Supplementary material

The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway

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Purification procedures

Buffers used in the purification procedure

Buffer A: 50 mM KCl, 20 mM HEPES pH 7.6, 5% glycerol. Buffer B: buffer A + 1M KCl. Buffer C: buffer A + 500 mM KCl. Buffer D: buffer C supplemented with 50 mM imidazole. Buffer E: buffer C without glycerol plus 1.8 M ammonium sulphate. Buffer F: buffer A + 10% glycerol. Buffer G: buffer A plus 0.1 mM PMSF, 0.1 mM EDTA, 1 mM 2-mercaptoethanol. Buffer H: buffer G + 1M KCl.

Enzyme purification

All purification steps were performed at 4°C. Human DHU-specific endonuclease activity was purified from 5 g of frozen-dry pellets of Hela S3 cells that were thawed in 5 mL of lysis buffer (buffer H plus 0,3% Nonidet P-40 and EDTA-free Complete™ protease inhibitors mix) and lysed by rocking at 4°C for 15 min. The suspension was then centrifuged for 1 hour at 100,000×g and the supernatant diluted by 10-fold with 20 mM HEPES pH 7.6. The resulting solution (Fraction I) was passed through a HiTrap Q-Sepharose anion exchange 5 mL column pre-equilibrated in buffer A. Fractions containing NIR activity (flow-through and wash) were pooled (Fraction II, 100 ml). The salt concentration was adjusted to 500 mM KCl and Fraction II was loaded on a HiTrap Ni^{2+}-Chelating Sepharose 1 mL column pre-equilibrated in buffer C. Active fraction (flow-through, Fraction III, 100 ml) was loaded on a HiTrap Cu^{2+}-Chelating Sepharose 1 mL column pre-equilibrated with buffer C. The column was washed with buffer C followed by a 15 ml linear gradient of buffer C to buffer D. Fractions were collected and analyzed for NIR activity. Active fractions were pooled (fractions 12-19, Fraction IV, 4 ml). Ammonium sulphate was added up to 1.8 M and the precipitate was removed by centrifugation at 20,800 g for 10 min. The supernatant was collected, the precipitate was resuspended in 4 ml of buffer A and a small aliquot from each fraction was analyzed for NIR activity after dialysis against buffer A. Active fraction (supernatant,

Fraction V, 4 ml) was loaded on a HiTrap Phenyl Sepharose HP 1 mL column pre-equilibrated in buffer E. The column was washed with buffer E followed by a 15 ml linear gradient of buffers E to F. Fractions were collected and analyzed for NIR activity. Active fractions (22-23, Fraction VI, 1 ml) were pooled and diluted with 20 mM HEPES pH 7.6 to obtain a conductivity equivalent to that of buffer A. Fraction VI was loaded on a HiTrap Heparin Sepharose 1 mL column pre-equilibrated in buffer G. The column was washed with buffer G followed by a 15 ml linear gradient of buffers G to H. Fractions were collected and analyzed for NIR activity. Active fractions (fractions 20-21, Fraction VII) were analyzed by SDS-PAGE with fractions I-VI and proteins were visualized by silver staining.

Assay Conditions for the Nucleotide Incision Activity

To purify a human DHU-specific endonuclease from the cell-free extract, we used a 3'-labeled DHU•G substrate. Previously we have detected a weak NIR activity in HeLa cell extracts towards DHU and DHT adducts in the presence of 0.1 and/or 2.0 mM EDTA, (Ischenko and Saparbaev, 2002). However, the NIR activity completely disappears after 2-3 chromatographic steps when using reaction buffer containing 0.1 mM EDTA and no divalent cations. Moreover, the activity was lost also when the HeLa cell-free extract was dialysed against buffer without divalent cations (data not shown). These observations strongly indicate that the purified enzymatic activity requires divalent metal ions. Therefore, to optimise reaction conditions we used incubation buffer supplemented with Mg^{2+} or Zn^{2+} as a divalent cation (0.3 mM $ZnCl_2$ or 1 mM $MgCl_2$). In these conditions, the NIR activity was greatly stimulated and became the major activity incising a DHU•G substrate in a crude lysate of HeLa cells as compared to DNA glycosylase/AP lyase (Figure 4).

Purification of the Endonuclease that incises DHU•G oligonucleotide

In order to estimate the molecular mass of the human endonuclease, we have used a gel filtration column. The NIR activity migrates as a single species and exhibits a mobility corresponding to a globular protein of 35-40 kDa (Supplementary figure 1, lane 8). As shown in Supplementary table and Supplementary figure 1 the human DHU-specific endonuclease activity was purified from HeLa S3 cells using five chromatographic steps including following columns: Q-Sepharose, Ni^{2+}- and Cu^{2+}-Chelating Sepharose, Phenyl Sepharose and Heparin Sepharose. In Fraction VII, the DHU-incision activity eluted from the heparin column as a single symmetrical peak. Starting from the 5g of cell pellets we have been able to

obtain 0.75 μg of the protein with specific activity 7500 fmol/min/μg (Supplementary table). Various column fractions containing the NIR activity were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Supplementary figure 1). The most active fraction after heparin chromatography (Fraction VII) was found to be purified to near apparent homogeneity and consisted of two polypeptides: a major species with an apparent size of 37 kDa and minor one with a size of 31 kDa (Supplementary figure 1, lane 8).

Substrate specificity of HeLa-purified Ape1

Previously, we have shown that the Nfo and Apn1 proteins nick DNA on the 5' side of various oxidatively damaged bases including DHT, DHU, 5ohU and me-FapyG residues. Conversely the *E. coli* Xth did not incise the DNA containing such lesions. Therefore, we examined whether the HeLa-purified protein (Fraction VII) can incise DNA containing various base lesions in a DNA glycosylase-independent manner. The DNA substrates used were 3'-labeled duplex substrates containing 8oxoG•C, αA•T, αT•A, DHU•G, DHT•A, me-FapyG•C, U•G, 5ohU•G, THF•G, εA•T and εC•G. The hOGG1, Nfo, Fpg, UDG and Ape1 proteins were used to generate size markers. The oligonucleotides containing αA, αT, DHU, DHT, 5ohU and THF adducts were readily incised by HeLa-Ape1 (Fraction VII) (Supplementary figure 4, lanes 6, 9, 16, 19, 25 and 28), whereas those containing 8oxoG•C, G•U, εA•T and εC•G were not (Supplementary figure 4, lanes 3, 22 and data not shown). A weak incision band was detected for me-FapyG•C. However, the gel mobility of the band was identical to that generated by Fpg, suggesting spontaneous cleavage of contaminating AP sites in the me-FapyG•C oligonucleotide. The HeLa-Ape1 protein did not incise the single-stranded oligonucleotides containing DHT (data not shown). Taken together the results suggest that the substrate specificity of HeLa-Ape1 is similar to that of Nfo and Apn1.

Supplementary table. Purification of human damage-specific endonuclease

Purification Step	Fraction	Total protein Amount	Specific Activity[a] (fmol/min/µg)	Total Activity (fmol/min)	Purification Factor
Crude extract	Fraction I	150 mg	3	480000	1
Q Sepharose	Fraction II	80 mg	5	400000	1.7
Ni^{2+} Chelating	Fraction III	40 mg	9	372000	3
Cu^{2+} Chelating	Fraction IV	0,6 mg	500	300000	166
Ammoniun Sulfate precipitation	Fraction V	40 µg	1160	46400	386
Phenyl Sepharose	Fraction VI	6 µg	2283	13700	761
Heparin Sepharose	Fraction VII	0.75 µg[b]	7500	5625	2500

[a]Nucleotide incision activity was measured using a 3´-[^{32}P]-dCMP labelled DHU•G duplex as a substrate.

[b]The amount of proteins was estimated from comparison with the band intensities of a known amount of marker proteins (silver staining) after 10% SDS-PAGE separation.

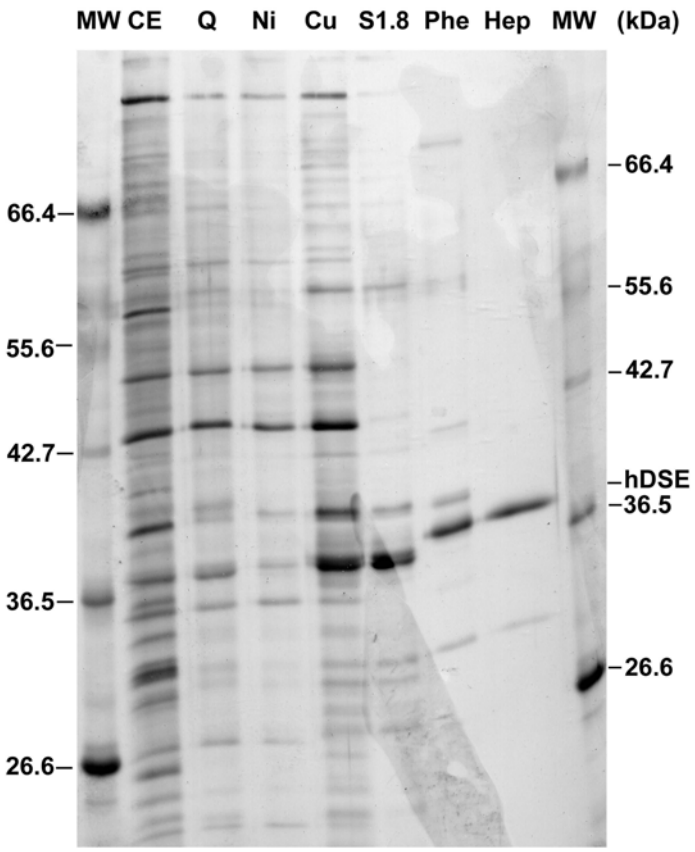

Figure S1. SDS-PAGE analysis of the fractions containing NIR activity. Proteins from each purification step of hDSE1 (1,5 µg-15 ng) were analyzed by 10% SDS-PAGE. After electrophoresis the gel was fixed and stained with Silver Plus Reagent. *Lanes*: **MW**, molecular weight markers; **CE**, crude cell extracts (Fraction I); **Q**, flow-through fraction of Q Sepharose (Fraction II); **Ni**, flow-through fraction of Ni^{2+} Chelating Sepharose (Fraction III); **Cu**, eluate of Cu^{2+} Chelating Sepharose (Fraction IV); **S 1.8**, supernatant of Fraction IV after 1.8M ammonium sulfate precipitation (Fraction V); **Phe**, eluate of Phenyl Sepharose (Fraction VI); **Hep**, eluate of Heparin (Fraction VII).

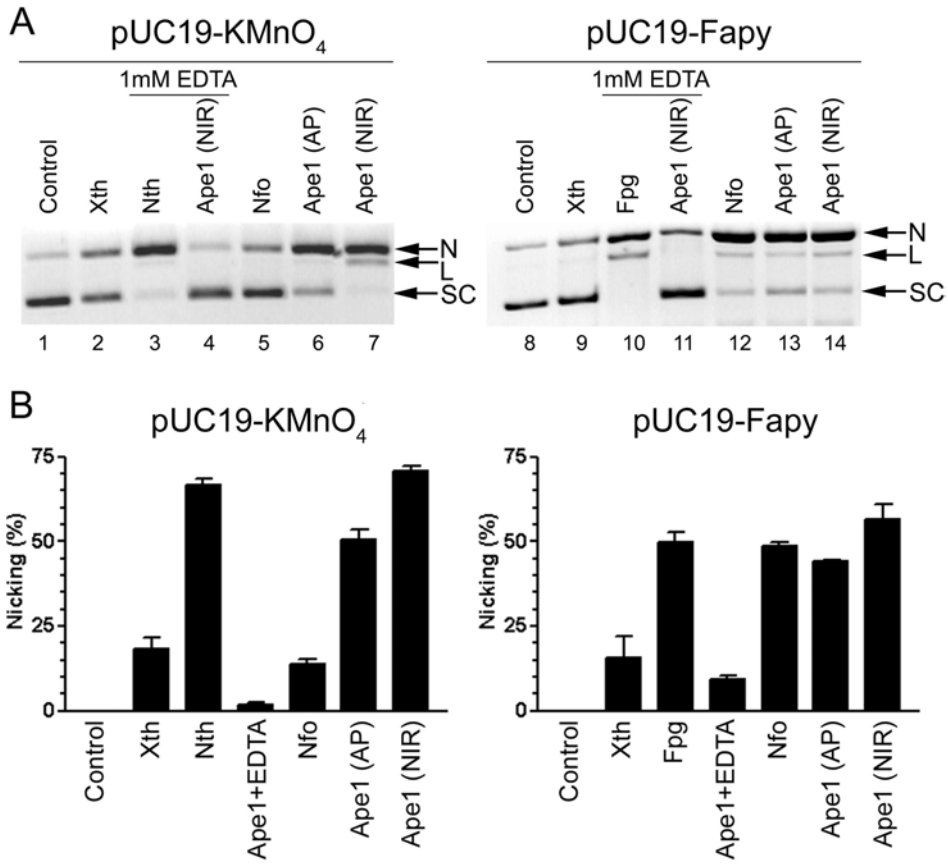

Figure S2. Incision of a damaged, supercoiled plasmid DNA by various DNA repair proteins. For the incision assay, 100 ng of chemically modified pUC19 and 10 nM of an enzyme were incubated in 20 µl in appropriate buffer at 37°C for 10 min. The reaction products were separated by 0.8 % agarose gel electrophoresis in 0.5xTBE in the presence of ethidium bromide (0.5 µg/ml). The gel was photographed and quantified using Bio-Profil software. **A**, damaged plasmid DNA was incubated with different enzymes and analyzed by agarose gel electrophoresis. **B**, relative nicking activity of various enzymes.

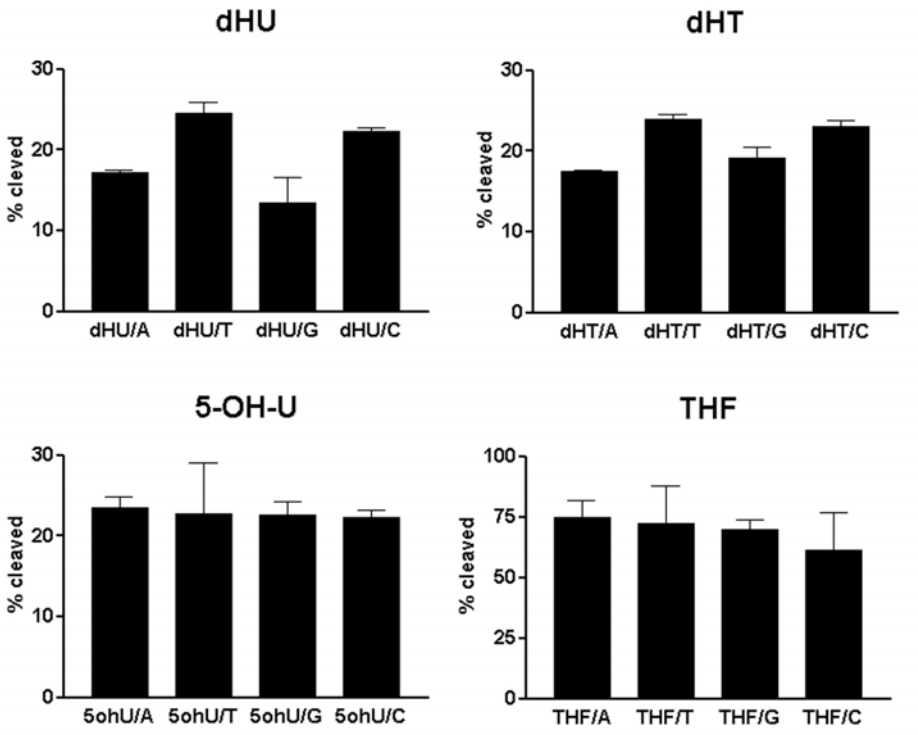

Figure S3. The influence of the base opposite the DHU, DHT, 5ohU and THF residues upon incision of the duplex oligonucleotides by Ape1.

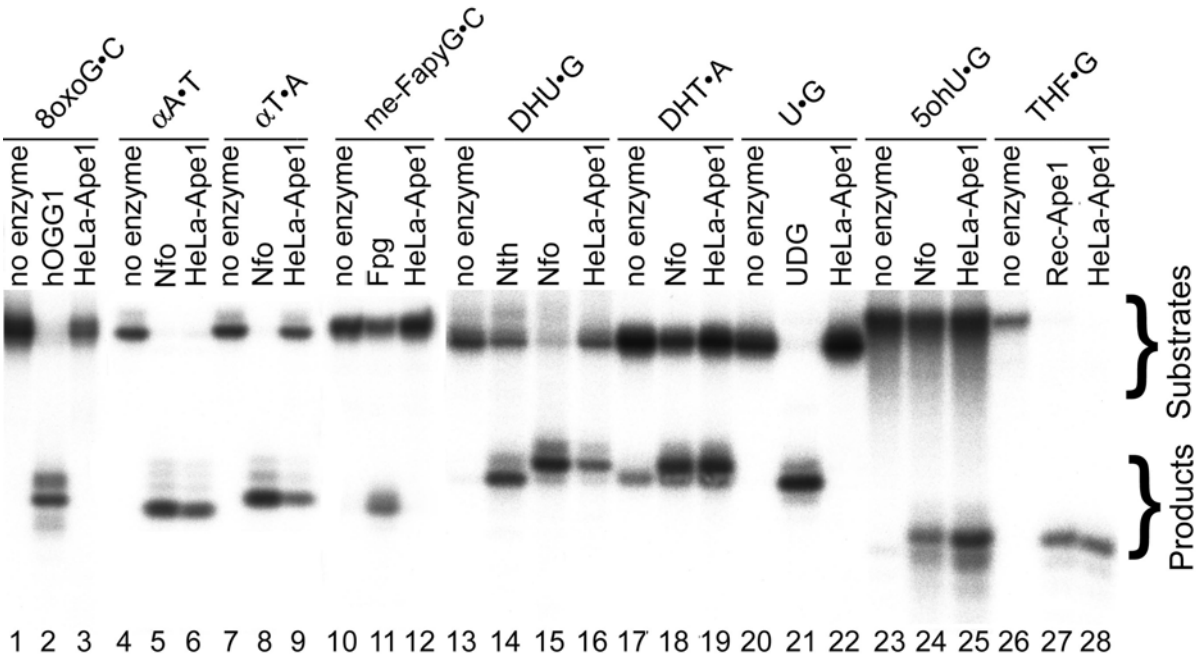

Figure S4. Either 3.0 ng of HeLa-purified Ape1 (Fraction VII) or 5 nM of control enzymes were incubated with 20 nM of 3'-[^{32}P]-labeled duplex oligonucleotide substrates carrying a single DNA lesion at 37°C for 5-30 min. The reaction products were analyzed as described in Materials and Methods. Substrates integrity was checked in absence of any enzyme (no enzyme) and control cleavages were performed with a repair enzyme specific for each kind of lesion (hOGG1, Fpg, Nfo, Nth, UDG and Rec-Ape1).