



Apex1 can cleave complex clustered DNA lesions in cells

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ABSTRACT

Current data indicate that clustered DNA damage generated by ionizing radiation contains 2–5 damages within 20 bps. The complexity of clustered damage is also believed to increase as the linear energy transfer of the radiation increases. Complex lesions are therefore biologically relevant especially with the use of carbon ion beam therapy to treat cancer. Since two closely opposed AP site analogs (furans) are converted to a double strand break (DSB) in cells, we hypothesized that breakage could be compromised by increasing the complexity of the cluster. We have examined the repair of clusters containing three and four lesions in mouse fibroblasts using a luciferase reporter plasmid. The addition of a third furan did reduce but not eliminate cleavage, while a tandem 8-oxo-7,8-dihydroguanine (8oxoG) immediately 5' to one furan in a two or three furan cluster decreased DSB formation by a small amount. *In vitro* studies using nuclear extracts demonstrated that the tandem 8oxoG was not removed under conditions where the furan was cleaved, but the presence of the 8oxoG reduced cleavage at the furan. Interestingly, a cluster of an 8oxoG opposite a furan did not form a DSB in cells. We have shown that Apex1 can cleave these complex clustered lesions in cells. This therefore indicates that Apex1 can generate complex DSBs from clustered lesions consisting of base damage and AP sites. Repair of these complex DSBs may be compromised by the nearby oxidative damage resulting in potentially lethal and biologically relevant damage.

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1. Introduction

Effects of ionizing radiation such as cell death, mutagenesis and tumorigenesis are largely attributed to the damage generated in cellular DNA. The types of damage induced include oxidative base damage, abasic (AP) sites, single strand breaks (SSBs) and DSBs [1]. This is also the signature of damage induced by reactive oxygen species (ROS). However, other types of ROS-generating damaging agents such as hydrogen peroxide are not as lethal as ionizing radiation: at an equivalent cytotoxic dose hydrogen peroxide generated 5 times more DSBs than X-rays [2], and 400 times more SSBs per lethal event than ionizing radiation [3]. Hence, Ward [4] proposed the existence of radiation-induced locally multiple damaged sites or clustered lesions, which are refractory to repair and consist of ≥ 2 damages within a 20 bp region [5]. Two opposing SSBs that form a DSB can be considered a clustered lesion. However, it has been

shown that clustered lesions exist that do not contain a DSB and they are generated by X-rays at 1 oxidized purine cluster to 0.9 oxidized pyrimidine cluster to 0.75 abasic site cluster to 1 DSB [6]. Radiation-induced DSBs can also occur within close proximity to other lesions: Datta et al. [7] demonstrated clustering of AP sites within 8–10 bps of an ¹²⁵I-induced DSB. These are called complex DSBs.

The complexity of ionizing radiation DNA damage is predicted to increase as the linear energy transfer of the radiation increases [8]. Monte Carlo models [9,10] predict that high LET radiation can generate clusters consisting of 10–25 lesions over a 100–200 bp DNA region. The relative biological effectiveness is also higher for high LET charged particles compared to X-rays [11] and so this supports the idea that complex DNA clusters, consisting of ≥ 3 damages, are biologically important lesions. With the use of carbon ion beam therapy for the treatment of tumors [12,13], it is important to understand how mammalian cells repair or attempt to repair these complex clusters. Even gamma-rays from a ¹³⁷Cs source are predicted by Monte Carlo simulations to generate complex clustered lesions: about 72% of the low LET clusters contain 2 lesions, 20% contain 3 lesions, 6% contain 4 lesions, and the remaining 2% of the clusters contain 5 or more lesions (personal communication, Dr. R.D. Stewart, Purdue University, IN).

Repair of oxidized base damage, AP sites and SSBs is predominantly performed by base excision repair (for review see 14).

Abbreviations: furan, tetrahydrofuran; oligonucleotide, oligodeoxyribonucleotide; bps, base pairs; AP, abasic; SSB, single strand break; DSB, double strand break; BER, base excision repair; Fpg, formamidopyrimidine DNA glycosylase; 8oxoG, 8-oxo-7,8-dihydroguanine; Apex1, major AP endonuclease in mouse cells; hApe1, major AP endonuclease in human cells.

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Initiation of repair of base damage requires a DNA N-glycosylase that removes the damaged base leaving an AP site. The majority of DNA glycosylases that remove oxidized base damage have an associated AP lyase activity that cleaves the phosphodiester backbone at the 3' side generating a 3' aldehyde or 3' phosphate. Initiation of repair of AP sites predominantly occurs by the major AP endonuclease in the cell, which cleaves on the 5' side of the AP site leaving a 3' hydroxyl and a 5' deoxyribose phosphate. The modified 3' and 5' termini generated at the SSBs/gaps in the initial steps of repair need to be removed prior to completion of repair by a DNA polymerase and DNA ligase. DNA polymerase β can remove the 5' deoxyribose phosphate terminus, while the 3' phosphate and aldehyde groups can be removed by polynucleotide kinase and the major AP endonuclease, respectively. In mouse cells, the predominant DNA glycosylase that removes 8oxoG is Ogg1, and Apex1 is the major class II AP endonuclease that can cleave at a furan. In fact, Apex1 has been implicated in cleaving closely opposed furan residues in cells [15]. The furan is an abasic site analog that cannot undergo β -elimination at the 3' side of the lesion [16]. This prevents cleavage by AP lyases, and short patch base excision repair cannot proceed as polymerase β is unable to remove the 5'-blocked terminus generated by Apex1. Long patch base excision repair is required to complete repair: DNA polymerase δ/ϵ extends the DNA, displacing the strand that contains the 5'-blocking lesion, Fen1 removes the "flap" structure and DNA ligase I seals the "nick". Other factors such as replication factor C and proliferating cell nuclear antigen are also required for this process.

The majority of the work on the repair of defined non-DSB containing clustered lesions has used substrates containing two damages *in vitro* with pure proteins or cell extracts (reviewed in 17 and 18; 19–22). In general, opposing lesions were converted to SSBs if they were positioned ≥ 3 bps apart. Work in bacteria and mammalian cells has shown similar results with respect to AP sites or the furan abasic site analog, and has demonstrated that opposing lesions can be converted to DSBs in cells [15,23]. Interestingly, studies examining closely opposed base damage in bacteria have shown that the lesions are not converted to DSBs, but rather enhance mutation frequency at the individual lesions in the cluster [24–27]. In this present study, we have examined two lesion clusters containing 8oxoG opposite to a furan and also did not find evidence of DSB formation in the mouse fibroblasts. The exception seems to be clustered uracil damage, where there is a difference in the results between bacteria, yeast and mammalian cells: in bacteria [28,29] and yeast [30] the opposing uracils are converted to DSBs, even in the absence of replication [29], yet in human cells opposing uracils did not form a DSB in the absence of replication [31].

There has been limited work performed *in vitro* [32–34] and in yeast [30] using defined clustered lesions containing ≥ 3 lesions within 20 bps. Published work has demonstrated that the cluster is not easily converted to a DSB. Our previous study [15] demonstrated that two opposing furans could be converted to a DSB in mouse cells, even in the presence of DSB repair by non-homologous end-joining (NHEJ). We therefore chose to increase the complexity of this clustered lesion to contain three furans, or two or three furans and an 8oxoG. We have utilized a luciferase reporter assay, in which the complex clustered damage was positioned within the firefly luciferase reporter coding region. Formation of a DSB and degradation or mis-repair of the plasmid results in a decrease in luciferase activity. This is the first study examining the repair of complex clustered lesions in mammalian cells and we provide evidence that Apex1, the major AP endonuclease in mouse cells, is able to convert non-DSB complex clustered lesions into complex DSBs, a proportion of which can be inaccurately repaired.

2. Materials and methods

2.1. Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (oligonucleotides) were purchased from Operon Technologies Inc. (Alameda, CA). The sequences of the double stranded oligonucleotides containing no damage or closely opposed tetrahydrofurans (furans, known as a d-spacer from Operon Technologies) and 8oxoG are shown in Table 1. These oligonucleotides contained a 5' phosphate and were purified using polyacrylamide gel electrophoresis. *In vitro* assays with endonuclease IV or Fpg confirmed that the furans and 8oxoG were present in each oligonucleotide.

Oligonucleotides purchased for PCR or sequence analysis were not purified and did not contain 5' phosphate termini. Four primer sets were used to amplify the luciferase region: Luc1 d(TGGATGGCTACATCTCG) and R d(TCATCGTCTTTCCGTGCT), Luc3 d(ATGTGGATTTTCGAGTCGTCTT) and Luc10 d(TGGCCACGAATTCCACGATCTCTTTTCCG), Luc3 and Luc5 d(GCTGTATCTTTTATAG), CMV3 d(GGTAAGTATCAAGTTAC) and Luc5. Sequence analysis of repair products was performed using primer R. Primers pAC1 d(CATCGCAGTACTGTTG) and pAC2 d(CAATGAAAGACGGTGA) were used for amplification of the fragment of pACYC184.

2.2. Plasmids

pACYC184 (New England Biolabs, Beverly, MA) is a low copy vector with a p15A origin of replication that encodes resistance to chloramphenicol (34 $\mu\text{g}/\text{ml}$). pRL-CMV (Promega, Madison, WI) expresses renilla luciferase in mammalian cells and encodes carbenicillin resistance. pCMV3'luc inactive [15] is a derivative of pCMV3'luc that expresses firefly luciferase from a CMV promoter and encodes carbenicillin resistance. pCMV3'luc inactive contains an insertion at the Pac I site that results in expression of a protein that does not have luciferase activity. This eliminates potential background activity from the re-circularization of partially digested plasmid used in ligation reactions. Digestion with Pac I and Cla I and insertion of a 45 bp double stranded oligonucleotide (Table 1) regenerates the pCMV3'luc sequence and expression of active firefly luciferase.

2.3. Cell line

A mouse fibroblast cell line (42WT) was kindly provided by Dr. D. Chen (University of Texas Southwestern Medical Center, Dallas, USA). This cell line was used in our previous study [15]. Cells were maintained in α -minimal essential medium (α -MEM) (HyClone, Logan, UT) containing 10% FBS, 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin (GIBCO Invitrogen Corporation, Carlsbad, CA) in a humidified atmosphere at 37 °C in 5% CO₂.

2.4. Insertion of double stranded oligonucleotides into pCMV3'luc

This was performed according to Malyarchuk et al. [15]. Briefly, double stranded molecules were prepared by annealing equimolar amounts of complementary oligonucleotides. Purified Pac I and Cla I linearized pCMV3'luc inactive was quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Ligation reactions containing 1.4 pmol of linear plasmid DNA (~5.5 μg), 7 pmol double stranded oligonucleotide, 0.5 mM ATP, 15 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 5 mM dithiothreitol, 2.5% polyethylene glycol and 15 units of T4 DNA ligase (Promega Corporation, Madison, WI) were incubated overnight at 4 °C. The salts were then removed using the Qiaquick Nucleotide Removal kit (Qiagen Inc., Valencia, CA) and the DNA eluted in 30 μl 10 mM Tris (pH 8.5). Three of these reactions for each type of insert were performed and pooled to

Table 1
Oligonucleotides used to form the insert between Pac I and Cla I in pCMV3'luc.

Name of lesion	Position of the F(s) relative to the F on the NT strand (base pairs apart)	Position of the O relative to the F on the NT strand (5' or 3')	Sequence
Undamaged	–	–	5' TAAATACAAAGGATATCAGGTGGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTATAGTCCACCGGGGGCGACTTAACCTTAGC 5'
A	2	–	5' TAAATACAAAGGATATCAGG F GGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTATAG F CCACCGGGGGCGACTTAACCTTAGC 5'
B	5	–	5' TAAATACAAAGGATATCAGG F GGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTA F AGTCCACCGGGGGCGACTTAACCTTAGC 5'
C	2 and 5	–	5' TAAATACAAAGGATATCAGG F GGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTA F AG F CCACCGGGGGCGACTTAACCTTAGC 5'
D	2	5'	5' TAAATACAAAGGATATCAG O FGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTATAG F CCACCGGGGGCGACTTAACCTTAGC 5'
E	2	3'	5' TAAATACAAAGGATATCAGG FO CCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTATAG F CCACCGGGGGCGACTTAACCTTAGC 5'
G	5	5'	5' TAAATACAAAGGATATCAG OF GGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTA F AGTCCACCGGGGGCGACTTAACCTTAGC 5'
H	5	3'	5' TAAATACAAAGGATATCAGG FO CCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTA F AGTCCACCGGGGGCGACTTAACCTTAGC 5'
I	2 and 5	5'	5' TAAATACAAAGGATATCAG OF GGCCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTA F AG F CCACCGGGGGCGACTTAACCTTAGC 5'
J	2 and 5	3'	5' TAAATACAAAGGATATCAGG FO CCCCCGCTGAATTGGAAT 3' (NT) 3' TAATTTATGTTTCCTA F AG F CCACCGGGGGCGACTTAACCTTAGC 5'

F designates the position of a furan and O designates the position of an 8oxoG in a damage-containing oligonucleotide. Two oligonucleotides were annealed to form a clustered lesion. Clustered lesions examined contained two or three furans in opposing DNA strands situated 5' to each other. The NT strand is the non-transcribed strand in pCMV3'luc. Each lesion has a designated letter to aid in identification in the figures. Six lesions are not shown: lesions K, L, M and N contain a furan and an 8oxoG in opposite strands 1, 3, 4 and 6 bp apart, respectively, and lesions P and Q contain two furans on 1 strand opposite to an 8oxoG on the NT strand. The furans are 1 and 4 bp away from the 8oxoG in lesion P and 3 and 6 bp away from the 8oxoG in lesion Q.

obtain one sample of ligation product. A similar sample of DNA was also generated as a control from ligation reactions that did not contain oligonucleotide (control ligation).

2.5. Introduction of DNA into the mouse fibroblasts

To transfect DNA into the cells we used the Nucleofector™ (AMAXA Biosystems, Gaithersburg, MD). 10 µl of ligation products (~1.8 µg of DNA) and 10 ng of pRL-CMV or 100 ng of pACYC184 were co-transfected into 2–3 × 10⁶ mouse fibroblast cells using the MEF Nucleofector™ Kit 2, according to the manufacturer's instructions using program T-20. pRL-CMV was used when cell-free extracts were prepared to measure luciferase activities, while pACYC184 was used for experiments where the DNA was re-isolated from the cells.

2.6. Measurement of firefly and renilla luciferase activities

Cells were transfected with DNA and incubated for 6 h at 37 °C and 5% CO₂. Cell-free extracts were prepared, and firefly and renilla luciferase activities measured as described in Malyarchuk et al. [15]. The results were expressed as a ratio of firefly luciferase/renilla activity. In each experiment, an average ratio was obtained from triplicate transfections of the undamaged sequence and set at 100%. The ratio from each transfection in the experiment was then expressed as a percentage of this average ratio for the undamaged sequence. This was also calculated for the transfections of the undamaged sequence to allow us to determine the level of variation for the undamaged sample. At least five transfections were performed for each type of ligation and the results were used to calculate the average percentage of luciferase expression compared to the undamaged sequence and the standard error for each construct. The InStat3 program and the unpaired *t*-test with Welch correction was used to determine statistical differences between different types of constructs. Trans-

fection of the control ligation resulted in negligible firefly luciferase activity.

2.7. Plasmid survival assay and PCR analysis of re-isolated DNA

This was performed according to Malyarchuk et al. [15]. Ligation reactions were co-transfected with 100 ng pACYC184 into 42WT cells to allow for normalization of the transfection efficiency, the re-isolation of the DNA and the transformation into the bacteria. Each re-isolated DNA sample was transformed in duplicate into Max efficiency DH5α™ chemically competent cells (Invitrogen Corporation, Carlsbad, CA) and after overnight growth a ratio of the carbenicillin-resistant (Carb[®]) colonies/chloramphenicol-resistant (Cm[®]) colonies was calculated for each transformation. The ratios were used to calculate the percentage of plasmid survival for each type of ligation compared to the undamaged sequence. The InStat3 program and the unpaired *t*-test with Welch correction was used to determine statistical differences between different types of constructs.

Carb[®] colonies were re-grown and a small amount of the bacteria used in a PCR reaction to examine the repair product. A PCR reaction with Luc1 and R primers was performed as previously described [15] to determine the presence and size of deletions or insertions within the Pac I–Cla I region. The predicted product size was 236 bps from pCMV3'luc and 292 bps from pCMV3'luc inactive. A small percentage of colonies examined generated products at the size of pCMV3'luc inactive. These were eliminated from the analysis.

Plasmid DNA containing deletions or that did not generate a PCR product was prepared from colonies using the Wizard Plasmid Mini Kit (Promega, Madison, WI). Further PCR analysis was performed on DNA using primer sets Luc3–Luc5 (annealing temperature 47 °C) and Luc3–Luc10 (annealing temperature 57 °C), and CMV3 and Luc5 (annealing temperature 52 °C). These reactions were expected to generate products of 740, 1800 and 2820 bps, respectively, from

pCMV3'luc. DNA that failed to generate a product was subjected to restriction digestion with HpaI and XbaI (NEB, Beverly, MA, USA) to determine the size of the deletion. To examine the sequence across the junctions of plasmid that contained deletions, DNA was sequenced by the DNA facility at the University of Iowa (Ames, IA, USA).

2.8. Southern analysis

Ligation reactions were co-transfected with 100 ng pACYC184 into 42WT cells and DNA re-isolated as previously described [15]. The DNA was subjected to electrophoresis through a 0.8% (w/v) agarose gel and transferred [35] to nylon (N⁺, GE Healthcare). The membrane was hybridized separately with two ³²P-labeled DNA probes: the undamaged oligonucleotides (Table 1) and a 415 bp fragment of the *cat* gene from pACYC184. The oligonucleotides were 5'-end labeled using T4 polynucleotide kinase (Invitrogen Corporation, Carlsbad, CA) and [γ -³²P]ATP (Perkin Elmer, Waltham, MA), and established the amount of pCMV3'luc in the samples. The fragment of pACYC184 was generated by PCR using primers pAC1 and pAC2, and pACYC184 DNA with a 55 °C annealing temperature. This fragment was labeled using a random prime kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's recommendations, and was used to normalize the samples for transfection efficiency, DNA re-isolation and for the amount of DNA on the membrane. Hybridization was performed at 42 °C overnight in 50% (v/v) formamide, 5× Denhardt's solution, 5× SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 1% (w/v) SDS and 10% (w/v) dextran sulfate. An image was obtained by autoradiography, and the signal for the DNA quantitated using a Storm phosphoimager (Molecular Dynamics, Amersham Biosciences Corp., Piskataway, NJ). A ratio was calculated for the signal for the oligonucleotides divided by the signal for the pACYC184 fragment. This ratio was used to compare the amount of pCMV3'luc in the samples.

2.9. Down-regulation of Apex1 using siRNA

siGENOME SMART pool against mouse APEX1 and siCONTROL RISC-free siRNA (negative control, scrambled siRNA) was purchased from DHARMACON Inc. (Chicago, IL). siRNA (100 pmol or 1.5 μ g) were transfected into 42WT cells using the standard conditions described above. Three sets of transfections were performed at the same time: mock (no siRNA), negative control (scrambled siRNA) and a sample to decrease Apex1 (siRNA designed against APEX1). Treated cells were then transfected after 72 h with the pCMV3'luc constructs (as described above).

Cell-free extracts were prepared and Western analysis performed for Apex1 after 72 h, as previously described [15].

2.10. Preparation of nuclear extract from 42WT cells

Nuclear extract was prepared according to Okouchi et al. [36] with modifications. Briefly, cells ($\sim 1.4 \times 10^7$) at $\sim 75\%$ confluency were harvested and resuspended in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, 1 μ g/ml Pepstatin A). After centrifugation, the cell pellet was resuspended with 3 times the packed cell volume of hypotonic buffer. The cells were mixed and incubated for 20 min, prior to centrifugation and resuspension in 1 ml of hypotonic buffer containing 0.1% IGEPAL. The cells were incubated on ice until >95% were lysed. The nuclei were obtained by centrifugation, and resuspended in 100 μ l of low salt buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, 1 μ g/ml Pepstatin A), followed by dropwise addition of 100 μ l of hypertonic buffer (10 mM HEPES, pH 7.9,

0.1 mM EDTA, 50 mM KCl, 0.3 M NaCl, 10% glycerol, 0.5 mM PMSF, 0.5 mM DTT, 1 μ g/ml Pepstatin A). After 30 min of rocking at 4 °C, the nuclear extract was obtained by centrifugation. Proteins in the nuclear extract were precipitated with ammonium sulfate, dissolved in hypotonic buffer and dialyzed against hypotonic buffer for 1 h at 4 °C. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) and the nuclear extract was stored at -80 °C in aliquots.

2.11. In vitro DNA damage cleavage assays

To examine repair of a specific lesion, the oligonucleotide (5 pmol) containing the lesion was labeled on the 5' end with ³²P, using 50 μ Ci [γ -³²P]ATP and T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) in the exchange reaction buffer provided by the manufacturer. Unincorporated [γ -³²P]ATP was removed using a G25 spin column. The labeled oligonucleotide was mixed with the same unlabeled oligonucleotide at a ratio of 1:1. To generate duplex substrate, this DNA was annealed to 1.5 molar excess of unlabeled complementary strand in 10 mM Tris pH 8, 50 mM NaCl by heating the solution to >85 °C for 4 min and then slowly cooling the DNA to room temperature over a period of ~ 2 h. Duplex substrate was diluted to 10 fmol/ μ l using 10 mM Tris pH 8, 50 mM NaCl, and 1 μ l used in a 5 μ l reaction with either 0.03 units endonuclease IV (Trevigen, Gaithersburg, MD), 0.5 units Fpg (NEB, Beverly, MA, USA), 0.05–0.2 units hApe1 (Trevigen, Gaithersburg, MD), 0.25 or 0.5 units hOgg1 (NEB, Beverly, MA, USA), or 100 or 200 ng nuclear extract in 45 mM HEPES, pH 7.8, 70 mM KCl, 1 mM DTT, 2.5 mM MgCl₂. Reactions were also performed without MgCl₂ in 45 mM HEPES, pH 7.8, 70 mM KCl, 1 mM DTT, 2 mM EDTA. All reactions were incubated at 37 °C for 1–60 min and stopped by the addition of 5 μ l 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF. Reactions were heated to ~ 70 °C for 5 min and cooled on ice prior to electrophoresis through a 7 M urea, 12% polyacrylamide gel at 1200 V for 1.5–2 h. The gel was dried and the products visualized by autoradiography and quantified using a phosphoimager (Storm 860, Molecular Dynamics). Greater cleavage was seen when the products of hOgg1 reactions were boiled prior to electrophoresis. hOgg1 is known to have a weak AP lyase activity, and boiling breaks the AP sites generated by the DNA glycosylase. All reactions containing hOgg1 were therefore boiled for 15 min prior to electrophoresis.

3. Results

To determine whether clustered lesions can be converted to DSBs in mammalian cells, we used an assay in which the damage is placed within the 3' coding region of the firefly luciferase reporter in a mammalian expression vector (pCMV3'luc) and the DNA transfected into cells. Conversion of the clustered lesion to a DSB results in linearization of the plasmid, and plasmid degradation or inaccurate repair results in a decrease in luciferase activity. To prevent DSB formation by replication fork stalling and collapse at the furans, pCMV3'luc does not contain a mammalian origin of replication. Assays are also performed 6 h after transfection, so it is unlikely that time would be available for plasmid replication. It is possible to assay luciferase activity at 6 h due to the use of a Nucleofector™, which increases the amount of DNA delivered to the nucleus. This is also advantageous as the nucleus is the site of DNA repair.

Control ligations of linear pCMV3'luc without the double stranded Pac I–Cla I sequence were always performed and found to result in negligible firefly luciferase activity. Ligations were also examined that only contained the damage on one strand of the Pac I–Cla I sequence. These single lesions or two lesions in tandem did

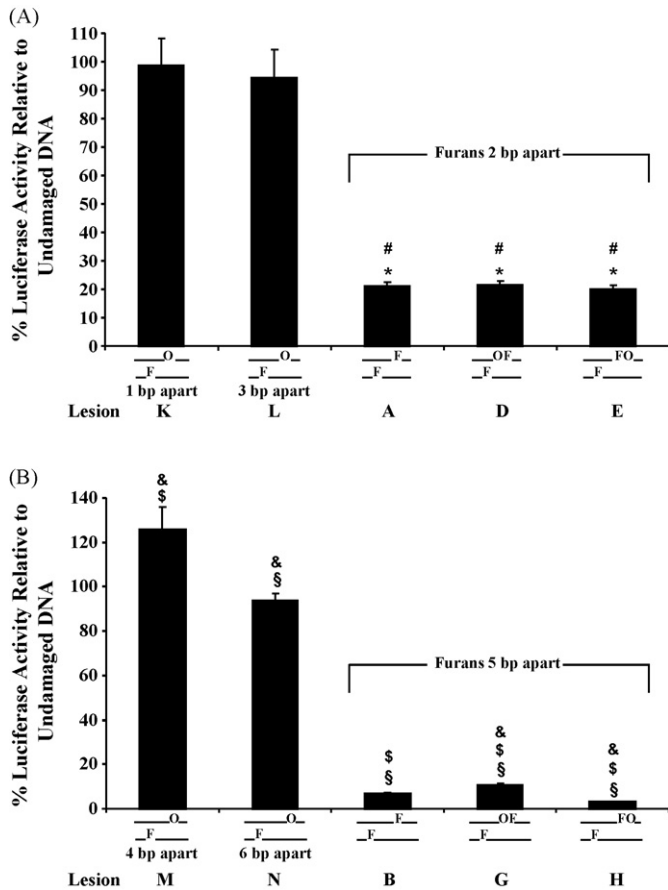


Fig. 1. An 8oxoG situated in close proximity to two closely opposed furans does not prevent cleavage of the two furans in cells. Cells were transfected with pRL-CMV and ligation reactions containing pCMV3'luc with clustered lesions consisting of an 8oxoG (O) opposite to a furan (F), two closely opposed furans 2 or 5 bps apart, two opposing furans with an 8oxoG situated in tandem 5' or 3' to one of the furans, or no damage. Firefly and renilla luciferase activities were measured from a cell-free extract after 6 h. The firefly/renilla ratio for each construct was expressed as a percentage of the average ratio of the undamaged sequence. At least five transfections of each type of ligation were performed. The average and the standard error are shown graphically in A for furans 2 bps apart and in B for furans 5 bps apart. *Statistical difference ($p < 0.001$) compared to lesion K. #Statistical difference ($p < 0.01$) compared to lesion L. §Statistical difference ($p < 0.01$) compared to lesion B. §Statistical difference ($p < 0.05$) compared to lesion M. §Statistical difference ($p < 0.05$) compared to lesion N.

not greatly reduce luciferase activity, even when the damage was present on the transcribed strand. The activities ranged from 72 to 125% of the undamaged sequence. It is therefore unlikely that decreases in activity from the complex lesion constructs were due to effects on transcription.

3.1. Effect of three clustered furan residues on firefly luciferase activity in cells

Cells were transfected with ligations containing no damage, two furans situated 5' to each other in opposite strands either 2 (lesion A) or 5 bps (lesion B) apart, or three furans (lesion C). This latter clustered lesion consisted of the furans used to generate lesions A and B, i.e. within this cluster there were two furans 2 bps apart and two furans that were 5 bps apart (Table 1). As previously found [15], the two furans situated 5 bps apart (lesion B) reduced luciferase activity to a greater extent than the two furans 2 bps apart (lesion A) resulted in 21% and lesion A 6.8% the luciferase activity of the undamaged sequence, Fig. 1). pCMV3'luc containing the three furans resulted in an intermediate level of luciferase activity that was significantly dif-

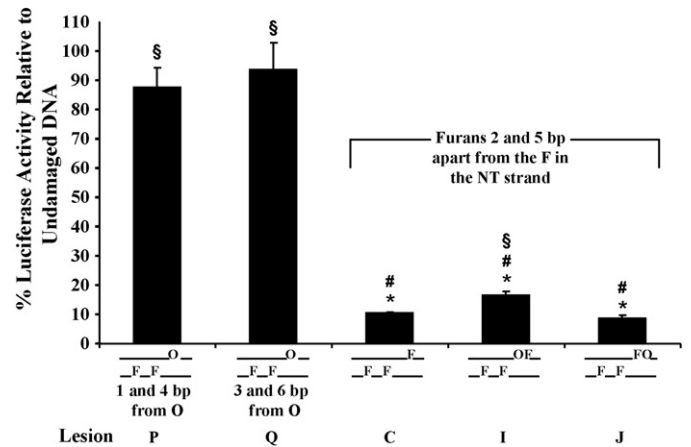


Fig. 2. A complex lesion containing four damages can be cleaved in cells. Cells were transfected with pRL-CMV and ligation reactions containing pCMV3'luc with no damage, or clustered lesions consisting of three furans, an 8oxoG opposite to two furans (Fs), or three furans with an 8oxoG (O). For clusters with two furans and an 8oxoG the number of base pairs separating each furan and 8oxoG is shown. Firefly and renilla luciferase activities were measured from a cell-free extract after 6 h. The firefly/renilla ratio for each construct was expressed as a percentage of the average ratio of the undamaged sequence. At least six transfections of each type of ligation were performed. The average and the standard error are shown graphically. *Statistical difference ($p \leq 0.0002$) compared to lesion P. #Statistical difference ($p \leq 0.0005$) compared to lesion Q. §Statistical difference ($p \leq 0.001$) compared to lesion C.

ferent from either of the two furan lesions (10.4% of the luciferase activity of the undamaged sequence, Fig. 2). Therefore, luciferase activity from pCMV3'luc was highest in the order of lesion A > lesion C > lesion B.

3.2. Effect of a clustered lesion consisting of an 8oxoG and one or two furans

In vitro experiments have demonstrated that an 8oxoG opposite to an AP site can be cleaved by pure enzymes or cell extracts if the AP site is ≥ 3 bp away [37–39]. However, an 8oxoG opposite to a uracil in bacteria (where the uracil is removed by uracil DNA glycosylase to generate an AP site; 25) was not converted to a DSB, even though closely opposed uracils did form a DSB [29]. We have now determined that a furan situated opposite and separated from an 8oxoG by 1, 3, 4 or 6 bps in the reporter construct does not result in a reduction of firefly luciferase activity following transfection into mouse fibroblast cells (Fig. 1), indicating a lack of DSB formation. The positioning of a second furan within these 8oxoG and furan clusters did result in loss of luciferase activity when the second furan was placed in the opposite strand to the first furan. Therefore loss of activity required two opposing furans. When the two opposing furans were situated 2 bps apart, the position of the 8oxoG with respect to the tandem furan, did not alter the luciferase activity in comparison with the cluster containing just the two opposing furans (Fig. 1A, lesions D and E compared to lesion A). However, when the furans were 5 bps apart an 8oxoG immediately 5' to the tandem furan on the same strand did result in a significant increase in luciferase activity (6.8% for lesion B and 10.5% for lesion G, Fig. 1B). This suggests that the 5' tandem 8oxoG decreases DSB formation at the opposing furans. There was also a slight decrease in activity when the 8oxoG was 3' to the furan (lesion H).

After allowing time for repair, we wanted to determine whether luciferase activity correlated with the amount of pCMV3'luc remaining in the cells. Therefore DNA was re-isolated after 2 and 6 h and subjected to Southern analysis. One band at approximately the size of supercoiled DNA was detected for the pCMV3'luc DNA,

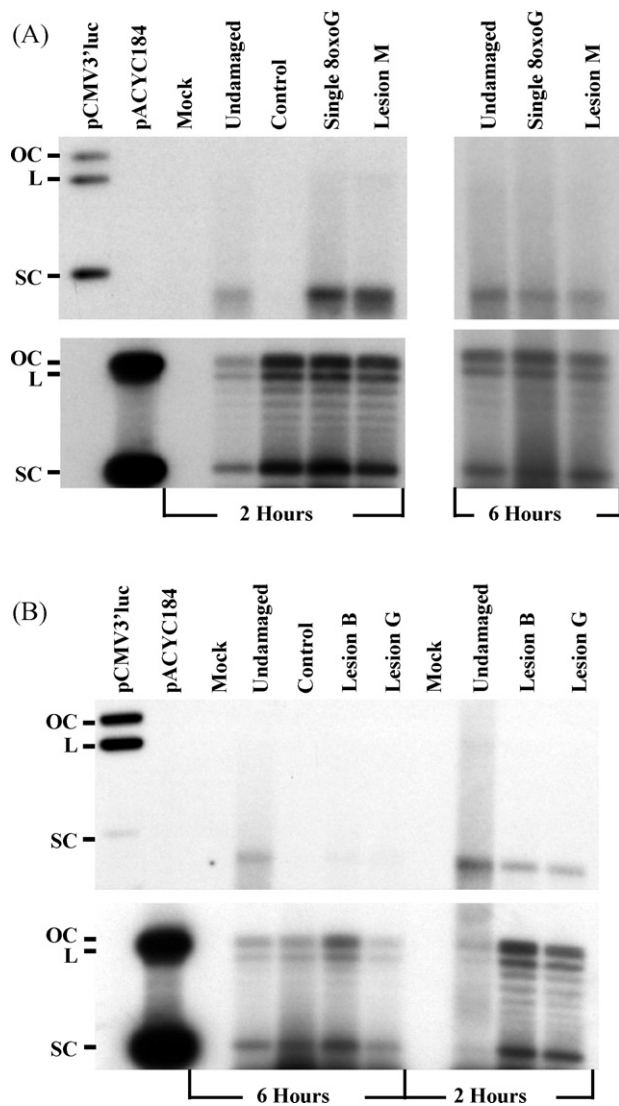


Fig. 3. Southern analysis of re-isolated DNA. DNA was re-isolated 2 and 6 h after transfection and subjected to electrophoresis through a 0.8% agarose gel. The DNA was transferred to a nylon membrane and hybridized with ^{32}P -labeled undamaged oligonucleotides (Table 1) to examine pCMV3'luc (top panel), or a fragment of the *cat* gene to examine pACYC184 (bottom panel), which was co-transfected with the pCMV3'luc ligation. Samples were visualized by autoradiography for DNA originally containing no damage, a single 8oxoG or lesion M (A) or lesions B and G (B). In each case samples were isolated from a mock transfection, where no DNA was added to the cells, and from a transfection of pACYC184 and a control ligation. The control ligation was a ligation that did not contain the Pac I–Cla I double stranded oligonucleotide. The bands corresponding to the supercoiled (SC), open circle (OC) and linear (L) forms of pCMV3'luc and pACYC184 are indicated on the gel.

while bands at open circle, linear and supercoiled DNA were found for the pACYC184 plasmid (Fig. 3). At 2 h, samples for the single 8oxoG and lesion M (an 8oxoG situated opposite to a furan) contained pCMV3'luc at 75 and 100% the level of the undamaged DNA, respectively, when samples were normalized using the signal for pACYC184. By 6 h a decrease was detected in both of these samples: the damaged DNA was reduced to ~40% the level of the undamaged DNA (Fig. 3A). Since a single 8oxoG resulted in a similar loss of DNA as lesion M, it is possible that loss was due to degradation of the plasmid DNA due to persistent SSBs, and not the formation of a DSB at the furan and 8oxoG.

For lesions B and G (Fig. 3B), a substantial decrease in the level of pCMV3'luc was found for both constructs: at 2 h lesions B and G samples were reduced to 20 and 30% the level of undamaged DNA,

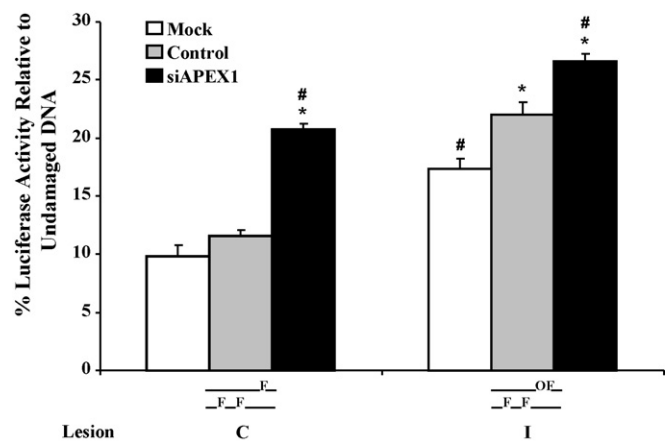


Fig. 4. Apex1 can cleave at complex clustered lesions. Cells were transfected with siRNA designed against APEX1, a scrambled control siRNA (siControl) or no siRNA (Mock) and incubated for 72 h. The cells were then transfected with ligation reactions containing pCMV3'luc with no damage, or clustered lesions consisting of three furans, or three furans and an 8oxoG. Firefly and renilla luciferase activities were measured from a cell-free extract after 6 h. The firefly/renilla ratio for each construct was expressed as a percentage of the average ratio of the undamaged sequence. At least five transfections for each condition and ligation type were performed. The average and the standard error are shown graphically. *Statistical difference ($p < 0.01$) compared to the mock transfection. #Statistical difference ($p < 0.01$) compared to the control siRNA.

respectively, while at 6 h this decreased to 10% for lesion B and 20% for lesion G.

The results for the single 8oxoG and lesions B, G and M do indicate that the luciferase activity does correlate with the surviving pCMV3'luc DNA at 2 h. For lesions B and G, the percentage of the remaining plasmid is not exactly the same as the percentage of luciferase activity compared to the undamaged sequence. This maybe due to DNA sequences being detected by Southern analysis that are deleted or altered in the coding region, resulting in production of inactive luciferase protein.

3.3. Effect of increasing the complexity of the cluster to four lesions

As can be seen from Fig. 2, clusters (lesions P and Q) containing two furans in tandem on one strand and positioned opposite to an 8oxoG did not decrease luciferase activity compared to the undamaged construct, again suggesting a lack of DSB formation. However, when a third furan was introduced, situated in tandem with the 8oxoG and hence 2 and 5 bps apart from the two furans in the opposite strand (lesions I and J), a reduction in luciferase activity was seen. Interestingly, as found in Fig. 1B, the positioning of the 8oxoG immediately 5' to the tandem furan resulted in significantly higher luciferase activity than the cluster containing just the furans (16.5% for lesion I compared to 10.4% for lesion C). This was a 1.5 times increase as found for lesions B and G.

3.4. Effect on firefly luciferase activity by pre-treating cells with siRNA against mouse APEX1 mRNA

Cells were transfected with scrambled siRNA (control), siRNA against APEX1 (siAPEX1) or no siRNA to obtain a mock sample and incubated for 72 h. Our previous experiments have demonstrated that this treatment decreases Apex1 by ~80% [15]. Cells were then re-transfected with ligations containing the undamaged sequence or the clustered lesion. As can be seen from Fig. 4, cells treated with siRNA against APEX1 had significantly higher luciferase activity compared to the mock and control samples: activity was increased by 2.1 and 1.5 times for lesions C and I, respectively, compared

Table 2
PCR analysis of colonies obtained from DNA re-isolated from mammalian cells.

Construct	Total colonies analyzed	Type of PCR product			
		No alteration in size	<50 bp deletion	50–500 bp deletion	>501 bp deletion
Undamaged	226	186 (82%)	23 (10%)	11 (5%)	6 (3%)
Single furan (T strand)	86	52 (61%)	8 (9%)	20 (23%)	6 (7%)
Single 8oxoG (NT strand)	123	86 (70%)	18 (14%)	12 (10%)	7 (6%)
Furan and an 8oxoG, lesion M	132	86 (65%)	15 (11%)	14 (11%)	17 (13%)
Triple furan, lesion C	94	24 (25.5%)	30 (32%)	23 (24.5%)	17 (18%)
Triple furan with an 8oxoG, lesion I	92	25 (27%)	15 (16%)	43 (47%)	9 (10%)

Double stranded oligonucleotides that contained no damage, a single damage or a clustered lesion were ligated to pCMV3' luc between the Pac I–Cla I sites. Single damages were situated on either the transcribed (T) strand or the non-transcribed (NT) strand. Ligations were transfected into 42WT cells and the DNA re-isolated after 6 h. Carbenicillin-resistant colonies were obtained following bacterial transformation of the re-isolated DNA. A small sample of the bacterial colony was resuspended in water and used in a PCR reaction with primers situated outside the Pac I–Cla I region. The PCR product was subjected to electrophoresis and the deletion size determined. In some cases plasmid DNA was isolated and subjected to restriction digestion to determine the size of the deletion (for details see Section 2).

to the mock sample. This implicates Apex1 in DSB formation at complex clustered lesions containing at least two closely opposed furans.

3.5. Examination of re-isolated DNA from cells

To examine the outcome of repair, DNA was re-isolated from cells after 6 h. Samples were analyzed that originally contained a single lesion (a single 8oxoG or a single furan), a double lesion (a furan opposite an 8oxoG, lesion M), a triple lesion (three furans, lesion C) or a cluster consisting of four damages (three furans and an 8oxoG, lesion I). This DNA was compared to the undamaged sequence. Re-isolated DNA was transformed into DH5 α bacteria and carbenicillin-resistant colonies were analyzed using PCR to determine whether repair products contained deletions. The DNA from the undamaged sequence showed that 82% of plasmids analyzed contained the correct size of DNA at the Pac I–Cla I region

(Table 2). This was very similar to that seen in previous experiments [15]. It is likely the remaining products were due to ligation of linear DNA present in the ligation reaction. For a single lesion (a single 8oxoG or a furan) or a double lesion that did not show loss of luciferase activity (lesion M, Fig. 1), 60–70% of the plasmids had the correct size for the Pac I–Cla I region (Table 2). This loss of integrity compared to the undamaged sequence is likely due to the introduction of SSBs at a single lesion, increasing the chance of deletion formation within the plasmid. Southern analysis did detect a decrease in the signal for this region for the single 8oxoG and lesion M at 6 h (Fig. 3). Evidence was also found for cleavage at the 8oxoG when repair products were sequenced (Table 3). The more complex clustered lesions that caused a decrease in luciferase activity (Fig. 2) also caused a greater decrease in plasmid containing the correct sequence size at the Pac I–Cla I region: 25.5% for lesion C and 27% for lesion I (Table 2). Plasmid survival for these constructs was 38% compared to the undamaged construct. Together

Table 3
Sequences of deletions encompassing the damage.

Lesion	Original sequence	Deletion size (bp)	Junction of inaccurate repair	Insertion at junction site	Microhomology region
Single 8oxoG (NT strand)	TTAATTAAATACAAAGGATATCAG O TGGCCCC AATTAATTTATGTTTCCTATAGTCCACCGGGG	22	TTATGG AATACC	–	–
F + O, lesion M	GACCGCTTGAAGTCTTTAAATTAATAACAAAGGATATCAG O TGGCC CTGCGCAACTTCAGAAATTAATTTATGTTTCCTA F AGTCCACCGG	36	GACGTG CTGCAC	–	–
Triple F, lesion C	AAAGGATATCAG F GGCCCCGCTGAATTGG TTTCC TAF AG F CCACCGGGGGACTTAACC	23	AAAATTGG TTTTAACC	–	A
	AAAGGATATCAG F GGCCCCGCTGAATTGGAA T GATAT T G TTTCCTA F AG F CCACCGGGGGCAGCTTAACCTTAGCTATAAC	31	AAAGGATAT T G TTTCCTATAAC	–	GATAT
Triple F + O, lesion I	TTGAAGTCTTTAAATTAATAACAAAGGATATCAG F GGCCCC AAC T TCAGAAATTAATTTATGTTTCCTA F AG F CCTCCGGGG	32	TTG GGG GGCCCC AAC CCC CCGGGG	GGG	–
	AAAGGATATCAG O FGGCCCCGCTGAATTGGAA T CGAT A ATTG TTTCCTA F AG F CCACCGGGGGCAGCTTAACCTTAGCTA T AAC	33	AAAGGAT T G TTTCCTAAC	–	AT
Triple F + O, lesion I	CCGC T TGAAGTCTTTAAATTAATAACAAAGGATATCAG O FGGCCCC GGCGAACTTCAGAAATTAATTTATGTTTCCTA F AG F CCA C CGGGG	35	CCGC GG GGCCCC GGCG C CCGGGG	G	–
	GGA T ATCAG O FGGCCCCGCTGAATTGGAA T CGATA T TGTTTA CCT A FAG F CCACCGGGGGCAGCTTAACCTTAGCTATAAC A AT	36	GGA AATCAG CTTA CCT TTAGTC GAA T	AATCAGC	–

Plasmid DNA was isolated from colonies that were found to contain deletions from the PCR analysis and the DNA was sequenced to identify the junction site. Examples of repair products that contained deletions in the original ligated oligonucleotides are shown above. F is the position of the furan, while O is the position of the 8oxoG in the original sequence. Sequences of microhomology at the junctions are underlined and a dashed line represents the site of the break point in the original sequence. Insertions at the junction are shown in bold and italics.

these results suggest DSB formation did occur at lesions C and I, and caused destruction of the plasmid DNA.

A greater percentage of plasmids with deletions were identified in the re-isolated DNA from lesions C and I compared to the other samples. This indicates that following DSB formation inaccurate repair occurred at the complex lesion. Lesions I and C differ by the addition of an 8oxoG, which was situated immediately 5' to a furan on the NT strand (Table 1). Interestingly, there seems to be a difference in the size of deletions present in the re-isolated DNA for the two clusters. Addition of the 5' 8oxoG in lesion I caused an increase in the amount of products with deletions of 50–500 bp and a decrease in the number with deletions of <50 bps compared to lesion C.

Inaccurate repair products containing small deletions within the Pac I–Cla I region were sequenced. Repair products were identified that contained deletions that encompassed the whole complex clustered lesion (lesions C and I, Table 3). As found in our previous study using a two furan cluster [15], deletions occurred and junctions formed at regions of microhomology, with one copy of the repeat sequence deleted during the ligation of the DNA. One product (Table 3, deletion size 31 bp) was also found in our previous study in DNA re-isolated from wild-type and Ku80^{-/-} cells. The frequency of detection of this product suggests that the location and size of the direct repeat must enhance the chance of ligation at this sequence. Junctions were also identified for lesions C and I that did not contain regions of microhomology, but contained small insertions. These types of junctions were not found in our previous study examining a cluster containing two furans [15]. Interestingly, junctions of repair products for the single 8oxoG and lesion M did not occur at regions of microhomology and did not contain insertions. This suggests a different process was used by the cell to repair these plasmids.

3.6. *In vitro* cleavage assays

To try to understand which damages were being cleaved in the complex clustered lesions in cells, we performed *in vitro* cleavage assays using the 5' ³²P-labeled oligonucleotides and 42WT nuclear extract.

3.6.1. Cleavage at the 8oxoG and furan tandem lesion

The NT strands of lesion G (8oxoG immediately 5' to a furan, Table 1), lesion B (single furan), and lesion M (single 8oxoG) were labeled with ³²P, and each annealed to the undamaged complementary strand. Substrate was incubated with Fpg, hOgg1, hApe1 or nuclear extract. Fpg is known to remove 8oxoG and cleaves on the 3' and 5' side of the AP site leaving a product with a 3' phosphate, whereas hOgg1 results in a DNA with a 3' aldehyde group following beta-elimination. As can be seen from Fig. 5A, Fpg (lane 1) cleaves the single 8oxoG and generates a product with faster mobility than hOgg1 (lanes 3 and 4) as expected. We were unable to detect 8oxoG removal using 200 ng of nuclear extract (lanes 5 and 6) even though the samples were boiled to reveal AP sites that were not cleaved by AP lyases or AP endonucleases in the extract. Previous studies [38] have used 2.5–10 μg of a Chinese hamster nuclear extract to remove 8oxoG from a double stranded oligonucleotide.

The substrate containing the tandem 8oxoG and furan was cleaved by the nuclear extract, as well as by hOgg1 and hApe1 (Fig. 5A). Since the hOgg1 and hApe1 products could not be distinguished by size, we performed reactions in buffer containing either MgCl₂ or 2 mM EDTA. The major AP endonuclease is known to require Mg²⁺ and this was confirmed with hApe1 for substrates with the tandem lesion (lanes 14 and 15) and a single furan (lanes 19 and 20). hOgg1, however, performed equally well under the two reaction conditions both with the substrate containing a single 8oxoG (lanes 3 and 4) and the tandem lesion (lanes 8 and 9).

The nuclear extract required Mg²⁺ to cleave both the tandem lesion (lanes 10–13) and the single furan (lanes 17 and 18). Cleavage of the tandem lesion by the extract furan could not be detected in the absence of Mg²⁺ even when the reaction products were boiled to break any AP sites generated by Ogg1 (lanes 12 and 13). Results from Fig. 5A therefore indicate that it is AP endonuclease activity in the mouse nuclear extract that is able to cleave the tandem lesion and that cleavage is therefore at the furan and not the 8oxoG. From our work with siRNA (Fig. 4), Apex1 is likely to be the enzyme cleaving the tandem lesion.

A time course using 200 ng nuclear extract with the single furan and the tandem lesion substrates was performed and this revealed that although the enzyme in the extract could cleave both substrates, cleavage occurred more readily and at a faster rate at a single furan than at a furan situated immediately downstream and in tandem with an 8oxoG (Fig. 5B). Reactions were therefore repeated using levels of enzyme or extract that were below that needed for maximum substrate cleavage. Interestingly, hOgg1 cleaved the tandem lesion greater than the single 8oxoG (Fig. 5C), while hApe1 and the nuclear extract cleaved the single furan greater than the tandem substrate. This provides further evidence that Apex1 is the enzyme in the extract cleaving the tandem lesion.

3.6.2. Cleavage at the furans in lesion C

The structure of lesion C is seen in Fig. 6A. It was possible that cutting at F₂ and F₃ could occur and so inhibit F₁ cleavage, or that a DSB could occur by cleavage at F₁ and F₃, or F₁ and F₂. In order to determine the order of cleavage, two substrates were prepared using the oligonucleotides in lesion C: one was labeled on the 5' terminus of the NT strand (Fig. 6B) and the other was labeled on the 5' terminus of the T strand (Fig. 6C). It is evident that the F₁ lesion within lesion C could be cleaved by purified endonuclease IV or hApe1 but was cleaved at a lower frequency by the nuclear extract (Fig. 6B). The nuclear extract could cleave at the F₁ lesion much more efficiently when the F₁ furan was annealed with undamaged DNA. The presence of the opposing furans therefore reduced cleavage at F₁.

In Fig. 6C, lanes 1, 2, 11 and 12 contained the single furans annealed to undamaged DNA and these were used as markers for the cleavage products. When the strand containing F₂ and F₃ was annealed to undamaged DNA, the predominant product seen after reaction with endonuclease IV (lane 3) and hApe1 (lane 13) was the F₂ product. Even if the enzyme was able to cleave both products at the same time, the F₂ would be the detected product as this was the shorter product. Products in lanes 4 and 14 therefore indicate that endonuclease IV and hApe1 cleave at F₃ preferentially when the strand is annealed to an NT strand containing F₁. A similar result was found when the substrate was incubated with nuclear extract: cleavage was detected at F₃ (lanes 6–8 and lanes 16–18) but not substantially at F₂ when the opposing strand contained F₁, and at F₂ (lane 10) when the opposing strand did not contain damage. Increasing the nuclear extract from 100 ng (lanes 6–8) to 200 ng (lanes 16–18) did not result in increased cleavage at F₂. The higher level of extract only seemed to degrade the F₃ product by 1 nucleotide. These results indicate that DSB formation in cells could preferentially occur by cleavage of F₁ and F₃, potentially resulting in a DSB with a 3' overhang containing F₂.

4. Discussion

When considering the repair of a complex non-DSB cluster, the following questions need to be addressed: firstly can the cluster be converted to a complex DSB, secondly can base excision repair remove the damage near the break termini and thirdly can

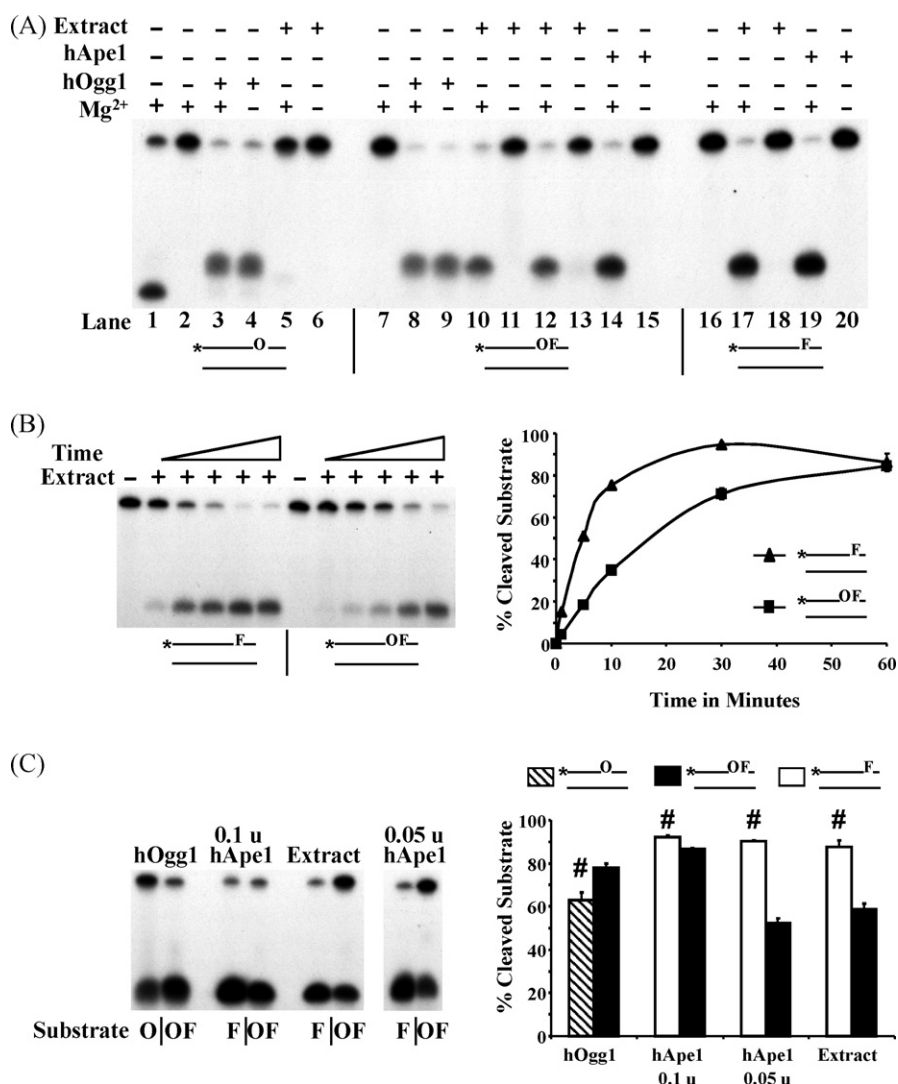


Fig. 5. An 8oxoG situated immediately 5' to a furan decreases cleavage at the furan *in vitro*. Oligonucleotides containing a single 8oxoG (O), a single furan (F) or an 8oxoG and a furan (OF) were labeled on the 5' terminus with ³²P (*). The oligonucleotides were annealed to an undamaged complementary strand and 2 nM DNA substrate was incubated with Fpg (0.5 units, lane 1), hApe1 (0.2 units), hOgg1 (0.5 units) and nuclear extract (200 ng) at 37 °C for 60 min in buffer containing MgCl₂ (Mg²⁺) or 2 mM EDTA (A). Samples in lanes 2–6, 8 and 9, and 12 and 13 were boiled before electrophoresis. In B, substrate was incubated with 200 ng nuclear extract for 1–60 min. Reactions were performed in triplicate. The average and standard deviation for the amount of cleavage is shown graphically. In C, substrate was incubated with 0.25 units hOgg1, 0.05 and 0.1 units of hApe1, or 100 ng nuclear extract for 60 min. Five reactions were performed and the average and standard error for the amount of cleavage is shown graphically. #Statistical difference (*p* < 0.05) compared to the tandem lesion (OF).

DSB repair mechanisms rejoin the “dirty” termini? This work has predominantly tried to answer the first question: can complex non-DSB clustered lesions be converted to complex DSBs in mammalian cells?

We have examined clustered lesions consisting of furans (a stable AP site analog) and 8oxoG. Previously we established that two opposing furans could be converted to a DSB in mouse cells by Apex1 [15], even though two closely opposed uracils did not form DSBs in HeLa cells [31]. The requirement for long patch repair at the furan lesions versus short patch repair at the uracils may contribute to DSB formation at the opposing furans following cleavage by Apex1. Future work is planned to determine the difference between the clustered uracils and the clustered furans. In this study, we have utilized the DSB formation at the opposing furans to determine whether Apex1 is able to cleave more complex lesions. Paap et al. [34] have shown *in vitro* that pure hApe1 is inhibited by positioning two abasic sites on one DNA strand and 5' to a third AP site on the opposite strand. We examined cleavage in cells of a cluster

with a similar positioning of furans (Fig. 6A and Table 1). Interestingly, the cluster with the three furans (lesion C) did not result in as great a reduction in luciferase activity as the two furans separated by 5 bps (lesion B), suggesting that the F₂ furan (Fig. 6A) positioned between F₁ and F₃ could have inhibited DSB formation. It was also possible that cleavage at F₂ and F₃ could have generated a gap in the DNA strand that also inhibited cleavage at F₁ and prevented DSB formation. Since it was not possible to determine the order of lesion repair in cells, we examined cleavage *in vitro* with a nuclear extract. It was very clear that the enzymes in the extract more readily incised the DNA at the 5' side of F₃ > F₁ > F₂. This was not due to a preference for a particular furan due to surrounding sequence context, at least for F₂ and F₃, as the extract readily cleaved F₂ when F₁ was not present in the opposite strand. Work *in vitro* and in cells therefore indicates that when DSBs formed in cells from lesion C cleavage likely occurred at F₁ and F₃, which were positioned 5 bps apart. By using siRNA against APEX1, we confirmed that Apex1 was the enzyme generating the DSB at the complex cluster in cells. Pre-

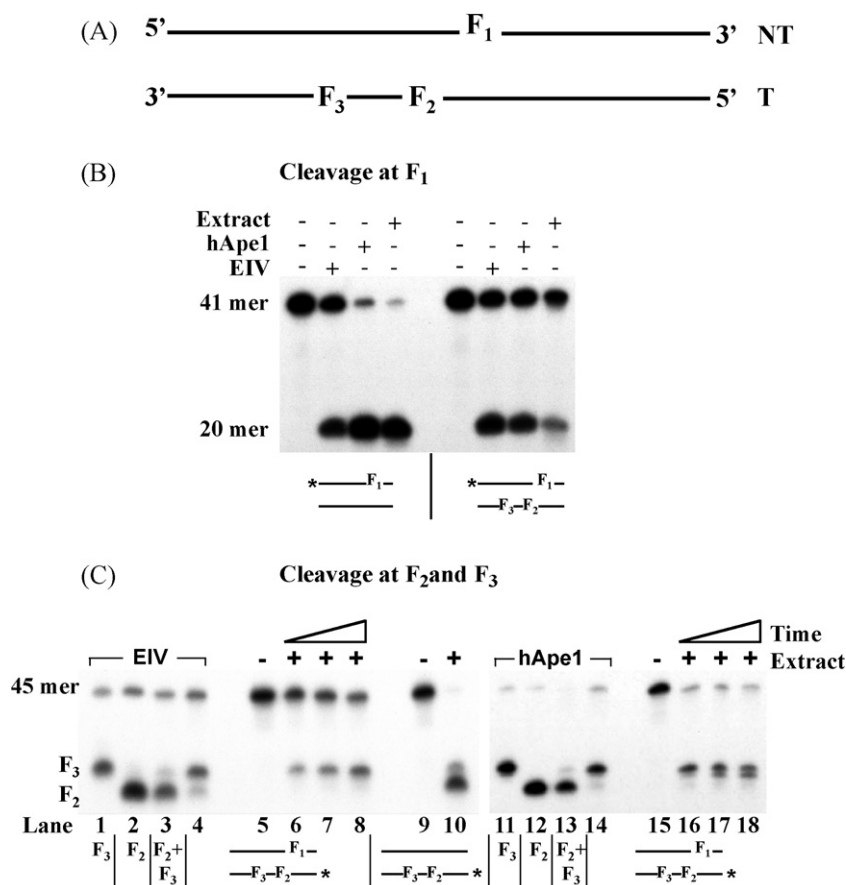


Fig. 6. Removal of furans from a clustered lesion *in vitro*. Lesion C (A) was prepared from two oligonucleotides. These oligonucleotides were annealed after one of the strands was labeled on the 5' terminus with ³²P (*): the NT strand was labeled to detect cleavage of F₁ and the T strand was labeled to detect cleavage at F₂ and F₃. Oligonucleotides containing each of the single lesions or F₂ and F₃ on the T strand were annealed to an undamaged strand as controls. Reactions containing 2 nM substrate and 0.03 units endonuclease IV (EIV), 0.2 units hApe1 or nuclear extract were performed at 37 °C for 60 min. Cleavage at F₁ with the pure enzymes or 200 ng nuclear extract is shown in B. Cleavage at F₂ and F₃ is shown in C. Lanes 1–3 with EIV and lanes 11–13 with hApe1 show reactions containing substrates of oligonucleotides with F₃, F₂ and F₂ + F₃ annealed to an undamaged strand and reacted with 100 ng nuclear extract. Reactions with lesion C contained 100 ng nuclear extract (lanes 6–8) or 200 ng nuclear extract (lanes 16–18) and were performed for 20–60 min.

vious studies have shown that hApe1 requires double stranded DNA of at least 4 bps on the 5' side and 3 bps on the 3' side of the lesion [40]. It is possible that the structure of this three furan lesion is disrupted by F₂ within the 4 bps 5' to F₁, so compromising the ability of Apex1 to cleave at F₁ and decreasing DSB formation. This work is in agreement with the *in vitro* studies using hApe1 and three AP site clusters [34]. However, this cluster (lesion C) appears to have been more readily converted to a DSB than those studied *in vitro* by Paap et al. [34]. The difference maybe the positioning of the opposing furans. The F₂ in our study is separated by 2 bps from the F₁. The equivalent AP site in the Paap et al. [34] study was immediately 5' to the opposing AP site, which has been shown to greatly inhibit hApe1.

8oxoG is a stable lesion produced by ionizing radiation and it is predominantly removed from DNA by Ogg1 in mammalian cells. We first examined a cluster consisting of an 8oxoG closely opposed to a furan (lesion M). Results from the luciferase reporter assay, Southern analysis and PCR analysis of re-isolated DNA indicate that this type of clustered lesion with a base damage and an opposing furan was not converted to a DSB in mammalian cells. This therefore is similar to the situation in bacteria, where a clustered lesion of an 8oxoG opposite a uracil is not converted to a DSB [25], and to yeast, where complex clustered lesions that contained 3 or 4 base damages and a nucleotide gap were not converted to DSBs [30]. Our *in vitro* studies using an 8oxoG-containing oligonu-

cleotide and nuclear extract did not detect removal of the 8oxoG under conditions where the furan lesions were readily cleaved. David-Cordonnier et al. [38] also previously noted that the XRS5 nuclear extract was more efficient at removal of an AP site than an 8oxoG. It is therefore likely in the mouse cells that the expression level of Apex1 and high reactivity with the furan caused removal of the furan, and probably repair, prior to removal of the 8oxoG in the opposite strand. Sequencing of repair products from constructs that had contained a single 8oxoG or a furan opposite an 8oxoG did show deletions occurring at the 8oxoG (Table 3). This does suggest that given time (6 h) cleavage did occur at the 8oxoG. Eot-Houillier et al. [32] did demonstrate that different types of base damage were processed *in vitro* by human cell extracts at different rates and suggested that this could prevent DSB formation in cells.

To determine whether an 8oxoG could inhibit DSB formation at a cluster containing two or three furans, clusters were generated with an 8oxoG positioned immediately 5' or 3' to a tandem furan and in opposition to one or two furans. Work in cells determined that the 8oxoG did not alter DSB formation if the two opposing furans were 2 bps apart (lesions D and E, Fig. 1A). However, when the opposing furans were 5 bps apart (Fig. 1B) or when the cluster contained three furans (Fig. 2), an 8oxoG situated 5' to a tandem furan caused a 1.5 times increase in luciferase activity. This suggested a small decrease in DSB formation. We performed *in vitro* cleavage assays comparing the ability of nuclear extract to cleave

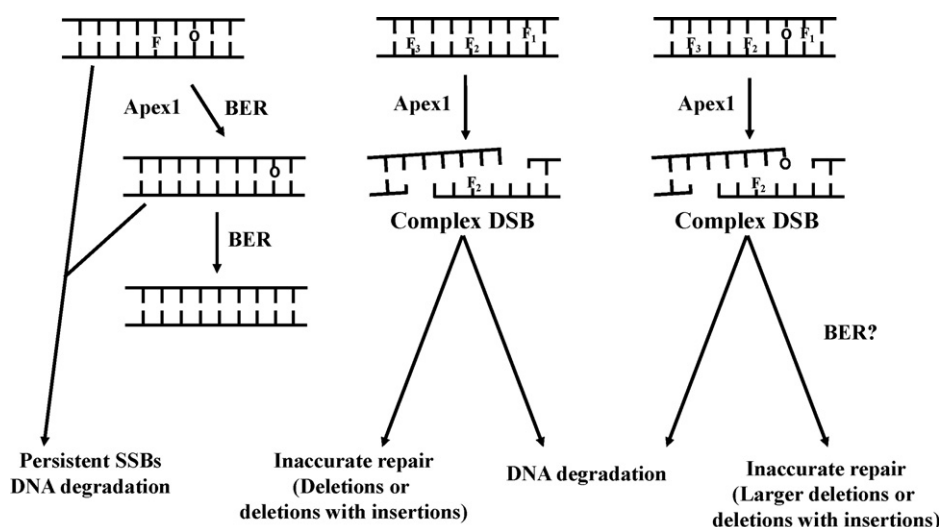


Fig. 7. Repair of complex clustered lesions in cells. A clustered lesion consisting of a furan (F) and an 8oxoG (O) does not form a double strand break in cells. This was not influenced by the distance separating the lesions. It is likely that the furan is removed more rapidly by Apex1 and the strand break completely repaired by base excision repair (BER), prior to removal of the 8oxoG. Persistent SSBs following BER at the single lesions may cause DNA degradation. A complex lesion consisting of three furans can also be converted to a double strand break (DSB) by Apex1, although not as readily as two opposing furans. The most probable DSB is generated by the removal of F₁ and F₃. It is possible that some accurate repair occurs at the break. However, DNA degradation and inaccurate repair do occur resulting in loss of plasmid DNA or repair products containing deletions, or deletions with small insertions. The situation of an 8oxoG 5' to F₁ still results in DSB formation and inaccurate repair products contain larger deletions, suggesting NHEJ does not repair complex DSBs as efficiently when an 8oxoG is situated at the 3' terminus of one of the breaks.

at a single furan or at a furan with a tandem 8oxoG situated immediately 5' to it. This confirmed that the presence of the 8oxoG decreased cleavage at the furan. A similar result was found for hApe1. Previous studies using purified exonuclease III (the bacterial equivalent of Apex1) have demonstrated that cleavage at an AP site is compromised by a tandem 8oxoG situated immediately 5', but not 3', to the AP site [41]. Our work suggests that Apex1 also has reduced activity on an AP site situated immediately downstream in tandem with an 8oxoG, as siRNA studies demonstrated that Apex1 cleaves lesion I. Since we determined *in vitro* that the three furan cluster was more likely to form a DSB by cleavage at the two furans separated by 5 bps, and that the 8oxoG did not reduce cleavage of two opposing furans two bps apart (lesion D), this work suggests that in cells the formation of a SSB 2 bps away from an opposing furan is more inhibitory to cleavage at the remaining furan than a tandem 5' 8oxoG.

Work presented here indicates that Apex1 can cleave at complex clusters containing two furans/AP sites and 8oxoG and create a complex DSB in cells. Previous studies *in vitro* have shown that Ogg1 has reduced capacity for removing an 8oxoG situated near a 3' terminus and there is the potential for the Ogg1 binding to the 8oxoG and delaying the repair of the nearby strand break [42]. In fact, Neil1 [43] and Ape1 [44] have been implicated in the removal of 8oxoG at the 3' end of a SSB. A complication to the repair of the terminal 8oxoG or F₂ in our study is that these damages are situated in 3' overhang sequences once the DSB has formed. The lack of double stranded DNA would inhibit the majority of base excision repair enzymes. Plasmid DNA containing lesion I or C did show reduced plasmid survival compared to the undamaged DNA, indicating degradation of some linearized plasmid in the cell. Inaccurate repair of the DSBs did occur as deletions were found in re-isolated DNA. In fact, the clustered lesion containing the 8oxoG and three furans (lesion I) resulted in repair products with larger deletions than the cluster that contained three furans (Table 2). One possible explanation could be that DSB repair may be less efficient or may invoke further processing of 3' termini with an 8oxoG. Work *in vitro* suggests that 8oxoG near a DSB can decrease DNA ligation [45]. Examination of the junctions of inaccurately repaired plasmids also revealed evidence of joining at regions of microho-

mology with one copy of the direct repeat deleted during ligation. Interestingly, we also found products that had junctions where there was no evidence of a direct repeat but there was evidence of insertions at the site. This was common to the lesions containing the three furans and therefore was not related to the terminal 8oxoG at the DSB. Our work indicates that the F₂ will be present on the 3' overhang of the DSB of a three furan cluster. The insertions maybe related to the F₂, since no such insertions were previously found at repair junctions generated from two furan clusters [15].

In summary (Fig. 7), a clustered lesion consisting of an 8oxoG and a furan does not form a DSB in mammalian cells. Plasmid DNA may be degraded by persistent SSBs introduced at the individual lesions, or may be completely repaired. However, two closely opposed furans can be converted to a DSB by Apex1, even when there is further base damage and furans situated within the lesion. When three furans form a cluster, Apex1 can introduce a DSB by cleaving two of the furans. In the case of the cluster we examined, this results in cleavage at F₁ and F₃ generating a DSB with a furan on the 3' overhang. The addition of the third furan does reduce DSB formation, but does not completely inhibit breakage. The positioning of an 8oxoG in tandem with a furan can also reduce cleavage at the furan if it is situated immediately 5' to the 8oxoG. Apex1 incises the DNA prior to the removal of 8oxoG and so this suggests that a four lesion cluster consisting of three furans and an 8oxoG can be converted to a complex DSB that has an 8oxoG and a furan on the 3' overhangs. Such complex DSBs in our system are either degraded or are inaccurately repaired resulting in deletions and insertions. The presence of the 8oxoG on the 3' overhang may result in processing of the termini that creates larger deletions.

We have therefore provided the first evidence that complex DSBs can be generated in mammalian cells from clustered base damage and AP sites. However, to fully appreciate how cytotoxic these lesions are further studies need to examine how complex DSBs are processed by DSB repair. We previously implicated NHEJ in the repair of clustered lesions, and further work *in vitro* and in cells is required to understand how NHEJ processes these complex DSBs.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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