1866–1873 Nucleic Acids Research, 1994, Vol. 22, No. 10

Removal of 3′-phosphoglycolate from DNA strand-break damage in an oligonucleotide substrate by recombinant human apurinic/apyrimidinic endonuclease 1

Thomas A.Winters*, W.David Henner[1], Pamela S.Russell, Amanda McCullough[1] 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 and [1]Division of Hematology and Medical Oncology, Oregon Health Sciences University, Portland, OR 97201, USA

Received December 30, 1993; Revised and Accepted April 6, 1994

ABSTRACT

A recombinant human AP endonuclease, HAP1, was constructed and characterized with respect to its ability to recognize and act upon a model double-stranded 39-mer oligodeoxyribonucleotide substrate containing a strand break site with 3′-phosphoglycolate and 5′-phosphate end-group chemistries. This oligodeoxyribonucleotide substrate exactly duplicates the chemistry and configuration of a major DNA lesion produced by ionizing radiation. HAP1 was found to recognize the strand break, and catalyze the release of the 3′-phosphoglycolate as free phosphoglycolic acid. The enzyme had a V_{max} of 0.1 fmole/min/pg of HAP1 protein, and a K_m of 0.05 μM for the 3′-phosphoglycolate strand break lesion. The mechanism of catalysis was hydrolysis of the phosphate ester bond between the 3′-phosphoglycolate moiety and the 3′-carbon of the adjacent dGMP moiety within the oligonucleotide. The resulting DNA contained a 3′-hydroxyl which supported nucleotide incorporation by *E.coli* DNA polymerase I large fragment. AP endonucleolytic activity of HAP1 was examined using an analogous double-stranded 39-mer oligodeoxyribonucleotide substrate, in which the strand break site was replaced by an apyrimidinic site. The V_{max} and K_m for the AP endonuclease reaction were 68 fmole/min/pg of HAP1 protein and 0.23 μM, respectively.

INTRODUCTION

3′-Phosphoglycolate (3′-PG) is a major lesion produced at the sites of radiation-induced DNA strand breaks (1), and can act as both a block to initiation and an inhibitor of DNA polymerase (2). Thus, enzymatic removal of 3′-PG is thought to be required for DNA strand break repair to proceed *in vivo*.

We have constructed a 39-base-pair oligodeoxyribonucleotide (oligo) containing a single-strand break at a specific sequence position midway along the molecule. The strand break has 3′-PG and 5′-phosphate (5′-P) termini separated by a one-base gap, thus exactly duplicating a major form of radiation-induced break (1). The 3′-PG moiety is ^{3}H-labeled to allow qualitative and quantitative evaluation of its enzymatic removal.

We used this substrate to measure the 3′-PG removal ability of human apurinic/apyrimidinic endonuclease 1 (HAP1). HAP1 is the major apurinic/apyrimidinic (AP) endonuclease of human cells, and is probably the enzyme responsible for the majority of human AP site repair. This enzyme hydrolytically cleaves abasic sites, on the 5′ side of the 5′ phosphate group (i.e. Class II), to leave 3′-hydroxyl (3′-OH) and 5′-2-deoxyribose 5-phosphate (5′-dRp) termini (3). In addition to its AP endonuclease activity, HAP1 has also been shown to have 3′-diesterase activity on 3′-phosphoglycoaldehyde — a synthetic analog of 3′-PG (4). Although HAP1 cleavage of 3′-phosphoglycoaldehyde suggests a comparable activity on 3′-PG, direct demonstration of such activity has not been reported, primarily due to the unavailability of a suitable chemically-defined and readily quantifiable 3′-PG substrate.

The enzyme used in this study was produced by inserting the cloned HAP1 cDNA into an expression vector, and expressing the protein in *E.coli*. Recombinant enzyme was purified to homogeneity from this system.

The availability of both pure enzyme and pure 3′-PG strand break substrate allowed us to assess the ability of HAP1 to remove 3′-PG and the kinetics of the cleavage reaction. Furthermore, we were able to show that 3′-PG is released in the form of phosphoglycolic acid, and that the resulting 3′- deoxyribonucleotide terminus can prime DNA synthesis, indicating that the 3′-terminus was converted to a hydroxyl group. These results confirm that HAP1 can remove 3′-PG groups from the sites of DNA strand breaks and indicate hydrolytic cleavage of the

*To whom correspondence should be addressed

carbon-oxygen bond immediately 5′ to the 3′-PG termini, in a reaction analogous to its Class II AP endonuclease activity.

MATERIALS AND METHODS

Materials

Nucleotide solutions and *E.coli* DNA polymerase I large fragment (5,000 units/ml) were purchased from New England Biolabs (Beverly, MA). Avian myeloblastosis virus (AMV) reverse transcriptase (1000 units/ml), uracil-DNA glycosylase (1000 units/ml), T4 kinase (10,000 units/ml), and yeast tRNA were purchased from Gibco-BRL (Gaithersburg, MD). Taq DNA polymerase (5000 units/ml) and RNasin (40,000 units/ml) were purchased from Promega (Madison, WI). Sequenase™ T7 DNA Polymerase Version 2.0 (13,000 units/ml) was purchased from United States Biochemical (Cleveland, OH). Radiolabeled nucleotides were obtained from New England Nuclear (Boston, MA), with the exception of 5′-^{32}P 3′-P deoxyguanosine diphosphate which was prepared as previously described (1). dU-CE phosphoamidite was purchased from Glen Research (Sterling,

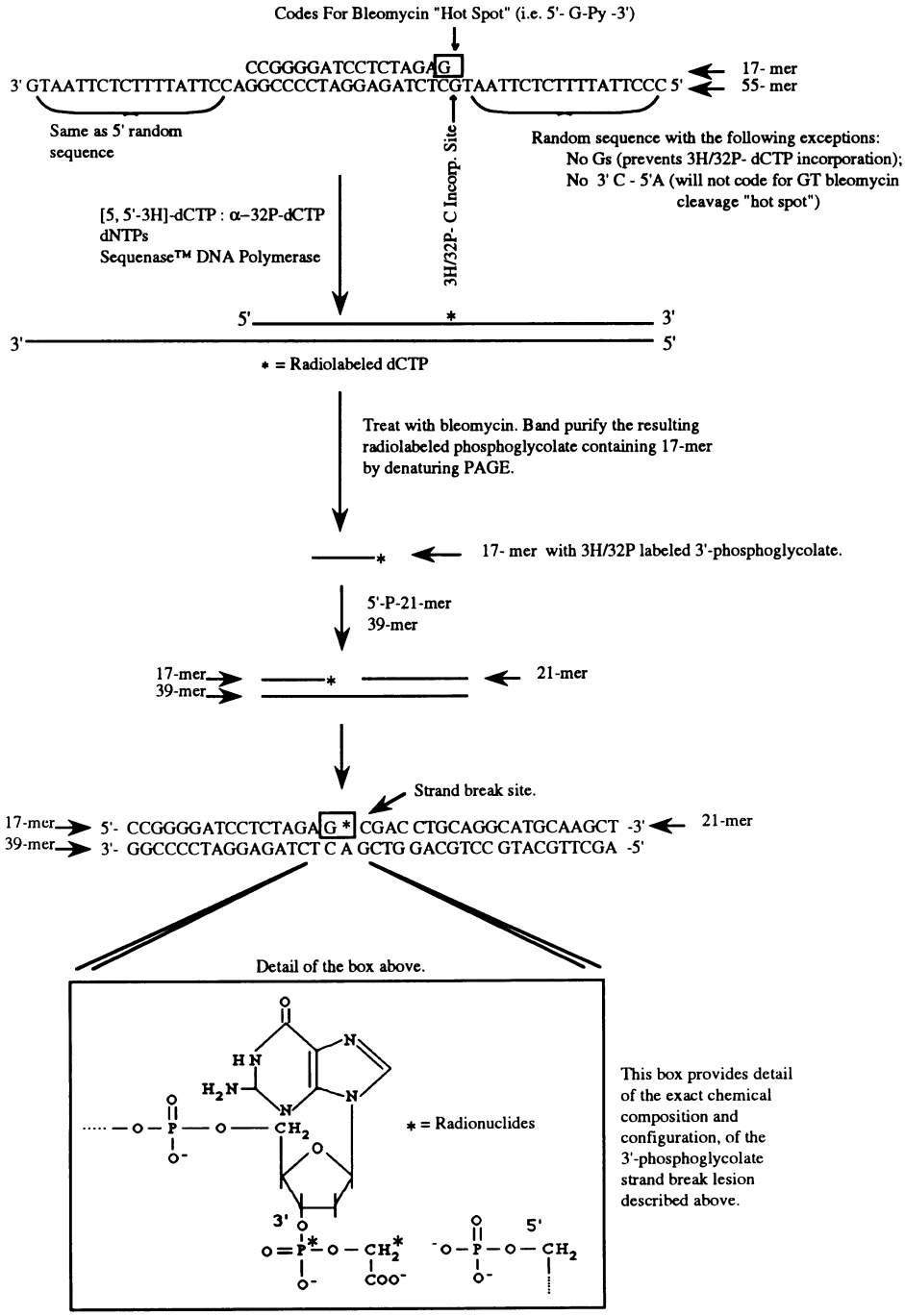

Figure 1. Flow diagram for the synthesis and construction of the double stranded 39-base-pair oligodeoxynucleotide strand break substrate.

VA). Plasmid pGEX-2T was purchased from Pharmacia LKB (Piscataway, NJ). Plasmid pQE-30 and nickel-nitrilotriacetic acid (Ni-NTA) resin were purchased from Qiagen (Chatsworth, CA). Sephadex G-25 Quick-Spin columns were purchased from Boerhinger Manheim (Indianapolis, IN). Bleomycin sulfate (Blenoxane™) was a gift from Bristol-Myers (Evansville, IN). Ferrous ammonium sulfate, phosphoglycolic acid, phosphonoformic acid, and phosphonoacetic acid were purchased from Sigma Chemical Company (St. Louis, MO). Triethylamine was obtained from Aldrich Chemical Company (Milwaukee, WI). Sep-Pak C18 cartridges were purchased from Waters Chromatography Division, Millipore Corporation (Milford, MA). Centricon SR3 concentrators were obtained from Amicon (Beverly, MA).

Preparation of 3′-phosphoglycolate double-stranded oligodeoxyribonucleotide substrate

The construction of a double-stranded 39-mer oligo substrate, containing a single-strand break midway along one strand with 3′-PG and 5′-P end groups, was achieved by the process depicted in Figure 1. Four oligos, a 55-mer, 39-mer, 21-mer, and 17-mer, were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. The 17-mer and 21-mer oligos (containing 5′-OH and 3′-OH end groups) were then modified as described below, to achieve the required end-group chemistries. The double-stranded 39-mer was produced by annealing the modified 17-mer and 21-mer to the complementary 39-mer.

Radiolabeled 3′-PG 17-mer construction. The 3′-PG oligo was prepared by extending a 17-mer with Sequenase™ Version 2.0 T7 DNA polymerase, radiolabeled dCTP, and unlabeled TTP, dGTP, and dATP, along a defined complementary 55-mer template oligo. Since the template coded for a single dCTP (at the 18th position), only one position was radiolabeled. The 17−18 sequence (i.e. G-C) is a 'hot spot' for bleomycin cleavage, consequently, subsequent treatment with bleomycin resulted primarily in production of a 17-mer with a terminally radiolabeled 3′-PG. The 17-mer was isolated from other damage products by polyacrylamide gel electrophoresis (PAGE) (Fig. 2) and purified. A schematic of the synthetic procedure for the 3′-PG 17-mer strand break substrate is shown in Figure 1. The details of the procedure are described below.

80 pmole of a 17-base oligodeoxyribonucleotide (17-mer primer) and 48 pmole of a complementary 55-base oligodeoxyribonucleotide (55-mer template) were annealed in 10 μl of 40 mM Tris−HCl pH 7.5, 20 mM $MgCl_2$, and 25 mM NaCl, by incubating at 65°C for two min, followed by slow cooling, over 30 min, to 35°C. Extension and labelling of the annealed primer-template was done in a final volume of 25.5 μl containing 3.9 mM DTT; 2.5 nmole each of dATP, dGTP, and TTP; 200 pmole [5,5′-^{3}H] dCTP (30 Ci/mmole at 5′-position) [Note: Only the C-5′-position contributes to labeling 3′-PG, because the C-5-position is lost from the cleavage site in the form of cytosine propenal following bleomycin treatment (5).], 1 pmole [α-^{32}P] dCTP (400 Ci/mmole), and 3.25 units Sequenase™ Version 2.0 T7 DNA polymerase. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by heating at 65°C for 5 min. The samples were cooled on ice and then treated with bleomycin. 1 μl of a 10 mg/ml solution of bleomycin was added to the sample followed by 1 μl of 0.1 mM ferrous ammonium sulfate. The sample was incubated for 30 min at 37°C, cooled on ice, and lyophilized to dryness. The resulting oligos were resuspended in loading buffer (0.001% xylene cyanol, 0.001% bromophenol blue, 80% formamide in TEB buffer (100 mM Tris, 114 mM boric acid, 2 mM EDTA, pH 8.3)) and subjected to PAGE on a 20% acrylamide/7M urea denaturing gel in which the DNA fragments were identified by autoradiography. The band which corresponded to a 3′-PG 17-mer was cut from the gel and extracted with 1M triethylammonium bicarbonate pH 7.5 (TEAB). The extract was desalted and concentrated by running over a Sep-Pak C18 cartridge and eluting with 30% acetonitrile, 100 mM TEAB. The 3′-PG oligo was concentrated and buffer was exchanged to distilled deionized water using a Centricon SR3 concentrator. The yield of 3′-PG 17-mer from this procedure is approximately 8 pmole, and can easily be scaled up 100-fold by running three large preparative gels.

The sequence and length of the final purified product, and the 3′ position of the ^{32}P radiolabel, were confirmed by standard Maxam & Gilbert DNA sequencing techniques (6). The chemistry of the 3′-terminus was confirmed to be 3′-PG by digesting the purified 17-mer down to mononucleotides and confirming that the radioactivity from the digest migrated on PAGE in the exact position of 5′-P,3′-PG deoxyguanosine (Fig. 3) — the expected terminal nucleotide — as previously described (1,7).

5′-P 21-mer. The 21-mer with 5′-P was prepared by a simple 5′-phosphorylation reaction of the 21-mer (Fig.1) with T4 polynucleotide kinase and ATP (8).

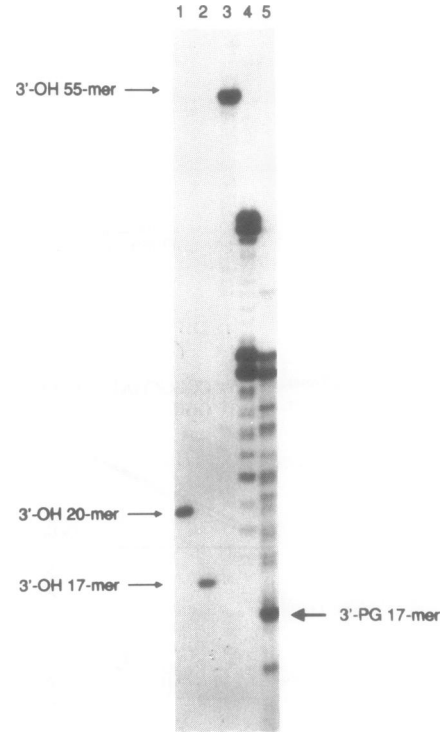

Figure 2. Isolation of 17-mer oligo with 3′-[^{3}H,^{32}P]-PG using denaturing PAGE. Lanes 1,2, and 3 have 5′-^{32}P-labeled size marker oligos of 20, 17, and 55 bases, respectively. Lane 4 contains the primer extension reaction products prior to bleomycin treatment, and shows a full-length extension band (36-mer) and three truncated extension bands. Lane 5 shows the extension reaction following bleomycin treatment. The full-length and truncated extensions all contribute bleomycin cleavage products to the 3′-PG 17-mer band, which is excised from the gel and purified.

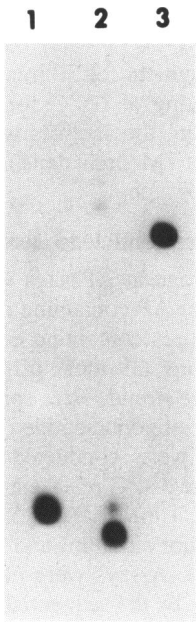

Figure 3. Confirmation of radiolabeled 3′-PG end group of isolated 17-mer. The purified 3′-PG 17-mer was chemically cleaved down to mononucleotides and run on a gel with 5′-^{32}P 3′-P deoxyguanosine diphosphate (lane 1) and α-^{32}P-ATP (lane 3). The radiolabeled reaction product ran ahead of these two markers in the exact position reported for 5′-P, 3′-PG deoxyguanosine, the expected terminal mononucleotide diphosphate.

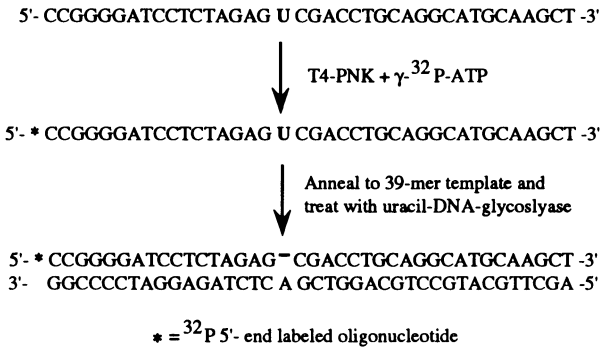

Figure 4. Flow diagram for the synthesis and construction of the double stranded 39-base-pair oligodeoxynucleotide substrate containing an AP site.

Preparation of a double-stranded oligodeoxyribonucleotide substrate containing an AP site

The construction of a double stranded 39-mer oligonucleotide substrate containing a single AP site is illustrated in Figure 4. A 39-mer oligonucleotide containing a uracil at position 18 was synthesized on an Applied Biosystems 391 DNA synthesizer. 5′-^{32}P-end labeled uracil-containing 39-mer was prepared using a standard T4 kinase reaction containing 10 units T4 kinase, 2 pmoles 39-mer DNA, 40 μCi γ-^{32}P ATP, 50 mM glycine, 10 mM $MgCl_2$, and 5 mM DTT. The reaction was incubated at 37°C for two hours and the unincorporated ATP was removed by centrifugation through a G-25 spin column.

The uracil containing 39-mer (diluted in TE, pH 8.0) was treated with uracil-DNA glycosylase (1 unit/100 pmoles DNA) for 15 min at 37°C generating an apyrimidinic site at position 18. This results in greater than 99% removal of the uracil and conversion to an apyrimidinic site as determined by piperidine cleavage. The double-stranded oligonucleotide AP substrate was created by annealing the AP-containing 39-mer described here, to the complimentary 39-mer oligonucleotide described above for the construction of the strand break substrate (Fig. 1).

Overexpression and purification of N-terminal histidine tagged HAP1 protein

HAP1 cDNA was amplified from total HeLa cell RNA using reverse transcriptase PCR (9). 1 μg total RNA was incubated in a total volume of 50 μl at 37°C for 45 min in the presence of 10 mM Tris−HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl; 1 mM each of dATP, dGTP, dCTP, and TTP; 100 pmoles random hexamers, 100 U RNasin, and 500 U AMV-reverse transcriptase. 20 μl of this first strand synthesis mixture were used in 30 cycles of PCR, with each cycle consisting of 2 min at 94°C to denature, 1 min at 55°C to anneal and 1.5 min at 72°C to extend, in 10 mM Tris−HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin; 200 μM each of dATP, dGTP, dCTP, and TTP; 1 μM each primer and 2.5 U Taq DNA polymerase, in a total volume of 100 μl. The primers contained *Bam*HI sites to allow cloning into the *Bam*HI site of pGEX-2T (10). Primer sequences were as follows: forward primer: 5′-T-TCGTAACGGGATCCCCGAAGCGTGGG; reverse primer: 5′-GCTCAAAGGGATCCAGGGGTGGTG.

The 992 bp PCR product was isolated from an agarose gel, digested with *Bam*HI and cloned first into the GST-fusion vector, pGEX-2T, to give pAS2. The vector for the expression of N-terminal histidine-tagged HAP1 protein was subsequently prepared by digesting pAS2 with *Bam*H1 and cloning the insert into the *Bam*H1 site of pQE-30 (11), to give pKJ176. The plasmid insert was sequenced by standard dideoxynucleotide methods (12).

The protein was expressed in *E.coli* M15 cells by growing the cells to an OD_{600} of 0.6 and inducing with 0.4 mM IPTG for 3.5 h. Bacterial cells were pelleted and washed in 50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10 mM βME, and frozen at −70°C until used for purification of the recombinant protein.

The N-terminal his-tagged HAP1 protein was purified by a modification of the procedure for native purification of cytoplasmic proteins, as described by Qiagen (QIAexpress system). Cell pellets were thawed, sonicated 30 sec (Branson sonifier 450, output setting 2, duty cycle 90%) and centrifuged at 50,000×g for 20 min. Proteins were bound to nickel-nitrilotriacetic acid (Ni-NTA) resin in a batch procedure by adding cell extract supernatants to the resin (4 ml resin/liter bacterial culture starting material) and gently rocking at 4°C for 1 h. The protein/resin complex was packed into a column and washed with three volumes of wash buffer A (50 mM $NaPO_4$ pH 6.0, 300 mM NaCl, 10% (v/v) glycerol) followed by three volumes of wash buffer B (wash buffer A containing 40 mM imidazole). The his-tagged HAP1 protein was eluted from the column at a flow rate of 0.36 ml/min with elution buffer (wash buffer A containing 120 mM imidazole) and the fractions containing protein were pooled. Pooled fractions (3 mg protein/run) were diluted into four volumes of TMEN buffer (50 mM Tris−HCl pH 7.5, 1 mM βME, 1 mM EDTA, 50 mM NaCl), and loaded onto a Pharmacia Mono S HR 5/5 column equilibrated in TMEN. The column was eluted with a linear NaCl gradient (20 ml) from 0.05 M to 1.0 M. The his-tagged HAP1 protein eluted from the

column as a single peak between 0.34–0.43 M NaCl. Fractions comprising the HAP1 peak were pooled and dialyzed against 10 mM Tris–HCl pH 7.5, 100 mM imidazole, and stored at 4°C. Protein concentrations were determined by the method of Bradford (13). Specific activity was determined on depurinated pUC18 DNA as previously described (14). One unit of AP endonuclease activity is defined as the amount of enzyme required to produce one pmole of nicks per minute under the standard assay conditions.

3′-Phosphoglycolate release assay and product analysis

Enzymatic removal of 3′-PG end groups from DNA strand breaks was measured using the double stranded 39-mer 3′-PG oligo substrate described above. Reaction mixtures contained equimolar concentrations of the 3′-radiolabeled-PG 17-mer, 5′-P 21-mer and complementary 39-mer; 20 mM Tris–HCl pH 8.0, 10 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 10 μg heat inactivated bovine serum albumin, and the enzyme sample, in a final volume of 50 μl.

Assays were performed at 37°C and stopped on ice by the sequential addition of 25 μg sheared calf thymus DNA, 0.1 volume 3 M NaCl, and 2 volumes of ice-cold ethanol (final volume 181.5 μl). After storage overnight at −20°C, the precipitate was pelleted by centrifugation at 16,000×g for 10 min. ^3H radioactivity in 150 μl of the supernatant was determined by liquid scintillation counting.

Products released from the oligonucleotide strand break substrate were identified by thin layer chromatography (TLC). TLC was performed by a modification of the method of VanScoy and coworkers (15). Whatman silica gel 60A K6F TLC plates were spotted with samples to be tested, along with standards consisting of either deoxynucleotide monophosphates (dNMPs), deoxynucleosides (dNs), phosphonoformic acid (PFA), phosphonoacetic acid (PAA), or phosphoglycolic acid (PGA). The plates were developed in methanol:NH_4OH:H_2O (6:3:1). Following development the plates were dried and stained for phosphate esters according to the method of Hanes and Isherwood (16). The plates were heated at 70°C for 5 min to remove traces of excess solvent and sprayed with staining solution (3% (w/v) ammonium molybdate, 0.1 M HCl, 3% (v/v) perchloric acid). The plates were dried in a current of warm air to remove excess water, then heated to 85°C for 10 min to hydrolyze phosphate esters. After cooling to 25°C, the plates were suspended in a glass tank containing dilute H_2S gas until color was fully developed. Lanes containing enzyme assay supernatants were sectioned by scraping (0.5 cm×1.0 cm sections) and ^3H activity in the recovered silica gel was determined by liquid scintillation counting.

Nucleotide incorporation assay

The 39-mer double-stranded oligo substrate described above, containing a strand-break with a 3′-PG and 5′-P, was incubated either with or without HAP1 prior to assaying for nucleotide incorporation by DNA polymerase I large fragment. A 39-mer double-stranded oligonucleotide with a strand break containing a 3′-OH and a 5′-P was used as a positive incorporation control. 10 pmole of oligo strand-break substrate was incubated with or without 50 ng of HAP1 for 30 min at 37°C in a buffer containing 40 mM Tris–HCl pH 8.0, 20 mM NaCl, 2 mM EDTA, 10 mM $MgCl_2$, and 400 μg/ml BSA. The reaction was stopped by incubating for 5 min at 65°C. 1 pmole of the oligo substrates used in the above reaction were then incubated in a mixture containing 4.5 mM DTT; 3 nmole each of dATP, dCTP, dGTP, and TTP; 3 pmole ^{32}P-dCTP; and 0.6 units of *E.coli* DNA polymerase I large fragment for 30 min at 37°C. The reactions were stopped by heating at 65°C for 5 min. Following the polymerization reaction, the samples were subjected to PAGE on a 20% acrylamide/7M urea denaturing gel. Results were analyzed by autoradiography.

Oligonucleotide AP endonuclease assay

Endonucleolytic cleavage at AP sites was measured using the double stranded 39-mer AP-containing oligo substrate described above. Reaction mixtures containing equimolar concentrations of the AP-containing 39-mer oligonucleotide and the complementary 39-mer strand, were spiked with 5′-end labeled AP-containing 39-mer oligonucleotide (8,000–10,000 cpm per reaction). Reactions were conducted in the buffer system described above for the 3′-PG release assay (20 mM Tris–HCl pH 8.0, 10 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 10 μg heat inactivated bovine serum albumin, and the enzyme sample in a final volume of 50 μl). Assays were performed at 37°C for 5 min and stopped on ice by the sequential addition of 5 μg tRNA, 0.1 volume 3 M sodium acetate, and 2 volumes ice-cold ethanol. Following 30 min at −70°C, the precipitate was pelleted by centrifugation at 16,000×g for 10 min. The sample was resuspended in 5 μl of gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and the products analyzed on a 15% acrylamide/7M urea gel. Following autoradiography the full length and cleaved product DNA bands were cut from the gel and quantitated by liquid scintillation counting.

RESULTS

Preparation of DNA strand break substrates containing 3′-PG and 5′-P

A major problem with studying repair of radiation-damaged DNA is the complexity of the substrate. One way to circumvent the problem is to substitute DNA treated with radiomimetic chemicals. These reagents usually produce a more limited DNA damage spectrum than ionizing radiation; however, few produce single lesions. For example, osmium tetroxide produces thymine glycol, but smaller amounts of cytosine glycol are also produced (17); and bleomycin treatment can generate apurinic sites in addition to 3′-PG (18). Analogs of true radiation-induced lesions can sometimes be used (4), but interpretation of such studies is complicated because it is unknown whether the enzyme recognizes and acts on the analog in the same manner as the natural lesion.

To address problems of substrate heterogeneity, we designed a double-stranded oligo strand-break substrate. This substrate contains a strand-break lesion, at a defined position within the oligo, having exactly the same chemistry as a major lesion produced by ionizing radiation (i.e. 3′-PG/5′-P; Fig. 1). Furthermore, our radiolabeling protocol produced a mixture of 3′-PG termini labeled with either ^3H or ^{32}P, at a relative molar ratio of 200:1, respectively. The ^{32}P label provided a high energy β^- tracer that allowed the 3′-PG 17-mer produced by bleomycin cleavage, to be identified by autoradiography and purified from other damage products by denaturing PAGE. The ^3H label provided a long-lived radioisotope and easily quantifiable enzyme substrate.

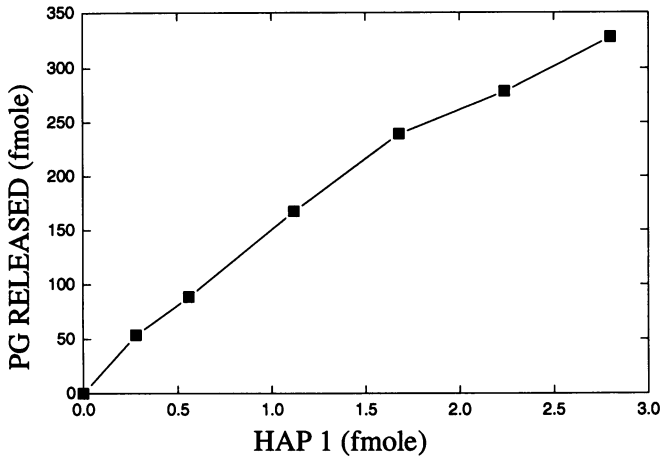

Figure 5. Enzymatic activity of HAP1 on a model DNA strand break substrate containing a 3'-PG/5'-P strand break lesion. 0.7 picomoles of the double stranded 39-mer oligonucleotide strand break substrate was incubated for 15 min at 37°C in the presence of increasing concentrations of HAP1. Ethanol soluble radioactivity was determined as described in 'Materials and Methods'.

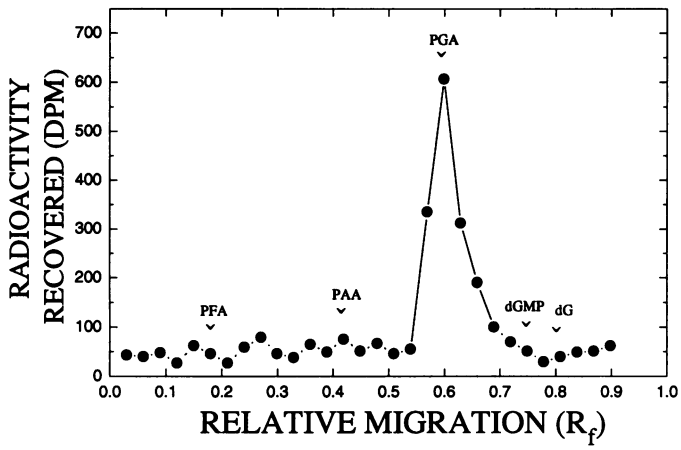

Figure 6. Identification of phosphoglycolic acid as the product released by HAP1 from the 3'-PG/5'-P double stranded oligonucleotide DNA strand break substrate. 14 fmole (0.5 ng) of HAP1 was incubated with 0.5 pmole of oligonucleotide strand break substrate under standard release assay conditions for 30 min. Following precipitation of the oligonucleotide, supernatant was subjected to thin layer chromatography as described in 'Materials and Methods'. The arrows indicate the migration positions for phosphonoformic acid (PFA); phosphonoacetic acid (PAA); phosphoglycolic acid (PGA); deoxyguanosine monophosphate (dGMP); deoxyguanosine (dG).

Cloning and purification of HAP1

The cDNA sequence published by Robson and Hickson (19) was used to generate PCR primers that allowed amplification of the coding sequence for HAP1 from the RNA of HeLa cells. The PCR product was first cloned into the pGEX-2T vector to yield plasmid pAS2. The coding sequence was then transferred into the vector pQE30 in the correct orientation and sequenced (plasmid pKJ176). The nucleotide sequence matched exactly that published by Cheng and coworkers (20), and also the sequence of the coding exons of the human gene (21). This expression vector links the cDNA coding sequence of HAP1 to an amino terminal sequence which includes coding for six consecutive

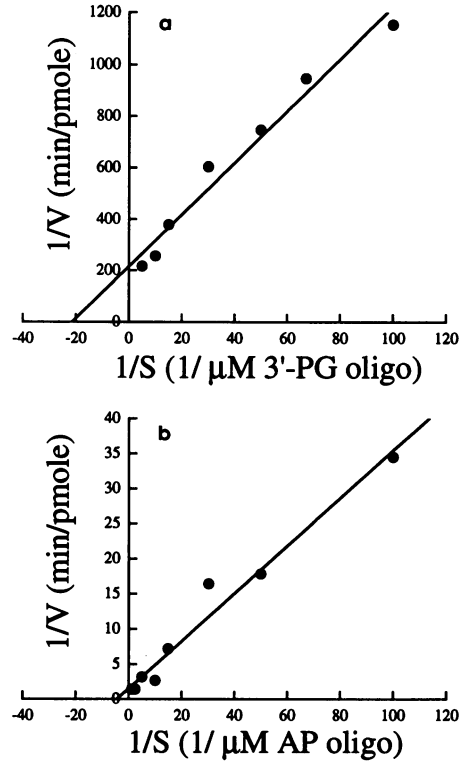

Figure 7. Lineweaver–Burk Plots: a) 1.4 fmole (0.05 ng) of HAP1 was incubated for 15 min at 37°C in the standard 3'-PG release assay reaction mixture containing the following concentrations of double stranded 39-mer oligonucleotide strand break substrate: 0.4 µM, 0.2 µM, 0.1 µM, 0.067 µM, 0.033 µM, 0.02 µM, and 0.01 µM. Under these conditions less than 50% of the substrate was degraded for each reaction. The K_m of HAP1 for 3'-PG was determined to be 0.05 µM and the V_{max} was 0.1 fmole/min/pg HAP1 protein. b) 0.28 fmole (0.01 ng) of HAP1 was incubated for 5 min at 37°C in the oligonucleotide AP endonuclease assay reaction mixture containing the following concentrations of double stranded 39-mer oligonucleotide AP substrate: 0.8 µM, 0.4 µM, 0.2 µM, 0.1 µM, 0.067 µM, 0.033 µM, 0.02 µM, 0.01 µM. The K_m of HAP1 for this AP lesion was 0.23 µM, and the V_{max} was 68 fmole/min/pg HAP1 protein.

histidines. Therefore, the wild type amino terminal methionine of HAP1 is replaced by the sequence Met-Arg-Gly-Serine-$(His)_6$-Gly-Ser. Following IPTG induction of pKJ176 the his-tagged HAP1 was purified by affinity chromatography on a Ni-NTA column. The protein was greater than 95% homogenous by SDS-PAGE at this stage. Following Ni-NTA chromatography, the HAP1 preparation was subjected to ion exchange chromatography on a Pharmacia Mono S cation exchange column. Approximately 2 mg of pure HAP1 protein (>98% on SDS-PAGE) was recovered from the Mono S column, per liter of bacterial culture extracted. The enzyme was stable for at least 6 months following dialysis against Tris/imidazole buffer (described in 'Materials and Methods'), when stored at 4°C. The his-tagged HAP1 protein recovered from this procedure had a specific activity of 1.5×10^5 units/mg protein when assayed on depurinated pUC18 DNA. This result is consistent with the specific activities reported for non-recombinant human (HAP1), or bovine (BAP1) apurinic endonucleases (14).

HAP1 catalyzes the release of 3'-PG from a model DNA strand break lesion

By incubating the recombinant HAP1 with the 39-mer strand-break oligo substrate in the standard 3'-PG release assay, it was

possible to demonstrate that HAP1 catalyzed the release of ethanol soluble radioactivity (Fig 5). Product formation was dependent upon enzyme and increased linearly with increasing enzyme concentration. Furthermore, the enzyme was only active on the double-stranded substrate, and was incapable of releasing radioactive products from the single stranded 3'-PG 17-mer. Since HAP1 lacks exonuclease activity ((22), and Fig.8), this suggested that the release product might be phosphoglycolic acid (PGA). To confirm this, reaction products were subjected to TLC. The TLC system is capable of separating PGA from two closely related analogs, phosphonoformic acid (PFA) and phosphonoacetic acid (PAA), as well as from two potential lesion-containing exonuclease reaction products (i.e. dGMP and dG). The product of the HAP1 reaction with the oligo substrate co-migrated with authentic PGA (Fig. 6).

The experiments described above demonstrated the ability of HAP1 to remove 3'-PG residues from DNA. Therefore, we determined the V_{max} and K_m of HAP1 for this lesion by constructing a double reciprocal plot (Lineweaver−Burk Plot; Fig. 7a). The apparent K_m of HAP1 for 3'-PG residues determined by this method was 0.05 μM. The V_{max} was 0.1 fmole/min/pg of HAP1 protein.

The identification of phosphoglycolic acid as a product of the reaction between HAP1 and the 3'-PG/5'-P strand break lesion, suggests hydrolysis of the phosphate ester bond between the 3'-PG and the adjacent dGMP residue within the 17-mer as the reaction mechanism. A hydrolysis mechanism such as this should result in a 3'-OH terminus at the site of cleavage. The formation of 3'-OH would be biologically significant, since this would permit DNA polymerase to recognize the site as a substrate and replace the nucleotide lost during the formation of the lesion.

Consequently, we examined the ability of the 39-mer oligo strand-break substrate to support nucleotide incorporation following treatment with HAP1 (Fig. 8). The untreated 3'-PG/5'-P oligo strand-break substrate was incapable of acting as a substrate for nucleotide incorporation by *E.coli* DNA polymerase I large fragment. However, following treatment with HAP1, the oligo did serve as a substrate and a full length 39-mer was synthesized. Since DNA polymerase I has a specific requirement for 3'-OH termini to prime polymerization, these results demonstrate that the DNA product of the HAP1 reaction contains a 3'-OH which can act as a substrate for DNA polymerase and permit repair synthesis. Furthermore, the absence of HAP1 mediated degradation of either the control oligo containing a 3'-OH 17-mer, or the oligo containing the 3'-PG 17-mer, indicates that HAP1 lacks exonuclease activity. Thus, the 3'-OH termini created in the DNA product of the HAP1 reaction must be immediately 5' of the 3'-PG phosphate group. This results in the formation of a 17-mer within the oligo substrate which possesses a terminal dGMP residue with a 3'-OH. This assumption has subsequently been proven by the observation that following reaction with HAP1, the denaturing PAGE migration pattern of the strand-break substrate 3'-PG 17-mer, shifts to that of a 3'-OH 17-mer (data not shown).

AP endonuclease kinetics of HAP1

A direct comparison between the enzymatic activity of HAP1 at an AP site, and at the site of a 3'-PG strand break lesion, required kinetic analysis on analogous substrates containing these lesions. In order to accomplish this we prepared an oligonucleotide substrate with the same 39-mer sequence as that used to prepare the strand break substrate, only containing an apyrimidinic site at position 18 in place of the 3'-PG strand break lesion (Fig. 4). Kinetic parameters for HAP1 AP endonuclease activity were determined by double-reciprocal plot (Fig. 7b), using the AP oligo substrate in the same manner as described above for kinetic determinations on the 3'-PG oligo strand break substrate.

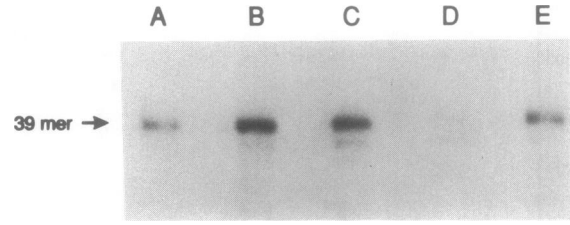

Figure 8. Nucleotide incorporation on double stranded 39-mer oligonucleotide strand break substrate. 10 pmole of double stranded 39-mer oligo in which the 17-mer component contained either a 3'-OH (to act as a positive control for nucleotide incorporation), or a 3'-PG, was incubated at 37°C for 30 min in the presence or absence of 140 fmole of HAP1. 1 pmole of the oligos treated in this manner were tested for their ability to support nucleotide incorporation by *E.coli* DNA polymerase I as described in 'Materials and Methods'. Lane A, 5'-end labeled 39-mer size marker; lane B, double stranded 39-mer oligo containing a 3'-OH 17-mer, untreated with HAP1; lane C, double stranded 39-mer oligo containing a 3'-OH 17-mer, treated with HAP1; lane D, double stranded 39-mer oligo containing a 3'-PG 17-mer, untreated with HAP1; lane E, double stranded 39-mer oligo containing a 3'-PG 17-mer, treated with HAP1.

The extent of AP site cleavage was determined by resolving and quantitating the full length 39-mer and the 17-mer cleavage product by denaturing PAGE (23). The apparent K_m and V_{max} for the AP lesion were 0.23 μM and 68 fmole/min/pg HAP1 protein, respectively. As indicated by the K_m values, HAP1 has approximately 5-fold greater affinity for the 3'-PG strand break lesion than for the AP lesion. In contrast, the enzyme's reaction rate is much higher for the AP lesion, being 680-fold the rate for 3'-PG cleavage.

DISCUSSION

The chemistry of 3'-PG sites indicates that these radiation-induced strand breaks involve base loss, since the lesion cannot be formed without the loss of at least one base residue. Thus, radiation-induced strand breaks cannot be repaired with fidelity without template directed replacement of the missing base. However, all of the known DNA polymerases require substrates with a 3'-OH as a primer for synthesis, and for their 3'→ 5' exonuclease activities. Consequently, strand-break lesions possessing a 3'-PG can not directly serve as a substrate for DNA polymerase. Kow and coworkers (24) have shown that 3' ends lacking hydroxyl moieties are blocks to DNA polymerization and strand break repair. They also showed that strand breaks with blocked 3' ends were twice as toxic as unblocked 3'-OH ends in an *E.coli* phage-rescue assay. Furthermore, recent reports by others indicate that 3'-PG strand break lesions are highly toxic in mammalian cells (7,25,26). All of this suggests that human cells must contain at least one end-group-modifying enzyme that can convert 3'-PG to the 3'-OH that is required for nucleotide incorporation by DNA polymerase.

There are several reports of human enzymes with putative 3'-PG removal ability. We have previously reported the existence of three chromatographically distinct human enzymes capable of

removing 3′-PG from DNA (27), however, none of these enzymes were found to be HAP1 (unpublished data). In a previous report by Chen and coworkers (4), HAP1 partially purified from HeLa cells was shown to possess a 3′-diesterase activity capable of releasing 3′-phosphoglycoaldehyde — a synthetic analog of 3′-PG — from a poly dA-T copolymer. This result suggested that the enzyme may also be capable of removing 3′-PG end groups from DNA; a supposition which we have confirmed with the data presented here. Consequently, at least four human enzyme activities exist which are capable of functioning as repair activities for strand breaks containing 3′-PG termini. It remains to be determined whether any of these four, or some as yet to be identified enzyme activity, serve as the primary repair activity for 3′-PG lesions *in vivo*.

The expression of HAP1 as a recombinant protein and the construction of an oligo strand-break substrate that is identical to the natural lesion, has allowed us the rare opportunity to study the mechanism and kinetics of a human DNA repair enzyme using an *in vitro* system with both pure enzyme and pure substrate. Our results for HAP1 in this system, show that the enzyme catalyzes the release of free PGA from the strand break site in our oligo substrate.

The defined nature of the oligo strand-break substrate also permitted us to determine that the DNA product of the HAP1 reaction can serve as a substrate for DNA polymerase, and has the configuration of a template bound primer possessing a 3′-OH group. The demonstration of the DNA leaving group to be a 3′-OH confirms hydrolysis to be the mechanism of catalysis for this reaction.

In addition, the construction of an analogous AP endonuclease oligonucleotide substrate, permitted direct comparison of HAP1 enzymatic activity on this substrate, with that on the 3′-PG strand break substrate. The kinetic results indicate that HAP1 displays a higher affinity for 3′-PG strand breaks (K_m= 0.05 μM) than for AP sites (K_m= 0.23 μM). Conversely, the reaction rate at AP sites (V_{max}= 68 fmole/min/pg HAP1 protein) was much greater than at 3′-PG strand break sites (V_{max}= 0.1 fmole/min/pg HAP1 protein). This suggests that under conditions of ample substrate, HAP1 acts primarily as an AP endonuclease. This may explain previous reports suggesting that the primary intracellular activity of HAP1 is its AP endonuclease activity even though it has the capacity to remove 3′-blocking groups (4,19,22).

ACKNOWLEDGEMENTS

The authors would like to acknowledge Kara Johnson for her excellent technical assistance. This work was supported by grants CA48716 (T.J.J.), CA55232 (W.D.H.), CA61717 (A.M.), and CA51008 (Macromolecular Sequencing/Synthesis Laboratory; Lombardi Cancer Center Core Grant) awarded by the National Cancer Institute of the National Institutes of Health, U.S. DHHS. T.A.W. was supported by American Cancer Society Fellowship Award PF-3782.

REFERENCES

1. Henner, W.D., Rodriguez, L.O., Hecht, S.M. and Haseltine, W.A. (1983) J. Biol. Chem. 258, 711–713.
2. Niwa, O. and Moses, R.E. (1981) Biochem. 20, 238–244.
3. Levin, J.D. and Demple, B. (1990) Nucleic Acids Res. 18, 5069–5075.
4. Chen, D.S., Herman, T. and Demple, B. (1991) Nucleic Acids Res. 19, 5907–5914.
5. Povirk, L.F. and Austin, M.J.F. (1991) Mutat. Res. Rev. Genet. Toxicol. 257, 127–143.
6. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
7. Weinfeld, M. and Soderlind, K.-J.M. (1991) Biochem. 30, 1091–1097.
8. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual, pp. 1.1–18.86, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
9. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. (1987). Current protocols in molecular biology, John Wiley and Sons, New York.
10. Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
11. Stuber, D., Matile, H. and Garotta, G. (1990) in Immunological Methods Vol. IV, Lefkovits, I. and Pernis, B. Eds. pp. 121–152, Academic Press, New York.
12. Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
13. Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
14. Henner, W.D., Kiker, N.P., Jorgensen, T.J. and Munck, J.N. (1987) Nucleic Acids Res. 15, 5529–5544.
15. VanScoy, M., Loghman-Adham, M., Onsgard, M., Szczepanska-Konel, M., Homma, S., Knox, F.G. and Dousa, T.P. (1988) Am. J. Physiol. 255(5 pt2), F984–994.
16. Hanes, C.S. and Isherwood, F.A. (1949) Nature 164, 1107–1112.
17. Dizdaroglu, M., Holwitt, E., Hagan, M.P. and Blakely, W.F. (1986) Biochem. J. 235, 531–536.
18. Sugiyama, H., Xu, C., Murugesan, N. and Hecht, S.M. (1985) J. Amer. Chem. Soc. 107, 4104–4105.
19. Robson, C.N. and Hickson, I.D. (1991) Nucleic Acids Res. 19, 5519–5523.
20. Cheng, X., Bunville, J. and Patterson, T.A. (1992) Nucleic Acids Res. 20, 370.
21. Zhao, B., Grandy, D.K., Hagerup, J.M., Magenis, R.E., Smith, L., Chauhan, B.C. and Henner, W.D. (1992) Nucleic Acids Res. 20, 4097–4098.
22. Demple, B., Herman, T. and Chen, D.S. (1991) Proc. Natl. Acad. Sci. USA 88, 11450–11454.
23. Sanderson, B.J.S., Chang, C.-N., Grollman, A.P. and Henner, W.D. (1989) Biochem. 28, 3894–3901.
24. Kow, Y.W., Faundez, G., Melamede, R.J. and Wallace, S.S. (1991) Radiat. Res. 126, 357–366.
25. Dedon, P.C., Jiang, Z.-W. and Goldberg, I.H. (1992) Biochem. 31, 1917–1927.
26. Decuyper, J., Piette, J., Merville, M.-P. and Van de Vorst, A. (1986) Biochem. Pharmacol. 35, 1345–1350.
27. Winters, T.A., Weinfeld, M. and Jorgensen, T.J. (1992) Nucleic Acids Res. 20, 2573–2580.