis. Although the incorporation seen with Pol α and ϵ was low, it was still above background. The addition of the polymerase accessory proteins, PCNA and RF-C, did not significantly influence the level of repair synthesis by Pol δ or ϵ (data not shown). The synthesis by all of the polymerases was dependent on DSP2 and a uracil-containing oligonucleotide.

To address further the possibility that DNA polymerases other than Pol β may be able to function in base excision repair synthesis, a fraction containing the bulk of the polymerase activity found in the crude extract was tested. The initial step in the fractionation scheme presented in this paper (Fig. 1), involves chromatography on a DEAE-cellulose column. While most Pol β flows through this column, 99% of the total polymerase activity found in the crude extract binds to the column (Table 1). The distribution of Pol β during this chromatographic step was examined by immunoblotting. Three times as much Pol β was detected in the flow-through fraction (DE1) compared with the bound fraction (DE2). These fractions were also tested for their ability to complement DSP2 in the repair reaction. DSP2-complementing activity was approximately equally divided between DE1 and DE2. This division of repair activity reflects neither the Pol β nor the total DNA polymerase distribution. The

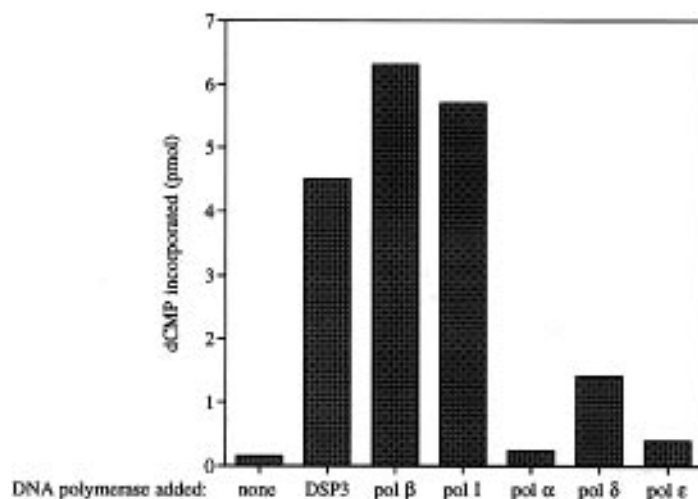


Figure 3. Activity of various DNA polymerases in the base excision repair assay. The ability of various purified DNA polymerases to complement DSP2 in the base excision repair assay was tested. Each reaction contained DSP2 (1.3 μ g) and equal amounts (35 polymerase units) of the indicated DNA polymerase. DNA polymerase activity of each polymerase was determined on activated DNA using repair conditions rather than the standard polymerase assay conditions. One polymerase unit incorporated 1.0 pmol of dTMP in 60 min at 37°C. DSP3 was purified through the SP Sepharose step. Recombinant rat Pol β was purified from *E. coli*. The *E. coli* PolI large fragment (Klenow fragment) lacking 5'→3' exonuclease was used. Pol α and Pol δ were purified from HeLa cells. Pol ϵ was purified from calf thymus.

DSP2-complementing activity in DE2 is most likely contributed to by both the 25% of Pol β which bound to the column and one or more of other polymerases found in this fraction.

Table 1. Distribution of polymerase and repair activities in DEAE-cellulose fractions

Fraction	Polymerase activity ^a	Pol β ^b	Repair activity ^c
DE1 (flow-through)	1%	75%	48%
DE2 (bound)	99%	25%	52%

^aDNA polymerase activity was quantitated using activated DNA as a template.

^bRelative Pol β levels were determined by quantitative immunoblotting.

^cRepair activity refers to the ability to complement DSP2 in the uracil base excision repair assay.

In order to relate our findings with purified polymerases to the situation in crude extracts, a neutralizing antibody against Pol β was developed. Crude rabbit anti-serum directed against Pol β did not contain a high enough titer of Pol β -neutralizing antibodies to be useful. However, when this serum was purified on a Pol β affinity column and concentrated, it proved to be a highly specific inhibitor of Pol β . This Pol β antibody inhibited the repair reaction with DSP2 and Pol β by 89% at the highest level of antibody (Fig. 4A). The Pol β antibody inhibited repair in crude extracts by 67% at maximal antibody levels (Fig. 4B). Thus the Pol β antibody only inhibits repair synthesis in crude extracts ~75% as well as it inhibits the reconstituted reaction containing purified Pol β . This suggests that another polymerase may be responsible for the synthesis in crude extracts which is unaffected by the Pol β antibody. A rabbit anti-PCNA antibody, purified on a PCNA affinity column, served as a control and had no significant effect

on repair synthesis in the reconstituted system or with crude extracts (Fig. 4A and B). In order to further demonstrate that the Pol β antibody was exerting its inhibitory effect on repair synthesis by specifically blocking Pol β , exogenously-added Pol β was used to saturate the antibody and overcome the inhibition. Addition of purified Pol β to reactions containing Pol β antibody fully restored repair synthesis to levels observed in the absence of antibody (Fig. 4C).

Chemical inhibitors of eukaryotic DNA polymerases were used to assess further the relative contribution of the various polymerases to repair synthesis in crude extracts (Fig. 5). The Pol β inhibitor, ddCTP, reduced repair synthesis by ~50%, while the Pol $\alpha/\delta/\epsilon$ inhibitor, aphidicolin, suppressed the reaction by ~20%. A combination of ddCTP and aphidicolin inhibited repair in crude extract by ~80%. The specificity of these inhibitors is demonstrated by their effect on repair reactions using purified polymerases in combination with DSP2. While ddCTP strongly inhibited repair assays utilizing Pol β , aphidicolin had no effect either alone or in conjunction with ddCTP. Conversely, aphidicolin markedly reduced repair synthesis catalyzed by Pol δ , but ddCTP had little or no effect on the reaction. Taken together, these results clearly indicate that multiple polymerases contribute to base excision repair synthesis in HeLa cell extracts.

DISCUSSION

In this report, we have initiated the identification of enzymes involved in the repair of uracil-containing DNA by fractionating and purifying factors required to reconstitute repair synthesis. Ion exchange chromatography was used to separate the HeLa cell crude extract into two required fractions. One of these fractions was extensively purified and appears to be identical to Pol β based on several criteria: (i) co-purification of repair and polymerase activities; (ii) the co-purifying polymerase is the same size as Pol β ; (iii) is immunologically-related to Pol β ; and (iv) has the same inhibitor sensitivity as Pol β ; (v) purified, recombinant Pol β can substitute for this fraction. This represents the first time Pol β has been purified as a factor required for DNA repair. Additionally, inhibition experiments indicate that Pol β is required for the majority of the repair synthesis in crude extracts. This important role for Pol β in base excision repair is consistent with what is known of this enzyme. For example, Pol β has been shown previously to efficiently repair DNA templates with short gaps *in vitro* (17,30,31). Also, Pol β is present in most cells regardless of their proliferative state, and Pol β mRNA levels are increased in response to alkylating agents (32,33).

Although Pol β appears to be the major base excision repair polymerase in HeLa cell extracts, our results indicate that other polymerases can and do function in this process. Other DNA polymerases, including *E.coli* PolI and human Pol δ , substituted for Pol β in reconstituting repair in a partially purified system. Secondly, the initial fractionation of the crude extract yielded one fraction enriched for Pol β and another fraction enriched for Pol $\alpha/\delta/\epsilon$ (containing only 25% of the total Pol β). Each fraction contributed equally to satisfying the polymerase requirement in the reconstituted repair reaction. Thirdly, a neutralizing antibody against Pol β was unable to completely abolish repair synthesis in crude extracts. Lastly, repair in extracts exhibited a strong but incomplete sensitivity to dideoxynucleotides and a partial sensitivity to aphidicolin. A combination of these inhibitors led to a greater effect than either alone.

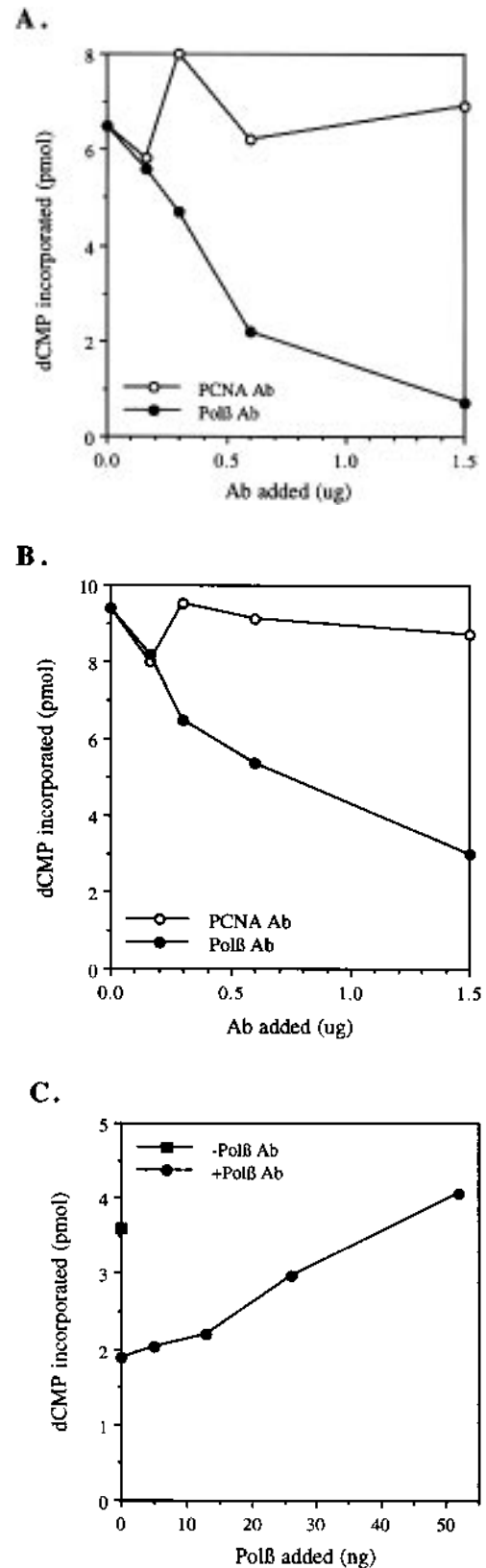


Figure 4. Effect of anti-Pol β antibodies on base excision repair. The effect of affinity-purified rabbit anti-Pol β or control, anti-PCNA antibodies was tested on repair synthesis using (A) DSP2 (1.3 μ g) and Pol β (2.6 ng) or (B) crude extract (13 μ g). (C) The effect of exogenously-added Pol β to a repair reaction containing crude extract and anti-Pol β antibody (1.5 μ g).

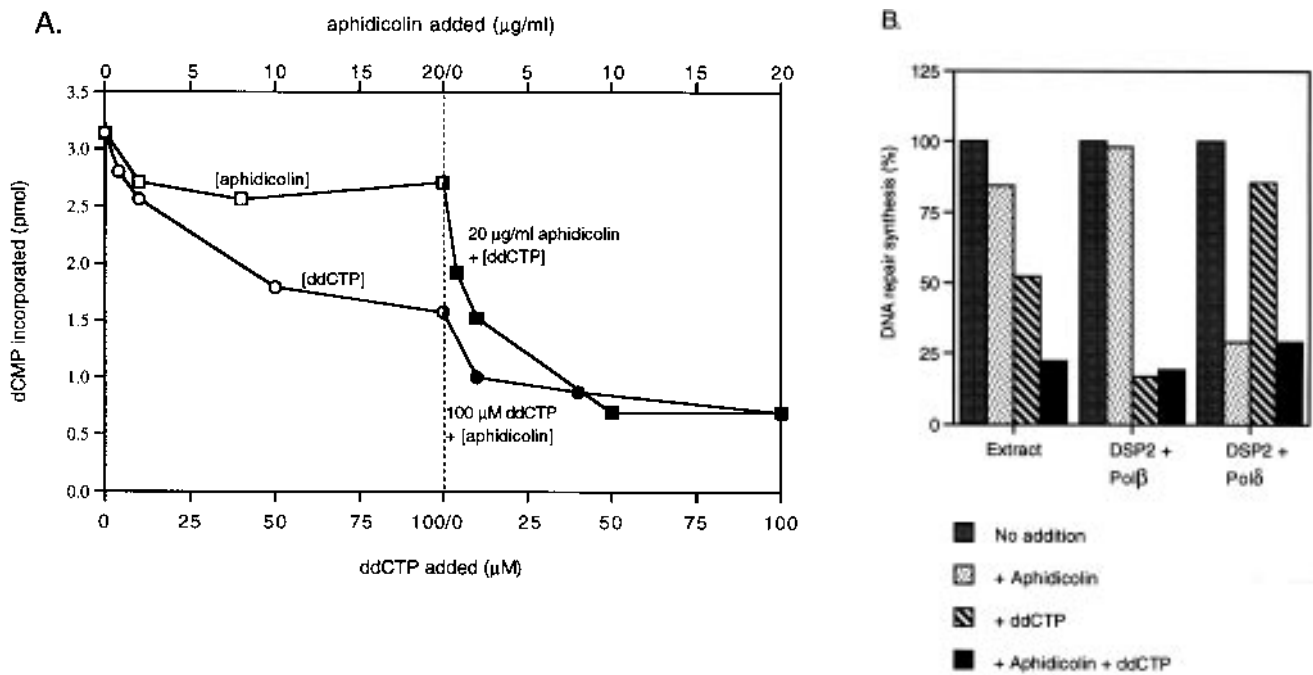


Figure 5. Effect of polymerase inhibitors on base excision repair. (A) Base excision repair synthesis was measured using crude extract in the presence of varying amounts of aphidicolin or ddCTP or a combination of both, as indicated. (B) Repair synthesis was assayed in crude extract or DSP2 plus Pol β or DSP2 plus Pol δ , as indicated. In each case, DNA repair synthesis in the absence of inhibitors is defined as 100%. The level of dCMP incorporated in the absence of inhibitors was: Extract, 2.43 pmol; DSP2 plus Pol β , 3.18 pmol; DSP2 plus Pol δ , 0.97 pmol. The effect of addition of aphidicolin (40 μ g/ml) or ddCTP (100 μ M) or a combination of both is shown. In order to control for the fact that aphidicolin was dissolved in DMSO, reactions were supplemented such that they all contained a final concentration of 1.0% DMSO.

Studies with other organisms indicate that Pol β , δ and ϵ may each participate in base excision repair. In combination with fractions from a *Xenopus* ovarian extract, both Pol β and Pol δ are able to support repair of DNA containing natural AP sites (13). In *Saccharomyces cerevisiae*, base excision repair is dependent on Pol δ and/or Pol ϵ , and Pol β does not appear to be required (11,29,34). This polymerase dependency may reflect the fact that yeast Pol β differs significantly from the mammalian enzyme and mitotically-growing yeast cells contain little if any Pol β compared to meiotic cells (35). Genetic evidence suggesting a role for Pol δ/ϵ in base excision repair comes from the study of the *Drosophila* mutant mus209, which is extremely sensitive to alkylating agents and ionizing radiation. The mus209 gene encodes the Pol δ/ϵ accessory factor, PCNA (14,15).

However, other studies in mammalian cells have concluded that Pol β alone is responsible for catalyzing base excision repair. Wiebauer and Jiricny (10) reported that base excision repair of DNA containing a G-T mismatch by human cell extracts was inhibited by a polyclonal antibody against Pol β . However, reversal of this inhibition by exogenously-added Pol β was not demonstrated and the specificity of this inhibition has been questioned (11). Dianov *et al.* (3) used DNA polymerase inhibitors to address the question of which polymerase was involved in base excision repair of uracil-containing DNA in human cell extracts. They found that 100 μ M ddTTP, an inhibitor of Pol β , reduced repair synthesis by 67%. On the other hand, 100 μ g/ml aphidicolin, an inhibitor of Pol α , δ and ϵ , diminished synthesis by ~40%. These data were used to suggest that Pol β carried out repair synthesis in this system. Alternatively, it could be argued that a combination of

dideoxynucleotide-sensitive and aphidicolin-sensitive polymerases were responsible for the repair synthesis.

Wilson and co-workers (11,12) have presented evidence that Pol β is essential for base excision repair of uracil-containing DNA in mammalian cell extracts and that this polymerase requirement cannot be satisfied by Pol α , δ or ϵ . Differences in the method of extract preparation may explain the disparity with the results presented here. The 0–40% ammonium sulfate precipitation step used for nuclear extract preparation by the Wilson laboratory (11,12) may have resulted in the removal of Pol α , δ , ϵ and accessory factors from the extract. Reconstitution of repair by the addition of pure Pol α , δ or ϵ may have failed because of the loss of accessory proteins such as deoxyribophosphodiesterase or exonuclease during extract preparation. Pol β , on the other hand, contains an intrinsic deoxyribophosphodiesterase activity and thus might not require this factor to be present in the extract (36).

Sobol *et al.* (12) also present genetic evidence that Pol β is involved in base excision repair *in vivo*. They found that a mouse cell line lacking the Pol β gene exhibited normal viability and growth characteristics but was moderately hypersensitive to alkylating agents. They concluded that Pol β was essential for base excision repair since most alkylation damage is believed to be repaired by this process. However, it has been estimated that mammalian cells acquire at least 10 000 mutagenic and cytotoxic 'base lesions' per day per genome. It seems unlikely that cells lacking Pol β would grow normally if their base excision repair was completely shut down. Additionally, it would be surprising if Pol δ can participate in base excision repair in *Xenopus*, *Drosophila* and yeast but not at all in mammals.

It appears, therefore, that multiple polymerases can catalyze base excision repair in mammals as well as lower eukaryotes. The abundance and availability of the various polymerases in a given cell may in part determine their relative contribution to base excision repair in that particular cell. In the HeLa cell extracts used here, Pol β appears to catalyze ~75% of the base excision repair synthesis while other polymerases (in particular Pol δ) are responsible for the remaining 25%.

Additionally, the nature of the base damage may also influence which DNA polymerase is used. In yeast, for example, Pol δ has been reported to repair methylated DNA while Pol ϵ was shown to repair thymine glycol-containing DNA (29,34). In the *Xenopus* system, both Pol δ and Pol β could participate in the repair of natural AP sites, but only Pol δ efficiently repaired synthetic AP sites (13). It is possible that the proteins and mechanisms involved in repairing these various types of DNA damage may differ significantly.

We believe that there may be two base excision repair pathways which differ in the polymerases used, the mechanisms of removal of the deoxyribose phosphate residue, and the ligases used. Recently, Frosina *et al.* (9) provided evidence for two pathways in the repair of AP sites in mammalian extracts. One pathway resulted in a repair patch of ~7 nt and was sensitive to inhibition by a PCNA antibody. The other pathway was PCNA-independent and produced a single nucleotide repair patch. Our findings are consistent with this model and provide the first direct evidence that both Pol δ and Pol β can function in base excision repair in mammals. The fact that Pol δ can load onto short linear templates in the absence of PCNA and RF-C explains the lack of a requirement for these proteins in our system. In contrast, Frosina *et al.* (9) used large circular duplex DNA containing a single AP site. The finding that Pol β exhibits intrinsic deoxyribosephosphodiesterase activity (36) also suggests that Pol δ will require different proteins to carry out the complete repair reaction.

Whether Pol β is the only base excision repair polymerase or simply the major one is an important distinction. There is currently interest in inhibiting DNA repair pathways in conjunction with standard chemotherapy protocols (37,38). It is crucial to know whether there is a single pathway or multiple sub-pathways when choosing targets to inhibit. This report indicates that there may be multiple pathways to achieve base excision repair and represents an important first step in reconstituting these pathways with purified proteins.

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