# Mutation Frequencies and Spectra in DNA Polymerase η–Deficient Mice

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## Abstract

**The low-fidelity polymerase η (polη) is required for bypass of UV-induced pyrimidine dimers inserting adenine nucleotides opposite these lesions. Mutations in the *polη* gene are responsible for the genetic defect in xeroderma pigmentosum variant patients. To study if the lack of polη significantly elevates spontaneous mutation frequency in various organs and tissues of the mouse, we crossed polη-deficient mice with transgenic mice harboring a chromosomally integrated lacZ-plasmid reporter construct. In cultured embryonic fibroblasts from the lacZ-polη$^{-/-}$ mice, 2.5 J/m$^2$ UV irradiation induced ~5-fold more mutations than in cells from lacZ control mice, in which an ~3-fold increase in mutation frequency was found compared with the normal level. Whereas untreated cells harbored mainly 1-bp deletions, UV induced both transitions and transversions, with the latter type more highly represented in the polη-null cells than in the controls. No difference in mutation induction between the polη-null cells and the wild-type cells was observed after treatment with *N*-ethyl-*N*-nitrosourea. Having shown the validity of the lacZ model to accurately identify polη-associated mutagenesis, we then determined the mutant frequency at the lacZ locus in liver, spleen, and small intestine of 12-month-old animals. No differences were found between polη-null, heterozygous, or littermate control mice. We conclude that the polη defect is specific for UV damage and has no effect on *in vivo* mutagenesis in mice.** [Cancer Res 2008;68(7):2081–4]

## Introduction

DNA polymerase η (polη) is a Y polymerase capable of replicating past cyclobutane pyrimidine dimers (CPD), the major lesion induced in DNA by UV radiation. The importance of polη in reducing mutagenesis in human skin, preventing skin cancer from sun exposure, is illustrated by the xeroderma pigmentosum variant syndrome. Patients afflicted with this disease lack polη expression and are more than 1,000 times more susceptible to skin cancer than normal individuals (1).

Polη knockout mice have been generated and are viable and fertile and do not show any obvious spontaneous defects during the first year of life (2). To test if polη-defective mice accumulate spontaneous mutations at a more rapid pace than normal mice, we crossed these animals with transgenic mice harboring a *lacZ* reporter gene. Being part of a plasmid construct, this reporter can be recovered from its integrated state and amplified in *Escherichia coli* to determine mutant frequencies and spectra. We show that although UV irradiation of lacZ-polη$^{-/-}$ embryonic fibroblasts readily induced a great excess of mutations over the UV-irradiated control cells, no effect was observed *in vivo* in several organs of the polη-null mice up until 24 months.

## Materials and Methods

**Transgenic animals.** polη$^{+/-}$ mice in a C57BL/6J background were crossed with C57BL/6J pUR288-(*lacZ*)-transgenic mice, line 30 (integration site on chromosome 11; ref. 3) and bred among each other to generate polη$^{-/-}$ animals hemizygous for pUR288 (*lacZ*). The polη$^{+/-}$ and polη$^{+/+}$ lacZ littermate animals served as controls. The animals were maintained in the animal facilities of the Buck Institute for Age Research. The mice were maintained on a 12-h light/12-h dark cycle at a standard temperature of 23°C. Standard lab chow (Harlan Teklad) and water were supplied ad libitum. Animals were sacrificed by $CO_2$ inhalation followed by cervical dislocation at 2, 12, and 24 mo of age. Tissues were removed from the animal, snap frozen in liquid nitrogen, and stored at −80°C until required.

**Cell isolation and culture.** Mouse embryonic fibroblasts (MEF) were isolated from day 13.5 embryos generated from the aforementioned F1 crosses between the polη$^{+/-}$ and lacZ$^{+/-}$ animals. MEF isolation has been described previously (4). Experiments were conducted on cells at passage 3.

**Treatment of MEFs with UV radiation or *N*-ethyl-*N*-nitrosourea.** lacZ MEFs prepared from individual embryos of polη$^{-/-}$, polη$^{+/-}$, or polη$^{+/+}$ genotypes were plated in 10-cm dishes ($10^6$ cells) in the presence of 10% serum and 1% penicillin/streptomycin and incubated for 24 h. For UV irradiation, proliferating cells were washed twice with PBS, covered with a thin layer of PBS, and irradiated in lidless culture dishes using a germicidal lamp (254 nm, 15 W; General Electric). The PBS was removed and fresh medium was provided to the cells before returning them to culture. For *N*-ethyl-*N*-nitrosourea (ENU) treatment, cells were washed twice with PBS and then incubated with 3 mmol/L ENU (Sigma-Aldrich, Inc.) in medium without serum for 2 h at 37°C. After the treatment period, the cells were washed twice with PBS and fresh medium was provided. The cells were returned to culture. Control cells were mock treated. Cells were harvested 72 h after treatment.

**Plasmid rescue and mutation analysis.** DNA was isolated by routine phenol/chloroform extractions. Complete protocols for plasmid rescue, mutant frequency determinations, and mutant analysis with this model have been described elsewhere (3–5). To characterize the mutations, the complete *lacZ* gene of 10 mutants per condition was sequenced. Sequence reactions of purified plasmids were outsourced to Davis Sequencing. The returned chromatograms were analyzed with Sequencher (Gene Codes). The primers used for the sequencing reactions were the same as previously described (4).

**Statistical analysis.** Unpaired *t* test was used for all statistical analyses using the statistical program GraphPad InStat (GraphPad Software). $P < 0.05$ was considered significant.

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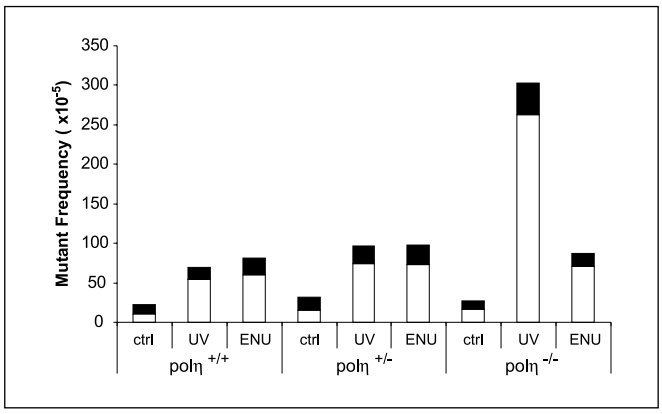


**Figure 1.** Mutant frequencies and spectra of polη wild-type, heterozygous, and knockout MEFs harvested 72 h after exposure to 3 mmol/L ENU or 2.5 J/m$^2$ UVC radiation. *White areas,* frequency of no-change mutations; *black areas,* genome rearrangements.

## Results

**Mutant frequencies and spectra in MEFs following exposure to ENU and UVC radiation.** To confirm that mutations associated with the polη deficiency can be accurately detected in the lacZ reporter system, we prepared MEFs from the embryos of polη wild-type, heterozygous, and knockout mice and subjected these cells to either 2.5 J/m$^2$ UVC radiation or 3 mmol/L ENU. lacZ mutant frequencies were measured 72 h after exposure. In keeping with our previous results (6), we found that the overall mutant frequencies in cells derived from wild-type animals were increased by 3.2- and 3.7-fold following exposure to UV or ENU treatment, respectively (Fig. 1). The cells derived from polη heterozygous mice behaved similarly to those from the wild-type mice. In the knockout mice, however, whereas the ENU-treated cells behaved no differently from those of wild-type and heterozygous mice, the UV-irradiated cells showed a dramatic 11-fold increase in mutant frequency when compared with untreated cells (Fig. 1).



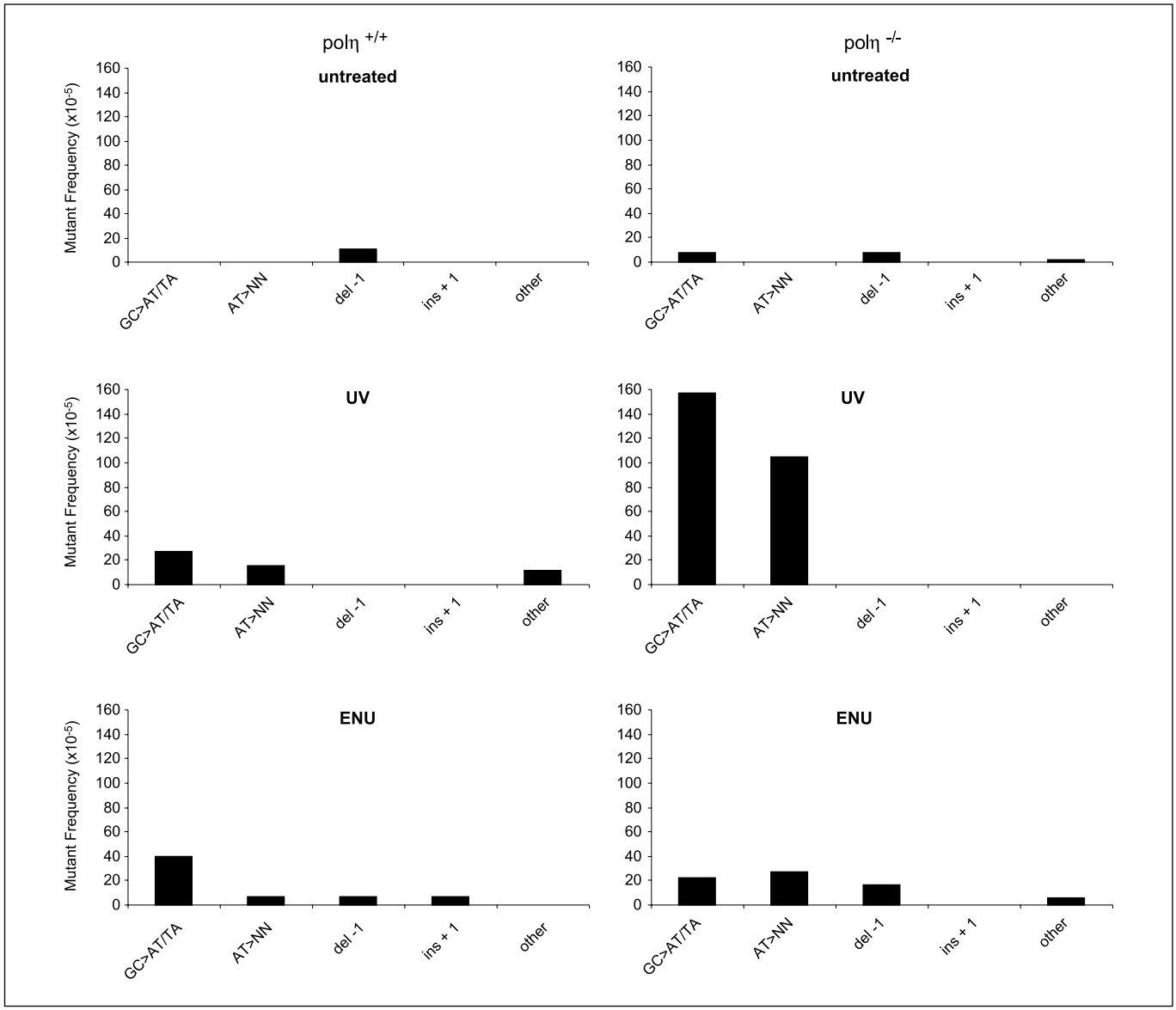


**Figure 2.** Point mutational spectra of polη-proficient and polη-deficient MEFs following exposure to UVC radiation or ENU. NN represents GC, CG, or TA.

To further characterize the types of mutations induced by UV or ENU in polη-proficient and polη-deficient cells, we characterized the lacZ mutants recovered from the cells. lacZ-plasmids showing no change in size after restriction digestion (no-change mutants) are generally point mutations, whereas those plasmids that show a size change after digestion (size-change mutants) are generally genome rearrangements with one break point within the lacZ and another elsewhere in the mouse genome (7). As expected, most of the UV- or ENU-induced mutations in all of the genotypes studied were of the no-change class (Fig. 1).

To further investigate the nature of the point mutations induced in polη wild-type and knockout MEFs after exposure to UV or ENU, we sequenced 10 lacZ mutants of the no-change class for each experimental group. In the cells deficient for polη, the spontaneous mutation spectrum consisted mostly of 1-bp deletions, similar to the control cells (Fig. 2; Table 1). UV, in both mutant and control cells, was found to induce base pair substitutions. The polη-deficient cells were different from the controls by a relatively higher fraction of transversion mutations (Table 1). This shift to more transversion mutations in polη-deficient cells has also been reported by others (8). Interestingly, following UV treatment in both wild-type and polη-deficient cells, we did not observe any persisting 1-bp deletions. The ENU spectrum was not significantly different between the mutant and wild-type cells.

**Mutant frequencies in tissues of polη-deficient mice.** Figure 3 shows the total spontaneous mutant frequencies in liver, spleen, and small intestine of polη wild-type, heterozygous, and knockout mice. The average mutant frequency in the liver of 12-month-old wild-type mice was $8.4 \times 10^{-5}$, with no significant difference when livers of heterozygous ($9.2 \times 10^{-5}$) or knockout ($8.1 \times 10^{-5}$) animals were studied (Fig. 3*A*). Similarly, no significant difference in mutant frequency was observed in the spleens of 12-month-old polη wild-type ($7.2 \times 10^{-5}$), heterozygous ($7.0 \times 10^{-5}$), or knockout ($7.6 \times 10^{-5}$) mice (Fig. 3*B*). We did observe a higher mutant frequency in the small intestine of similarly aged polη knockout mice ($25.5 \times 10^{-5}$) when compared with wild-type ($16.6 \times 10^{-5}$) or heterozygous ($15.5 \times 10^{-5}$) controls. However, it is obvious that the SD in this case was very high (Fig. 3*C*), which is due to a very high mutant frequency in one animal. Such occasional outliers are not unusual in this kind of experiment, but we nevertheless decided to follow this up by determining the mutant frequency in the small intestine of 24-month-old polη mice. Figure 3*D* shows that even at 2 years of age there was no effect of the polη deficiency on the spontaneous mutant frequency in the small intestine. Although an extensive life span study was not performed on these animals, we did notice that there seems to be no phenotypic effect of the polη deficiency. Of the seven animals remaining after our 12-month determination point, none died prematurely and the two not sacrificed at 24 months were still alive at 28 months.

**Table 1.** Main categories of point mutations in UV- or ENU-treated polη wild-type and knockout MEFs

| Treatment | polη | Mutant frequency ($\times 10^{-5}$) | | | |
|---|---|---|---|---|---|
| | | Transitions | Transversions | 1-bp deletions | Other |
| Untreated | Wild-type | 0 | 0 | 10.98 | 0 |
| | Knockout | 1.84 | 5.51 | 7.37 | 1.84 |
| UV | Wild-type | 38.62 | 15.77 | 0 | 0 |
| | Knockout | 131.16 | 131.16 | 0 | 0 |
| ENU | Wild-type | 19.8 | 26.4 | 6.9 | 6.9 |
| | Knockout | 16.31 | 37.59 | 17.02 | 0 |

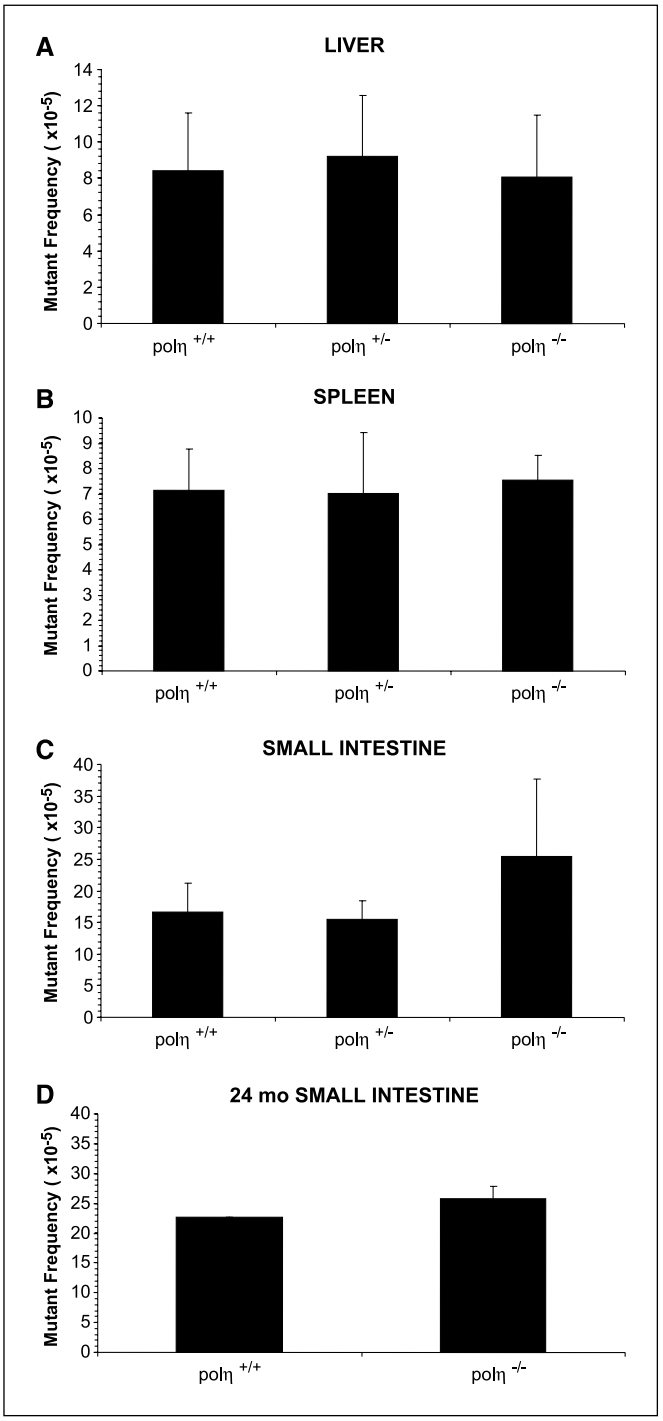


**Figure 3.** Spontaneous mutant frequencies in the liver (*A*), spleen (*B*), and small intestine (*C*) of 12-mo-old polη wild-type, heterozygous, and knockout mice. *D,* mutant frequencies in the small intestine of 24-mo-old wild-type and knockout mice.

## Discussion

The Y polymerase polη, encoded by the *POLH* gene, is able to insert the correct A base across a cyclobutane thymidine dimer, the main UV-induced lesion. When encountering replication-blocking lesions, the use of such enzymes increases the chance of survival while maintaining genome integrity. The results presented in this article indicate that the loss of this enzyme in mouse cells is associated with a dramatic increase in UV-induced mutations. This result is very similar to what has been observed in human *POLH* fibroblasts, lacking this same polymerase, using the hypoxanthine phosphoribosyltransferase (HPRT) selectable locus assay (9). Like in the HPRT assay, we also observed a shift to primarily transversion mutations. Interestingly, after treatment with the powerful mutagen ENU, we did not see any difference in mutation induction between polη-defective cells and their wild-type controls. Hence, these results validate the lacZ system in accurately measuring mutations associated with polη deficiency.

There is some evidence that, at least *in vitro*, polη can bypass several other DNA lesions that seem to be structurally unrelated. For example, it has been shown that yeast and human polη replicate DNA containing 8-oxoguanine efficiently and accurately by inserting a cytosine across the lesion and by proficiently extending from this base pair (10). In that study, spontaneous mutations were found to increase in the absence of polη in yeast, suggesting a possibly more general role for polη in the suppression of spontaneous mutations that can give rise to human internal cancers. The results of our present work, which was undertaken to investigate the possible mutagenic effect of polη deficiency *in vivo*, do not support this. Indeed, the spontaneous mutant frequencies at the lacZ locus in the polη-deficient mice do not exceed those normally observed in these tissues. Even as late as 24 months in highly mitotically active tissue from the small intestine, no evidence was obtained for an increased level of spontaneous mutations. This is in spite of the fact that we have previously reported a considerable age-related increase of point mutations in this tissue that most likely results from replication errors (11).

Meanwhile, our present data do not directly refute the possibility that oxidative damage, such as 8-oxoguanine, is bypassed by polη. However, if such bypass would occur and the presence of 8-oxoguanine would give rise to increased mutant frequencies *in vitro*, we should have observed higher spontaneous mutant frequencies in the polη-defective cells due to oxygen present during cell culture. As shown in Fig. 1, this was not the case. Although we routinely culture cells at 3% and not at 20% oxygen, some oxidative damage is unavoidable (e.g., during isolation of the cells, which is at ambient oxygen). Indeed, previous results from our laboratory revealed 8-oxoguanine signature mutations in MEFs, which were significantly elevated at 20% oxygen (4).

Thus, our data support the concept that polη is a highly specific enzyme that evolved during evolution solely for translesion synthesis across CPDs. This begs the question as to why polη seems to be equally proficient in mouse cells as in human cells in its role of suppressing CPD-associated mutations. Indeed, whereas in humans polη is obviously of great importance, its utility in mice is unclear. Mice are night animals with a fur and are therefore unlikely to ever encounter significant amounts of UV. Indeed, mice and rats almost completely lack global nucleotide excision repair (12, 13), the pathway that is critically important to repair CPDs before these can give rise to mutations. It is therefore somewhat surprising that they have maintained proficient polη-mediated CPD translesion synthesis. Of note, all Y polymerases are present in vertebrates. It is therefore conceivable that translesion synthesis is a highly cost-effective way to prevent mutations and that mice simply have not lost this enzyme, possibly because they occasionally still have some exposure to sunlight through their ears and eyes.

Finally, although in this present study we focused on the possibility that polη deficiency could affect spontaneous mutagenesis *in vivo*, lacZ-pol$\eta^{-/-}$ hybrid mice have other applications too. For example, they could be used in pursuing interesting questions related to the role of polη in generating different types of mutations during somatic hypermutation of immunoglobulin genes in B cells (14).

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