

Determination of human DNA polymerase utilization for the repair of a model ionizing radiation-induced DNA strand break lesion in a defined vector substrate

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ABSTRACT

Human DNA polymerase and DNA ligase utilization for the repair of a major class of ionizing radiation-induced DNA lesion [DNA single-strand breaks containing 3'-phosphoglycolate (3'-PG)] was examined using a novel, chemically defined vector substrate containing a single, site-specific 3'-PG single-strand break lesion. In addition, the major human AP endonuclease, HAP1 (also known as APE1, APEX, Ref-1), was tested to determine if it was involved in initiating repair of 3'-PG-containing single-strand break lesions. DNA polymerase β was found to be the primary polymerase responsible for nucleotide incorporation at the lesion site following excision of the 3'-PG blocking group. However, DNA polymerase δ/ϵ was also capable of nucleotide incorporation at the lesion site following 3'-PG excision. In addition, repair reactions catalyzed by DNA polymerase β were found to be most effective in the presence of DNA ligase III, while those catalyzed by DNA polymerase δ/ϵ appeared to be more effective in the presence of DNA ligase I. Also, it was demonstrated that the repair initiating 3'-PG excision reaction was not dependent upon HAP1 activity, as judged by inhibition of HAP1 with neutralizing HAP1-specific polyclonal antibody.

INTRODUCTION

DNA strand breaks are a consequence of a variety of different processes, including exposure to environmental DNA damaging agents, oxidative metabolism and DNA base damage processing (1–4). Strand breaks formed as a result of exposure to ionizing radiation are structurally representative of a large proportion of the breaks produced in all of these processes (5–7). Therefore, ionizing radiation-induced DNA strand breaks serve as an excellent model for DNA strand breaks resulting from a variety

of different cellular processes and insults. Also, the observation that DNA single-strand breaks (SSBs) are the most prevalent specific lesion type directly induced in DNA by ionizing radiation (8) and recent findings showing that these are mutagenic lesions (9–11) illustrate the importance of understanding the biochemical mechanisms by which cells process these lesions for repair.

Ionizing radiation-induced DNA SSBs are chemically defined and consist of two equally distributed forms. The lesion is composed of a 1 nucleotide gap containing a 5'-phosphate (5'-P) and either a 3'-phosphoglycolate (3'-PG) or a 3'-phosphate (3'-P) (5,12). The loss of coding information and the lack of a 3'-OH in ionizing radiation-induced SSBs suggests that these lesions are processed by a repair pathway similar to that of base excision repair or AP site repair. Presumably the 3'-end of the lesion is converted to a 3'-OH, the lost coding information is restored in a template-dependent manner by DNA polymerase and repair is completed by the action of DNA ligase. In the case of base excision and AP site repair, these reactions have been shown to be catalyzed by HAP1 and DNA polymerase β in association with DNA ligase I or DNA ligase III and XRCC1 (13–18) and/or by DNA polymerases δ or ϵ in association with DNA ligase I (19–22). Enzymes capable of initiating ionizing radiation-induced SSB repair have recently been identified and characterized (23–26); however, the polymerase(s) responsible for the polymerization step have not been determined, nor have the DNA ligases responsible for resolving the lesion been identified.

In this study, we have used a defined vector substrate containing a site-specific SSB, consisting of a 1 nucleotide gap flanked by a 5'-P and 3'-PG, to investigate the roles of the human DNA polymerases, ligases and HAP1 in SSB repair. The 3'-PG-containing lesion was chosen because it has been implicated as being the more toxic of the two ionizing radiation-induced SSB forms (27) and because it is the lesion for which enzymes capable of initiating SSB repair have been characterized. The ability to effect repair of the model SSB vector substrate by the individual α , β and δ/ϵ DNA polymerases from extracts of human HeLa cells was assessed by differential DNA polymerase inhibition. DNA ligase

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utilization was assessed by enhanced polymerase-specific repair in the presence of chromatographically separated HeLa cell DNA ligases. In addition, the role of HAP1 (also known as APE1, APEX, Ref-1), a postulated SSB repair initiating enzyme, was examined for its ability to initiate the repair reaction by specific inhibition with a neutralizing polyclonal anti-HAP1 antibody.

MATERIALS AND METHODS

Materials

M13mp19, *Escherichia coli* strain DH5 α F' and fetal bovine serum were obtained from Life Technologies (Gaithersburg, MD). Minimal Essential Medium with Eagle's salts and Spinner Modification (SMEM) were obtained from Biofluids, Inc. (Rockville, MD). Aphidicolin was purchased from United States Biochemical (Cleveland, OH). Poly(dA), poly(A), oligo(dT)₁₂₋₁₈ and ddTTP were purchased from Pharmacia (Piscataway, NJ). BuPdGTP was a generous gift from Dr George E. Wright (University of Massachusetts, Worcester, MA). Anti-HAP1 polyclonal antibody and recombinant HAP1 were a generous gift from Dr W. David Henner (Oregon Health Sciences University, Portland, OR). Anti-PCNA clone PC-10 monoclonal antibody was purchased from Oncogene Science (Cambridge, MA). Non-radioactive nucleotides, aprotinin, leupeptin, pepstatin A, chymostatin, bestatin, PMSF, were obtained from Sigma Chemical Co. (St Louis, MO). [5-³H]Thymidine triphosphate (TTB; 77.9 Ci/mmol) and [γ -³²P]adenosine triphosphate (6000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Oligonucleotides bearing 3'-PG were synthesized by and obtained from Eppendorf USA, Inc. (Madison, WI). All other oligonucleotides were obtained from the Lombardi Comprehensive Cancer Center's Macromolecular Synthesis/Sequencing Core Facility, Georgetown University, Washington, DC.

Cell growth conditions and nuclear isolation

HeLa S₃ Cells were grown as suspension cultures at 37°C in SMEM containing 10% (v/v) fetal bovine serum, 5% (v/v) glutamine, 1% (v/v) Pen/Strep (10 000 U/ml penicillin, 10 000 U/ml streptomycin sulfate) and 25 mM HEPES-KOH (pH 7.2). In experiments for which nuclear extracts were used, nuclei were isolated from 1 × 10⁹ freshly harvested, logarithmically growing cells, by a modification of the method previously described (28). Cells were harvested by centrifugation (4°C and 1000 g), then washed and re-pelleted twice in ice-cold PBS. The cell pellet was resuspended in 2.5 vol of cell lysis buffer [CLB; 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM EDTA, 0.2 mM PMSF, 100 μg/ml chymostatin, 40 μg/ml bestatin, 1 μg/ml pepstatin, 1 μg/ml aprotinin and 1 μg/ml leupeptin] and swollen on ice for 10 min. The cells were then lysed in a pre-cooled 15 ml dounce homogenizer [10 strokes with the B (loose) pestle]. Lysis was estimated to be ≥90% by Trypan Blue dye exclusion.

The homogenate was brought to 250 mM sucrose and nuclei were recovered by centrifugation at 1000 g at 4°C for 2 min. The nuclei were resuspended in an equal volume of CLB containing 250 mM sucrose and re-pelleted at 2000 g for 10 min at 4°C. Nuclear pellets were stored at -70°C until used for extraction.

HeLa whole cell extracts were prepared by a modification of the method of Wobbe *et al.* (29). This modification results in differential extraction of DNA polymerase β from DNA polymerases α and δ/ε. Briefly, 30 g (wet weight) of HeLa cells

were harvested, washed twice with PBS and frozen until used. The frozen cell pellet was thawed on ice and the cells were washed once in 100 ml of hypotonic lysis buffer [20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 1.5 mM MgCl₂, 1 mM DTT]. This step is designed to swell, but not rupture, freshly harvested cells. However, since the cells used here had previously been frozen, this step constitutes a very gentle partial extraction. This extract (designated E1) was separated from the cell mass by centrifugation at 500 g for 5 min at 4°C and, with the exception of dounce homogenization, was subjected to the procedures described below for the cell pellet.

The cell pellet from the preceding step was resuspended in 30 ml of hypotonic lysis buffer and placed on ice for 10 min. Lysis was completed by dounce homogenization (20 strokes, B pestle) in a pre-cooled 40 ml homogenizer. The homogenate (designated E2) along with E1 from above, was brought to 200 mM NaCl, allowed to stand on ice for 10 min, then centrifuged for 30 min at 50 000 g and 4°C. The supernatants were dialyzed [20 mM HEPES-KOH (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol; 3 h with one buffer change], cleared by centrifugation at 50 000 g for 30 min and stored as aliquots at -70°C until needed.

This procedure resulted in differential polymerase extraction, with DNA polymerase α and δ/ε activities preferentially in extract E1 and DNA polymerase β activity preferentially in extract E2.

Nuclear and cell extract fractionation

All procedures were performed at 4°C. Isolated nuclei, prepared as described above, were resuspended in nuclear extraction buffer [NEB 20 mM Tris-HCl (pH 7.6), 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol, 500 mM NaCl, 0.2 mM PMSF, 40 μg/ml bestatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin] and lysed by sonication on ice for 1 min [four 15 s pulsed bursts with 30 s cooling periods (Branson sonifier 450, setting 4, 70% duty cycle)]. Following incubation on ice for 30 min with gentle stirring, the extract was clarified by centrifugation at 25 000 g for 30 min at 4°C.

Nuclear extracts or whole-cell extracts (E1 and E2) were brought to 200 mM NaCl with NEBD buffer (NEB less NaCl) and loaded onto a Pharmacia HR 16/10 DE52 cellulose column equilibrated in NEBD + 200 mM NaCl. The column was washed with 3 vol NEBD + 200 mM NaCl and eluted with a 6 vol linear gradient from 200 to 500 mM NaCl followed by a 2 vol wash at 500 mM NaCl. Fractions were assayed for DNA polymerase α, β, δ/ε, HAP1, 3'-PG release, non-specific nuclease, DNA ligase and PCNA. Enzymes were pooled (separately where appropriate), dialyzed against NEBD buffer and stored at -70°C as aliquots until needed.

DNA ligases I and III were identified based upon differential elution from hydroxyapatite (Bio-Rad, Hercules, CA) as previously described (30). Briefly, active fractions from DE52 chromatography were loaded onto an HR 5/5 column pre-equilibrated in 20 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.5 mM DTT, 1 mM KPO₄. After washing with equilibration buffer and a second wash with 50 mM KPO₄ + 0.5 mM DTT, DNA ligase I was eluted from the column by washing with 150 mM KPO₄. DNA ligase III was subsequently eluted by washing at 400 mM KPO₄. Following chromatography, fractions were dialyzed [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT], assayed, and active fractions were pooled and stored at -70°C until needed.

DNA polymerase assays

DNA polymerase activities were determined by modification of the procedures described by Matsumoto *et al.* (19). DNA polymerase δ/ϵ activity was measured at 37°C on a poly(dA)/oligo(dT) template/primer (molar ratio of 5:1) at 0.5 μg total polymer per 25 μl reaction. Reaction mixtures contained the following: 50 mM bis-Tris-HCl (pH 6.5), 10 mM KCl, 6 mM MgCl_2 , 0.4 mg/ml heat inactivated BSA, 1 mM DTT, 5% (v/v) glycerol, 20 μM TTP (1000 d.p.m. ^3H -TTP per reaction; specific activity 0.455 Ci/mmol TTP), 100 μM ddTTP and 10 μM BuPdGTP. DNA polymerase α activity was determined as equivalent to the difference between DNA polymerase δ/ϵ reactions and the activity observed in the absence of the α polymerase-specific inhibitor BuPdGTP.

DNA polymerase β activity was measured at 25°C on poly(rA)/oligo(dT) template/primer (molar ratio of 1:1) at 2 μg total polymer per 25 μl reaction, containing 50 mM Tris-HCl (pH 8.8), 100 mM KCl, 0.5 mM MnCl_2 , 0.4 mg/ml heat inactivated BSA, 1 mM DTT, 5% (v/v) glycerol, 20 μM TTP (1000 d.p.m. ^3H -TTP per reaction; specific activity 0.455 Ci/mmol TTP) and the enzyme sample. One unit of polymerase activity was defined as the amount of enzyme required to incorporate 1 pmol of TMP (thymidine monophosphate) into acid-precipitable material in 30 min.

3'-PG release assay

Enzymatic removal of SSB 3'-PG end groups was measured using the double-stranded 39mer 3'-PG oligo substrate described previously (22). Reaction mixtures contained equimolar concentrations of 3'-radiolabeled-PG 17mer, 5'-P 21mer and complementary 39mer; 20 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM EDTA, 5 mM MgCl_2 , 10 μg heat inactivated BSA and the enzyme sample, in a final volume of 50 μl .

Assays were performed at 37°C and stopped by ethanol precipitation as described previously. One unit of enzyme activity is defined as the amount of enzyme required to release 1 pmol of 3'-PG as ethanol soluble material in 30 min.

Ligase and exonuclease assays

Non-specific nuclease activity and DNA ligase activity were measured by determining the ability of a sample to either increase or decrease *EcoRI* linearized M13 RF migration in agarose gels. Reaction mixtures contained: 20 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM MgCl_2 , 1 mM DTT, 2 mM ATP, 0.1 mg/ml heat inactivated BSA, 150 ng *EcoRI* linearized M13 RF and the enzyme sample in a final volume of 10 μl . The relative conversion of linearized M13 RF to higher or lower molecular weight forms was assessed densitometrically using NIH Image and a Fotodyne (Hartland, WI) Foto/eclipse CCD digital imaging system. One unit of DNA ligase activity was defined as the amount of enzyme required to convert 1 ng of *EcoRI* cut M13 RF DNA to higher molecular weight forms in 30 min at 37°C.

Western blotting

Proteins separated by electrophoresis in 10% polyacrylamide gels (Tris-glycine-SDS buffer system) were transferred to nitrocellulose by the method of Towbin (31). Blots were immunostained with the Bio-Rad immunoblot kit using rabbit anti-HAP1 polyclonal

antibody (18919, 1:4000 dilution) or monoclonal PCNA PC-10 antibody as the primary antibody and alkaline phosphatase conjugated goat anti-rabbit antibody as the secondary antibody. Relative band intensities were determined densitometrically with NIH Image.

Substrate preparation

Substrate DNA containing a defined SSB was constructed by a modification of the procedure described by Gentil *et al.* for the construction of vectors containing site-specific AP lesions (32). M13mp19 was propagated in *E.coli* DH5 α F'. (+)M13 genomic DNA and M13 RF DNA were purified from virions and cell pellets, respectively. An aliquot of 50 μg of M13 RF DNA was digested with *KpnI* and *HindIII* releasing a 35mer oligonucleotide from the multiple cloning site (MCS). The 6985 bp fragment was separated from the 35mer by spin column chromatography on Sepharose CL-4B, extracted once with phenol:chloroform (1:1), once with chloroform, ethanol precipitated, and resuspended in TE pH 8.0.

Gapped heteroduplex DNA was formed by mixing 50 μg of *KpnI/HindIII* cleaved M13 RF with 300 μg of (+)M13 genomic DNA (molar ratio 1:12) in 1 \times SSC at a final concentration of 25 $\mu\text{g}/\text{ml}$ and heating to 98°C for 10 min. The DNA mixture was slow cooled to 65°C, held at this temperature for 1 h, then allowed to cool slowly to room temperature. Following ethanol precipitation, the mixture was brought to 720 mM NaCl in TE pH 8.0. Gapped heteroduplex DNA was isolated by filtration through 0.45 μm nitrocellulose (equilibrated in TE pH 8.0 + 1 M NaCl) and ethanol precipitation of the filtrate.

Vector DNA containing a SSB consisting of a 1 nucleotide gap flanked by a 3'-PG and a 5'-P opposite the adenosine residue within the *Sall* site of the M13 MCS, was formed by annealing and ligating the 5'- ^{32}P -phosphorylated 17mer oligodeoxynucleotides (specific activity 750 Ci/mmol), 5'-pCCGGGGATC-CTCTAGAG-PG-3' and 5'-pCGACCTGCAGGCATGCA-3', to the purified gapped heteroduplex DNA (Fig. 1). Annealing was done in T4 DNA ligase buffer at 70°C for 20 min (10:1, oligo:heteroduplex DNA molar ratio), followed by slow cooling to room temperature. The reaction was supplemented with 1 mM ATP and ligated at 16°C overnight with T4 DNA ligase. The vector construct was recovered by chromatography on an HR 10/30 Sepharose CL-4B column equilibrated in TE pH 8.0 + 150 mM NaCl, concentrated by ethanol precipitation and resuspended in TE, pH 8.0.

Repair reactions

The standard repair reaction was performed at 25°C for 30 min. Reaction mixtures (25 μl) consisted of 20 mM HEPES (pH 7.5), 50 mM KCl, 10 mM MgCl_2 , 2 mM DTT, 2.5% (v/v) glycerol, 2.5 mM *p*-nitrophenylphosphate, 2 mM ATP, 0.5 mM NAD, 1 mM GMP, 0.5 mM EDTA, 200 μg BSA, 50 μM each of dATP, dCTP, dGTP and TTP, the vector substrate and the enzyme sample. Repair reactions were stopped by the addition of 0.4% SDS and incubation at 65°C for 15 min. DNA was recovered by extraction with phenol:chloroform (1:1), and ethanol precipitation with 0.5 μg tRNA as carrier. After resuspension in restriction buffer [10 mM bis-Tris-propane-HCl (pH 7.0), 10 mM MgCl_2 , 1 mM DTT], the DNA was digested for 1 h at 37°C with 4 U of *AluI*. Repair products were analyzed by autoradiography following electrophoresis in 20% polyacrylamide gels with 8 M urea.

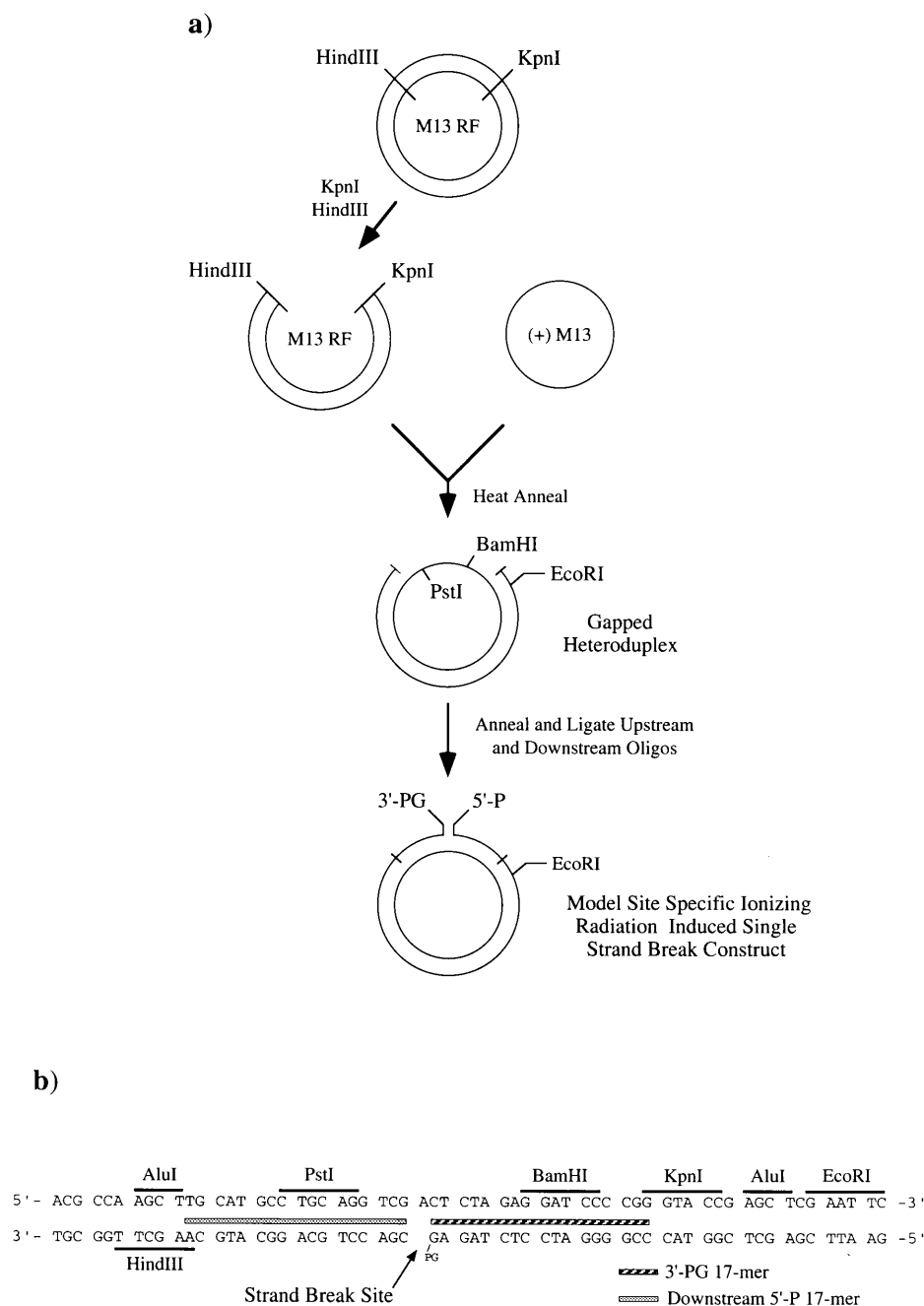


Figure 1. (a) Construction flow chart for the defined site-specific 3'-PG/5'-P containing SSB vector substrate. (b) Ionizing radiation-induced SSB vector substrate lesion detail. The position of the 3'-PG containing 17mer is indicated by the cross hatched bar. The position of the downstream 5'-P containing 17mer is indicated by the shaded bar. The location of the missing nucleotide is indicated by the arrow. Restriction enzyme recognition sites are indicated by black bars.

RESULTS

Strand break vector construction

Ionizing radiation causes a wide spectrum of DNA lesions, including numerous specific base modifications, AP sites and double- and single-strand breaks. This high degree of complexity makes determining the mechanism of repair for a specific lesion type nearly impossible in irradiated DNA. In order to define the mechanism of repair for a specific ionizing radiation-induced

DNA lesion, an approach must be developed to study the lesion in isolation.

We have previously reported the construction of a defined double-stranded oligonucleotide 3'-PG strand break substrate based upon the M13 MCS (23). This substrate has been used for the characterization of nucleases capable of releasing 3'-PG from strand break lesions. However, the oligonucleotide substrate is not well suited to studies in which the goal is to examine complete repair of ionizing radiation-induced SSBs in cell-free systems. This is due to the oligo's small size, susceptibility to exonucleolytic

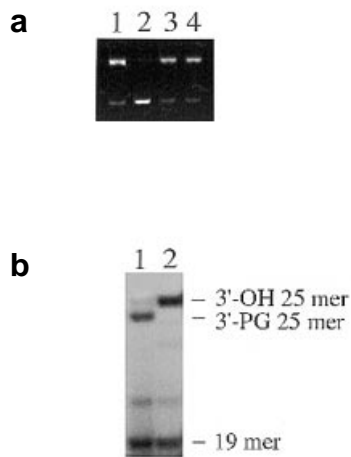


Figure 2. M13mp19 heteroduplex and vector substrate analysis. (a) Restriction enzyme analysis of M13mp19 heteroduplex DNA construction. Lane 1, heteroduplex DNA; lane 2, *EcoRI* cut heteroduplex DNA; lane 3, *BamHI* cut heteroduplex DNA; lane 4, *PstI* cut heteroduplex DNA. The gapped heteroduplex construct is depicted in Figure 1a. (b) Ionizing radiation-induced SSB vector substrate construction analysis. Lane 1, 20% denaturing PAGE of *AluI* cut vector substrate construct; lane 2, 20% denaturing PAGE of recombinant HAP1 treated vector substrate construct cut with *AluI*.

degradation and the potential for repair polymerases acting at the break site to displace the downstream 21mer, all of which may not reflect the true state of events during repair in large DNA molecules (23,26,33).

Consequently, we constructed a vector-based DNA substrate with the same configuration and chemical composition as an authentic ionizing radiation-induced SSB. The method used to construct the vector substrate is depicted in Figure 1. A gapped heteroduplex was generated by annealing the M13 RF *KpnI/HindIII* large restriction product with M13 genomic DNA. To assess for correct annealing, gapped heteroduplex DNA was reacted with a panel of restriction endonucleases that either recognized sites in the double- or single-stranded region of the molecule. Correct heteroduplex annealing was confirmed by cutting with *EcoRI*, which recognizes the double-stranded region, while *BamHI* and *PstI*, which recognize the single-stranded region, were unable to cut (Fig. 2a).

Vector substrate construction was completed by annealing and ligating complementary upstream and downstream 17mers to the 35 nucleotide gap of the heteroduplex (Fig. 1b). Correct construction of the vector substrate was confirmed by restriction analysis with *EcoRI*, *BamHI* and *PstI* (data not shown) and by cleavage with *AluI* to generate a radiolabeled 3'-PG 25mer and a radiolabeled 19mer (Fig. 2b, lane 1). To establish the presence of the 3'-PG end group on the 25mer, the vector substrate was treated with recombinant HAP1 prior to *AluI* digestion and electrophoresis. HAP1 has a very low efficiency capacity to release phosphoglycolic acid from 3'-PG strand break sites in oligonucleotide substrates (23,24,26). Therefore, following HAP1 treatment, the 25mer produced by *AluI* digestion migrates more slowly at the position of a 3'-OH 25mer (Fig. 2b, lane 2).

Repair of the strand break lesion in the vector substrate results in a covalently-closed M13 RF molecule which upon cleavage with *AluI* will yield a ³²P-radiolabeled 45mer repair product (Fig. 1b).

HeLa cell extract enzymatic characterization

In order to examine the enzymatic activities involved in repair of the model ionizing radiation-induced SSB substrate, nuclear extracts of human HeLa cells were prepared and characterized with respect to various potential repair enzyme activities. The repair pathway for ionizing radiation-induced SSBs is postulated to consist of at least three steps; a nuclease activity to remove 3'-PG (or 3'-P) and generate a 3'-OH, DNA polymerase activity to replace the missing nucleotide and DNA ligase activity to seal the resulting nicked DNA.

Therefore, the HeLa cell nuclear extracts were assessed for nuclease activity, DNA polymerase α , β and δ/ϵ activities and DNA ligase activities following DE52 cellulose chromatography (Fig. 3). In addition, the elution profiles for HAP1 and PCNA were determined by western blotting. When appropriate, fractions containing distinct activities were pooled separately.

DNA polymerase α , β and δ/ϵ activities in the pooled polymerase fraction were distinguished based upon their differential sensitivities to the inhibitors butylphenyldeoxyguanosine triphosphate (BuPdGTP), aphidicolin and dideoxythymidine triphosphate (ddTTP; Table 1). BuPdGTP is a highly specific inhibitor of DNA polymerase α . At 10 μ M BuPdGTP, DNA polymerase α is inhibited $\geq 95\%$ (34). In contrast, under the same conditions DNA polymerases δ/ϵ are only slightly affected with inhibition of $\leq 20\%$. While DNA polymerase β does display sensitivity to BuPdGTP, it is only moderately inhibited ($\leq 40\%$) at 10 μ M. Aphidicolin is a specific inhibitor of DNA polymerases α and δ/ϵ . 100 μ M aphidicolin inhibits DNA polymerases α and δ/ϵ $\geq 95\%$, but has negligible effect ($< 10\%$ inhibition) on DNA polymerase β (34,35; Table 1). In contrast, DNA polymerase β is inhibited $\geq 95\%$ by 100 μ M ddTTP, while DNA polymerases α and δ/ϵ are essentially unaffected under these conditions.

Table 1. DNA polymerase inhibition of HeLa cell nuclear extract by polymerase inhibitors alone or in combination

DNA Polymerase (Substrate) ^a	Inhibitor ^b	ddTTP	Aphidicolin	BuPdGTP
Pol α and/or δ/ϵ (Poly (dA)/ Oligo (dT))	ddTTP	1.17 \pm 0.05 ^c	--	--
	Aphidicolin	0.16 \pm 0.01	0.19 \pm 0.03	--
	BuPdGTP	0.54 \pm 0.05	0.16 \pm 0.08	0.76 \pm 0.1
Pol β (Poly (A)/ Oligo (dT))	ddTTP	0.0 \pm 0.07	--	--
	Aphidicolin	0.0 \pm 0.05	0.77 \pm 0.16	--
	BuPdGTP	0.0 \pm 0.08	0.48 \pm 0.11	0.61 \pm 0.1

^aIncorporation specific for DNA polymerase α , δ or ϵ was measured using the polymerase-specific substrate Poly(dA)/Oligo(dT) as described in the Materials and Methods. The Poly(A)/Oligo(dT) substrate is specific for DNA polymerase β incorporation.

^bThe inhibitor concentrations were 100 μ M ddTTP, 100 μ M aphidicolin and 10 μ M BuPdGTP. In those cases for which the rows and columns of a single inhibitor converge, only that inhibitor was present at the indicated concentration. In all other cases, both inhibitors were present at the indicated concentrations.

^cActivity is normalized to an activity of 1.0 in the absence of inhibitors \pm the standard deviation for three determinations.

No general class of nuclease-specific inhibitors exists. Therefore, specific identification of nuclease activities (with the exception of

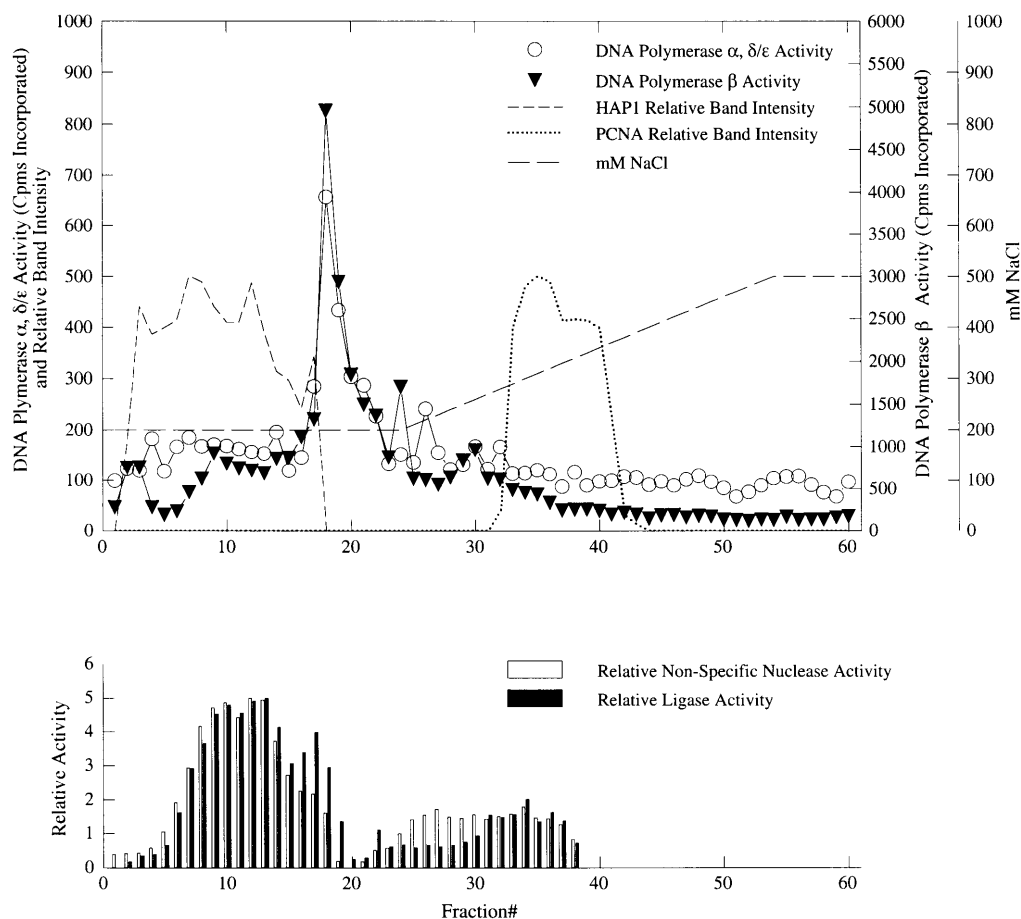


Figure 3. HeLa cell nuclear extract DE52 chromatography profile. DNA polymerase α , δ/ϵ activity is indicated by the open circles. DNA polymerase β activity is indicated by the closed triangles. HAP1 western blot relative band intensities are indicated by the short dashed line. PCNA western blot relative band intensities are indicated by the dotted line. NaCl concentration is indicated by the long dashed line. Non-specific nuclease activity is indicated by the open bars and DNA ligase activity is indicated by the shaded bars. Fractions (1.0 ml) 6–30 were pooled and constitute the DNA polymerase active fraction. Fractions 31–41 were pooled and constitute the PCNA containing fraction.

HAP1) was not attempted. Identification of specific DNA ligase activities was dependent upon differential elution from hydroxyapatite and is discussed below.

Repair enzyme identification

The initial step of SSB repair requires the 3'-PG end of the break to be converted to a 3'-OH. Although HAP1 has been postulated to have a role in this step of the repair pathway (36–38), other human enzyme activities have been identified that also possess 3'-phosphodiesterase activity (24,39). In order to assess the role of HAP1 during initiation of SSB repair, specific inhibition was attempted with anti-HAP1 polyclonal antibody. Following purification on protein A-Sepharose to remove contaminating serum nucleases, the anti-HAP1 antibody was demonstrated to neutralize HAP1's ability to release 3'-PG from SSBs in an oligonucleotide substrate (Fig. 4). Based upon the neutralization assay for recombinant HAP1 and western blot quantification of the partially purified HeLa cell nuclear extract HAP1 content, enzyme fractions were pre-incubated with sufficient anti-HAP1 neutralizing antibody (≥ 2 -fold excess) to completely inhibit all HAP1-specific 3'-PG releasing activity. No reduction in strand

break repair was noted under these conditions. Rather, pre-incubation of the pooled polymerase fraction with anti-HAP1 antibody resulted in an increase of 45mer repair product (Fig. 5, lanes 1–4), suggesting that HAP1 may competitively bind the SSB lesion and hinder access to other factors in the polymerase fraction.

DNA polymerase utilization for HeLa cell nuclear extract repair reactions was determined by differential polymerase inhibition (Fig. 5). In the absence of protein from the pooled PCNA fractions, the nuclear extract DE52 chromatography polymerase pool did not produce a 45mer repair band in excess of that produced when all three DNA polymerase inhibitors were present (Fig. 5, lanes 1 and 2, and 5 and 6). However, rather than reflecting a need for PCNA, this probably indicates the need for a nuclease or other protein in the PCNA fraction that is involved in making the site accessible to an initiating enzyme. This is supported by the observation that the polymerase pool contains a small quantity of PCNA, and DNA polymerase activity that is resistant to BuPdGTP and ddTTP, indicating that there is DNA polymerase δ or ϵ with sufficient PCNA cofactor for activity. Also, addition of the PCNA containing mixture to the polymerase pool does not stimulate increased DNA polymerization (data not

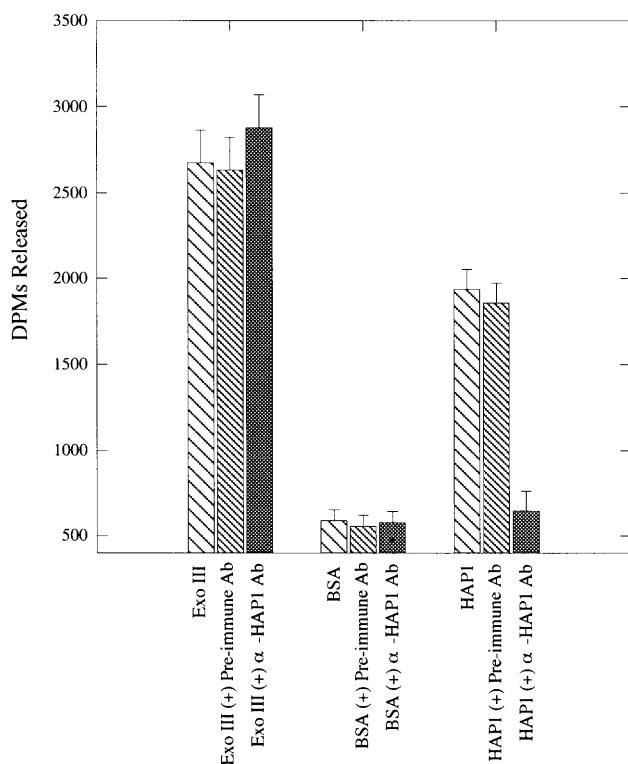


Figure 4. HAP1 neutralization. Protein A purified pre-immune rabbit antiserum or anti-HAP1 polyclonal antiserum (1 μ l), was pre-incubated for 10 min at 25°C with either 40 U of *E. coli* exonuclease III (positive control), 50 ng BSA (negative control) or 20 ng of recombinant HAP1 protein. Following pre-incubation, 3'-PG release assay oligonucleotide substrate was added and reactions were carried out as described in the Materials and Methods.

shown). In contrast, addition of the PCNA containing fraction to repair reactions results in a greater yield of 45mer repair product (Fig. 5, lanes 1–4).

In order to determine which of the DNA polymerase activities present in the nuclear extract were responsible for repair, reactions were performed in the presence of DNA polymerase-specific inhibitors. Minimal repair was observed in the presence of all three inhibitors (aphidicolin, BuPdGTP, ddTTP; Fig. 5, lanes 5 and 6), whereas when all but DNA polymerase β is inhibited, 67% of the maximal repair in the absence of inhibitors is observed. This value remains essentially unchanged at 60% of maximal repair in the presence of neutralizing anti-HAP1 antibody (Fig. 5, lanes 7 and 8). In the presence of the DNA polymerase β -specific inhibitor ddTTP (with or without addition of the PCNA containing pool), repair is diminished to ~27% of maximal, but not eliminated. Upon addition of BuPdGTP to ddTTP containing reactions, no additional reduction in repair was observed. These results indicate that DNA polymerase δ and/or ϵ , but not DNA polymerase α , can also contribute to the repair reaction and that the sum of repair for the DNA polymerase β and DNA polymerase δ/ϵ reactions is approximately equal to the maximum repair observed in the absence of inhibitors (Fig. 5, lanes 9–12). Taken together, these results indicate that HAP1 is not required to initiate SSB repair and that DNA polymerase β is the polymerase primarily responsible for incorporation of the missing nucleotide, although DNA polymerase δ/ϵ is also capable of fulfilling this role.

Repair pathway enzyme utilization

Studies of base excision repair pathway enzyme utilization indicate a polymerase dependence and distribution similar to what we have observed for ionizing radiation-induced SSB repair (19–21,35,40–42). Furthermore, DNA ligase I and DNA ligase III have been implicated in the ligation step of base excision repair in a polymerase-dependent manner (13–15,18,22). In order to establish if this is also the case for ionizing radiation-induced SSB repair, DNA polymerases α , δ/ϵ and β , as well as DNA ligases I and III, were differentially extracted from HeLa cells and partially purified for examination of inter-relationships via repair assays.

The HeLa whole cell extraction procedure resulted in differential extraction of DNA polymerases α , δ/ϵ and DNA polymerase β . The majority of activity in extract E1 is DNA polymerases α and δ/ϵ , with only a small amount of DNA polymerase β (~5:1 within the pooled fraction). The majority of polymerase activity in extract E2 is DNA polymerase β , with comparatively small amounts of DNA polymerases α and δ/ϵ (~10:1 within the pool). E1 was determined to possess 2-fold more DNA polymerases α and δ/ϵ than E2, while E2 possesses ~25-fold more DNA polymerase β activity than E1. In addition, this extraction procedure segregated most of the DNA ligase activity with DNA polymerase β in extract E2. DNA polymerase activity and 3'-PG releasing activities along with DNA ligase activities were separated from one another in both E1 and E2 by DE52 chromatography (Fig. 6). The isolated E1 DNA polymerase α and δ/ϵ activities and isolated E2 DNA polymerase β were pooled separately. However, in the case of E2, fractions 27–31 contained an RNaseH-like activity, or an inhibitor of DNA polymerase β , that eliminated activity in the β polymerase assay. Therefore, fractions 27–31 were excluded from the DNA polymerase β DE52 pooled active fraction. Upon DE52 chromatography of E2, the majority of 3'-PG releasing activity and DNA ligase activity were eluted in the flowthrough and wash fractions as was observed earlier for DE52 chromatography of the HeLa cell nuclear extract (Fig. 6b). These fractions were pooled and chromatographed on hydroxyapatite to isolate DNA ligases I and III according to the method of Tomkinson *et al.* (30). 3'-PG releasing activity eluted from hydroxyapatite in the flowthrough, and was pooled and dialyzed under the same conditions as the DNA ligases prior to use in repair reactions.

Having identified and separated the potential ionizing radiation-induced SSB repair enzymes into separate pools, combinatorial repair assays were conducted to specifically identify the enzymes involved in repair of the lesion, as well as to confirm our results with the HeLa cell nuclear extracts.

When E1 (α , $\delta/\epsilon > \beta$) was the polymerase source, maximal repair occurred in the presence of 3'-PG releasing activity, the DNA ligase I containing fraction and the DNA polymerase β inhibitor ddTTP (Fig. 7a, lane 2). This activity decreased in the presence of the DNA polymerase α -specific inhibitor BuPdGTP, but still exceeded the level of repair observed in the absence of added DNA ligase activity, indicating DNA ligase I-dependent DNA polymerase δ/ϵ -mediated repair. A nearly equivalent amount of repair product was generated by the E1 polymerase pool in the presence of 3'-PG releasing activity and DNA ligase III (Fig. 7a, lane 4). However, the DNA ligase III-dependent repair activity was eliminated by the DNA polymerase α -specific inhibitor BuPdGTP and was equivalent to the inhibition observed

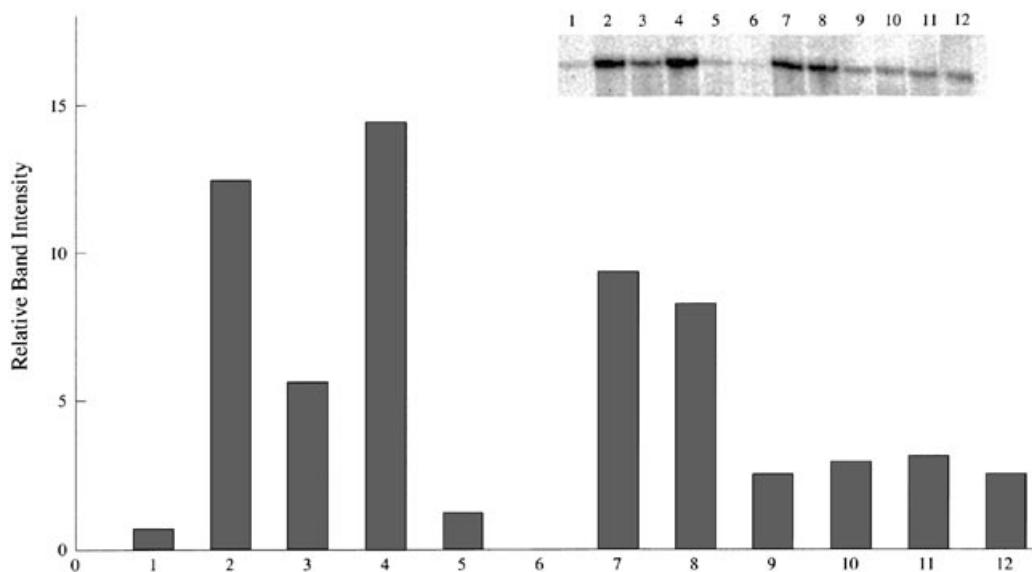


Figure 5. HeLa cell nuclear extract ionizing radiation-induced SSB repair reactions. DE52 partially purified HeLa cell nuclear extract DNA polymerase (Pol) and PCNA containing fractions were used to conduct repair reactions. All reactions contained the DNA polymerase fraction (5 μ l) and 3.5 ng vector substrate (570 dpms/ng), and one or more of the following; PCNA containing fraction (2 μ l), 100 μ M aphidicolin, 10 μ M BuPdGTP, 100 μ M ddTTP or anti-HAP1 (2 μ l), as indicated below. Inset, 45mer repair product bands. The bar graph depicts the relative band intensity normalized to the negative polymerase control (lane 6). The repair reactions are as follows: lane 1, Pol; lane 2, Pol + PCNA; lane 3, Pol + anti-HAP1 Ab; lane 4, Pol + PCNA + anti-HAP1 Ab; lane 5, Pol + 100 μ M aphidicolin + 10 μ M BuPdGTP + 100 μ M ddTTP; lane 6, Pol + 100 μ M aphidicolin + 10 μ M BuPdGTP + 100 μ M ddTTP + PCNA; lane 7, Pol + 100 μ M aphidicolin + 10 μ M BuPdGTP; lane 8, Pol + 100 μ M aphidicolin + 10 μ M BuPdGTP + anti-HAP1 Ab; lane 9, Pol + 100 μ M ddTTP + PCNA; lane 10, Pol + 100 μ M ddTTP; lane 11, Pol + 100 μ M ddTTP + 10 μ M BuPdGTP; lane 12, Pol + 100 μ M ddTTP + 10 μ M BuPdGTP + PCNA.

upon addition of aphidicolin (Fig. 7a, lanes 5 and 6). Therefore, under these reaction conditions, DNA polymerase α can serve as the repair polymerase in a DNA ligase III-dependent manner. However, the nuclear extract results suggest that DNA polymerase α probably does not act as a repair polymerase in the presence of the complete nuclear protein complement.

When E2 ($\beta > \alpha$, δ/ϵ) served as the source of DNA polymerase, maximal repair occurred in the presence of 3'-PG releasing activity, the DNA ligase III containing fraction and the DNA polymerase α , δ/ϵ inhibitor aphidicolin (Fig. 7b, lane 4). Upon addition of the DNA polymerase β -specific inhibitor ddTTP, repair product was reduced to background levels. The DNA ligase I containing fraction also appeared capable of supporting repair in the presence of E2, aphidicolin and 3'-PG releasing activity; however, this combination resulted in \sim 4-fold less repair product than the reactions containing the DNA ligase III fraction. Therefore, DNA polymerase β -mediated SSB repair is more efficient with DNA ligase III.

One possible reason for the apparent dominance of DNA polymerase β during the polymerization step of the repair reactions conducted with the HeLa cell nuclear extract is the proportionately high level of DNA polymerase β to DNA polymerase α , δ/ϵ activities in the extract. Therefore, the preferential DNA polymerase utilization observed with the nuclear extract may not reflect an actual bias for one repair pathway over the other, but may represent a dose loading effect. This question was addressed by repair reconstitution reactions combining equal units of E1 DNA polymerase activities with equal units of E2 DNA polymerase activities, excess 3'-PG releasing activity and either DNA ligase I or III plus DNA polymerase inhibitors as appropriate (Fig. 7c). In the presence of the DNA ligase I active fraction, repair was \sim 3-fold above the

background activity observed in the absence of added DNA ligase activity. Repair was abrogated in the presence of either aphidicolin or ddTTP and by a combination of aphidicolin, BuPdGTP and ddTTP. However, in the presence of a combination of ddTTP and BuPdGTP, DNA ligase I associated repair returns to approximately twice the background level (Fig. 7c, lanes 1–6). This confirms the observation that DNA polymerase δ/ϵ is capable of repair product formation in the presence of DNA ligase I. However, it also suggests that DNA polymerase α may interfere with the repair reaction under the conditions of the assay.

In the presence of the DNA ligase III containing fraction, the E1, E2 and 3'-PG releasing activity combination results in repair product formation in excess of 12 times the background repair activity observed in the absence of added DNA ligase activity (Fig. 7c, lane 7). This is \sim 4 times the repair observed for this combination of enzyme activities in the presence of the DNA ligase I containing fraction. In the presence of aphidicolin, repair was reduced by 37%. In the presence of ddTTP, repair was reduced by 60%. However, upon the addition of BuPdGTP to ddTTP-containing reactions, repair was completely inhibited (Fig. 7c, lanes 8–11), again suggesting that DNA polymerase α may act in conjunction with DNA ligase III. These results are consistent with the results observed for repair using the isolated E1 and E2 fractions as well as for the distribution of repair observed for the DNA polymerase δ/ϵ and DNA polymerase β -specific assays conducted with the partially purified HeLa cell nuclear extract. Since the reactions contained equal units of E1 (DNA polymerases α , δ/ϵ) and E2 (DNA polymerase β) polymerase activities, these results suggest that the DNA polymerase bias observed with HeLa cell nuclear extracts is not simply a DNA polymerase dose effect, but may represent an actual bias for the DNA polymerase β -dependent repair pathway.

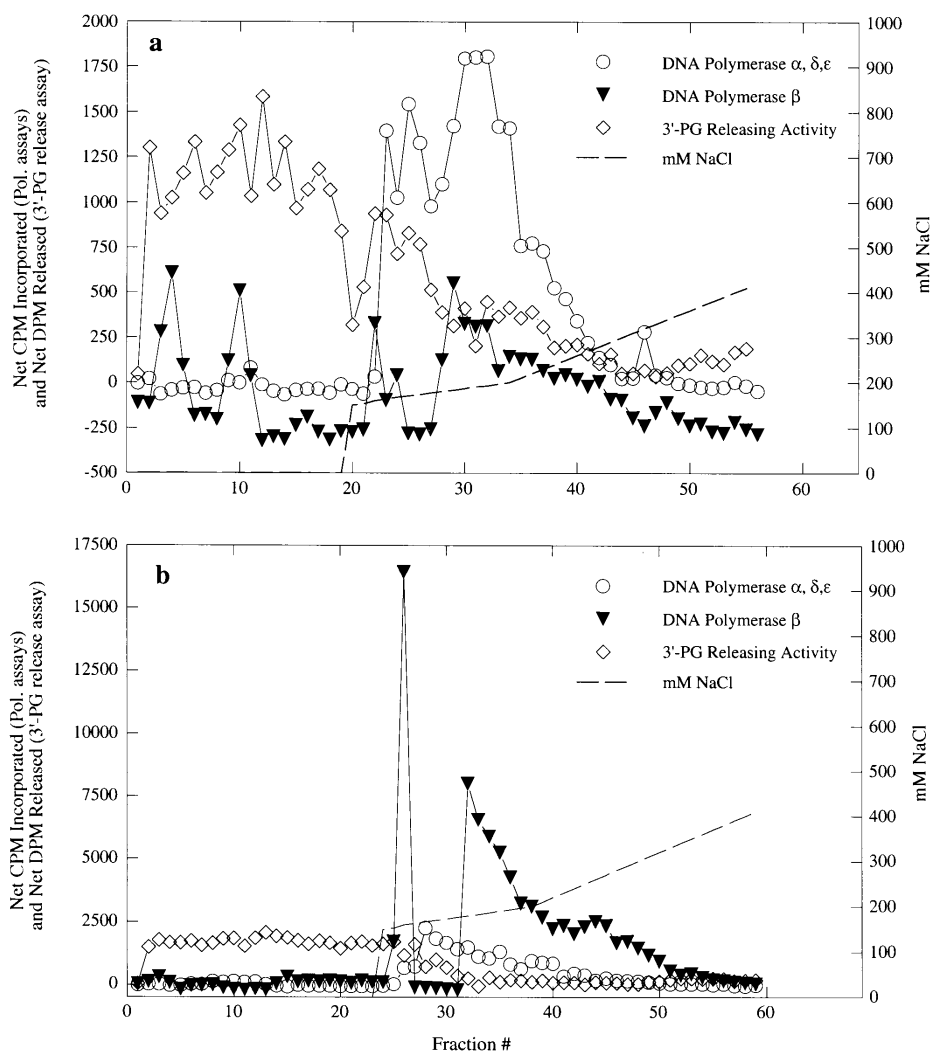


Figure 6. HeLa cell extracts E1 and E2 DE52 chromatography. DNA polymerase α , δ/ϵ activity is indicated by the open circles. DNA polymerase β activity is indicated by the closed triangles. 3'-PG releasing activity is indicated by the open diamonds. NaCl concentration is indicated by the dashed line. (a) HeLa cell extract E1 DE52 chromatogram. Fractions 23–37 were pooled (concentrated 4 \times) and constituted the E1 DNA polymerase active fraction. The E1 DNA polymerase active fraction contains 3.5 U/ μ l total polymerase activity, 2.5 U/ μ l DNA polymerase δ/ϵ activity, when assayed as described in the Materials and Methods (DNA polymerase δ and ϵ cannot be distinguished under the assay conditions). (b) HeLa cell extract E2 DE52 chromatogram. Fractions 25 and 26 were pooled with fractions 32–47 (fractions 27–31 were excluded), concentrated 2 \times and constituted the E2 DNA polymerase active fraction. The E2 DNA polymerase active fraction contains 14.3 U/ μ l DNA polymerase β -specific incorporation activity. Fractions 3–12 included DNA ligase activity and were pooled separately for hydroxyapatite chromatography. 3'-PG releasing activity, DNA ligase I and DNA ligase III activities, were separated by hydroxyapatite chromatography as described in the Materials and Methods. The hydroxyapatite 3'-PG releasing activity pooled active fraction contains 10.5 U 3-PG releasing activity/ μ l. The hydroxyapatite DNA ligase I pooled active fraction contains 0.24 U/ μ l and the DNA ligase III pooled active fraction contained 0.31 U/ μ l.

DISCUSSION

In order to define the enzymatic requirements of ionizing radiation-induced SSB repair, it was necessary to develop an assay system which allowed examination of the repair process at an isolated and defined ionizing radiation-induced SSB lesion. We addressed this problem by constructing a vector based site-specific SSB substrate of defined chemistry and configuration.

By using partially purified HeLa cell nuclear extracts as the source of repair enzyme activities and polyclonal antibody that is neutralizing for HAP1 3'-PG hydrolysis activity, we have demonstrated that repair of ionizing radiation-induced SSBs is not dependent upon HAP1 activity. This is not surprising in light of recent enzymatic studies showing that although HAP1 binds tightly to gapped 3'-PG oligomeric substrates, it is several hundred

times less efficient at hydrolyzing 3'-PGs than AP sites (23,24,26). In addition, the enzyme has been shown to have even lower catalytic efficiency on plasmid substrates, such as the one used here, than on oligomeric substrates (26,43). Also, a recent study by Sandigursky *et al.* (33) demonstrates that AP site repair mediated by *E.coli* extracts proceeds differently and results in longer patch sizes than repair reactions conducted with oligonucleotide substrates (44,45), suggesting that in addition to lesion structure, large-scale DNA structure may also directly influence repair processing of specific damages. Furthermore, a recent study by Strauss *et al.* (46) demonstrates that the 3'- β -elimination product of AP sites is a potent inhibitor of HAP1. These data provide direct evidence that 3'-damages are tightly bound by HAP1 and poorly processed or released and are consistent with the observation that HAP1 may hinder efficient repair in our assay system and that another

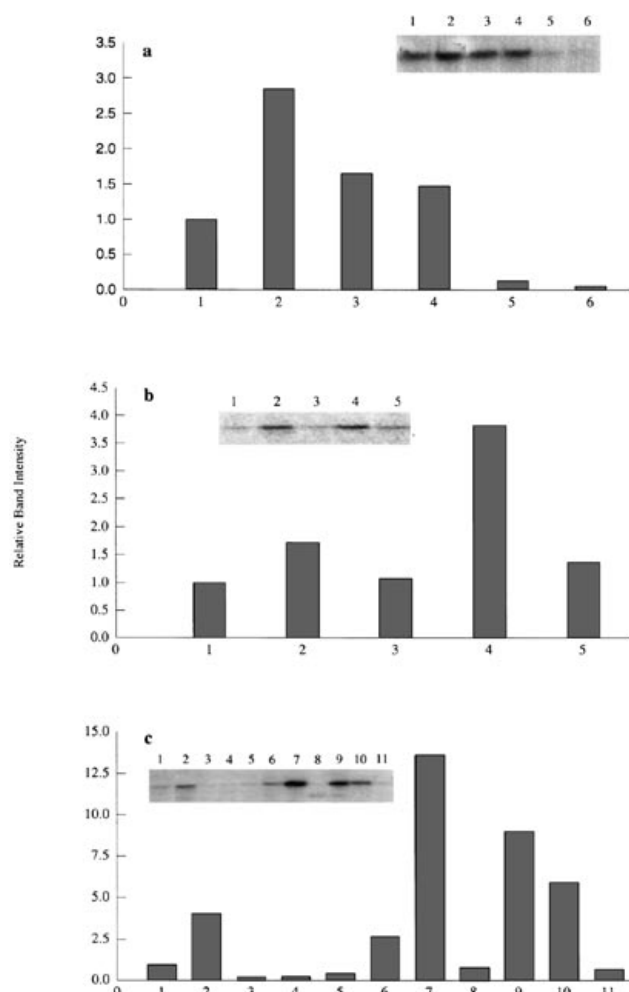


Figure 7. Repair reconstitution. All repair reactions contained 3.5 U of E1 and/or E2 DNA polymerase activity, 18 U of 3'-PG releasing activity (3PG) and 1.5 U of DNA ligase active fraction as indicated below. **(a)** E1 DNA polymerase active fraction reconstitution reactions. All lanes contained E1, 3PG and 100 μ M ddTTP plus the following additions: lane 1, no additional components; lane 2, DNA ligase I (L1); lane 3, L1 + 10 μ M BuPdGTP; lane 4, DNA ligase III (L3); lane 5, L3 + 10 μ M BuPdGTP; lane 6, L3 + 10 μ M BuPdGTP + 100 μ M aphidicolin. **(b)** E2 DNA polymerase active fraction reconstitution reactions. All lanes contained E2, 3PG and 100 μ M aphidicolin plus the following additions: lane 1, no additional components; lane 2, L1; lane 3, L1 + 100 μ M ddTTP; lane 4, L3; lane 5, L3 + 100 μ M ddTTP. **(c)** DNA polymerase E1 plus E2 reconstitution reactions. All reactions contained 3PG activity and equal units of E1 and E2 DNA polymerase activity as indicated above, plus the following additions: lane 1, no additional components; lane 2, L1; lane 3, L1 + 100 μ M ddTTP + 100 μ M aphidicolin + 10 μ M BuPdGTP; lane 4, L1 + 100 μ M aphidicolin; lane 5, L1 + 100 μ M ddTTP; lane 6, L1 + 100 μ M ddTTP + 10 μ M BuPdGTP; lane 7, L3; lane 8, L3 100 μ M ddTTP + 100 μ M aphidicolin + 10 μ M BuPdGTP; lane 9, L3 + 100 μ M aphidicolin; lane 10, L3 + 100 μ M ddTTP; lane 11, L3 + 100 μ M ddTTP + 10 μ M BuPdGTP. Insets in (a, b and c) depict the 45mer repair product. The bar graphs indicate the relative band intensities for each repair reaction normalized to the negative repair control given in lane 1 of each repair set plus an adjustment factor of 1.0.

3'-diesterase may be required to initiate SSB repair. However, these results are in contrast to reports for *in vivo* HAP1 antisense RNA expression which demonstrate hypersensitivity to alkylating agents, as well as various oxidizing agents (36,37). While many of the genotoxic agents used in these studies produce SSBs, they may also produce base damage and AP sites. Since the antisense expressing

cells were generally less sensitive to oxidative agents such as bleomycin than to the alkylating agent MMS, this may reflect increased sensitivity to lesions other than SSBs. Furthermore, a recent report by Wilson *et al.* (47) demonstrated that *trans* complementation of *apn1⁻* yeast with HAP1 did not protect against hydrogen peroxide challenges. Therefore, the increased sensitivity of the antisense expressing cells to hydrogen peroxide and redox cycling agents may reflect disruption of signaling pathways, such as the SAPK/JNK kinase pathway, due to a decrease in HAP1-mediated reductive activation of the AP-1 transcription factor (48–53), and not to a reduction in HAP1-mediated SSB repair.

Repair reactions using partially purified HeLa cell nuclear extracts in the presence of highly specific DNA polymerase inhibitors established that the DNA polymerase utilization pattern for SSB repair followed the dual pathway paradigm previously established for base excision repair and AP site repair (17,19,20,35,40–42,54,55). In addition, the distribution of DNA polymerase activities involved in the repair reaction was essentially the same as that observed by Nealon and co-workers for AP site repair (21). DNA polymerase β is the primary polymerase acting during SSB repair, incorporating up to 70% of the missing nucleotides at SSB sites, while either DNA polymerase δ or ϵ (these polymerases cannot be distinguished under the conditions used) incorporate up to 30% of nucleotides during SSB repair.

Differential DNA polymerase utilization during base excision and AP site repair has been associated with polymerase-dependent DNA ligase usage. Specifically, completion of repair reactions mediated by DNA polymerase β may involve either DNA ligase I or DNA ligase III (13–17,56). In contrast, repair reactions mediated by DNA polymerase δ/ϵ appear to be dependent upon DNA ligase I (57–59).

In order to confirm if this were the case for ionizing radiation-induced SSB repair, HeLa cell extracts that had been differentially extracted for DNA polymerases α and δ/ϵ , versus DNA polymerase β , DNA ligase and 3'-PG releasing activity were partially purified to produce isolated enzyme pools for use in reconstitution experiments. These experiments confirmed that the DNA polymerase-specific DNA ligase usage observed in base excision repair pathways also holds for the repair of ionizing radiation-induced SSBs. Several recent reports have demonstrated base excision repair mediated by a complex of DNA polymerase β and DNA ligase I (13,40,60). Although we observed a small amount of repair by DNA polymerase β in the presence of DNA ligase I, our results indicate that SSBs are preferentially repaired by DNA polymerase β in combination with DNA ligase III. This result is consistent with observations that DNA polymerase β physically interacts with XRCC1 which binds DNA ligase III (14,15,17,56). In addition, the reconstitution experiments indicate that DNA polymerase δ/ϵ -mediated SSB repair is dependent upon DNA ligase I, which is consistent with previous reports of interactions between these enzymes during DNA replication and repair reactions (22,57,59).

Our results are consistent with the known physical interactions between human DNA polymerases and DNA ligases. A physical interaction between HAP1 and DNA polymerase β has also recently been reported (16), suggesting that at least for some types of damage, downstream repair pathway selection may be directly linked to lesion recognition. A similar interaction between DNA polymerase β and a 3'-diesterase specific for 3'-blocked SSBs may also exist. Suggestive evidence for such an interaction has recently been reported by Karimi-Busheri *et al.* (61) for the repair

of SSBs containing 3'-P and 5'-OH groups by purified polynucleotide kinase (PNK), recombinant DNA polymerase β and recombinant DNA ligase I. The authors found that the presence of DNA polymerase β stimulated the 5'-end phosphorylation activity of PNK, which was further stimulated by addition of DNA ligase. They also demonstrated that on a gapped substrate containing 3'- and 5'-OH termini, DNA polymerase β required the presence of PNK to incorporate the missing nucleotide (probably to phosphorylate the 5'-end of the gap) and that this activity was stimulated by addition of DNA ligase. These results not only imply potential protein-protein interactions between the enzymes used to repair the lesion, but that the initial reaction rate is stimulated by the proteins involved in the later steps of repair.

This effect may account for the increased repair observed in the reconstitution assays presented here (Fig. 7). Although ligase-dependent repair activity was observed for the reactions with isolated polymerase pools and different DNA ligases, proportionately more repair was observed when the two polymerase pools were mixed in the presence of the other isolated enzyme fractions. This suggests that a factor(s) in one or both of the polymerase pools may stimulate repair. Therefore, in order to fully understand the protein-protein interactions responsible for ionizing radiation-induced SSB repair, the nuclease(s) required to initiate the repair reaction and its interactions with the other enzymes of the repair pathway need to be determined.

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