Isolation of cDNA clones encoding an enzyme from bovine cells that repairs oxidative DNA damage *in vitro*: homology with bacterial repair enzymes

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ABSTRACT

Ionizing radiation and radiomimetic compounds, such as hydrogen peroxide and bleomycin, generate DNA strand breaks with fragmented deoxyribose 3′ termini via the formation of oxygen-derived free radicals. These fragmented sugars require removal by enzymes with 3′ phosphodiesterase activity before DNA synthesis can proceed. An enzyme that reactivates bleomycin-damaged DNA to a substrate for Klenow polymerase has been purified from calf thymus. The enzyme, which has a M_r of 38,000 on SDS-PAGE, also reactivates hydrogen peroxide-damaged DNA and has an associated apurinic/apyrimidinic (AP) endonuclease activity. The N-terminal amino acid sequence of the purified protein matches that reported previously for a calf thymus enzyme purified on the basis of AP endonuclease activity. Degenerate oligonucleotide primers based on this sequence were used in the polymerase chain reaction to generate from a bovine cDNA library a fragment specific for the 5′ end of the coding sequence. Using this cDNA fragment as a probe, several clones containing 1.35 kb cDNA inserts were isolated and the complete nucleotide sequence of one of these determined. This revealed an 0.95 kb open reading frame which would encode a polypeptide of M_r 35,500 and with a N-terminal sequence matching that determined experimentally. The predicted amino acid sequence shows strong homology with the sequences of two bacterial enzymes that repair oxidative DNA damage, ExoA protein of *S. pneumoniae* and exonuclease III of *E. coli*.

INTRODUCTION

Reactive oxygen species such as the superoxide anion and the hydroxyl radical are formed in cells by normal oxidative metabolism (1–3). These species damage cellular DNA producing a spectrum of lesions, including strand breaks, base loss or damage, and fragmentation of the deoxyribose moiety (for reviews see 4, 5). Spontaneous base loss occurs at an estimated frequency of between 10^3 and 10^4/cell/day, and as such represents a major challenge to the integrity of DNA under physiological conditions (6, 7).

A number of DNA damaging agents also exert their effects via the formation of reactive oxygen species. For example, ionizing radiation produces a variety of lesions in DNA including strand breaks with atypical 3′ termini such as 3′ phosphate and 3′ phosphoglycolate (PGA) groups (4). The glycopeptide antibiotic, bleomycin, is regarded as a radiomimetic agent and produces apurinic/apyrimidinic (AP) sites as well as strand breaks with 3′ PGA termini (8, 9). For strand gaps to be refilled by DNA polymerases, a 3′ hydroxyl terminus is required. Consequently, the blocked 3′ termini of strand breaks generated by ionizing radiation and bleomycin must be removed by 3′ phosphodiesterases before repair can be completed by polymerases and ligases.

E. coli contains at least two enzymes able to repair a fragmented sugar from the blocked 3′ terminus of a DNA strand break (reviewed in 10). The major enzyme, exonuclease III, is the product of the *xth* gene and can remove 3′ fragments generated by both ionizing radiation and hydrogen peroxide (11). The residual activity present in *xth* mutants is largely dependent upon the product of the *nfo* gene, endonuclease IV (12). This latter enzyme is of major importance in the repair of bleomycin induced damage (14) and its synthesis is induced by the redox active agent paraquat (13). Both exonuclease III and endonuclease IV exhibit an AP endonuclease activity operating via a class II mechanism (cleaving 5′ to the AP site) (10).

To characterise the mammalian cell enzymes active in the repair of DNA strand breaks with blocked 3′ termini produced by bleomycin, we have purified the major activity present in calf thymus. We have isolated cDNA clones encoding this enzyme and determined the complete sequence of one of them. The predicted amino acid sequence shows strong homology with the sequences of bacterial DNA repair enzymes that recognise oxidative DNA damage, including exonuclease III of *E. coli*.

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MATERIALS AND METHODS

Reactivation of bleomycin-damaged DNA

This assay was used to monitor the purification of an enzyme able to reactivate bleomycin treated DNA to a template for Klenow enzyme. The 'standard' reaction mixture in a final volume of 100 μl contained 0.0175% Triton X 100, 0.25 M Sucrose, 10 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 150 μg/ml sheared calf thymus DNA, 30 μg/ml $FeSO_4$, 10 μg/ml bleomycin and 5 μl of an appropriate column fraction. The purified enzyme was assayed using pre-damaged DNA. Calf thymus DNA was treated as described above and the DNA then precipitated with ethanol, resuspended in H_2O and dialysed against H_2O. The mixture was incubated at 37°C for 15 mins, and then at 75°C for 10 mins to inactivate enzymes. After cooling on ice, 50 μl of Klenow reaction buffer and 0.2 u of Klenow enzyme were added, and the level of ^{32}P-dCTP incorporation into TCA-precipitable DNA determined.

Enzyme purification

Calf thymus (200 g) was suspended in TME buffer (50 mM Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, 1 mM EDTA) and homogenised. A 0.7 M NaCl extract of the homogenate (Fraction I) was diluted in TME and passed down a DEAE cellulose column to remove nucleic acid. Proteins not retained (Fraction II) were applied to a phosphocellulose P11 column and eluted with a 0.05−0.7 M NaCl gradient. Fractions able to reactivate bleomycin treated DNA, eluting at approximately 0.6 M NaCl (Fraction III), were applied to a hydroxylapatite column. Active fractions eluting at approximately 0.26 M NaCl (Fraction IV) were applied to a heparin sepharose column. Active fractions eluted at approximately 0.2 M NaCl (Fraction V). At this stage the fractions contained a major protein of M_r approximately 38,000 but also some minor contaminating proteins. These were removed by chromatography on FPLC Mono-S (active fractions eluting at approximately 0.2 M KPO_4) and FPLC phenyl superose (active fractions eluting at approximately 0.56M $(NH_4)_2SO_4$). The purified enzyme (Fraction VI) was stored at −80°C in buffer containing 50% glycerol.

Hydrogen peroxide treatment of DNA

Calf thymus DNA (0.5 mg/ml) was dissolved in 50 mM KPO_4 buffer, pH 7.2. To this was added H_2O_2 to a final concentration of 100 mM and the mixture incubated at 20°C for 24 hours. The reaction was stopped with EDTA and the DNA precipitated with ethanol. The DNA pellet was washed with 70% ethanol, resuspended in water and dialysed against water. The assay for reactivation of H_2O_2 treated DNA was carried out under the conditions described above for reactivation of bleomycin-damaged DNA (except that bleomycin and $FeSO_4$ was omitted).

Preparation of DNA containing AP sites and O_SO_4-damaged DNA

To induce AP sites, purified Form I (covalently closed circular) plasmid DNA (in 0.1 M sodium acetate, pH 5.5) was incubated at 70°C for 15 mins. The solution was then neutralised by addition of 1 M Tris HCl, pH 8 to a final concentration of 0.1 M and the DNA desalted on Sephadex G50 equilibrated with TE buffer. DNA containing thymine glycol residues was prepared by incubation of Form I plasmid DNA with 1 mM O_SO_4 for 15 mins at 25°C.

AP endonuclease assay

The assay mixture in a final volume of 10 μl contained 66 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 0.5 μg of Form I plasmid DNA containing AP sites. This mixture was incubated with purified enzyme (Fraction VI) at 37°C for 30 mins before addition of 10 μl of sample buffer containing 30% sucrose, 100 mM EDTA and 0.01% bromophenol blue. The samples were run on a 0.7% agarose gel, stained with ethidium bromide and photographed under UV illumination. Photographic negatives were scanned by using an LKB 2202 Ultrascan laser densitometer.

Exonuclease activity

The reaction mixture contained 66 mM Tris HCl, pH 7.5, 0.66 mM $MgCl_2$, 1 mM β mercaptoethanol and 0.5 μg/ml DNA, end-labelled at the 5′ or 3′ end, in a final volume of 20 μl. Where appropriate, the DNA was heated to 95°C for 5 minutes before rapid cooling on ice to render it single stranded. This mixture was incubated with the purified bovine enzyme (Fraction VI) or with *E. coli* exonuclease III for 1 hr at 37°C. 80 μl of 0.1 mg/ml calf thymus DNA and 500 μl cold 10% trichloroacetic acid were added and the solution left on ice for 20 minutes. This was then spun for 10 minutes in an Eppendorf microfuge, and radioactivity liberated into 500 μl of supernatant measured by scintillation counting.

Amino acid sequencing

Freeze-dried protein was dissolved in 50 μl 50% aqueous isopropanol acidified with 2 μl concentrated HCl. 12 μl of this solution was applied to an 8 mm Sequelon-DITC membrane (Millipore) and evaporated dry over 10 min at 50°C. The covalently bound peptide was then washed with water and methanol before sequence analysis using a MilliGen 6600 solid-phase sequenator (15). Raw data were reduced to a semilog plot of Log [PTH yield] versus [Cycle No.] and a line fitted by regression analysis. The initial yield (2.6 pmol) was obtained from the intercept, and the average repetitive sequencing yield (93%) derived from the slope of the fitted line.

Oligonucleotides

Oligonucleotides were prepared on an Applied Biosystems 391 DNA synthesiser and processed according to the manufacturer's recommendations.

Isolation of cDNA clones

Degenerate oligonucleotides defined from N-terminal amino acids 1−7 (for the sense strand) and 14−19 (for the antisense strand) were used to amplify, by PCR, a 57 bp fragment from a bovine lymphocyte cDNA library (kindly provided by Dr D. Simmons). 1 ng of this purified product was subjected to 10 cycles of amplification using [α-^{32}P] dCTP to generate a probe of high specific activity. Bacteria representing the bovine cDNA library were plated at high density (~100,000 colonies per 20 cm^2 filter) and colonies were transferred to Hybond-N membrane (Amersham). Hybridisation and filter washing were performed according to manufacturers' instructions with a final wash of 0.1 × SSPE, 0.1% SDS at 65°C for 10 minutes. Secondary and tertiary rounds of screening were conducted similarly, with densities of 50−500 colonies per 82 mm filter.

DNA sequencing

DNA sequencing was carried out by the dideoxy chain termination method using Sequenase (USB Corp). Multiple synthetic oligonucleotide primers were used to sequence both strands completely.

Homology analyses

Alignments were generated using the IBI MacVector protein scoring matrix.

RESULTS

Purification of an enzyme that reactivates bleomycin treated DNA

DNA treated with bleomycin is a poor substrate for Klenow enzyme due to the presence of fragmented sugar groups blocking the 3′ terminus of DNA strand breaks (16). An enzyme that reactivates bleomycin-treated DNA to a template for Klenow enzyme was detected in crude extracts from calf thymus and purified by sequential chromatography on DEAE cellulose, phosphocellulose P11, hydroxylapatite, heparin sepharose and FPLC Mono-S and phenyl superose. Active fractions from FPLC phenyl superose contained a single protein of M_r 38,000 (Figure 1). The preparation was adjudged to be greater than 95% pure. Approximately 0.3mg of purified protein was obtained from 1.75g of salt-extracted nuclei obtained from 200g of calf thymus.

Properties of the purified enzyme

The purified enzyme was able to reactivate both bleomycin-damaged and hydrogen peroxide-damaged DNA to a template for Klenow enzyme (Table I). Reactivation was dependent upon both damage to the substrate and the presence of native enzyme.

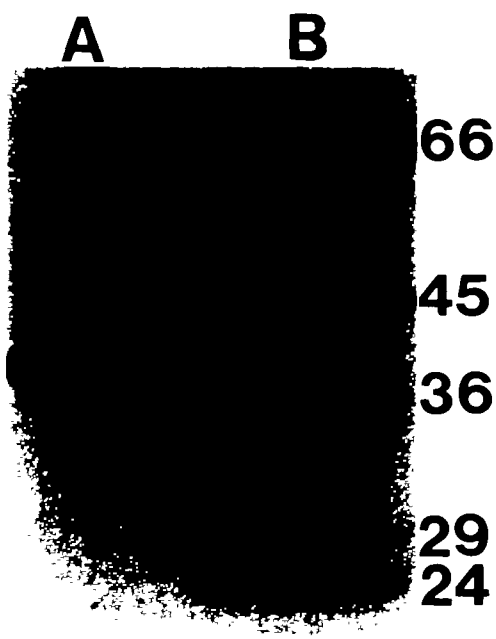

Figure 1. M_r of the purified AP endonuclease enzyme. Purified enzyme and molecular weight standards were electrophoresed on a 12% SDS polyacrylamide gel and stained with Coomassie Blue. Track A, Purified enzyme (2.5 μg): Track B, Protein standards (Sigma). The M_r in thousands of the standards is shown on the right.

No significant activity was seen with heat-inactivated enzyme or in the presence of EDTA.

The purified enzyme had an endonuclease activity specific for supercoiled DNA containing AP sites, but had no significant activity against native supercoiled DNA or supercoiled DNA containing thymine glycol residues (following treatment with osmium tetroxide) (Table II). The AP endonuclease activity was heat-sensitive and could be inhibited by EDTA (Table II).

Amino acid sequencing

The purified protein was covalently attached to an isothiocyanate-substituted membrane and the N-terminal amino acid sequence determined by solid-phase sequence analysis. Sequence data were obtained in the low-picomole range (initial yield of approximately 3 picomoles) and is shown below (upper line). Assignments after the first 12 residues were made below 1 picomole, and more tentative assignments are shown in parentheses. Residue 1 could not be identified as it is rendered 'silent' by the covalent attachment procedure. Comparison of this sequence with those of other known DNA repair enzymes revealed a close match to the N-terminal sequence (below, lower line) of a previously purified enzyme from calf thymus purified on the basis of AP endonuclease activity (17).

	Reference
X-K-R-G-K-K-G-A-V-(V)-E-D-A-E-(E)-(G)-K-X-E	(This work)
P-K-R-G-K-K-G-A-V- V -E-D-A-E- E- P -K-T-E	(17)

Isolation of cDNA clones encoding the bovine DNA repair enzyme

Based on the N-terminal sequence, fully degenerate oligonucleotides were synthesized for use in the polymerase chain reaction (PCR). A single PCR product was generated using a bovine cDNA library as template. This product was cloned into pUC18 and sequenced. The sequence of this product is shown below.

CCG AAA CGG GGC AAG AAG GGA GCG GTG GTC GAA GAC GCG GAA GAG CCC AAA ACC GAA

This DNA fragment would encode a peptide closely matching (this work) or identical to (17) the sequence derived experimentally from the N-terminus of the purified protein. We therefore used this specific probe to isolate cDNA clones from the bovine library. Approximately 2×10^5 bacterial colonies

Table I. Activity on bleomycin and H_2O_2-damaged DNA

a) Bleomycin-damaged DNA	f moles ^{32}P-dCTP incorporated into TCA-precipitable DNA
(i) Bleomycin-damaged DNA alone	0.87
(ii) DNA plus purified enzyme	7.5
(iii) DNA plus heat-treated enzyme (95°C, 5 mins)	0.51
(iv) DNA plus enzyme plus 5mM	0.38
b) H_2O_2- damaged DNA	
(i) H_2O_2-damaged DNA alone	1.9
(ii) Native DNA alone	3.2
(iii) Native DNA and enzyme	2.9
(iv) H_2O_2-treated DNA and enzyme	10

All experiments were performed under 'standard' reaction conditions (see Materials and Methods), unless otherwise stated. Where indicated 100ng of purified enzyme (Fraction VI) were included in the reactions.

Table II. Activity against DNA containing AP sites or thymine glycol residues

(i) AP substrate DNA alone	5.8
(ii) AP substrate DNA and enzyme	33
(iii) AP substrate DNA and heat-treated enzyme	8.0
(iv) AP substrate DNA and enzyme plus 5mM EDTA	11
(v) OsO_4 – treated DNA and enzyme	< 1

Value represents the percentage of the total plasmid DNA in the form of relaxed (Form II) molecules. Values have been corrected for the percentage seen with undamaged plasmid. Where indicated 0.25ng of purified enzyme (Fraction VI) were included in the reaction.

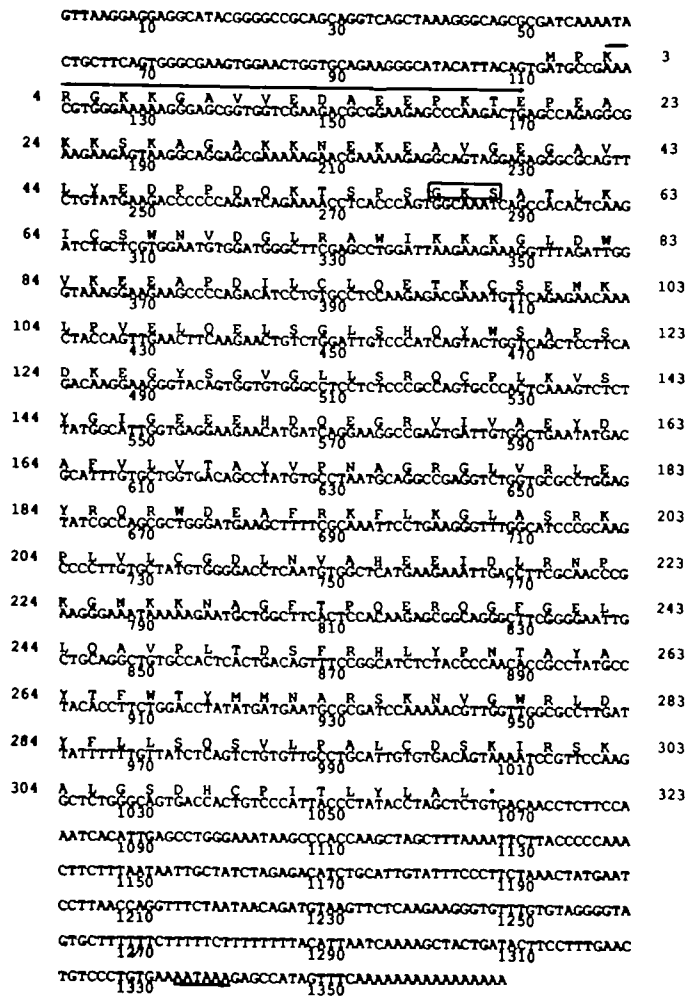

Figure 2. Nucleotide sequence of the Bovine AP endonuclease cDNA and the predicted amino acid sequence. The N-terminal amino acid sequence determined experimentally from purified enzyme is overlined. The amino acid residues corresponding to a potential adenine nucleotide binding site are boxed (see text for details). The putative polyadenylation signal is underlined. The nucleotide sequence will appear in the EMBL Database under the accession number X56685.

were screened and 12 independent positive clones identified. Restriction analysis of plasmid DNA from 4 of these colonies revealed a common 1.35 kb cDNA insert. The complete nucleotide sequence of one of these was determined. The 1367 bp sequence (Figure 2) contains a single long open reading frame of 954 bp (positions 112 to 1065) which would encode a protein comprising 318 amino acids (including the N-terminal methionine residue which was absent from the mature protein) and with a

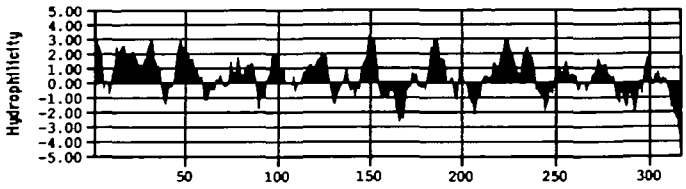

Figure 3. Hydrophilicity profile for the predicted amino acid sequence encoded by the *BAP1* cDNA. The profile was obtained using the Kyte-Doolittle analysis programme, IBI MacVector.

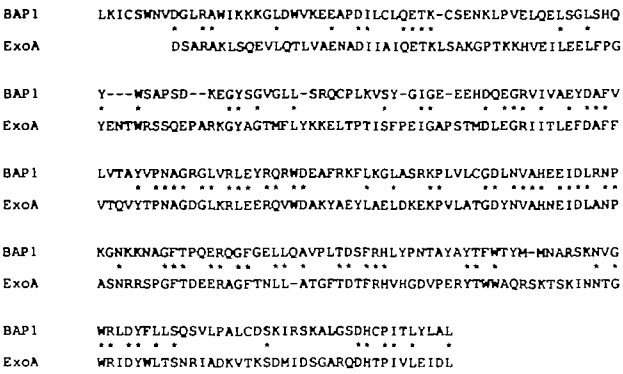

Figure 4. Alignment of the BAP1 protein sequence with that of the ExoA protein of *S. pneumoniae* (18). The BAP1 and ExoA sequences begin at residues 62 and 19 respectively. Identical residues are indicated by an asterisk.

calculated M_r of 35,547. The first ATG initiation codon is followed by a sequence closely matching that of the PCR product used as a probe and which would encode the amino acid sequence previously identified. The cDNA insert terminates with a 16 bp poly(A) tail preceded 13 bp upstream by a polyadenylation signal sequence. We propose to designate the enzyme encoded by this cDNA sequence BAP1 (*B*ovine *AP* endonuclease *1*).

Analysis of the predicted BAP1 amino acid sequence

The hydrophilicity profile for the encoded protein (Figure 3) reveals a high proportion (30%) of charged residues. The predicted protein sequence contains 43 acidic (aspartic acid and glutamic acid) and 51 basic (lysine, arginine, histidine) residues and a net charge of +8. The predicted pI of the protein is 8.6.

The predicted amino acid sequence of the BAP1 enzyme was compared with the protein sequences representing DNA repair enzymes from bacteria and yeast. Strong homology was seen between the sequence of BAP1 and those of two bacterial repair enzymes, ExoA protein of *S. pneumoniae* (18) and exonuclease III of *E. coli* (11). Figure 4 shows the best alignment of the sequences of BAP1 and ExoA protein. The sequences show significant similarity over their entire lengths, with the exception of the N-terminal 68 residues (excluding the cleaved methionine) of BAP1, for which no equivalent domain was seen in ExoA or exonuclease III. Where conserved, the sequences of BAP1 and ExoA show 36% identical residues and 50% similar (if conservative changes are included). Equivalent figures for the comparison between BAP1 and exonuclease III are 25% identical and 43% similar. No significant homology was seen between the BAP1 protein sequence and those of *E. coli* endonuclease IV (19) or the major AP endonuclease in yeast, APN1 protein (20).

There are two potential nuclear location signals near the N-terminus of the predicted BAP1 protein sequence. The motifs KRGKK (residues 2–6) and KKSK (residues 23–26) resemble the nuclear location sequences found in several proteins and match the consensus discussed by Roberts (21). There is a motif in the predicted BAP1 amino acid sequence which resembles sequences found in purine nucleotide binding proteins (22). This motif lacks the conserved glycine residue at the −2 position of the essential GKS/T triplet. The role, if any, of this sequence in modulating enzymatic activity is unclear at this stage.

Exonuclease activity

Because of the sequence homology between the BAP1 protein and bacterial AP endonucleases with associated exonuclease activity, we assayed the purified BAP1 enzyme for exonuclease activity against single- and double-stranded linear DNA end-labelled at either the 3′ or 5′ ends. For comparison, purified *E. coli* exonuclease III was similarly tested. Exonuclease III showed the expected degradative activity against 3′ end-labelled DNA, whereas the BAP1 enzyme lacked any significant exonuclease activity against single- or double-stranded DNA labelled at either the 3′ or 5′ end (data not shown).

DISCUSSION

We have isolated and sequenced a cDNA clone encoding the major AP endonuclease enzyme present in calf thymus. We have designated this cDNA *BAP1* and the enzyme BAP1. The enzyme appears to be involved in the repair of oxidative DNA damage in bovine cells. This form of DNA damage, in addition to being generated continuously during aerobic metabolism, is also induced by several important DNA damaging agents including ionizing radiation, bleomycin and peroxides.

There can be difficulties in using highly degenerate oligonucleotides based upon amino acid sequence to probe cDNA libraries. To overcome this, we used PCR to generate a specific probe representing the 5′ end of the BAP1 cDNA. The sensitivity of PCR allows the use of even fully degenerate primers (as in this case) and very short primers, with the result that relatively short stretches of peptide sequence are required.

The predicted open reading frame of the *BAP1* cDNA would encode a protein with a calculated M_r of 35,500, in close agreement to the M_r of the purified protein determined by SDS-PAGE. Moreover, nucleotide residues 115–171 of the cDNA would encode a peptide matching the experimentally determined N-terminal sequence of the purified protein (17 and this work).

The N-terminal amino acid sequence of the BAP1 protein matches that of an enzyme purified previously (17) on the basis of AP endonuclease activity. We have shown that in addition to AP endonuclease activity, the BAP1 enzyme exhibits an activity capable of removing blocking groups from the 3′ terminus of DNA strand breaks induced by hydrogen peroxide and bleomycin. These blocking groups prevent DNA strand elongation by DNA polymerases. Considering this inhibitory effect on DNA synthesis of 3′ fragmented sugars (23), and the mutagenic nature of AP sites (24, 25) it is likely that this enzyme is involved in both protection against cell lethality and suppression of mutations. Isolation and study of mutants deficient in this enzyme would help to confirm this.

The predicted amino acid sequence of the BAP1 protein shows strong homology to the ExoA protein of *S. pneumoniae* (18) and exonuclease III protein of *E. coli* (11). In both cases, the sequence similarity is around 50% and extends over almost the full length of the BAP1 protein sequence. Only the N-terminal 68 residues are unique to the BAP1 protein. This region has a somewhat atypical sequence composition being highly charged (38% overall, 50% for the first 40 residues). Whether this charged domain of the BAP1 protein is essential for interaction with other repair enzymes in bovine cells remains to be determined. An alternative hypothesis for the existence of this domain in the mammalian but not the bacterial proteins, is that it contains putative nuclear location signal sequences. Despite the sequence conservation between BAP1 and the bacterial enzymes, no exonuclease activity associated with the BAP1 protein could be demonstrated, in contrast to exonuclease III protein which exhibited its characteristic 3′ → 5′ exonuclease activity.

The degree of sequence conservation between BAP1, and the ExoA and exonuclease III proteins, is more striking than that reported between several other DNA repair enzymes in mammalian cells and bacteria. This holds even for enzymes with highly specialised roles and narrow substrate specificities, such as O^6-methylguanine DNA methyltransferase (26–28) and 3-methyladenine DNA glycosylase (29).

The BAP1 enzyme also has activities similar to those of other enzymes purified from both bacteria and yeast. In addition to exonuclease III, *E. coli* contains at least one other enzyme with combined 3′ phosphodiesterase and AP endonuclease activity, endonuclease IV (19). A yeast gene encoding a 3′ PGA diesterase activity has recently been cloned and shown to exhibit homology to endonuclease IV (20). However, the predicted BAP1 protein sequence shows no significant homology to either endonuclease IV or the yeast 3′ PGA diesterase, APN1.

It seems, at least in bacteria, that two or more enzymes with overlapping but not identical substrate specificities are necessary to protect against oxidative DNA damage. Moreover, it has been shown recently that the UvrABC excision complex, previously thought to be active only on bulky DNA adducts, can also utilise small lesions such as AP sites (30). Oxidative damage, either as a result of its toxic or its mutagenic potential, is deemed sufficiently dangerous to warrant multiple repair systems to counteract it. Whether mammalian cells contain more than one enzyme active against oxidative DNA damage is currently under investigation.

We have recently isolated, using PCR, cDNAs encoding the human homologue of the BAP1 enzyme. There is marked homology between the predicted bovine and human sequences. Lenz *et al.* (31) recently reported the isolation of cDNA clones encoding a protein from human cells that recognises AP sites. It is not clear at this stage whether those cDNAs also encode the human homologue of BAP1.

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