

# DNA repair of clustered lesions in mammalian cells: involvement of non-homologous end-joining

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## ABSTRACT

**Clustered lesions are defined as  $\geq$ two lesions within 20 bps and are generated in DNA by ionizing radiation. *In vitro* studies and work in bacteria have shown that attempted repair of two closely opposed lesions can result in the formation of double strand breaks (DSBs). Since mammalian cells can repair DSBs by non-homologous end-joining (NHEJ), we hypothesized that NHEJ would repair DSBs formed during the removal of clustered tetrahydrofurans (furans). However, two opposing furans situated 2, 5 or 12 bps apart in a firefly luciferase reporter plasmid caused a decrease in luciferase activity in wild-type, Ku80 or DNA-PKcs-deficient cells, indicating the generation of DSBs. Loss of luciferase activity was maximal at 5 bps apart and studies using siRNA implicate the major AP endonuclease in the initial cleavage. Since NHEJ-deficient cells had equivalent luciferase activity to their isogenic wild-type cells, NHEJ was not involved in accurate repair of clustered lesions. However, quantitation and examination of re-isolated DNA showed that damage-containing plasmids were inaccurately repaired by Ku80-dependent, as well as Ku80-independent mechanisms. This work indicates that not even NHEJ can completely prevent the conversion of clustered lesions to potentially lethal DSBs, so demonstrating the biological relevance of ionizing radiation-induced clustered damage.**

## INTRODUCTION

Reactive oxygen species generate a variety of DNA damage in cells that includes oxidative base damage, abasic (AP) sites and single strand breaks (SSBs). Ionizing radiation introduces similar types of damages, but due to the production of low energy electrons, clusters of ionizations can result in  $\geq 2$  lesions within 20 bps (1). These are called clustered lesions; the most well-known of which is a double strand break that can be formed from

two closely opposed SSBs. The yields of ionizing radiation damage are in the order of base damage  $>$ SSBs  $>$ DSBs (2) and hence  $\sim 80\%$  of  $\gamma$ -ray-induced clustered lesions contain oxidized base damage, while only 20% are DSBs (3). AP sites also exist in clusters generated by ionizing radiation: a ratio of 1 DSB to 1.5 AP site clusters has been measured following  $\gamma$ -irradiation of DNA (4), and heat-labile sites that can be converted to DSBs have been detected in mammalian cells post-irradiation (5). AP sites are heat-labile and so this again suggests that closely opposed AP sites are generated in cells by ionizing radiation and are biologically relevant lesions.

Previous studies *in vitro* have demonstrated that clustered lesions containing base damage and AP sites in opposite strands can be partially processed by the base excision repair enzymes to generate DSBs (6,7) and it has been hypothesized that these clustered lesions maybe biologically highly significant due to the inability of cellular enzymes to completely repair the clustered lesions (8,9). In fact, complete repair of base damage and SSBs within a cluster can be inhibited or occur at a reduced rate *in vitro* due to closely opposed lesions (10–18).

The DSB generated from a partially processed clustered lesion can be repaired by two main pathways in mammalian cells: homologous recombination and non-homologous end-joining (NHEJ; 19–21). Homologous recombination predominantly occurs in the S and G2 phases of the cell cycle and uses the sister chromatid or homologous chromosome to perform accurate repair, while NHEJ occurs in all phases of the cell cycle and can be error-prone. During NHEJ, the Ku70 and Ku80 proteins form a heterodimer which binds to the DNA ends. This then attracts the DNA protein kinase catalytic subunit (DNA-PKcs) to the termini, activating the protein kinase. Incompatible termini are processed by accessory proteins and the compatible ends are joined by the DNA ligase IV/XRCC4 complex, which is stimulated by the XLF/Cernunnos protein. In the absence of NHEJ, microhomology-mediated end-joining (MMEJ) can occur, which involves joining DNA termini at regions of microhomology that flank the DSB with one copy of the homologous sequence deleted. Ku80 has also been implicated in this type of repair (22). Single strand annealing (SSA)

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produces a similar product to MMEJ and is a subset of homologous recombination that is error prone. Even though the products of repair are similar, SSA and MMEJ use different proteins and mechanisms of repair.

In this work, we have examined the repair of clusters consisting of two tetrahydrofurans (furans) in opposite DNA strands in mouse cells. A furan is a stable AP site analog and so this is a biologically relevant clustered lesion. Furans can be cleaved on the 5' side by class II AP endonucleases (23), but unlike an AP site, furans cannot undergo  $\beta$ -elimination at the 3' side of the lesion (24). Hence AP lyases are unable to cleave at a furan, and short patch base excision repair cannot proceed as polymerase  $\beta$  is unable to remove the 5'-blocked terminus generated by a class II AP endonuclease. Ape1 (or Apex1 in mouse cells) is therefore the likely enzyme to initiate repair of a furan in cells and long patch repair would be required to complete repair (25). *In vitro* studies have demonstrated that human Ape1 can cleave closely opposed AP sites, but the extent of cleavage is determined by the positioning of the two lesions in the cluster. Ape1 is severely inhibited when an opposing AP site or base damage is situated within 3 bps, especially when the second lesion is positioned 5' to the AP site (10,12,16). Long patch base excision repair requires DNA polymerase  $\delta/\epsilon$  to extend the DNA, displacing the opposite strand that contains the 5'-blocking lesion. Fen1 then removes the 'flap' structure and DNA ligase I seals the 'nick' completing repair. Other factors such as replication factor C and proliferating cell nuclear antigen are also required for this process. Previous *in vitro* studies have shown that both short patch (14,15) and long patch repair of a clustered lesion can be inhibited by closely opposed lesions (13). This is dependent on the orientation of the lesions: Budworth *et al.* (13) demonstrated that strand displacement and flap removal were inhibited during the repair of an AP site if an 8-oxo-7,8 dihydroguanine was situated in the opposite strand 3' to the incised AP site. We have therefore positioned the furans 5' to each other in our system so the strand displacement from either furan would not encounter a lesion in the DNA. Hence, we would not expect to inhibit long patch repair.

Previous studies of defined clustered lesions in cells have been limited and the majority of work has used pure enzymes or cell-free-extracts. In bacteria, we demonstrated that two opposing uracils (which are converted to AP sites by uracil DNA glycosylase) or furans situated  $\leq 7$  bps apart were converted to a DSB (26,27). However, two closely opposed uracils did not result in a DSB in HeLa cells (28). Two processes could have prohibited the detection of DSBs in the HeLa cells: firstly, limited uracil removal could have prevented the generation of clustered AP sites so eliminating the chance of producing DSBs, and secondly, DSBs may have been repaired in the cells. We therefore chose to examine furans to eliminate the step of uracil removal.

The aim of this study was to determine whether Apex1 could cleave two closely opposed furans in cells, forming a potentially lethal DSB that could escape NHEJ. NHEJ is the likely relevant DSB repair pathway for a non-replicating plasmid entering a non-synchronized cell population by transfection. We positioned two furans 5' to each other 2, 5 or 12 bps apart within the firefly luciferase coding region of a plasmid. Formation of a DSB and degradation or mis-repair of the plasmid results in a decrease in luciferase activity. Repair was examined in mouse fibroblasts, which allowed the use of isogenic cell lines that were wild-type or deficient in NHEJ proteins. We have determined that two closely opposed furans can be cleaved in cells and a fraction of the lesions avoid DSB repair. We provide evidence that a defined clustered lesion in a plasmid in mammalian cells can be inaccurately repaired by NHEJ. This therefore suggests that clustered lesions converted to a DSB in chromosomal DNA could also be inaccurately repaired by NHEJ.

## MATERIALS AND METHODS

### Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (oligonucleotides) were purchased from Operon Technologies Inc. (Alameda, CA, USA). The sequences of the double-stranded oligonucleotides containing no damage or two tetrahydrofurans (known as a d-spacer from Operon Technologies) are shown in Table 1. These oligonucleotides contained a 5' phosphate and were purified using polyacrylamide gel

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

Position of two damages relative to each other (base pairs apart)	Sequence		
Undamaged	5'	TAAATACAAAGGATATCAGGTGGCCCCCGCTGAATTGGAAT	3'
	3'	TAATTTATGTTTCCTATAGTCCACCGGGGGCGACTTAACCTTAGC	5'
2	5'	TAAATACAAAGGATATCAGGF <sup>F</sup> GGCCCCCGCTGAATTGGAAT	3'
	3'	TAATTTATGTTTCCTATAG <sup>F</sup> CCACCGGGGGCGACTTAACCTTAGC	5'
5	5'	TAAATACAAAGGATATCAGGF <sup>F</sup> GGCCCCCGCTGAATTGGAAT	3'
	3'	TAATTTATGTTTCCTA <sup>F</sup> AGTCCACCGGGGGCGACTTAACCTTAGC	5'
12	5'	TAAATACAAAGGATATCAGGF <sup>F</sup> GGCCCCCGCTGAATTGGAAT	3'
	3'	TAATTTATG <sup>F</sup> TTCCTATAGTCCACCGGGGGCGACTTAACCTTAGC	5'

**F** designates the position of a furan in a damage-containing oligonucleotide. Two oligonucleotides were annealed to form a clustered lesion. Clustered lesions examined contained two furans in opposing DNA strands situated 5' to each other. The distances (in bps) separating the damages are shown.

electrophoresis by Operon Technologies. *In vitro* assays did confirm that the furan in each oligonucleotide could be cleaved by endonuclease IV, but not Fpg, and that the oligonucleotide solution did not contain shorter synthesis products. A furan is an abasic site analog and was chosen because it has greater stability than a natural abasic site.

To prepare double-stranded molecules, equimolar amounts of complementary oligonucleotides were incubated in 10 mM Tris HCl (pH 7.5), 50 mM NaCl at 85°C for 5 min, prior to cooling the DNA to <30°C during a 1 h time period.

Oligonucleotides purchased for PCR or sequence analysis were not purified and did not contain 5' phosphate termini. Three primer sets were used to amplify the luciferase region: Luc1 d(TGGATGGCTACATTCTG) and R d(TCATCGTCTTTCCGTGCT), Luc3 d(ATGTGG ATTTTCGAGTCGTCTT) and Luc10 d(TGGCCACGAA TTCCACGATCTCTTTTCCG), Luc3 and Luc5 d(GC CTGGTATCTTTATAG). Sequence analysis was performed using primer R and Luc1.

To create a version of pCMV3'luc with an insert at the Pac I site (pCMV3'luc inactive), oligonucleotides containing 5' phosphates were annealed and ligated into the Pac I site: Inactive1 d(TAAATAACCTCAGCACCGCGGAA GCGGCCGCAAGCTAGCACCCGGGTGCATTTCA AT) and Inactive2 d(TGAAATGCACCCGGGTGCTA GCTTGC GGCCGCTTCCGCGGTGCTGAGGTTATT TAAT).

### Plasmids

pACYC184 (New England Biolabs, Beverly, MA, USA) is a low copy vector with a p15A origin of replication that encodes resistance to chloramphenicol (34 µg/ml). pRL-CMV (Promega, Madison, WI, USA) expresses renilla luciferase in mammalian cells and encodes carbenicillin resistance. pCMV3'luc (28) is a derivative of p3'luc (26) and expresses firefly luciferase from a CMV promoter and encodes carbenicillin resistance. Pac I and Cla I are unique restriction sites in the 3' end of the luciferase coding region that allow insertion of a 45 bp double-stranded oligonucleotide (Table 1). A double-stranded oligonucleotide prepared from Inactive 1 and 2 (see above) was ligated into the Pac I site to eliminate potential background luciferase activity from the re-circularization of partially digested pCMV3'luc. The sequence was designed to destroy the downstream Pac I site, so that digestion with Pac I and Cla I removed a 98 bp insert. The orientation of the insert was confirmed by sequencing. The resultant plasmid was called pCMV3'luc inactive and was used to generate the linear vector DNA used in the ligations with oligonucleotides in Table 1 (see below). Pac I and Cla I digestion and then insertion of the oligonucleotides in Table 1, regenerates the pCMV3'luc sequence and expression of firefly luciferase.

### Cell lines

Spontaneously immortalized mouse fibroblast cell lines that were wild-type (42WT and PK34N, called WT1 and WT2, respectively, in this article), Ku80<sup>-/-</sup> or DNA-PKcs<sup>-/-</sup> (PK33N; 29) were kindly provided by Dr D. Chen (University of Texas Southwestern Medical Center, Dallas,

TX, USA). WT1 and Ku80<sup>-/-</sup> were isogenic, as well as WT2 and DNA-PKcs<sup>-/-</sup>. Cells were maintained in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) (HyClone, Logan, UT, USA) containing 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin (GIBCO Invitrogen Corporation, Carlsbad, CA, USA) in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>.

### Insertion of double-stranded undamaged or furan-containing oligonucleotides into pCMV3'luc

This was performed according to Malyarchuk and Harrison (28) with modifications. pCMV3'luc inactive was digested using Pac I and Cla I restriction enzymes, subjected to electrophoresis through a 0.8% agarose gel and the linear plasmid isolated from the gel using the Qiaquick Gel Extraction kit (Qiagen Inc., Valencia, CA, USA). The concentration of the linear DNA was quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA). 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 MgCl<sub>2</sub>, 5 mM dithiothreitol, 2.5% polyethylene glycol and 15 units of T4 DNA ligase (Promega Corporation, Madison, WI, USA) were incubated overnight at 4°C. The salts were then removed using the Qiaquick Nucleotide Removal kit (Qiagen Inc., Valencia, CA, USA) 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 obtain one sample of ligation product. In order to have three different samples of ligation products for each type of insert, the procedure was performed three times using three different annealing reactions. A similar sample of DNA was also generated as a control from ligation reactions that did not contain oligonucleotide (control ligation).

### Introduction of DNA into the mouse fibroblasts

Prior to transfection, cells were plated so that they would reach a similar level of confluency (60–70%) in 42 h. A cell-free extract was prepared at the time of each transfection to perform western analysis for Apex1 as described below. To transfect DNA into the cells we used the Nucleofector<sup>TM</sup> (AMAXA Biosystems, Gaithersburg, MD, USA). Ligation products of 10 µl (~1.8 µg of DNA), unless otherwise stated, and 10 ng of pRL-CMV or 100 ng of pACYC184 were co-transfected into 2–3 × 10<sup>6</sup> mouse fibroblast cells using program T-20 and the MEF Nucleofector<sup>TM</sup> Kit 2, according to the manufacturer's instructions. pRL-CMV was used when cell-free extracts were prepared to measure firefly and renilla luciferase activities, while pACYC184 was used for experiments where the DNA was re-isolated from the cells.

### Measurement of firefly and renilla luciferase activities

Cells were nucleofected with DNA and incubated for 6 h at 37°C and 5% CO<sub>2</sub>. Cell-free extracts were prepared by scraping the cells from the dish in 1X Passive Lysis Buffer (PLB; Promega, Madison, WI, USA). A 20 µl of the extract was used to measure firefly and renilla luciferase activities using the Dual-Luciferase Reporter Assay

System (Promega, Madison, WI, USA) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) as described by the manufacturers. The results were expressed as a ratio of firefly luciferase/renilla activity. An average ratio was obtained in each experiment 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 six 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. To determine statistical differences between different types of constructs and the same construct from different cell lines, the data was analyzed using the InStat3 program and the unpaired *t*-test with Welch correction or the Mann-Whitney test. A control ligation was performed for each experiment and negligible firefly luciferase activity was measured in all cell lines.

#### Plasmid survival assay and analysis of re-isolated DNA

Three different ligation reactions for each type of construct were nucleofected in duplicate into the cells with 100 ng pACYC184. After 6 h at 37°C and 5% CO<sub>2</sub>, plasmid DNA was re-isolated using the E.Z.N.A Plasmid Mini Kit I (OMEGA Bio-Tek Inc., Doraville, GA, USA) and DNA from the duplicate transfections were pooled to form a sample. Each DNA sample was then transformed in duplicate into Max efficiency DH5 $\alpha$ <sup>TM</sup> chemically competent cells (Invitrogen Corporation, Carlsbad, CA, USA) and allowed to grow for 1 h. Cultures were then plated in triplicate on solid medium containing either 100  $\mu$ g/ml carbenicillin or 34  $\mu$ g/ml chloramphenicol. After overnight growth, colonies were counted and a ratio of the carbenicillin-resistant (Carb<sup>R</sup>) colonies/chloramphenicol-resistant (Cm<sup>R</sup>) colonies calculated for each transformation. The pACYC184 plasmid therefore allowed for normalization of the transfection efficiency, the re-isolation of the DNA and the transformation into the bacteria. The ratios were used to calculate the percentage of plasmid survival for each type of ligation compared to the undamaged sequence. To determine statistical differences between different types of constructs and the same construct from different cell lines, the data was analyzed using the InStat3 program and the unpaired *t*-test with Welch correction.

To examine the products of repair, colonies were re-grown individually on solid medium containing carbenicillin. A small amount of the bacterial colony was resuspended in 20  $\mu$ l water and 5  $\mu$ l of bacteria was used in a PCR reaction with LucI and R primers to determine the presence and size of deletions or insertions within the PacI-Cla I region. PCR reactions were performed using 40 nM of each primer in a 25  $\mu$ l reaction containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl<sub>2</sub>, 200  $\mu$ M dGTP, dCTP, dATP and dTTP and 0.5 units of Taq DNA polymerase

(Promega, Madison, WI, USA). The reactions were performed for thirty cycles using an annealing temperature of 55°C. 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 predominantly found in the control ligations and so were eliminated from the analysis.

Plasmid DNA containing either deletions, insertions or that did not generate a PCR product was prepared from colonies using the E.Z.N.A Plasmid Mini Kit I (OMEGA Bio-Tek Inc., Doraville, GA, USA). Further PCR analysis was performed on DNA using primer sets Luc3-Luc5 (annealing temperature 47°C) and Luc3-Luc10 (annealing temperature 57°C), which were expected to generate products of 740 and 1800 bps, respectively, from pCMV3/luc. DNA that failed to generate a product from the Luc3-Luc10 reaction 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).

#### Down-regulation of Apex1 protein using siRNA

siGENOME SMART pool against mouse APEX1 and siCONTROL RISC-free siRNA (negative control, scrambled siRNA) was purchased from DHARMACON Inc. (Chicago, IL, USA). The sequences for the sense and antisense duplexes of APEX1 pool were, respectively: duplex 1 (CAAUGUGGCUCAUGAAGAAUU and 5'-PUUCUUCAUGAGCCACAUUGUU), duplex 2 (GGACUUACAUGAUGAAUGCUU and 5'-PGCAUUCAUCAUGUAAGUCCUU), duplex 3 (CAGAAAGCCUCUAGUGCUAUU and 5'-PUAGCACUAGAGCUUCUGUU) and duplex 4 (CAACACUGCUUACGCUUACUU and 5'-PGUAAGCGUAAGCAGUGUUGUU).

WT1 cells were nucleofected with 100 pmol (1.5  $\mu$ g) of siRNA using the standard conditions described above. Three sets of nucleofections 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 used after 72 h for nucleofection with the pCMV3/luc constructs (as described above), or after 48 h and 72 h to prepare cell-free extracts.

#### Western analysis

To prepare extracts, cells were scraped into cold PBS buffer, harvested by centrifugation at 4°C and re-suspended in ice-cold lysis buffer (0.3 M NaCl, 10 mM Tris HCl pH 7.5, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF) prior to sonication and centrifugation at 4°C for 10 min at 10 000 r.p.m. to remove debris. Protein concentrations were determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). Protein (50  $\mu$ g) was subjected to electrophoresis through a 12% Tris-Glycine SDS polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA, USA) and transferred by

electroblotting to 0.2  $\mu$ m nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Hybridizations with antibodies were performed according to the manufacturer's recommendations. The primary antibodies used were monoclonal anti-human/mouse/rat APE antibody (1:1000, R&D Systems, Inc., Minneapolis, MN, USA), or anti-actin (1:5000, BD Biosciences, San Jose, CA, USA). Bound primary antibody was visualized using autoradiography following incubation with a secondary horseradish peroxidase antibody (1:3000, Amersham Piscataway, NJ, USA) and chemiluminescent substrate (ECL-plus substrate, Amersham Piscataway, NJ, USA).

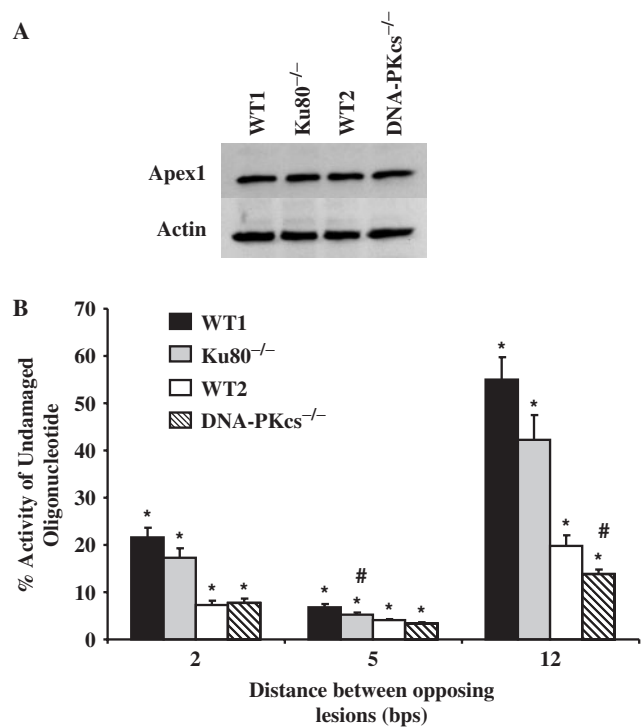
## RESULTS

To examine the repair of clustered furans in mammalian cells, we used an assay where the damage is situated in a plasmid within the coding region of the firefly luciferase reporter sequence between two unique restriction enzyme sites (PacI and ClaI). Conversion of the clustered lesion to a DSB results in linearization of the plasmid and a decrease in luciferase activity unless accurate repair occurs. The plasmid (pCMV3'luc) was adapted from p3'luc, which we previously used to demonstrate that clustered uracils as well as furans form DSBs in bacteria (26). To prevent the formation of DSBs from the collapse of replication forks at the clustered furans, pCMV3'luc does not contain a mammalian origin of replication. We have also modified the assay by using a nucleofector to deliver the DNA to the nucleus. Luciferase activity is therefore measured 6 h after plasmid introduction, which is unlikely to allow time for plasmid replication. This form of transfection also has the advantage that it delivers the DNA to the nucleus, which is the site of DNA repair. We have also optimized the assay for use with spontaneously immortalized cells isolated from wild-type and knock-out mice so that we are able to use cells that are deficient in specific repair proteins.

### Effect of clustered furan residues on firefly luciferase activity in wild-type cells

We examined two closely opposed furan lesions situated 5' to each other and separated by 2, 5 or 12 bps (Table 1). The oligonucleotides containing the single furans used to generate these clustered lesions were tested to make sure that they did not substantially reduce luciferase activity compared to the undamaged sequence. In wild-type cells (WT1) single lesions ranged from 80%  $\pm$  11 (average  $\pm$  SD) to 120%  $\pm$  18 of the activity obtained for the undamaged sequence (data not shown), so demonstrating that the single furans did not disrupt transcription of the luciferase sequence.

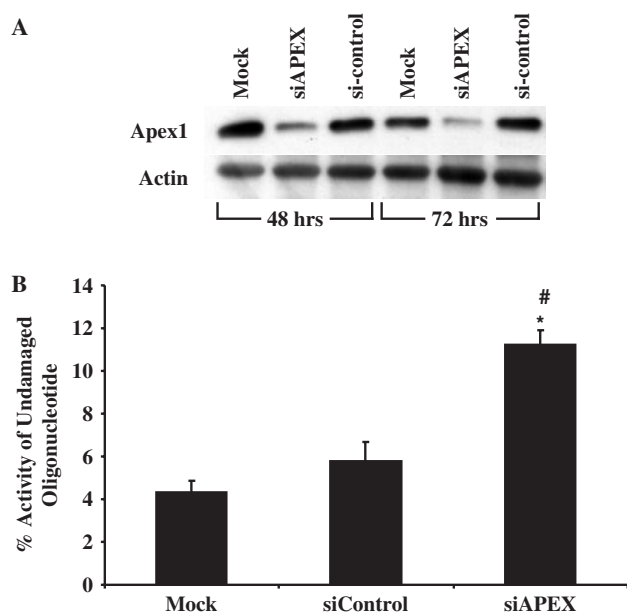
In mouse cells, Apex1 is the major class II AP endonuclease and the expression of this protein has been shown to change with cell cycle and growth (30). Cells were therefore plated to attain 60–70% confluency and cell-free extracts were prepared and western analyses performed to ensure that Apex1 protein expression was similar from experiment to experiment, and comparable between the different cell lines examined (Figure 1A).



**Figure 1.** Repair of two opposing furans in cells proficient and deficient in NHEJ. Cells, either wild-type for NHEJ (WT1 and WT2) or knocked-out for the Ku80 (Ku80<sup>-/-</sup>) or DNA-PKcs (DNA-PKcs<sup>-/-</sup>) gene, were grown in culture for 42 h to obtain cultures that were 60–70% confluent. WT1 is the isogenic wild-type for the Ku80<sup>-/-</sup>, while WT2 is isogenic to DNA-PKcs<sup>-/-</sup>. A portion of the cells was used to prepare a cell-free extract and western analyses performed to determine the level of Apex1 (A). The remaining cells were nucleofected (B) with ligation reactions containing pCMV3' luc with two closely opposed furans or no damage, and pRL-CMV. 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 obtained for transfections of three different ligations with the undamaged sequence performed at the same time. At least six transfections were performed in each cell line using different ligation reactions for each type of clustered lesion. The average and the standard error are shown graphically. The number of base pairs separating the two furans is indicated. \*represents a statistical difference ( $P < 0.05$ ) compared to the undamaged plasmid for that cell line. # represents a statistical difference ( $P < 0.05$ ) for that particular type of clustered lesion compared to isogenic cells wild-type for NHEJ.

Two wild-type cell lines (WT1 and WT2) were used, and in both cell lines two opposing furans substantially reduced the activity compared to the undamaged sequence when they were positioned 2, 5 and 12 bps apart (Figure 1B). The greatest loss of activity was measured when the furans were 5 bps apart and the activity actually increased as the distance between the furans increased to 12 bps. The luciferase activity therefore varied with the distance between the lesions. Interestingly, WT2 resulted in a greater loss of luciferase activity compared to WT1 for each type of lesion, even though they express a similar level of Apex1. It is possible that the expression of other enzymes involved in long patch base excision repair may be different in these two cell types, resulting in different levels of accurate repair.

To determine whether the amount of ligation used for the transfection altered the level of furan repair, two



**Figure 2.** A decrease in the major AP endonuclease (Apex1) results in an increase in luciferase activity from a construct containing two closely opposed furan lesions. WT1 cells were nucleofected with siRNA designed against APEX1, a scrambled control siRNA or no siRNA and incubated for 48 or 72 h. A cell-free extract was prepared and subjected to SDS-PAGE. The protein was transferred to nitrocellulose and western analysis performed using an antibody to Apex1 or actin (A). The nucleofection for the siRNA was repeated and after 72 h the cells were nucleofected with ligation reactions, containing pCMV3' luc with two closely opposed furans 5 bp apart or no damage, and pRL-CMV. After 6 h luciferase assays were performed on cell-free extracts. The firefly/renilla ratio for each construct was expressed as a percentage of the average ratio obtained for the undamaged construct. Three experiments and a total of nine transfections were performed for the mock and siAPEX and two experiments with a total of five transfections were performed for the siControl. The average and standard error are shown (B). \*represents a statistical difference ( $P < 0.05$ ) compared to the mock transfection. # represents a statistical difference ( $P < 0.05$ ) compared to the control siRNA.

different amounts (2 and 10  $\mu$ l) of the undamaged DNA and the cluster with furans separated by 2 bps were nucleofected into WT1 and Ku80<sup>-/-</sup> cells. The firefly luciferase activity increased approximately linear with the amount of DNA. However, when compared to the appropriate amount of undamaged sequence, a similar percentage of DSB formation was found for the two different amounts of damage-containing DNA (data not shown). We were therefore not exceeding the capacity of the cells to cleave or repair the clustered furans.

#### The effect of pre-treating cells with siRNA against mouse APEX1 mRNA

To determine whether Apex1 was the enzyme responsible for the strand breakage at the furans, WT1 cells were pre-treated with a pool of four different siRNAs designed to decrease expression of Apex1. As can be seen from Figure 2A, Apex1 protein expression was substantially decreased only by the siRNA designed against Apex1 and not the scrambled control siRNA (siControl). By 72 h expression was reduced by 80%. WT1 cells were

therefore pre-treated with the siRNA against Apex1, the siControl and no siRNA to obtain a mock sample. After 72 h the cells were nucleofected with ligations containing the undamaged sequence or the clustered lesion with two opposing furans situated 5 bps apart. The mock pre-treated cells or those pre-treated with siControl had equivalent luciferase activity compared to the undamaged sequence, while the cells pre-treated with the siRNA against APEX1 had 2–3 times more activity (Figure 2B). This indicates that cells with reduced Apex1 protein did not cleave the clustered lesion as well as those with higher expression levels.

#### Effect of clustered furan residues on firefly luciferase activity in cells deficient in NHEJ

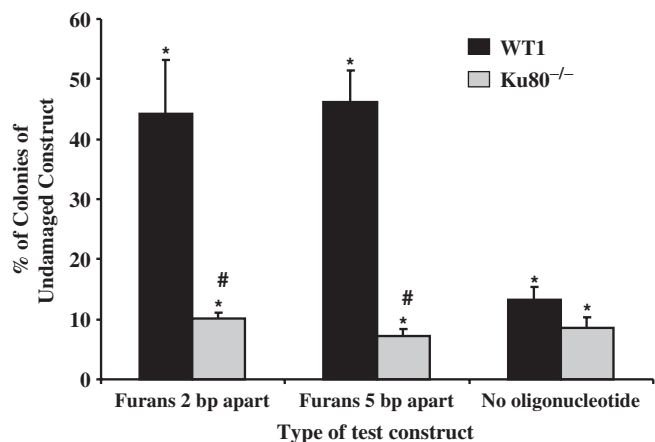
The clustered furans, especially those situated 2 and 12 bps apart, did not completely eliminate luciferase activity in wild-type cells. The remaining activity measured from the constructs could have been due to cleavage and accurate repair, or due to inhibition of the incision at the second furan after the generation of the SSB at the first furan. We therefore repeated the experiment with clustered furans 2, 5 and 12 bps apart using Ku80<sup>-/-</sup> or DNA PKcs<sup>-/-</sup> mouse fibroblasts to determine whether NHEJ could accurately repair DSBs generated at the clustered furans. As can be seen from Figure 1B, compared to the undamaged sequence the luciferase activity was significantly decreased by all the clustered lesions and a similar trend was seen in the Ku80 or DNA-PKcs-deficient cells as found in the wild-type, i.e. undamaged >12bps apart >2 bps apart >5 bps apart. However, there was very little difference between each NHEJ-deficient cell line and its respective isogenic wild-type strain.

#### Plasmid survival assay

A decrease in luciferase activity indicates that the plasmid was converted to a DSB and either destroyed or inaccurately repaired. Nucleofections with the ligations containing clustered furans 2 and 5 bps apart were repeated with WT1 and Ku80<sup>-/-</sup> cells, using pACYC184, which encodes chloramphenicol resistance in bacteria. After 6 h, the DNA was re-isolated and used to transform DH5 $\alpha$  bacteria. The culture was grown on carbenicillin or chloramphenicol-containing solid medium and the colonies counted. A decrease in the ratio of carbenicillin/chloramphenicol colonies compared to the undamaged sequence indicates that the pCMV3'luc construct was destroyed. To examine the background level of plasmid recovery that could occur due to re-circularization of linear plasmid, control ligations that did not contain the PacI–ClaI insert were nucleofected. Compared to the undamaged sequence 13.2%  $\pm$  2.1 (SEM) and 8.5%  $\pm$  1.8 colony-forming DNA was recovered for WT1 and Ku80<sup>-/-</sup>, respectively, and this background was not significantly different for the two cell lines (Figure 3). However, for the constructs containing clustered furans 2 or 5 bps apart there was ~45% recovery of carbenicillin-resistant colonies in wild-type cells. This high level of DNA recovery indicates that the furans were processed in the mammalian cells, since our previous studies showed

that two opposing uracils situated  $\leq 5$  bps apart or furans 5 bps apart resulted in plasmid destruction and recovery only at the level of background for the assay (26,27). This data (Figure 3) indicates that  $\sim 50\%$  of the plasmid DNA containing a clustered lesion was destroyed in wild-type cells.

Plasmid survival of the clustered lesion constructs was reduced to  $\sim 10\%$  of the undamaged construct when the lesions were processed in  $Ku80^{-/-}$  cells (Figure 3). There was therefore a significant decrease of  $\sim 4$  times compared to WT1. This suggests that even though the  $Ku80^{-/-}$  cells



**Figure 3.** Plasmid survival in WT1 and  $Ku80^{-/-}$  cells. Ligation reactions, containing pCMV3' luc with two closely opposed furans or no damage, were co-transfected with pACYC184 into cells wild-type for NHEJ (WT1) or cells deficient in  $Ku80$  ( $Ku80^{-/-}$ ). After 6 h the DNA was re-isolated and transformed into DH5 $\alpha$  bacteria. Three different ligations were transfected and the DNA re-isolated for each type of construct. Each re-isolated DNA sample was transformed twice. Bacteria were grown on carbenicillin or chloramphenicol containing solid medium and the colonies counted after overnight growth. The ratio of carbenicillin/chloramphenicol-resistant colonies was calculated for each transformation and the percentage compared to the undamaged sequence was determined. The average and standard error are shown graphically. \*represents a statistical difference ( $P < 0.05$ ) compared to the undamaged plasmid for that cell line. # represents a statistical difference ( $P < 0.05$ ) for that particular type of construct between  $Ku80^{-/-}$  and the wild-type cells.

could complete the equivalent amount of cleavage and/or accurate repair as WT1 (Figure 1B), WT1 could perform a greater amount of inaccurate repair of the DSBs generated from the cleavage of the furans.

Plasmid survival was independent of the distance between the furans in the cluster in WT1 and  $Ku80^{-/-}$  cells (Figure 3). This was unexpected as the luciferase activity data (Figure 1) predicted that there would be a decrease in plasmid survival of  $\sim 3$  times by moving the furans from 2 to 5 bps apart. Since luciferase expression requires accurate coding sequence, the plasmid survival results suggested that inaccurate repair had occurred. PCR analysis was therefore performed to determine the accuracy of the sequence across the PacI and ClaI sites. As expected the majority of the DNA re-isolated from samples that had been ligated with undamaged sequence had the correct insert size between PacI and ClaI (Table 2). Plasmid was however detected that contained deletions, the majority of which were  $< 1$  kb and sequencing determined that only 1 colony contained a deletion that was less than the size of the PacI–ClaI insert. Deletions were also seen in the control ligation. These background deletions were reduced in the  $Ku80$ -deficient cells and this suggests that some of these products were generated in the cells as well as *in vitro* by T4 DNA ligase. These background deletions did not, however, mask the effect of the double lesion on the types of plasmid recovered. The presence of a clustered lesion caused a decrease in the plasmid that contained the correct size in the region of PacI–ClaI and an increase in plasmid containing deletions of  $< 1$  kb (Table 2). Constructs with the furans separated by 5 bps resulted in a higher percentage of plasmid recovered with deletions than those with the furans situated 2 bps apart. This therefore agrees with the loss of luciferase activity (Figure 1B) and provides further evidence that more DSBs formed from a clustered lesion when the furans were 5 versus 2 bps apart.

To examine the junctions of inaccurate repair 45 samples were sequenced from WT1 (29 from the construct with the furans separated by 2 bps and 16 where the furans were 5 bps apart) and 16 from  $Ku80^{-/-}$  (6 from

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

Cell strain	Construct	Total colonies analyzed	Type of PCR product			
			No alteration in size	$< 1$ kb deletion	1–2.5 kb deletion	Insertion
WT1	Undamaged	94	69 (73%)	23 (25%)	2 (2%)	0
	Furans 2 bp apart	69	12 (17%)	51 (74%)	4 (6%)	2 (3%)
	Furans 5 bp apart	50	4 (8%)	43 (86%)	3 (6%)	0
	No oligonucleotide	60	0	34 (57%)	26 (43%)	0
$Ku80^{-/-}$	Undamaged	49	47 (96%)	2 (4%)	0	0
	Furans 2 bp apart	31	20 (65%)	9 (29%)	2 (7%)	0
	Furans 5 bp apart	18	2 (11%)	16 (89%)	0	0
	No oligonucleotide	10	0	10 (100%)	0	0

Double-stranded oligonucleotides that contained no damage or a clustered lesion (two opposing furans 2 or 5 bp apart and 5' to each other) were ligated to pCMV3' luc between the Pac I–Cla I sites. A control ligation that did not contain an oligonucleotide was also prepared. Ligations were transfected into WT1 (wild-type) or  $Ku80^{-/-}$  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 the Methods section).

**Table 3.** Sequences of deletions encompassing the clustered lesion

Cell strain	Bp apart	Original sequence	Deletion size (bp)	Junction of inaccurate repair	Micro-homology region
WT1	2	TTAATTAATACAAA;GGATATCAGGFGGCCCGCTGAATT;GGAATCG AATTAATTTATGTTT;CCTATAGFCCACCGGGGGCGACTTAA;CCTTAGC	26	TACAAAGGAATCG ATGTTTCCTTAGC	GGA
	2	TTAA;TTAAATACAAAGGATATCAGGFGGCCCGCTGAATT;TGGAATCGA AATT;AATTTATGTTTCCTATAGFCCACCGGGGGCGACTTA;ACCTTAGCT	36	TTAATGGAATCGA AATTACCTTAGCT	T
	2	ATC;AGGFGGCCCGCTGAATTGGAATCGATATTGTTACAACACCCCAACATCTT;CG TAG;FCCACCGGGGGCGACTTAACCTTAGCTATAACAATGTTGTGGGGTTGTAGAA;GC	52	ATCCG TAGGC	none
	5	TTGAA;GTCTTTAATTAATAACAAGGATATCAGGFGGCCCGCTGAA;TTGGAAT AACTT;CAGAAATTAATTTATGTTTCCTAFAAGTCCACCGGGGGCGACTT;AACCTTA	43	TTGAAATGGAAT AACTTAACCTTA	TGAA
	5	AATACAA;AGGATATCAGGFGGCCCGCTGAATTGGAATCGATATTGTTACAA;CA TTATGTT;TCCTAFAAGTCCACCGGGGGCGACTTAACCTTAGCTATAACAATGTT;GT	46	AATACAACA TTATGTTGT	TACAA
	5	AAAG;GATATCAGGFGGCCCGCTGAATTGGAATC;GATATTGTT TTTC;CTAFAAGTCCACCGGGGGCGACTTAACCTTAG;CTATAACAA	31	AAAGGATATTGTT TTTCCTATAACAA	GATAT
	Ku80 <sup>-/-</sup>	5	AAAG;GATATCAGGFGGCCCGCTGAATTGGAATC;GATATTGTT TTTC;CTAFAAGTCCACCGGGGGCGACTTAACCTTAG;CTATAACAA	31	AAAGGATATTGTT TTTCCTATAACAA

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. Sequences of micro-homology at the junctions are underlined and a dashed line represents the site of the break point in the original sequence.

constructs with the furans 2 bps apart and 10 from constructs with the furans 5 bps apart). These products were found to contain deletions of <150 bps according to the PCR analysis. Comparative samples were also sequenced from the DNA recovered from the control ligation (20 from WT1 and 8 from Ku80<sup>-/-</sup>) and the undamaged samples (10 from WT1 and 1 from Ku80<sup>-/-</sup>). One type of product that was specific for damage-containing constructs was small deletions in the sequence that occurred near or around the site of the furans. Examples of these are shown in Table 3. Deletions of this type were not found in the DNA from the undamaged sequence or control ligation and so they are unlikely to be due to 'background'. The majority were present in samples from wild-type cells: 10 such deletions were found in WT1 samples (22% of sequenced samples) compared to only 1 in the Ku80<sup>-/-</sup> samples (6% of sequenced samples). The one product from the Ku80<sup>-/-</sup> samples was identical to a product found in the wild-type samples (Table 3). These samples were isolated and sequenced at different times and so could not be due to contamination.

Analysis of the junction sequences also revealed that most of the inaccurate repair had occurred at regions of microhomologies, where one copy of the homology region was deleted at the junction (Table 3). This was not however, specific for wild-type or Ku80<sup>-/-</sup> cells.

## DISCUSSION

We have utilized a model lesion consisting of two closely opposed furans to determine whether clustered lesions can be converted to DSBs in mammalian cells and to examine the involvement of NHEJ in preventing DSB formation. The damage was placed within the firefly luciferase coding region of a plasmid and nucleofected into cells. We determined that luciferase activity was significantly decreased by all types of clustered furans tested.

This indicates that a DSB can be formed from two closely opposed lesions in a plasmid even in wild-type cells where NHEJ proteins are available for repair.

Initiation of repair of a furan is performed by a class II AP endonuclease, which introduces a strand break on the 5' side of the damage. There are two class II AP endonucleases in mammalian cells: Ape1 and Ape2. Even though Ape1 has greater AP endonuclease activity than Ape2 *in vitro* (31), both enzymes have been implicated in DSB formation from opposing AP sites during immunoglobulin class switch recombination in B cells (32). Due to the higher AP endonuclease activity of Ape1, the mouse Ape1 (Apex1) was the likely candidate for causing the formation of the DSBs at the clustered furans in the mouse fibroblasts. Using siRNA we were able to implicate Apex1 in the formation of the DSB, but since we were still able to detect breakage when Apex1 was reduced to 20% its normal level, it is possible that the mouse Ape2 can also cleave at the clustered lesion, especially under conditions of reduced Apex1.

The level of luciferase activity measured from the clustered lesion constructs varied with the distance between the lesions: 12 bps apart > 2 bps apart > 5 bps apart. These results therefore indicate that less cleavage or greater repair occurred when the furans were 2 bps apart compared to 5 bps apart. *In vitro* studies examining only the initiation of repair have shown that a SSB can inhibit the removal of a lesion if it is situated in the opposite strand  $\leq 3$  bps away (6,7), and that removal of one AP site can inhibit the removal of an opposing AP site, especially when it is situated 2 bps away and 5' from the target AP site (10). The lower formation of DSBs at the furans separated by 2 bps could therefore be due to Apex1 only cleaving at one furan in the cluster, generating a SSB-repair intermediate that prevents further action of Apex1 at the second furan. When the distance between the furans increased to 5 bps apart, the inhibition was

lost/decreased and initiation of repair occurred at both furans, resulting in greater formation of DSBs and a further decrease in luciferase activity. Previous *in vitro* experiments have shown greater cleavage of lesions separated by  $\geq 5$  bps (6).

Increasing the lesions to 12 bps apart decreased the formation of DSBs as seen by the increase in firefly luciferase activity. This is likely due to an increase in the complete repair of the two single strand breaks introduced at the furans. It is possible that the hydrogen bonding created by the intervening 12 bps stabilized the DNA structure, decreasing the chance of DSB formation prior to complete repair. This is in agreement with our work on clustered lesions in bacteria, where using a similar assay and two opposing uracils we determined that moving the lesions from 7 to 13 bps apart increased luciferase activity and plasmid recovery (26,27). A similar situation in bacteria was also seen for two opposing furans situated 5 and 20 bps apart (27).

In wild-type cells all the proteins were available to repair DSBs and so the luciferase activity measured from the constructs could have been partially due to accurate repair of a fraction of the DSBs generated from the initiation of clustered lesion repair. Gulston *et al.* (33) implicated NHEJ in repair of  $\sim 10\%$  of the non-DSB clusters produced by  $\gamma$ -rays in Chinese hamster ovary cells, and suggested that these lesions may consist of two closely opposed AP sites. *In vitro* studies have also shown that the Ku70/80 protein can inhibit base excision repair by binding to an opposing SSB (34). We therefore examined repair in both Ku80<sup>-/-</sup> or DNA-PKcs<sup>-/-</sup> mouse fibroblasts. The activity of the furan constructs in these NHEJ-defective cells was very similar to that in the isogenic wild-type cells. This indicates that Ku80 did not disrupt the cleavage of the furans in the cluster, and that NHEJ is not involved in the accurate repair of the clustered lesions. It is unlikely that this negative result was due to a lack of activation of the DNA repair pathways, even though these experiments were performed without pretreatment of the cells with ionizing radiation. Ionizing radiation is known to generate DSBs and result in a cascade of signaling events that include DNA-PK and ATM activation (35,36). However, even the addition of linear DNA to cell extracts has been found to activate ATM (37) and damage-containing plasmids electroporated into cells can stimulate non-homologous as well as homologous recombination (38). Since our experiments use ligations that contain linear DNA as well as damaged circular DNA, it is likely that the presence of DSBs activates the repair pathways. Future experiments are planned to determine whether the outcome of the clustered lesions is altered by irradiation of the cells prior to nucleofection of the damaged plasmid.

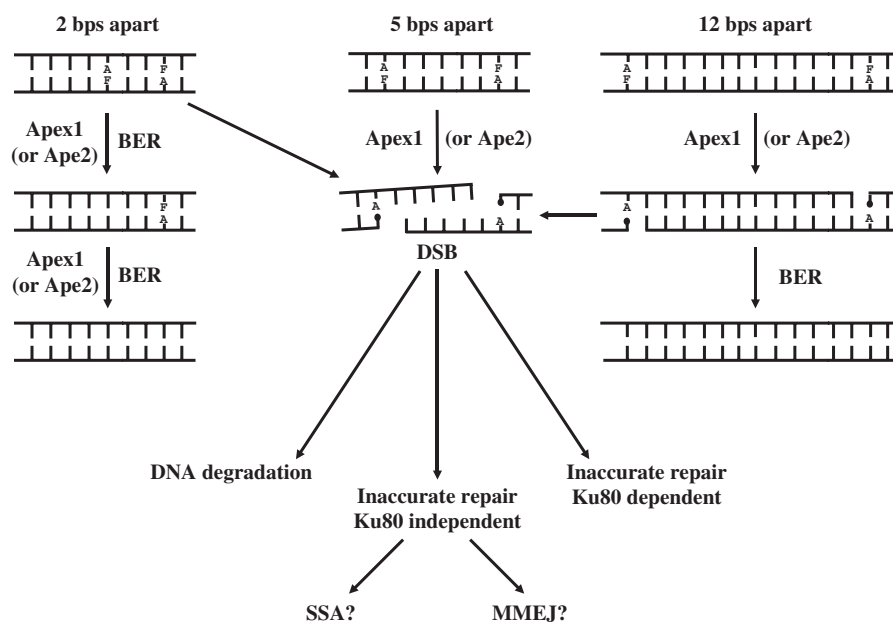
NHEJ is known to repair DSBs inaccurately (20). Re-isolation of the plasmid DNA from WT1 cells showed that there was  $\sim 45\%$  recovery of plasmid DNA containing furans separated by 2 or 5 bps, even though these lesions resulted in much lower luciferase activities compared to the undamaged sequence. This suggested that a portion of the plasmid did not contain the accurate luciferase coding sequence and was generated by

inaccurate repair. These same constructs resulted in  $\sim 4$  times less recovery of plasmid compared to the undamaged sequence from cells that were deficient in Ku80. This indicated that NHEJ or Ku80 in the wild-type cells was required for the inaccurate repair of the clustered lesions and prevention of DNA degradation.

PCR analysis did confirm that a fraction of the repair products contained deletions and fewer deletions were present in DNA isolated from Ku80<sup>-/-</sup> cells. Sequencing of the products revealed that deletions/inaccurate repair had frequently occurred at regions containing microhomology sequences, where one copy of the microhomology was deleted upon formation of the junction. Since deletions were detected in the Ku80<sup>-/-</sup> cells, these must have been generated by Ku80-independent inaccurate repair of the strand breaks at the clustered lesions. Previous repair studies using Ku80-deficient cell lines have identified DNA containing deletions with junctions at microhomology regions (39). This type of deleted product can be generated by microhomology-mediated end-joining (MMEJ) as well as single strand annealing (SSA; 40). Our data therefore implicates MMEJ and SSA in the repair of DSBs generated at clustered lesions. Ku80 is also believed to be involved in MMEJ: Ku80-deficient Chinese hamster ovary cells showed  $\sim 75\%$  reduction in MMEJ (22). This may explain why this type of product was detected in this study in wild-type cells and appeared to be reduced in the small number of samples processed from Ku80<sup>-/-</sup> cells. SSA requires Rad52, while Fen1 has been found to be involved in MMEJ (40). Parp-1 and DNA ligase III have also been implicated in a 'back-up' end-joining mechanism in the absence of functional DNA-PK mediated NHEJ (41,42). Although it is beyond the scope of this study, it will be interesting to examine the contributions of Rad52, Fen1, Parp-1 and DNA ligase III to the repair of clustered furans in both wild-type and Ku80<sup>-/-</sup> cells by the use of siRNA.

In summary (Figure 4), we have shown that clustered lesions consisting of two closely opposed furans can be converted to a DSB in wild-type cells and we have implicated Apex1 in mouse cells as the enzyme responsible for the initiation of repair and DSB formation. The formation of the DSB, escape from DSB repair and plasmid degradation was dependent on the distance separating the two lesions. Our studies indicate that NHEJ does not play a role in accurate repair of clustered lesions, but this work implicates Ku80 and hence NHEJ in the inaccurate repair of DSBs generated by Apex1 at clustered abasic sites. We also detected Ku80-independent repair that used microhomology sequences to join the termini, so implicating MMEJ and SSA in the repair of clustered lesions.

This work, using a model system where the clustered damage is situated in a plasmid, demonstrates that clustered lesions are biologically relevant damage, and suggests that the cell will attempt to repair clustered lesions in chromosomal DNA both accurately and inaccurately. However, we have also shown that repair is not always complete resulting in mutagenic lesions being converted to potentially lethal DSBs. This has implications for future development of adjuvant therapies for radiotherapy. Our work suggests that not only would it be beneficial to



**Figure 4.** Repair of clustered lesions. Two opposing furans (F) can be removed from the plasmid DNA by Apex1 and potentially Ape2, causing the generation of two single strand breaks (SSBs) that can interact to generate a double strand break (DSB). The 5' terminus of these breaks contain a 5'-blocking lesion (represented by a black circle at the break) which has to be removed by components of long patch base excision repair (BER) prior to complete repair. The distance between the two lesions greatly affects the probability of forming a DSB. Removal of one lesion when the furans are 2 bps apart can inhibit cleavage at the opposite furan and repair of the second lesion is likely to occur only when repair of the opposite strand is complete. This sequential repair does not result in a DSB. When the furans are situated 12 bps apart, each furan can be removed to form two opposing SSBs, but the probability of forming the DSB is reduced by the intervening hydrogen bonding in the sequence, again resulting in a high probability of complete repair. If a DSB occurs, the DNA can be degraded, or inaccurate repair can occur potentially by Ku80-independent and dependent mechanisms. Single strand annealing (SSA) and microhomology-mediated end joining (MMEJ) are two repair pathways that can occur in the absence of NHEJ, although Ku80 has also been implicated in MMEJ.

enhance AP endonuclease repair in irradiated tumors, but that inhibition of NHEJ would prevent the repair of AP endonuclease-generated DSBs and enhance cell killing.

Future work will focus on identifying the proteins involved in repair in cells and the biological consequences of more complex clustered lesions, which *in vitro* (43) have been shown to severely compromise repair.

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