Human HeLa cell enzymes that remove phosphoglycolate 3'-end groups from DNA

Thomas A. Winters, Michael Weinfeld¹ and Timothy J. Jorgensen*

Department of Radiation Medicine, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center, 3800 Reservoir Road NW, Washington DC 20007, USA and

¹Radiobiology Program, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta T6G 1Z2, Canada

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ABSTRACT

We have purified three chromatographically distinct human enzyme activities from HeLa cells, that are capable of converting bleomycin-treated DNA into a substrate for *E. coli* DNA polymerase I. The bleomycin-treated DNA substrate used in this study has been characterized via a ³²P-postlabeling assay and shown to contain strand breaks with 3'-phosphoglycolate termini as greater than 95% of the detectable dose-dependent lesions. The purified HeLa cell enzymes were shown to be capable of removing 3'-phosphoglycolates from this substrate. Also 3'-phosphoglycolate removal and nucleotide incorporation were enzyme dependent. In addition, all three HeLa cell enzymes have been determined to possess Class II AP endonuclease activity. The enzymes lack 3' → 5' exonuclease activity and are, therefore, dissimilar to exonuclease III—an *E. coli* enzyme that can remove 3'-phosphoglycolate.

INTRODUCTION

Strand breaks are a major class of DNA lesion produced by ionizing radiation and other genotoxic agents. DNA strand breaks have been studied both *in vitro* and *in vivo*, and their quantitation has been used as an index of overall DNA damage and repair occurring in cells. In general, characterization of strand breaks has been based entirely on biophysical disruption of the continuity in the DNA phosphodiester backbone (eg. single- vs. double-strand break), without knowledge of the exact chemistry of the lesion at the site of the break. Yet there is much evidence that even among single-strand breaks there can be different lesions with distinct chemical identities. These different lesions need to be considered individually in terms of their formation, repair, and biological effects (1).

DNA strand breaks produced by either ionizing radiation or bleomycin can have phosphoglycolate termini as the cleavage site 3'-end group. For DNA treated under aqueous aerobic conditions

in vitro, with either ionizing radiation (2) or bleomycin (3,4), 3'-phosphoglycolates (3'-PG) are present at approximately 50 or 100 percent, respectively, of the strand break sites. The 5'-end group of cleavage for both agents is a phosphate (5'-P).

The existence of 3'-PG and 5'-P at sites of strand breakage indicates that at least one base has been lost from the DNA at each site, since the destructive loss of at least one nucleoside is needed to produce this end-group configuration. It follows that repair of these types of strand breaks involves a DNA polymerase to replace the missing base(s). DNA polymerases, however, require 3'-hydroxyl groups (3'-OH) (5) and cannot use 3'-PG as a primer for synthesis. Prior conversion of 3'-PG to 3'-OH is, therefore, necessary for DNA strand-break repair to proceed.

We have purified three chromatographically distinct enzyme species from human cells, all of which (i) convert bleomycin-treated DNA to a substrate for DNA polymerase, (ii) remove 3'-PG from this substrate, (iii) possess Class II AP endonuclease activity and (iv) have no accompanying exonuclease activity. These results imply a central role for the enzymes in repair of both DNA strand breaks and AP sites, and suggest a common pathway for ultimate repair of both types of DNA lesions.

MATERIALS AND METHODS

Materials

The DE52 cellulose and P11 phosphocellulose chromatography matrices were purchased from Whatman (Hillsboro, OR). Mono P and HR columns were purchased from Pharmacia (Piscataway, NJ).

Snake venom phosphodiesterase (*Crotalus atrox*, type IV, 1 g/ml, 22 units/ml) and nuclease P1 (1 mg/ml, 340 units/ml) were purchased from Sigma Chemical Co. (St. Louis, MO). DNase I (10,000 units/ml) was from BRL (Burlington, ON). Calf alkaline phosphatase (1000 units/ml) was purchased from Boehringer Mannheim Canada (Dorval, PQ). *E. coli* DNA polymerase I large fragment (Klenow) (5,000 units/ml) and exonuclease III (100,000 units/ml) were purchased from New England Biolabs (Beverly, MA).

* To whom correspondence should be addressed

Non-radioactive nucleotides, aprotinin, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). [5-³H]-thymidine triphosphate (82 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [gamma-³²P]-ATP (4,500 Ci/mmol) was purchased from ICN Canada (Montreal, PQ).

Calf thymus DNA was purchased from Calbiochem (LaJolla, CA). pUC19 plasmid DNA was obtained from Pharmacia (Piscataway, NJ) and closed supercoiled plasmid (Form I) DNA was prepared using standard bacterial transformation and cesium banding purification techniques (6). Oligo(dT)₁₂ (5.0 A₂₆₀units/ml) was purchased from Pharmacia (Baie d'Urfé, PQ).

Fetal bovine serum was obtained from Gibco (Grand Island, NY). Minimal Essential Medium with Eagle's salts and Spinner Modification (SMEM) was purchased from Biofluids, Inc. (Rockville, MD). Bleomycin was provided in the form of bleomycin sulfate (Blenoxane) by Bristol-Myers Pharmaceutical and Nutrition Group (Evansville, IN).

The phosphoglycolate-bearing marker compounds, 2'-deoxynucleoside 3'-(phospho-2''-O-glycolic acid), were prepared by Dr. S.M.Hecht of the University of Virginia, Charlottesville, VA, and kindly provided by Dr. W.D.Henner of Oregon Health Sciences University, Portland OR.

Substrate Preparation

Bleomycin-treated DNA was prepared by incubating calf thymus DNA at 150 µg/ml, in the undiluted preincubation buffer mixture used in the nucleotide incorporation assay (12.5 mM Tris-HCl pH 8.0; 300 mM sucrose; 0.0188% (v/v) Triton X-100; 1.25 mM EDTA; 5 mM MgCl₂; 7.5 mM beta-mercaptoethanol (βME); 0.250 mg/ml of heat-inactivated BSA), with 0 to 10 µg/ml bleomycin and 2 µM ferrous ammonium sulfate at 37°C for 30 min. All enzyme purification was done with DNA substrate treated with bleomycin at 5 µg/ml.

pUC19 plasmid DNA containing AP sites was prepared as previously described (7). Briefly, depurinated DNA was prepared immediately before use by heating Form I pUC19 plasmid DNA in depurination buffer (20 mM NaCl, 20 mM sodium citrate, 20 mM NaH₂PO₄ pH 5.2) at 70°C for 80 min. The depurination reaction was stopped by rapid cooling on ice and neutralization to pH 8.25. pUC19 DNA treated in this way contains 3–4 apurinic sites per pUC19 molecule.

Nucleotide Incorporation Assay

The ability of enzyme to convert bleomycin-treated DNA into a substrate for DNA polymerase was quantitated by a two-step nucleotide incorporation assay. The first step involved preincubation of enzyme sample with bleomycin-treated DNA. The reaction mix was then supplemented with a mixture of DNA polymerase and nucleotide precursors, and incubated further. The preincubation reaction mixture contained the following in a total volume of 0.1 ml: 10 mM Tris-HCl pH 8.0; 240 mM sucrose; 0.015% (v/v) Triton X-100; 1 mM EDTA; 4 mM MgCl₂; 6 mM βME; 20 µg of heat-inactivated BSA; 15 µg bleomycin-treated, or untreated-control, calf thymus DNA; and the enzyme sample. The reaction mixtures were allowed to incubate at 37°C for appropriate lengths of time, and the reactions were terminated by incubation at 65°C for 5 min. After cooling on ice for at least 5 min, reactions were supplemented with 0.05 ml DNA polymerase reaction mixture, such that in a final volume of 0.15 ml the reaction mixtures contained: 50 mM Tris-HCl pH 8.0; 160 mM sucrose; 0.010% (v/v) Triton X-100; 1 mM EDTA;

5 mM MgCl₂; 4 mM βME; 20 µg BSA; 15 µg bleomycin treated, or untreated-control DNA; 150 µM each of dATP, dGTP, dCTP; 7.5 µM TTP; 0.375 µCi [³H]-TTP; and 0.75 units *E. coli* DNA polymerase I large fragment. The reaction mixtures were then incubated at 37°C for an additional 30 min. Reactions were terminated by incubation at 65°C for 5 min and placed on ice. 0.1 ml of each reaction mixture was pipetted onto a 2.5-cm diameter Whatman 3MM paper disc and washed twice with ice cold 5% (w/v) trichloroacetic acid/ 1% (w/v) sodium pyrophosphate, and once with 95% (v/v) ethanol. Following the ethanol wash the discs were dried and radioactivity was quantitated by liquid scintillation counting.

In this assay, the ability of a given enzyme sample to convert lesions containing non-3'-OH end groups to 3'-OH groups suitable for priming the DNA polymerase reaction, is indirectly measured as a function of the ability of *E. coli* DNA polymerase I large fragment to incorporate [³H]-TTP into the DNA substrate. Therefore, a unit of substrate priming nuclease activity was defined as the amount of enzyme required in the pre-incubation step, to achieve the incorporation of 1 pmole of [³H]-TTP per min at 37°C by 0.75 units of *E. coli* DNA polymerase I large fragment, under the defined assay conditions. Enzyme activity specific for bleomycin-induced DNA damage was assessed throughout the purification procedure, using the nucleotide incorporation assay.

Apurinic Nicking Assay

5 µl of enzyme was incubated with 1 µg of pUC19 plasmid DNA, containing or not containing heat/acid-induced AP sites, in 25 µl reaction buffer (100 mM HEPES pH 8.25/ 3 mM MgCl₂) for 30 min at 37°C. The reaction was stopped by incubation at 65°C for 10 min. The reaction mix was then run on a 1% agarose gel and stained with ethidium bromide. The stained gel was photographed under UV light to reveal the fast migrating supercoiled (Form I) and slower migrating nicked (Form II) plasmid bands.

3'-Phosphoglycolate Assay

The 3'-PG content of sample DNA was measured using a postlabeling assay as previously described (8). Samples (5 µg) of bleomycin-treated or untreated DNA were incubated overnight at 37°C with 0.4 units of DNase I, 0.04 units of snake venom phosphodiesterase, and 0.4 units of calf alkaline phosphatase in 40 µl of digestion buffer (10 mM Tris-HCl, pH 7.5, 4 mM MgCl₂). After a further incubation period of 3 h with an additional 0.02 units of snake venom phosphodiesterase and 0.2 units of phosphatase, the enzymes were precipitated by addition of three volumes of ice-cold ethanol and removed by centrifugation (10,000 × g, 15 min). The supernatants were evaporated and the resultant residues dissolved in 200 µl of distilled water, heated at 100°C for 10 min to inactivate residual nuclease and phosphatase activity, and then stored at -20°C.

Each phosphorylation reaction mixture (20 µl) contained kinase buffer (10 mM Tris-acetate, pH 7.5, 10 mM Mg acetate, 50 mM K acetate), 10 µl of enzyme-digested DNA, 5 µCi of [gamma-³²P]-ATP, and 5 units of polynucleotide kinase. The samples were incubated at 37°C for 1 h, and then the bulk of the excess ATP was consumed by incubation for a further 30 min with 1 µl of oligo(dT)₁₂ (5.0 A₂₆₀units/ml) and 2.5 units of the kinase. An equal volume of formamide loading buffer (90% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanol in 1×TBE) was added to each sample, and half of the reaction

mixture was loaded onto a 20% polyacrylamide/7 M urea gel. The gel electrophoresis equipment and conditions were the same as previously described (9). Radiolabeled products were visualized by autoradiography, and identified by comigrating marker compounds. Bands corresponding to nucleosides with 3'-PG termini were excised from the gel, and quantitated by scintillation counting.

Protein Assay

All protein determinations were made according to the method of Bradford (10), using bovine plasma gamma globulin as the standard.

Purification

HeLa cells were grown in suspension in SMEM supplemented with 10% fetal bovine serum, 25 mM HEPES (pH 7.0), 4 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. When the cultures reached 5×10^5 – 1×10^6 cells/ml they were harvested by centrifugation for 10 min at $850 \times g$. The cells were washed twice with 25 mM potassium phosphate (pH 7.0), 150 mM NaCl, 15 mM sodium citrate and stored at -70°C until used.

Figure 1. Phosphoglycolate 3'-end group at DNA strand break.

Cell pellets from multiple harvests were combined and approximately 10 g (wet weight) was extracted by stirring for 2 h at 4°C in 2 volumes of lysis buffer (20 mM Tris-HCl pH 7.0, 5 mM β ME, 1 mM EDTA, 0.2% (v/v) Triton X-100, 3 M NaCl). The extract was clarified by centrifugation at $70,000 \times g$ for 2 h at 4°C , and the supernatant was dialyzed overnight against TMEG buffer (20 mM Tris-HCl pH 8.0, 5 mM β ME, 1 mM EDTA, 10% (v/v) glycerol) + 0.15 M NaCl.

The dialysate was brought to 45% $(\text{NH}_4)_2\text{SO}_4$ saturation over a 30 min period with stirring on ice. After incubating an additional 30 min on ice with occasional stirring, the mixture was centrifuged at $10,000 \times g$ for 30 min at 4°C . The pellet was discarded and the supernatant was brought to 75% $(\text{NH}_4)_2\text{SO}_4$ saturation as described above. Proteins precipitated by this procedure were resuspended in TMEG and dialyzed overnight against the same buffer. The resulting mixture constituted the crude cell extract. This crude extract was subjected to column chromatography as described below. Protease inhibitors (leupeptin and aprotinin, each at 2 μ g/ml) were used throughout the purification.

DNA was removed from the crude extract by passing it through a DE52 DEAE-cellulose column equilibrated in TMEG + 400 mM NaCl. Bleomycin-treated DNA incorporation activity eluted in the unabsorbed fractions. These fractions were pooled, dialyzed against PMEG buffer (20 mM KPO_4 pH 7.5, 5 mM β ME, 1 mM EDTA, 10% (v/v) glycerol), and chromatographed on an HR 10/30 phosphocellulose column pre-equilibrated in the same buffer. The column was eluted with a linear NaCl gradient from 0–1.0 M, and nucleotide incorporation activity which eluted between 250 mM and 340 mM NaCl was pooled and dialyzed against DC/MP buffer, pH 8.6 (20 mM Tris-HCl pH 8.6, 2 mM β ME, 1 mM EDTA, 10% (v/v) glycerol). The dialysate was applied to an HR 5/5 double-stranded DNA cellulose column pre-equilibrated in DC/MP buffer and eluted with a linear NaCl gradient (0–0.5 M). Fractions (1 ml) were collected and assayed for nucleotide incorporation activity. Activity which eluted from the column between 160 mM and 290 mM NaCl was pooled and dialyzed against DC/MP buffer, pH 8.0 (20 mM Tris-HCl pH 8.0, 2 mM β ME, 1 mM EDTA, 10% (v/v) glycerol).

The dialysate was applied to an HR 5/20 Mono P chromatofocusing column pre-equilibrated in 25 mM triethanolamine-iminodiacetic acid pH 8.3. The Mono P column was eluted with a self-developing pH gradient from pH 8.0–5.0. Fractions (1 ml) were collected and assayed for nucleotide incorporation activity. Three species of bleomycin-treated DNA incorporation activity were eluted in the gradient at pH 8.30,

Table I. Purification

Procedure	Volume (ml)	Total Units	Protein (mg/ml)	Specific Activity	% Yield
Phosphocellulose	19.5	1303	0.3145	213 ^a	100
DNA-Cellulose	10	689	0.0015	6,562	52.9
Mono P:					
MPA	4.1	94	0.0018	12,808	7.2 ^b
MPB	3.5	88	0.0026	9,670	6.7 ^b
MPC	3.1	43	0.0039	3,584	3.3 ^b

^a Specific activity for the phosphocellulose fraction represents an upper limit since phosphocellulose and previous purification fractions are known to be contaminated with other nuclease activities.

^b Total Mono P yield of endonuclease activity = 17.2%.

Figure 2. Liquid column chromatography. Assays were performed using the nucleotide incorporation assay with either bleomycin-treated (open circles) or untreated (closed triangles) calf thymus DNA as a substrate. (A) Double-stranded DNA cellulose chromatography (—, NaCl gradient). (B) Mono P chromatography (— pH gradient).

7.35, and 6.35 respectively. Each species was pooled separately, and dialyzed against DC/MP buffer, pH 8.0. Following dialysis, the enzymes were stabilized by the addition of the protease inhibitors leupeptin and aprotinin (100 μ g/ml of each), and 0.1 ml aliquots were stored at -70°C until needed.

RESULTS

Purification

Bleomycin reacts with DNA in the presence of oxygen and iron, via a free radical mechanism, to release base propenal and leave 3'-PG termini at the resulting strand breaks ((3,4); Fig. 1). This end-group chemistry is the same as a major lesion produced by ionizing-radiation, but unlike radiation, bleomycin-induced

Table II. Nucleotide incorporation into apurinic plasmid DNA

	-AP sites ^a	+AP sites ^b
No enzyme:		
form I	0	137
form II	0	63
MPA:		
form I	0	110
form II	186	6720
MPB:		
form I	5	304
form II	362	8950
MPC:		
form I	0	279
form II	83	8868

^aDPM incorporated in the nucleotide incorporation assay using native pUC19 plasmid DNA in place of calf thymus DNA.

^bDPM incorporated in the nucleotide incorporation assay using pUC19 plasmid DNA containing ~2-3 AP sites/molecule. (All numbers represent the mean of duplicate samples.)

damage of DNA causes minimal base damage, other than accompanying loss of a base from the site of strand breakage (11). Therefore, bleomycin treatment was chosen as a means to produce a less complex substrate than irradiated DNA, in which the predominant lesion was DNA strand breaks with 3'-PG termini.

Since we were interested in enzyme activities which modify 3'-end groups to produce the 3'-OH required by DNA polymerase, we employed a purification assay which measured the conversion of bleomycin-treated DNA into a substrate for *E. coli* DNA polymerase I large fragment. In addition, the effect of enzyme and column fractions on control DNA (untreated with bleomycin) was determined in parallel assays. This approach allowed for the identification of damage-specific enzyme activity with respect to non-damage specific activity. The presence of large amounts of non-damage-specific activity in the early purification steps made calculation of damage-specific activity difficult. For this reason, yields and purification were determined relative to the phosphocellulose purification step (Table I). The greatest purification, however, occurs earlier in the ammonium sulfate precipitation and phosphocellulose chromatography steps, since these steps remove the bulk of contaminating protein (97%).

Double-stranded DNA cellulose chromatography revealed non-damage-specific activity contaminating a peak of damage-specific activity (Fig. 2A). To resolve these activities, Mono P chromatofocusing was employed. The resulting chromatograph (Fig. 2B) showed removal of the non-specific activity and revealed three peaks of damage-specific activity, having isoelectric points of 8.30, 7.35, and 6.35. These peaks were designated as MPA, MPB, and MPC, in order of their elution from Mono P and investigated further for possible differences in enzymatic properties. The small amount of enzyme activity on control DNA that coelutes with these three peaks (Fig. 2B) probably represents damage-specific activity acting on the small amounts of oxidative damage in the commercially obtained calf thymus DNA that was used in these assays. (Fig. 5 shows that even untreated calf thymus DNA contained some 3'-PG.) As shown below, there is no enzymatic activity on control unnicked plasmid DNA that was freshly purified by a method that does not employ phenol.

Figure 3. Superose 12 chromatography. A sample of the DNA cellulose chromatography pooled active fraction (0.2 ml) was chromatographed on a Pharmacia a HR10/30 Superose 12 analytical gel filtration column equilibrated in 20 mM Tris-HCl pH 8.0 and 50 mM NaCl (Chromatography was performed at 4°C). Fractions (0.2 ml) were assayed for nucleotide incorporation (open triangles, dashed line) or AP endonuclease activity (closed circles, solid line).

Apurinic Endonuclease Activity

E. coli (12–14), yeast (15–17), murine (18–20) and bovine (7,21,22) enzymes have been identified, which are able to convert bleomycin-treated DNA into a substrate for polymerase. These enzymes have also been observed to have endonuclease activity on AP-containing DNA. In order to determine whether the human enzymes also share this activity, the DNA cellulose preparation was run on a Superose 12 gel filtration column and fractions were assayed for nucleotide incorporation activity on bleomycin-treated DNA and endonuclease activity on AP-containing DNA. Despite poor yield and instability, it was determined that nucleotide incorporation and AP endonuclease activities comigrated as symmetrical peaks (Fig. 3), suggesting enzyme(s) of similar size had both activities (or that only one enzyme with both activities was recovered).

Since nucleotide incorporation and AP endonuclease activities had been measured on different substrates it was not clear if the cleaved AP-DNA was also a substrate for nucleotide incorporation by DNA polymerase. To address this question, Mono P fractions were tested for nucleotide incorporation on AP containing DNA. The ability of AP-containing DNA to serve as a substrate for both nicking and incorporation was measured within the same experiment. Freshly prepared closed supercoiled (Form I) pUC19 plasmid DNA was heat/acid depurinated. This DNA was then incubated with MPA, MPB, or MPC. Following heat treatment to inactivate the enzymes, the DNA polymerase incorporation reaction was run in the usual manner. The sample DNA was then run on a 1% (w/v) agarose gel to separate the supercoiled (Form I) and nicked (Form II) species, and stained with ethidium bromide to visualize the bands. The Form I and Form II bands were cut from the gel, dissolved in scintillation cocktail, and quantitated for radioactivity by liquid scintillation counting. Control DNA that lacked heat-depurinated AP sites was also assayed.

Results showed that all three enzyme fractions cleaved AP-containing DNA, converting it from Form I to Form II, and had no cleavage ability on DNA without AP sites (Fig. 4). Furthermore, the enzyme-cleaved DNA was a good substrate for DNA polymerase as measured by radioactive nucleotide

Figure 4. AP-nicking by mono P enzyme species. Supercoiled plasmid DNA containing AP sites was first incubated in duplicate with each Mono P fraction (MPA, MPB, or MPC) or no enzyme (–), then with DNA polymerase and nucleotide precursors, and run on an agarose gel. The gel was stained and photographed to reveal supercoiled (Form I; lower band) and nicked (Form II; upper band) plasmid. Bands from this gel were excised and counted as described in 'Results' so that relative nucleotide incorporation could be assessed (Table II).

incorporation into the Form II bands (Table II). These results indicate that the Mono P fractions have AP endonuclease activity, which cleaves AP sites on the 5' side of the 5'-phosphate group, leaving a 3'-hydroxyl group that subsequently can serve as a substrate for DNA polymerase.

3'-End Group Modifying Activity

Since DNA treated with bleomycin may contain AP sites (23), results from the AP experiment presented the possibility that the purified enzymes were merely AP endonucleases and had no 3'-end group modifying activity. These enzymes might simply have been recognizing the contaminating AP sites in our bleomycin-treated substrates with no activity on phosphoglycolate end groups. Therefore, it was necessary to characterize our substrate with respect to types of lesions, their relative abundance, and in particular, to determine which lesion was acted upon by the enzymes. To do this, we employed a postlabeling gel electrophoresis assay which can measure, among other lesions, 3'-PG termini in DNA.

Figure 5. Gel electrophoresis autoradiograph of ^{32}P -postlabeled bleomycin-treated DNA. Bleomycin-treated DNA was enzyme digested, phosphorylated, and run on a polyacrylamide gel. Oligo(dT) $_{12}$ was used to consume the bulk of the excess ^{32}P -ATP, thereby reducing the background radioactivity in the vicinity of the small products. A control lane (C) containing all reagents, but no sample DNA, was also run.

Figure 6. 3'-Phosphoglycolate production by bleomycin. Dose/response for 3'-phosphoglycolate formation in DNA treated with bleomycin, based on the quantitation of the gel shown in Fig. 5, as described in 'Materials and Methods'.

Preliminary experiments revealed that DNA treated with bleomycin according to our substrate preparation protocol, had a dose dependent increase in 3'-PG end groups (Fig. 5 & 6). Also, 3'-PG end groups represented greater than 95% of the dose-dependent lesions in this substrate, as assessed by the ^{32}P -postlabeling assay. Furthermore, the 5 $\mu\text{g}/\text{ml}$ bleomycin concentration that we used to prepare substrate for the nucleotide incorporation assay, resulted in approximately 700 fmole 3'-PG termini per μg of DNA and only minimal amounts of other lesions (Fig. 5 & 6). Using this substrate and the ^{32}P -postlabeling assay, the Mono P enzyme fractions were examined to determine what lesions were acted upon, and to what extent.

When 5 μg of DNA, treated with 5 $\mu\text{g}/\text{ml}$ bleomycin, was preincubated with 30 μl of MPA, MPB, or MPC, and then

Figure 7. Enzymatic removal of 3'-phosphoglycolate end groups. Assays were performed by incubating a fixed volume of enzyme in a Tris reaction buffer (10 mM Tris-HCl pH 8.0, 4 mM MgCl_2 , 6 mM βME , 10 μg BSA) containing 5 μg of DNA (pretreated with bleomycin at 5 $\mu\text{g}/\text{ml}$), and allowed to incubate at 37°C for 2 h. Reactions were stopped by incubation at 65°C for 10 min. The DNA was then subjected to the ^{32}P -postlabeling assay described in 'Materials and Methods'. Bands from the gel were excised and counted so that relative 3'-PG removal could be assessed (Table III). A negative control of bleomycin-treated DNA sample incubated without enzyme (-), and a positive control of bleomycin-treated DNA incubated with an excess of *E. coli* exonuclease III (6 units) are shown. A double negative control of DNA treated with neither bleomycin nor enzyme (C) is shown.

Table III. Enzymatic removal of 3'-phosphoglycolate end groups^a

Enzyme	Units Used ^b	% 3'-PG Removed ^c
MPA	0.688	75
MPB	0.754	82
MPC	0.416	36

^aRemoval of 3'-PG was as determined from the gel shown in Fig. 7.

^bUnits of each enzyme used in this experiment as determined by the nucleotide incorporation assay (See Materials and Methods).

^cIncreasing removal of 3'-PG correlates highly with increasing nucleotide incorporation (correlation coefficient = 0.99).

assayed with the ^{32}P -postlabeling assay, results showed that all three enzyme species removed a significant proportion of 3'-PG (Fig. 7). *E. coli* exonuclease III, a 3' → 5' exonuclease which is not inhibited by 3'-PG (8,12), was used in excess as a positive control. The amount of 3'-PG removal for each Mono P fraction correlated with the units of enzyme activity in the 30 μl enzyme volume, as determined by the nucleotide incorporation assay (Table III).

MPB was then used in a dose/response experiment to measure the effect of increasing enzyme on both 3'-PG removal and nucleotide incorporation simultaneously. DNA treated with 2 $\mu\text{g}/\text{ml}$ bleomycin was incubated with increasing amounts of MPB for 2 h. The reaction was then divided in two and assayed either for incorporation or 3'-PG removal. The results demonstrated enzyme-dependent 3'-PG removal and nucleotide incorporation with similar dose/response (Fig. 8). Since the ^{32}P -postlabeling assay is relatively insensitive and requires large amounts of enzyme for quantitation, only single point dose/response determinations were employed for the pooled MPB fractions.

Figure 8. Incorporation and 3'-phosphoglycolate removal. Nucleotide incorporation and 3'-phosphoglycolate removal are shown for DNA treated with 2 μ g/ml bleomycin. Varying amounts of MPB were incubated for 2 h with bleomycin-treated calf thymus DNA, and then the DNA was assayed for either nucleotide incorporation (open circles) or 3'-phosphoglycolate (closed squares) removal.

Based on previous experience, however, variability among replicate samples is no more than 10 percent.

Results from these studies show that the purified enzyme fractions could remove 3'-PG from bleomycin-treated DNA in addition to their AP endonuclease activity. Both activities were accompanied by increased nucleotide incorporation by DNA polymerase, suggesting that a 3'-OH end group was the product of these reactions.

DISCUSSION

Of the different types of damage produced in DNA, strand breaks have received the most attention. Yet, characterization and quantitation of strand breaks has been based solely on biophysical disruption of DNA double helix continuity, and not the chemical nature of the lesions responsible for the breaks.

Studies with model nucleotides, irradiated in aqueous solution, suggest a heterogeneous nature to the various end groups that might constitute radiation-induced DNA strand breaks (24). However, Henner and coworkers (2,25), working with purified plasmid DNA irradiated *in vitro*, have demonstrated that strand breaks produced under aerated aqueous conditions are predominantly of two types, present in roughly equal amounts. Both types have phosphates as 5' end groups, but the 3' end groups are either phosphates or phosphoglycolates. Bases and coworkers (26) have shown that strand breaks produced *in vivo* in irradiated mammalian cells have the same end groups.

Bleomycin, a radiomimetic compound (27), can produce DNA strand breaks with 3'-PG and 5'-P end groups. The end groups explain why bleomycin-induced DNA strand breaks cannot serve as primers for DNA synthesis, since the strand breaks lack the 3'-OH group required by DNA polymerase. These results also indicate a need for DNA polymerase in strand-break repair, since 3'-PG cannot be produced without the loss of at least one base.

Finally, they suggest that a 3'-end group modifying enzyme is required for strand break repair, in order to provide the 3'-OH group needed for DNA polymerization.

Despite the necessity of 3'-PG removal prior to DNA repair synthesis, and some prior claims of such removal activities in mammalian cells (18,21,28), no direct demonstration of 3'-PG removal by a mammalian enzyme has ever been reported. Previous claims of such activity have either used poorly characterized substrates or synthetic analogs of 3'-PG. We have purified three enzyme activities from human HeLa cells, which convert bleomycin-treated DNA into a substrate for DNA polymerase. Since we characterized our substrate and showed that 3'-PG represented >95% of the dose-dependent lesions produced under our treatment conditions, we anticipated that these enzymes might be responsible for modification of 3'-PG end groups at strand breaks. We subsequently showed that the purified enzymes removed 3'-PG from the DNA.

Characterization of the purified enzymes also revealed an AP endonuclease activity which produced an initiation site for DNA polymerase. Linn and coworkers have classified AP endonucleases according to their phosphodiester bond cleavage site (29). Our results suggested that the purified enzymes acted as Class II AP endonucleases (i.e. cleave on the 5' side of the 5' phosphate), since only Class II cleavage can produce the 3'-OH group required for nucleotide incorporation.

Other investigators have recently reported on murine (18–20,30) and bovine (21) enzymes which convert bleomycin-damaged DNA into a substrate for DNA polymerase. These enzymes were also reported to have AP endonuclease activity, however, no assessment of 3'-PG modifying activity was made. It is not clear whether the incorporation activity seen, in those systems, was due solely to cleavage at AP sites or whether strand-break 3'-end-group modification was also involved. In addition, Chen and coworkers (31,32) have recently reported two enzyme activities in HeLa cells, which possess Class II AP endonuclease activity, and the ability to release 3'-phosphoglycolaldehyde from a synthetic DNA substrate. The ability of these enzymes to remove bona fide 3'-PG was not determined.

There is precedence for cleavage capabilities at both strand breaks and AP sites in enzymes isolated from lower organisms. Both endonuclease IV and exonuclease III of *E. coli* and a yeast diesterase from *Saccharomyces cerevisiae* (15,16) have AP endonuclease, 3'-end group modifying activity, and incorporation activity on bleomycin damaged DNA. Exonuclease III also has a 3'→5' polyexonuclease activity. The human enzymes described here do not have either 3'→5' or 5'→3' polyexonuclease activity, as assessed by their inability to degrade nicked plasmid DNA. These human enzymes are more similar to endonuclease IV in this regard, and may be the human equivalent of this bacterial enzyme. Furthermore, a recent report by Robson and Hickson (33) in which the cDNA encoding the human analog of *E. coli* hI exonuclease III was expressed in *E. coli* mutants that lack the enzyme, indicated that this enzyme is fully capable of substituting for only exonuclease III, and not endonuclease IV, and that its primary role is in the repair of AP sites. These data suggest that the major Class II human AP endonuclease is responsible for only a small fraction of repair involving oxidatively-induced DNA strand breaks. Moreover, the data reported by Chen and coworkers (31) is consistent with this and suggests a model for the repair of oxidatively-induced DNA strand breaks in human cells that is similar to that emerging for their repair in *E. coli*. That is, the major cellular Class II AP endonuclease (exonuclease

III in *E. coli*, HAP1 in human cells), while being capable of removing 3'-deoxyribose fragments at low levels, is not sufficient for the repair of large amounts of damage, and is primarily responsible for the repair of AP sites. Conversely, one or more species of enzymes possessing Class II AP endonuclease activity exist (endonuclease IV in *E. coli*; this work, and Chen and coworkers in humans) which primarily act to remove 3'-deoxyribose fragments from DNA strand break sites.

The relationship between the three enzymes that we have reported upon here remains unclear. They may represent three discrete enzymatic species, all of which are capable of removing 3'-PG from bleomycin damaged DNA, but have some other strand break 3'-end group lesion as their preferred substrate. Multiple enzymes in human cells might be expected, since a number of uncharacterized 3'-end-modifying enzymes are known to exist even in *E. coli* (34). Alternatively, they may be isoenzymes or differently modified forms of the same gene product. We do not think that they represent proteolytic breakdown products, since care was taken to include protease inhibitors at all stages of the purification.

The relationship between the three enzymes described here and the two AP endonucleases recently reported by Chen and coworkers (31) is not clear. The chromatographic behavior of our enzymes suggests that they are different from the major AP activity that they describe. However, the second activity that they describe may represent one of the three reported here, but not enough is known yet about either enzyme.

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