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## **Ionizing Radiation Sensitivity of DNA Polymerase Lambda-Deficient Cells**

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## Ionizing Radiation Sensitivity of DNA Polymerase Lambda-Deficient Cells

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Vermeulen, C., Bertocci, B., Begg, A. C. and Vens, C. **Ionizing Radiation Sensitivity of DNA Polymerase Lambda-Deficient Cells.** *Radiat. Res.* 168, 683–688 (2007).

**Ionizing radiation induces a diverse spectrum of DNA lesions, including strand breaks and oxidized bases. In mammalian cells, ionizing radiation-induced lesions are targets of non-homologous end joining, homologous recombination, and base excision repair. *In vitro* assays show a potential involvement of DNA polymerase lambda in non-homologous end joining and base excision repair. In this study, we investigated whether DNA polymerase lambda played a significant role in determining ionizing radiation sensitivity. Despite increased sensitivity to hydrogen peroxide, lambda-deficient mouse embryonic fibroblasts displayed equal survival after exposure to ionizing radiation compared to their wild-type counterparts. In addition, we found increased sensitivity to the topoisomerase inhibitors camptothecin and etoposide in the absence of polymerase lambda. These results do not reveal a major role for DNA polymerase lambda in determining radiosensitivity *in vivo*.** © 2007 by Radiation Research Society

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### INTRODUCTION

Strand breaks and oxidized bases induced in the DNA by ionizing radiation are critical lesions that, if unrepaired, can lead to cell killing and mutation. Two complementary double-strand break (DSB) repair pathways exist in mammalian cells, non-homologous end joining (NHEJ) and homologous recombination (HR). In addition, ionizing radiation-induced lesions are targets of base excision repair (BER). Deficiencies in all of these pathways lead to increased sensitivity to ionizing radiation, indicating that all are important for genomic repair. Within these pathways, DNA polymerases are thought to play a critical role in repair of radiation-induced damage. At least 19 different DNA polymerases are expressed in mammalian cells. These polymerases differ from each other according to the specialized roles they have during replication, DNA repair, bypass of DNA damage, and somatic hypermutation. *In vitro*

studies have indicated a potential role of DNA polymerase lambda in DSB repair and/or BER, although the *in vivo* role is unknown.

DNA polymerase lambda is a member of the X family of DNA polymerases, comprising other enzymes involved in DNA repair processes such as DNA polymerase beta, polymerase mu, and terminal deoxy-nucleotidyl-transferase (TdT). Due to its bypass polymerase activity on templates with base lesions such as abasic sites, it has been implicated in translesion synthesis (TLS), a process allowing lesion bypass during replication. Other data demonstrating repair deficiency when lacking in a classical *in vitro* BER assay suggested a possible contribution in BER, as shown by Braithwaite *et al.* (1). However, in earlier studies, DNA polymerase lambda was shown to be capable of filling gaps and promoting end joining in an *in vitro* NHEJ assay (2, 3). Recent data indicate this involvement to be selective for DNA with complementary overhangs (4, 5). The observed interaction with ligase IV (LigIV) underlines its proposed role in NHEJ (6). Only a few studies have addressed DNA polymerase lambda's role *in vivo* or in cellular systems. *In vivo*, polymerase lambda-deficient mice were viable and fertile. These mice showed normal immunoglobulin hypermutation (7). Furthermore, recent data indicated a participation of polymerase lambda in heavy chain rearrangement (8). Polymerase lambda-deficient embryonic stem cells did not show hypersensitivity to H<sub>2</sub>O<sub>2</sub> or methyl methane sulfonate (MMS), ultraviolet (UV) light, or ionizing radiation (9). However, lambda-deficient mouse embryonic fibroblast cells have been found to be hypersensitive to oxidative damage produced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 5-hydroxymethyl-2'-deoxyuridine (HmdUrd), indicating a potential role in response to oxidative damage and BER (10). Our own studies have shown a significant role of BER in determining radiation response (11–13). We showed that BER deficiency, by DNA polymerase beta deficiency, resulted in sensitization to ionizing radiation. Consistent with lambda's proposed role in NHEJ or BER, Capp *et al.* showed increased radiosensitivity in cells expressing a dominant negative to DNA polymerase lambda (14). In contrast, primary lambda-deficient mouse embryonic fibroblasts were not found to have increased sensitivity to ionizing radiation or bleomycin in earlier studies (8). However, strongly reduced proliferation and a concomitantly reduced percentage of cells in S phase in these

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primary lambda-deficient cells could have influenced survival after exposure to ionizing radiation. This alteration in proliferation has been attributed to oxidative damage response (8). Since ionizing radiation induces a DNA damage spectrum that partly overlaps with the spectrum induced by oxidative damage, we questioned whether the oxidative damage-sensitive population might have been under-represented in the radiation response analysis.

Using transformed mouse embryonic fibroblasts with equal growth and cell cycle phase distribution patterns here, we show that, despite exhibiting H<sub>2</sub>O<sub>2</sub> hypersensitivity, lambda-deficient cells were not more sensitive to ionizing radiation. In addition, we show increased sensitivity to other non-oxidative DSB-inducing agents such as etoposide and camptothecin.

## MATERIALS AND METHODS

### Cell Lines

Polymerase lambda-deficient mice were generated by Bertocci *et al.* as described previously (7). RT-PCR analysis confirmed DNA polymerase lambda expression status. Transformed polymerase lambda-deficient (pol λ<sup>-/-</sup>) mouse embryonic fibroblasts and the isogenic wild-type (pol λ<sup>+/+</sup>) counterparts were generated by the 3T3 protocol from these mice, as described previously by Bertocci *et al.* (8). Pol λ<sup>+/+</sup> and pol λ<sup>-/-</sup> cells were grown at 37°C in a 95% air/5% CO<sub>2</sub> incubator in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Growth curves were obtained after seeding 0.5 × 10<sup>4</sup> and 10<sup>4</sup> cells/well in six-well dishes in triplicate and counting at various times.

### Colony-Forming Survival Assay

Colony-forming survival assays were performed as described previously (13). Briefly, cells were seeded and, after 6 h, irradiated using a <sup>137</sup>Cs irradiation unit at a dose rate of 0.9 Gy/min. For H<sub>2</sub>O<sub>2</sub>, camptothecin and etoposide sensitivity, cells were seeded and treated after 6 h with H<sub>2</sub>O<sub>2</sub> (Sigma) for 1 h or with camptothecin or etoposide for 2 h in medium without fetal calf serum and antibiotics. Exposure to serum-free medium for 2 h did not alter S-phase content or replication as measured by BrdU incorporation determined by flow cytometry (data not shown). After 1 or 2 h, fresh medium was added and plates were replaced in the incubator to allow colony formation. Nine days later, cells were fixed, stained and colonies consisting of >50 cells were counted either manually under a binocular microscope or using an automatic colony counter (ColCount™, Oxford Optronix, UK). Survival was expressed as colonies per plated treated cell colonies per plated untreated cell.

### In Vitro Cell Growth Inhibition Assay

Cytotoxicity was determined by growth inhibition assays as described previously (13). Cells were seeded in six-well dishes at a density appropriate to maintain exponential growth during the course of the experiment. The following day, cells were exposed to a range of concentrations of H<sub>2</sub>O<sub>2</sub> (Sigma) for 1 h or camptothecin (Sigma) or etoposide for 2 h in growth medium without fetal calf serum and antibiotics. After the indicated time of drug treatment, fresh medium was added and dishes were incubated for 4–5 days until untreated control cells were approximately 75% confluent. Cells (triplicate wells for each drug concentration) were trypsinized and counted using a cell counter (Casy TT-059, Schärfe System GmbH, Germany). The relative growth inhibition was calculated by normalizing the average cell number in the drug-treated wells to the control (percentage control growth). The SF<sub>50</sub> (dose needed to reduce the surviving fraction to 0.5), IC<sub>50</sub> (concentration that inhibited growth to

50% of control), and IC<sub>10</sub> (concentration that inhibited growth to 10% of control) were determined from polynomial or power curve fits.

### Cell Cycle Phase Determination

Cells were pulse labeled with 1 μM BrdU (Sigma) by incubating for 10 min at 37°C. Cells were then trypsinized, resuspended in 1 ml of PBS, and fixed by adding the cell suspension to 5 ml of ice-cold 70% ethanol. Anti-BrdU staining was performed as described previously (12). Measurement of the samples and data analysis were performed as described previously (13).

### Genomic Sequence Analysis

Genotyping of pol λ<sup>+/+</sup> and pol λ<sup>-/-</sup> cells was performed by PCR using primers that amplify the DNA polymerase lambda allele, resulting in a 250-bp fragment in the wild-type cells and a 500-bp fragment in lambda-deficient cells. Primers were as follows: 5' GCTCCATATGGTTGCTG GGC and 3' CAGTCCCCAGATGTTGGAG.

### Statistical Analysis

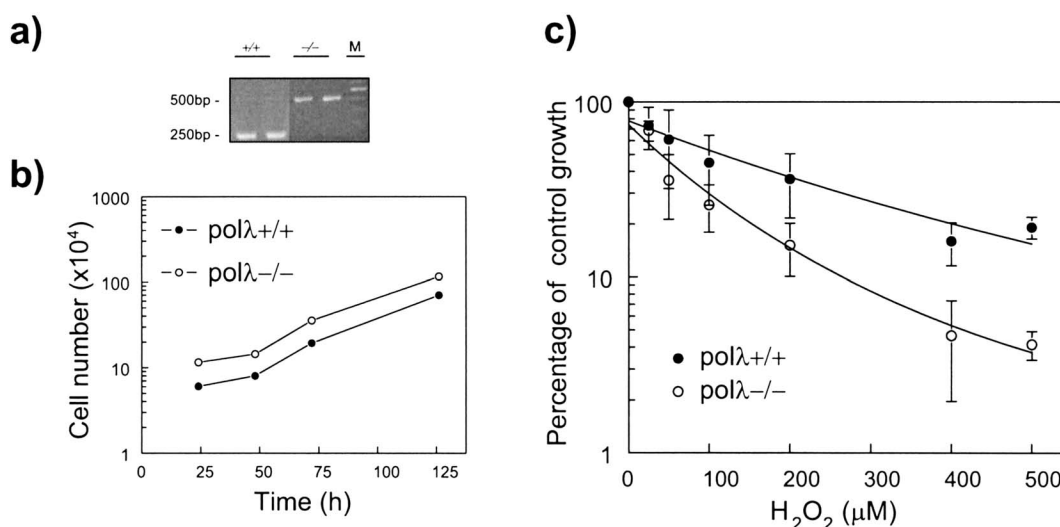
For comparison of differences between the groups, the *t* test was used with a significance level of *P* < 0.05.

## RESULTS

### DNA Polymerase Lambda Deficiency Did Not Influence the Sensitivity to Ionizing Radiation but Sensitized Cells to H<sub>2</sub>O<sub>2</sub>

We first characterized the transformed polymerase lambda-deficient (pol λ<sup>-/-</sup>) mouse embryonic fibroblasts and the isogenic wild-type (pol λ<sup>+/+</sup>) counterparts. A genomic PCR was performed to confirm the genotype of both cell lines. We found the fragment representing the wild-type allele (250 bp) in the pol λ<sup>+/+</sup> cells and the polymerase lambda gene targeted allele (500 bp) in pol λ<sup>-/-</sup> cells (Fig. 1a). Changes in growth or cell cycle phase distribution could indirectly result in survival differences. However, and in contrast to the primary cells, both cell lines showed similar growth rates, with doubling times of 13.4 ± 3.7 h for wild-type cells and 14.2 ± 1.3 h for pol λ<sup>-/-</sup> cells (mean values ± SD, *n* = 4) (Fig. 1b). Cell cycle phase distribution was determined by BrdU labeling. Data analysis showed an S-phase content of 41.2 ± 1.2% and 53.4 ± 4.1%, G<sub>1</sub> phase of 32.4 ± 1.4% and 33.7 ± 6.6%, and G<sub>2</sub> phase of 26.4 ± 0.3% and 12.9 ± 2.5% for wild-type and pol λ<sup>-/-</sup> cells, respectively. Taken together, no differences were observed in growth rate and only minor differences were observed in the cell cycle phase distributions of the cell lines. We next confirmed sensitivity to H<sub>2</sub>O<sub>2</sub> by determining growth inhibition. We found that pol λ<sup>-/-</sup> cells were more sensitive to H<sub>2</sub>O<sub>2</sub> than wild-type cells (Fig. 1c), consistent with its proposed role in the oxidative damage response. This is in good concordance with data published previously by Braithwaite *et al.* (10) from growth inhibition assays.

With ionizing radiation producing similar oxidative damage, we next determined the survival of polymerase lambda-deficient cells and wild-type cells after irradiation in a colony-forming survival assay. We found that pol λ<sup>-/-</sup> cells were not



**FIG. 1.** Characterization of pol  $\lambda^{-/-}$  cells. Panel A: Genotype of pol  $\lambda^{-/-}$  ( $-/-$ ) and wild-type ( $+/+$ ) cells. Genomic DNA was isolated from both cell lines, and genotyping was performed by PCR with simultaneous amplification of the wild-type allele (250 bp) and the gene-targeted allele (500 bp). The marker (M) is a 100-bp ladder (Invitrogen). Panel B: Growth of pol  $\lambda^{-/-}$  (○) and wild-type (●) cells. Cells were seeded, cultured and counted at the indicated times. A representative growth curve is shown. Panel C: Growth inhibition of pol  $\lambda^{-/-}$  (○) and wild-type (●) cells after hydrogen peroxide treatment. Cells were seeded into six-well plates and treated the following day with increasing concentrations of  $H_2O_2$  for 1 h. After 5 days, cells were counted and the number of  $H_2O_2$ -treated cells was normalized to that of control cells. Percentage control growth is plotted, with each data point representing the mean and standard deviation of three independent experiments with triplicate samples.

significantly more sensitive to ionizing radiation than the wild-type cells (Fig. 2a), confirming previous growth inhibition analyses (8). In contrast, lambda-deficient cells appeared to be more radioresistant. However, this was significant only at 6 Gy. We then questioned whether the lack of sensitization was caused by the assay used, with growth assays emphasizing growth inhibition rather than killing. We therefore repeated the  $H_2O_2$  sensitivity study with a colony-forming survival assay. The data confirmed the previous growth inhibition study, shown in Fig. 1b, with DNA polymerase lambda-deficient cells being significantly more sensitive to  $H_2O_2$  than wild-type cells, with  $SF_{50}$  values of  $89.1 \pm 12.2 \mu M$  and  $44.9 \pm 4.4 \mu M$  (mean values  $\pm$  SD;  $P = 0.004$ ) for wild-type and pol  $\lambda^{-/-}$  cells, respectively, giving a dose ratio of  $2.0 \pm 0.4$  (Fig. 2b). In summary, our data demonstrate no major involvement of DNA polymerase lambda in the response to ionizing radiation.

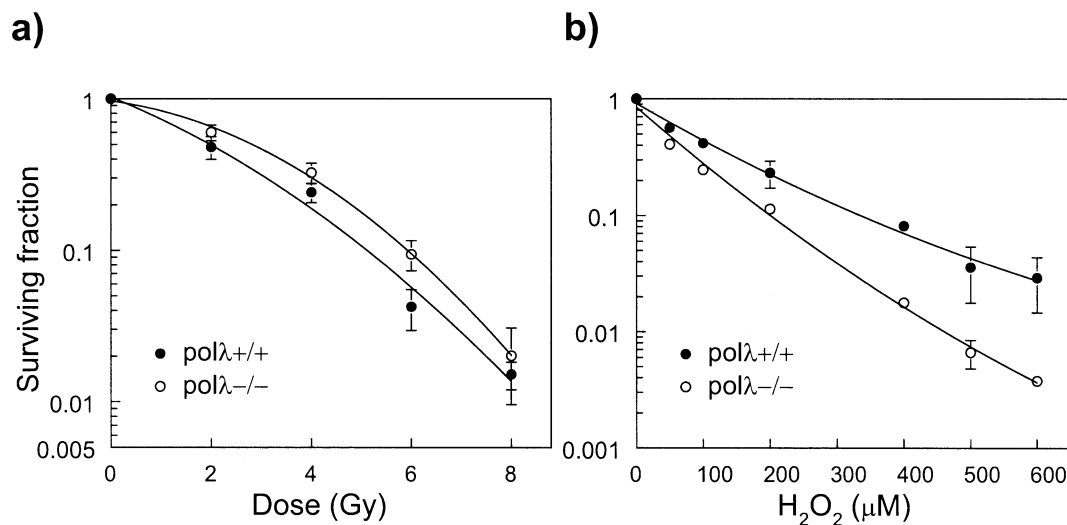
#### *Sensitivity of pol $\lambda^{-/-}$ Cells to Camptothecin and Etoposide*

Both damaging agents,  $H_2O_2$  and ionizing radiation, induce similar oxidative lesions. Nevertheless, lambda-deficient cells exhibited hypersensitivity to  $H_2O_2$  but surprisingly not to ionizing radiation. This discrepancy prompted us to analyze two other DNA strand break-inducing agents without inducing oxidized base damage. For this purpose, we incubated polymerase lambda-deficient and wild-type cells with topoisomerase inhibitors. Type I topoisomerase inhibitors such as camptothecin bind irreversibly to the DNA-topoisomerase I complex, thereby generating enzyme-trapped cleaved DNA. Etoposide, a type II topoisom-

erase inhibitor, enhances double-strand cleavage by complexation with topoisomerase II. We found that pol  $\lambda^{-/-}$  cells showed an increased sensitivity to camptothecin compared to wild-type cells, as determined by growth inhibition assays (Fig. 3a). The  $IC_{10}$  values were  $1.4 \pm 0.4$  and  $0.5 \pm 0.2 \mu M$  (mean values  $\pm$  SD;  $P = 0.02$ ) for wild-type and pol  $\lambda^{-/-}$  cells, respectively, with a dose ratio of  $3.2 \pm 0.9$ . Furthermore, pol  $\lambda^{-/-}$  cells were significantly more sensitive to etoposide than wild-type cells (Fig. 3b), with  $IC_{50}$  values of  $1.0 \pm 0.1$  and  $0.6 \pm 0.1 \mu M$  ( $P = 0.001$ ) for wild-type and pol  $\lambda^{-/-}$  cells, respectively, with a dose ratio of  $1.9 \pm 0.4$ . We repeated the growth inhibition studies for both drugs with a colony-forming survival assay and again found that DNA polymerase lambda-deficient cells were significantly more sensitive than wild-type cells (Fig. 3c). As with the growth assays, the surviving fractions of the cell lines diverged at greater killing after etoposide treatment. Taken together, these results suggest the involvement of polymerase lambda in the repair of DNA double-strand breaks of non-oxidative origin.

## DISCUSSION

In the present study, we investigated whether DNA polymerase lambda played a significant role in determining ionizing radiation sensitivity. Previous studies have shown contradictory results, with a lack of sensitization to ionizing radiation in lambda-deficient primary cells (8) and hypersensitivity in polymerase lambda dominant negative-expressing cells. However, studies on the deficient primary cells may have been hampered by the reduced growth rate



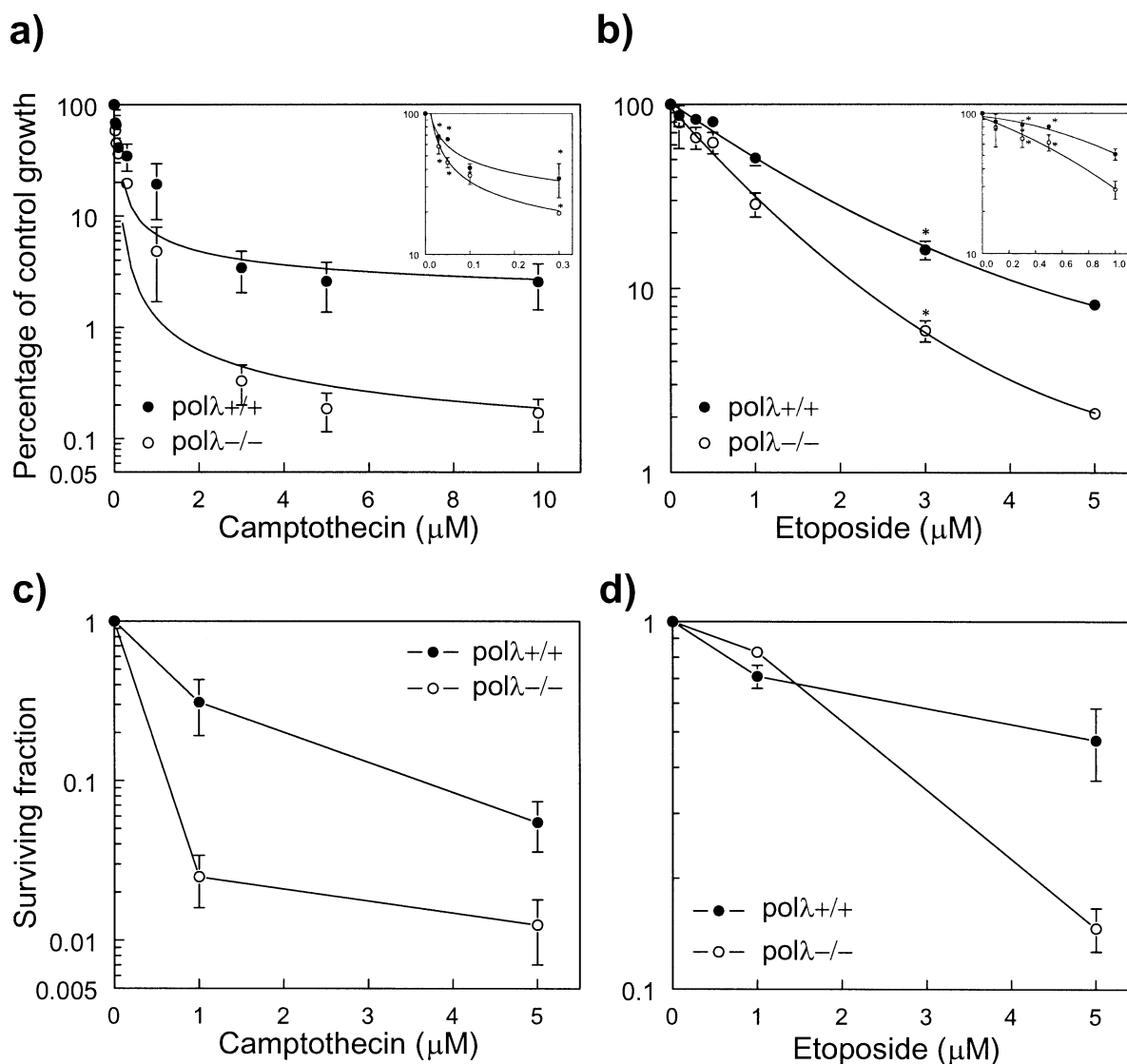
**FIG. 2.** Sensitivity of pol  $\lambda^{-/-}$  cells to ionizing radiation and H<sub>2</sub>O<sub>2</sub>. Panel A: Clonogenic survival of pol  $\lambda^{-/-}$  (○) and wild-type (●) cells after exposure to ionizing radiation. Panel B: Clonogenic survival of pol  $\lambda^{-/-}$  (○) and wild-type (●) cells after exposure to H<sub>2</sub>O<sub>2</sub> for 1 h. After irradiation or H<sub>2</sub>O<sub>2</sub> exposure, the cells were returned to 37°C to allow colonies to develop. Data points are the means and standard deviations of five or three independent experiments with triplicate samples for panels A and B, respectively.

and considerably increased senescence, in particular when analyzed by a growth inhibition assay. A potential radiosensitive phenotype of the DNA polymerase lambda-deficient cells, in particular in S phase, could have been masked by a reduction in the S-phase cell population in the lambda-deficient cells to half of the control level, as observed previously in the primary cultures. Transformed cells, on the other hand, probably due to loss of cell cycle checkpoints, showed similar cell cycle phase distributions and growth rates for wild-type and lambda-deficient cells (Fig. 1b). Here we show that these transformed cells did not exhibit differences in radiation sensitivity (Fig. 2a) despite increased hypersensitivity to hydrogen peroxide. This indicates that the cells did not greatly adapt (due to selection) to oxidative damage during culture, thereby altering the oxidative damage response and/or repair. It is worthwhile noting that even though there were differences between the assays used, the growth inhibition assay (Fig. 1c) and the colony-forming survival assay (Fig. 2b), the results were generally in good concordance with each other. Differences between the two cell lines were similar to the results of by Braithwaite *et al.* (10). We observed a slight increase in radioresistance in DNA polymerase lambda-deficient cells. Although not significant for most doses, this could have resulted from the additional 10% of S-phase cells, which are generally more resistant to ionizing radiation. In conclusion, these results suggest that a deficiency in polymerase lambda is associated with an increased sensitivity to oxidative damage and strand breaks but not when these lesions are induced by ionizing radiation.

Two other DNA break-inducing agents, camptothecin and etoposide, both produced more killing in lambda-deficient cells (Fig. 3). In contrast to our results (Fig. 3c), Capp *et al.* found an increased resistance to camptothecin in hamster cells

(CHO cells) expressing a catalytically inactive polymerase lambda (polλDN) (14). In addition, these cells were found to be more sensitive to ionizing radiation. Interestingly, sensitization reached levels that were comparable to that of NHEJ mutant cells. The inactive DNA polymerase lambda is expected to act in a dominant negative manner in the proposed repair pathways. It is likely that the mode of action is different from the lack of repair due to gene deficiency, in analogy with our polymerase beta dominant negative studies (11–13). The dominant negative might have the capacity to block alternative backup polymerases and pathways, or it could interfere in processes unrelated to its wild-type lambda protein. In addition, the origin of the cell lines, mouse fibroblasts and hamster ovary cells, could result in different responses to damaging agents.

Based on the data presented here, we can only speculate on the possible role of lambda. Assuming that the observed sensitivities result from involvement in a common pathway, it is feasible that the nature of the lesion dictates DNA polymerase lambda's interaction. Double-strand breaks produced by ionizing radiation are complex, often presenting as clustered lesions. In contrast, topoisomerase inhibition will cause breaks complexed to topoisomerases together with ligatable ends. Second, double-strand break induction by topoisomerase inhibitors and hydrogen peroxide are partly connected to replication. Replication could thereby present a mutual process in the lambda-dependent responses. Homologous recombination-deficient cells have been shown to be highly sensitive to camptothecin (15). Etoposide sensitivity showed a dependence on non-homologous end joining but also showed some dependence on homologous recombination (16, 17), indicating that the choice of pathway in response to the damaging agent might be related to DNA polymerase lambda dependence. Consistent with



**FIG. 3.** Sensitivity of *pol*  $\lambda^{-/-}$  cells to camptothecin and etoposide. Panel A: *Pol*  $\lambda^{-/-}$  ( $\circ$ ) and wild-type ( $\bullet$ ) cells were seeded in six-well plates and treated the following day with increasing concentrations of camptothecin for 2 h. Panel B: Growth inhibition of *pol*  $\lambda^{-/-}$  ( $\circ$ ) and wild-type ( $\bullet$ ) cells by etoposide was determined as described above. Percentage control cell number is plotted, with each data point, as described for Fig. 1b, representing the mean and standard deviation of four independent experiments with triplicate samples. An asterisk indicates that the dose was included in only two of the four experiments. The insets in each panel are the same data with an expanded scale to illustrate the differences at low drug doses. Panel C: Clonogenic survival of *pol*  $\lambda^{-/-}$  ( $\circ$ ) and wild-type ( $\bullet$ ) cells after exposure to camptothecin for 2 h. Panel D: Clonogenic survival of *pol*  $\lambda^{-/-}$  ( $\circ$ ) and wild-type ( $\bullet$ ) cells after exposure to etoposide for 2 h. Data points are the means and standard deviations of three independent experiments with triplicate samples for panels C and D.

such an interpretation, ionizing radiation response in most cell lines is less dependent on homologous recombination than on non-homologous end joining.

Repair of ionizing radiation damage is highly dependent on the non-homologous end-joining repair pathway, as shown by numerous studies, being dependent on the presence of non-homologous end-joining proteins such as Ku70/80, DNA-PK and LigIV. The lack of increased sensitivity to ionizing radiation of lambda-deficient cells found here therefore does not support a hypothesis of lambda's sole involvement in non-homologous end joining after exposure to ionizing radiation. Our data rather suggest the involvement of another redundant polymerase (18). In sum-

mary, DNA polymerase lambda does not play an important role in determining sensitivity to ionizing radiation, despite the observed hydrogen peroxide sensitivity and proposed role in repair of DNA double-strand breaks.

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