

A NEW APPROACH TO THE STUDY OF CHEMICALLY-INDUCED GERM-LINE  
MUTATIONS USING TRANSGENIC FISH

by

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(Under the Direction of Charles H. Jagoe)

ABSTRACT

A new approach using transgenic fish to quantify mutations directly in a genomically integrated mutation target gene has shown significant promise for improved analyses of germ-line mutations. Transgenic medaka carrying the  $\lambda$ cII target gene were exposed to the potent germ cell mutagen, ethylnitrosourea (ENU), and bred for 6 days before and after exposure to untreated non-transgenic females. Offspring carrying mutated cII genes were readily identified using the  $\lambda$ cII mutation detection assay. Mutant frequencies in the cII target gene of offspring from exposed males were elevated significantly above those from the same males before exposure, and the highest mutant frequency observed was  $2.62 \times 10^{-2}$ . Sequencing offspring with elevated mutant frequencies revealed identical mutations in all or multiple cells. Transgenic mutation assays are a powerful approach for detecting mutations *in vivo*, and this approach could make germ-line mutagenesis studies more efficient and economical.

INDEX WORDS: germ-line, transgenic, transgene, cII,  $\lambda$ cII, Japanese medaka, *Oryzias latipes*, mutant frequency, mutations, mutants, spermatozoa, mosaic, whole-body

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## DEDICATION

This thesis is dedicated to my parents, Jim and Jane for their support. I want to thank my brother, Matt, sister Julie, and sister-in-law Laura for their interactions during this endeavor. I also want to thank my grandparents, Annabel, Marion, Florabel and Joseph for giving me the monetary means as well as the drive to keep going. Lastly, I want to thank Taras for I cannot put into words my gratitude.

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## CHAPTER 1

### INTRODUCTION

Mutations are alterations in the sequence of DNA that introduce variation and novelty in biological systems (1). These alterations are ongoing and their fate in populations is determined under selective or nonselective conditions. Mutations can occur during replication, transcription, or translation and their types can range from base-pair point mutations to large-scale deletions or insertions involving multiple base pairs. Mutations are a leading cause of both heritable and non-heritable diseases. Furthermore, functions of cells, including proliferation, metabolism, toxicity, and DNA repair, can be better understood by studying mutational events.

Mutations can be heritable or non-heritable, and can occur in somatic or germ cells. Heritable mutations can happen in both somatic or germ cells but somatic mutations are not transmissible through the germ-line. Unrepaired somatic mutations may lead to cancer if they occur in critical parts of important genes (2). Mutations that occur in germ cells can be transmitted to subsequent generations. These mutations can alter interactions between heritable factors and genomic stability (two or more mutations appearing at the same base in one individual) leading to genetic diseases in future generations (3). By studying germ-line mutations, we can better understand processes that lead to genomic instability.

Male germ-line mutagenesis experiments have been carried out on cells from pre-meiotic (spermatogonia and spermatogonium) and post-meiotic (spermatocytes, spermatids and spermatozoa) germ cell stages (Appendix 2). Germ cells undergo cell division, and the time required (days) for germ cells to differentiate into the subsequent spermatogenic stage varies from species to species. For example, in Japanese medaka (*Oryzias latipes*) embryos collected

daily for up to 30 days following exposure to a mutagen each contain cells which stemmed from a particular type of sperm cell precursor that was present at the time of the exposure (4).

Collection of medaka embryos one to three days after exposure of the male parent result in offspring that stemmed only from mature spermatozoa, whereas collection of embryos 30 days after parent exposure result in offspring that stemmed only from exposed spermatogonium (Figure 1.1). In comparison, mouse embryos collected one to seven days after the exposure contain DNA that stemmed only from mature spermatozoa, whereas those collected > 42 days after the exposure of the male parent contain DNA that stemmed only from spermatogonium (5, 6, 7).

Various methods have been used to study mutations in different germ cell types. Assays that have been used to study mutations in the germ-line include the dominant lethal assay, the sex-linked recessive lethal test, the heritable translocation assay, the micronucleus test, the spermhead morphology assay, and various transgenic animal assays (2). Germ-line mutations have been identified *in vitro*, by using cell cultures, or by comparing pedigrees with genotyping or direct sequencing (2). Scientists have primarily used the specific locus test to detect the rate of germ-line mutations *in vivo*. The specific-locus test (SLT) was developed by W.L. Russell (8) and has served as the basis for studying *in vivo* mutations in germ-lines. This method was first developed on rodents, and uses animals that are homozygous recessive at up to seven loci (9). The loci are markers that, if mutated, will phenotypically change either the type or distribution of pigment in the coat, eye color, hair structure, or morphology of the external ear (9). This specially bred animal is mated with a wild-type animal after treatment with a mutagen. If a mutation occurs at the marker locus or loci, it can be phenotypically observed in their offspring (9, 10). The advantage of this technique is that mutant offspring can be easily detected, thus

many individuals can be screened in a relatively short amount of time. However, because such mutations are rare, thousands of individuals may have to be screened to determine mutation rates. For example, the SLT was used to study transmissible mutations in mouse spermatogonia following exposure to 250 mg/kg of ethylnitrosourea (ENU), a common laboratory mutagen. Male wild-type mice were exposed to ENU and bred 13 + weeks post-treatment with a standard specific-locus test strain of females homozygous for seven marker genes. Offspring were visually screened for mutations, and out of 7,584, only 35 harbored a visual mutation, a mutation rate of  $4.61 \times 10^{-3}$  per gamete, or  $6.59 \times 10^{-4}$  per locus. For offspring of non-exposed males, out of 531,500 offspring screened, only 28 contained a mutation, representing a mutation rate of  $5.27 \times 10^{-5}$  per gamete, or  $7.53 \times 10^{-6}$  per locus (11). Though the results indicated that the germ-line mutation rate increased about 100-fold after ENU exposure, the SLT method can be costly and time-consuming. Furthermore, it is specific only to the loci tested, and not applicable to other tissues (12).

Development of transgenic animal models was a revolutionary step in the study of genetic effects in diseases (13). Transgenic model species provided a major progression in our ability to rapidly identify tissue-specific mutations following chemical exposure (12). In 1981, the first transgenic animal was created, and the term “transgenic” was given to this mouse carrying foreign DNA incorporated into its genome (13). Genetically modified mice were used in a variety of tests in the early 1980s, including those on growth hormones, which contributed largely to the transgenic “technology” at that time (13). As techniques rapidly progressed in recent decades, other transgenic systems were developed including fish, chickens, pigs and plants. Development of tests based on transgenic fish were advantageous, because these type of tests are generally less time-consuming, use fewer animals, and are thus more cost-effective.

Considering the vast experience that researchers accumulated using transgenic mammals, fish have brought a new dimension to the study of mutagenicity because of their capacity as indicators in aquatic environments (14).

In this study we introduce a new approach for studying germ-line mutations. We combined a procedure used to study somatic mutations in the transgenic fish model, Japanese medaka (*Oryzias latipes*), with a new method for the detection of germ-line mutations. This method uses offspring of ENU-exposed individuals to determine mutant frequencies of the cII gene by using the  $\lambda$ cII mutation detection assay.

The cII gene is part of the  $\lambda$ LIZ (310) shuttle vector and has been permanently integrated into the genome of transgenic medaka. The cII gene is nonfunctional in the fish, and there are multiple copies of this gene per cell in a single animal. The assay allows maximum recovery of cII genes from the extracted DNA, and mutated and non-mutated genes can be readily identified using the assay. The cII mutant frequency is the rate of mutations that occurred in cells containing the cII gene. The cII mutant frequency is calculated for each fish by dividing the number of mutated cII genes by the total number of cII genes recovered (includes mutated and non-mutated). For example, if the number of mutated cII genes was 20 and the number of total cII genes recovered was 100,000 then the mutant frequency is  $2.00 \times 10^{-4}$  for that individual.

This approach seeks to improve current methods of germ-line mutation detection by making them more sensitive, efficient, and economical. It is more sensitive than current methods of germ-line detection because multiple cII genes per fish can be readily observed for mutations. The approach is efficient because of the high numbers of cII genes recovered per fish. This reduces the number of offspring that must be examined to detect mutations because hundreds of thousands to over a million targets (cII genes) can be screened in the same offspring. In contrast

to the SLT, where the maximum number of loci screened for germ-line mutations in one offspring is seven, a cII transgenic medaka contains approximately 75 cII genes per cell that can be screened for germ-line mutations. The  $\lambda$ cII mutation assay is economically efficient because it lowers overall laboratory costs, and reduces animal care expenses. This method can also potentially serve as a standard technique for detecting germ-line mutations caused by other agents by making the assessment process more routine.

#### $\lambda$ cII Transgenic Fish Development

The  $\lambda$ cII target gene is a part of the  $\lambda$ LIZ shuttle vector ( $\approx 45.5$  kb). To develop transgenic fish, the vector was injected into the one-cell stage of the developing embryo and became permanently integrated into the genome. Mutations in the cII gene are phenotypically identified *ex vivo* and are characterized by sequencing. In the transgenic medaka lineage that we used, each haploid cell contains approximately 75 copies of the cII gene. Transgenic fish are bred to homozygosity for the target gene to get 100% homozygous offspring that contain 150 copies of the target gene. The foreign DNA as well as the endogenous DNA is repaired during replication and transcription events. However, unlike the endogenous DNA that has capabilities of undergoing repair during translation, this foreign DNA is not translated, which makes it functionally irrelevant. This feature makes the cII gene very useful as a target for the detection of mutations. An additional advantage of the cII assay is that multiple targets can be screened each time in every fish, allowing for the reduction in a number of individuals that need to be screened to detect a single mutation event.

#### ENU Mutagenesis

ENU is a potent monofunctional-ethylating agent that scores positively in various types of mutagenicity test systems ranging from mammalian germ cells to viruses (15). It is the most

extensively studied genotoxic agent in male germ cell mutagenicity studies (16), and the most potent chemical mutagen in spermatogonial stem cells for mice and *Drosophila*. ENU has been shown to induce both pre-meiotic and post-meiotic cell mutations in mice, and has been established as a model compound for studying the effects of chemical mutagenesis on mice germ cells (15). In addition, ENU is used as the mutagen of choice for saturation mutagenesis studies in fish and rodent models. Male animals are treated with ENU to generate mutant offspring, which are then used in growth studies of gene function.

ENU is a direct alkylating agent that ethylates nucleic acids, and a number of reactive sites have been identified (15). These include the N-3, N-7 and N-1 of adenine (A), the N-7, O<sup>6</sup> and N-3 of guanine (G), the O<sup>2</sup>, O<sup>4</sup> and N-3 of thymine (T), the O<sup>2</sup> and N-3 of cytosine (C) (17-23). About 60% of the total ethylation product of ENU can be accounted for by the formation of ethyl phosphate triesters. ENU can induce high levels of alkylation at oxygens, such as the O<sup>6</sup> position of guanine and the O<sup>4</sup> position of thymine of DNA. Studies of various species exposed to ENU suggest that this compound causes mostly GC-AT transitions and, to a lesser extent, AT-GC, AT-CG, AT-TA, GC-CG and GC-TA base substitutions (15).

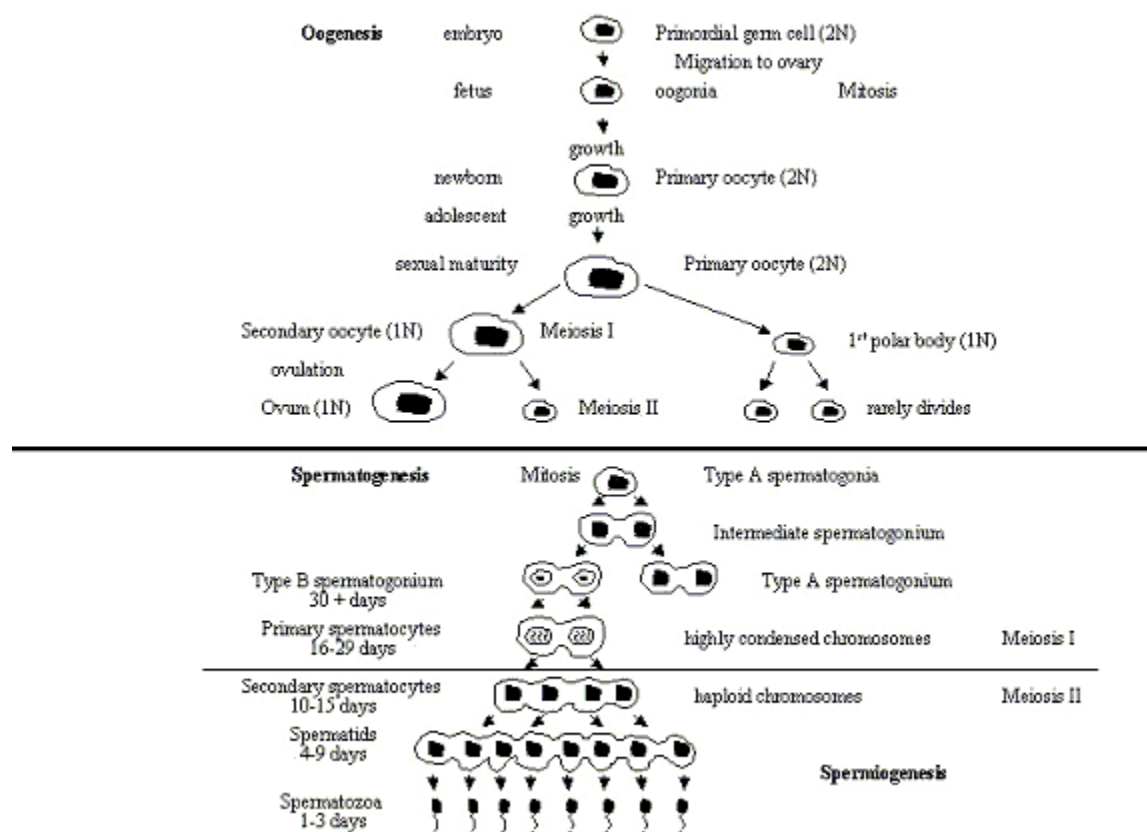
The assays used to study germ-line mutations in *Drosophila* have been mainly based on sex-linked recessive lethal (SLRL) tests and the specific-locus tests (15). Following ENU exposure, most mouse studies showed that post-spermatogonial germ cells had a much lower sensitivity than spermatogonia. However, several studies using *Drosophila* showed cells in both spermatogonia and post-spermatogonial stages were very sensitive to ENU (24-26). ENU induces both whole-body mutants and mosaic mutants. Mosaic mutants are those in which the F<sub>1</sub> progeny from treated parents contain both mutant and non-mutant cells (15). ENU has been used

widely in germ-line mutagenesis studies, and should provide positive results by inducing mutations in individuals at readily observable levels.

## OBJECTIVES

The objectives of this experiment were to assess the cII mutant frequency in juvenile medaka (seven to eight day old offspring) collected zero to six days after fertilization. Offspring were the result of crosses between  $\lambda$  transgenic males that had been exposed to ENU and untreated wild-type females. Each male served as its own control by collection of offspring from matings before ENU exposure. Mutant frequencies were measure using the  $\lambda$ cII mutation detection assay on individual offspring.





**Figure 1.1.** Gametogenesis. Oogenesis depicts the female germ cell development. Reproducible mature females contain only oocytes and contribute DNA present in these cells to their offspring. Spermatogenesis depicts the male germ cell development. Collections of Japanese medaka (*Oryzias latipes*) offspring from day one through 30+ days contain DNA present in a stem cell from a particular spermatogonial stage (37). It takes 30 days for spermatogonium to become mature sperm, and the DNA present in spermatogonium from day one is conserved to the mature sperm that will potentially fertilized an egg on the 30<sup>th</sup> day of collection.

## CHAPTER 2

### A NEW APPROACH TO THE STUDY OF CHEMICALLY-INDUCED GERM-LINE MUTATIONS USING TRANSGENIC FISH<sup>1</sup>

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<sup>1</sup>Audrey J. Majeske, Charles H. Jagoe, Travis C. Glenn, Michael H. Smith and Richard N. Winn.  
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## ABSTRACT

Germ-line mutations are alterations in a DNA sequence passed to subsequent generations. Until recently, insensitive, time-consuming, and costly methods have hampered the progress of studies of transmissible mutations. A new approach using transgenic fish to quantify mutations directly in a genomically-integrated mutation target gene has shown significant promise for improved analyses of germ-line mutations. Transgenic medaka (*Oryzias latipes*) carrying the  $\lambda$ cII transgene target were used to characterize the mutations in the offspring of mutagen-treated males. Fish were bred for 6 days to untreated non-transgenic females then exposed to the potent germ cell mutagen ENU and bred for 6 more days. Mutations in cII genes of 7-8 days old offspring from each male were readily detected using the  $\lambda$ cII mutation detection assay. Mutant frequencies in the cII target gene of offspring from ENU-exposed males were elevated significantly above that of the same males before ENU exposure. The highest mutant frequency observed in an offspring collected after treatment was  $2.62 \times 10^{-2}$ , compared to a mean of  $2.80 \pm 0.41 \times 10^{-5}$  for offspring of all males before treatment. The percentage of offspring with elevated mutant frequencies varied from 11% to 48% among male parents. Sequencing revealed identical mutations in all or multiple cells per individual. These results illustrate the utility of this approach for detecting inherited mutations.

## INTRODUCTION

Mutations are a leading cause of both heritable and non-heritable diseases, and can occur in somatic or germ cells. Heritable mutations can happen in both cell types but somatic mutations are not transmissible through the germ-line. If germ-line mutations are not repaired, they can cause DNA damage and alter interactions between heritable factors and genomic stability leading to genetic diseases in future generations (3). By learning about germ-line mutations, we can get a better understanding of processes that lead to genomic instability, when two or more mutations appear at the same base in an individual. Furthermore, functions of cells, including proliferation, metabolism, toxicity, and DNA repair, can be better understood by studying germ-line mutational events.

Germ-line mutations have been examined extensively in *Drosophila* and mice (27, 28). However, the amount of data that has been collected on germ cells in each stage of spermatogenesis varies among species. Most of the male germ-line mutation data collected for *Drosophila* were from cells in the post-meiotic stages (spermatocytes, spermatids, and spermatozoa), whereas for mice, results were predominantly from pre-meiotic (spermatogonia, spermatogonium) germ cell stages (29, 30). Due to recent developments, this situation has changed, because a larger database for germ-line mutations has been collected for both *Drosophila* and mice in general, and specifically more male germ-line data has been collected on post-meiotic germ cell stages in mice (28). Though there has been an increase in amount of data collected, one method has dominated germ-line mutation studies.

Scientists have primarily used the specific locus test (SLT) to detect germ-line mutations *in vivo*, and to calculate mutation rates. The SLT was developed by W.L. Russell and has served as the basis for studying *in vivo* mutations in germ-lines (8). This method was first developed on

rodents, and uses animals that are homozygous recessive at up to seven loci. The loci chosen are morphological markers that, if mutated, will cause observable phenotypic changes in either the type or distribution of pigment in the coat, eye color, hair structure, or morphology of the external ear (9). In this approach, these specially bred animals are mated with wild-type animals after treatment with a mutagen. If a mutation occurs at the marker locus, and is carried through the germ-line, it can be phenotypically observed in offspring (9, 10). Because an offspring carrying one or more phenotypic mutations can be easily detected, many individuals can be screened in a relatively short amount of time. However, only one locus per individual usually mutates, and these mutations are rare. Therefore, thousands of individuals typically have to be screened to estimate mutant frequencies. This can be a costly and time-consuming endeavor. In addition, this technique is highly tissue specific to only the particular loci tested, and not applicable to other tissues (12).

Our objective was to develop a new approach for the detection of germ-line mutations by modifying one used to study somatic mutations in a transgenic lineage of a model species, Japanese medaka (*Oryzias latipes*). Our approach uses offspring of males exposed to ethylnitrosourea (ENU) to determine germ-line mutant frequencies of the cII gene using the  $\lambda$ cII mutation detection assay. This approach represents an improvement of current methods of germ-line mutation detection because it is more sensitive, efficient, and economical. In contrast to the SLT, where the maximum number of loci screened for germ-line mutations in one offspring is seven, a cII transgenic medaka contains approximately 75 cII genes per cell that can be screened for germ-line mutations. Hundreds of thousands to over a million targets (cII genes) per fish can be screened for mutations in the same offspring, which reduces the number of offspring needed to screen mutations. This approach is cost efficient because the need for a smaller sample size

lowers the overall animal care costs. Furthermore, because multiple mutations can be screened at once, the need for hundreds of thousands of screens is eliminated. This approach can be used to routinely detect germ-line mutations caused by a variety of agents. Furthermore, it can also potentially serve to standardize approaches for assessing germ-line mutations caused by other agents by making the assessment of germ-line mutations more routine.

## MATERIALS AND METHODS

### Animal Exposure and Offspring Collection

Japanese medaka were obtained from our in-house breeding program. Transgenic medaka containing the  $\lambda$ LIZ shuttle vector from the 310 lineage and wild-type medaka were used in the study. Fish were fed newly hatched brine shrimp daily and kept in aerated dechlorinated fresh water tanks at 27 to 28°C.

Six month-old transgenic medaka males (n=6) were placed in a beaker containing aerated water (250 ml) and 100 mg/L of ENU at room temperature for one hour. The treated fish were then rinsed with clean water and transferred into another beaker containing aerated fresh water. A separate exposure of 100 mg/L of ENU was repeated after 24 hours for each animal. Individual fish were transferred to tanks containing five to seven untreated wild-type females.

Eggs were collected six days prior to the exposure, as well as zero to six days after the exposure. Eggs were examined for fertilization and non-fertilized eggs were removed. Dead embryos were removed and recorded throughout incubation. Live embryos were kept in an egg rearing solution (10% NaCl, 0.3% KCl, 0.4% CaCl<sub>2</sub>, 1.6% MgSO<sub>4</sub>) until hatched. Embryos that did not hatch and those with visible growth defects were frozen on the 18th day after their collection. Embryos, and seven to eight day old fry were frozen in liquid nitrogen and held in an -80° freezer until needed for DNA extraction.

### DNA Isolation

A DNA extraction method optimized to obtain high molecular weight DNA required for *in vitro* packaging was used to isolate DNA from the offspring (33). Whole fish were homogenized in buffer (1X SSC, 1% SDS), and then incubated with proteinase K (0.6 mg/ml) at 37°C for 30 minutes. Digestions were extracted with an equal volume of phenol:chloroform, and centrifuged at 4000 rpm for 10 minutes at 4°C, and potassium acetate (1M) was added followed by a final extraction with chloroform. DNA was then washed with ethanol and resuspended in TE buffer (10mM Tris, 1mM EDTA) overnight and kept at 4°C until analysis.

### $\lambda$ cII Mutation Detection Assay

Recovery of the  $\lambda$ LIZ target vector containing the  $\lambda$ cII gene (294-bp) was performed using modified methods previously described by Stratagene (35). DNA (5-10  $\mu$ g) was packaged over two 1.5 hr intervals. SM buffer (50 mM Tris, 10 mM MgSO<sub>4</sub>, 0.001% gelatin, 0.1 M NaCl, pH 7.5) was added (990  $\mu$ l) after the second packaging interval followed by a brief vortex, and samples were placed on ice. Packaged DNA was then mixed with G1250 *E. coli* bacteria, incubated, and plated. G1250 *E. coli* host strains contain hfl- genes that allow growth under conditions that favor lysogeny. The  $\lambda$  bacteriophage infects the *E. coli*, and colonies that contain the wild-type  $\lambda$ cII strain growing at 24°C for 40 hours undergo lysogenation and become part of the bacterial lawn. The  $\lambda$ cII strains survive and undergo the lytic cycle resulting in detectable plaques (31). Total number of plaque forming units (PFU) containing mutated and non-mutated cII genes was determined by plating 20  $\mu$ l of three separate 100-fold dilutions of packaged DNA onto three replicate titer plates, which were incubated overnight (16 hrs) at 37°C (non-selective conditions). The remaining packaged DNA was plated onto 10 screening plates (100  $\mu$ l per plate), and incubated under selective conditions (40 hrs at 24°C) to produce growth of phage

containing only mutant cII genes. The cII mutant frequency was calculated for each individual fry by dividing the total number of plaques on the screening plates by total number of PFU recovered. Mutant frequencies of offspring from males were obtained before exposure and after exposure (i.e., untreated and treated, respectively).

### Sequencing

Sequencing was performed on mutant cII genes recovered from offspring of the six untreated and treated males to verify mutant plaques and determine the mutational spectra. Males were divided into groups based on initial cII assay results: those with and without elevated mutant frequencies. For the untreated and treated group having non-elevated mutant frequencies, sequencing was performed for one offspring from each male with cII mutant frequencies ranging from  $1.4 \times 10^{-5}$  to  $4.5 \times 10^{-5}$ . For the treated group having elevated mutant frequencies, only offspring with a cII mutant frequency greater than  $1.2 \times 10^{-4}$  were sequenced (13 offspring). Individual plaques were cored (approximately 20 plaques per offspring) from the mutant screening plates, and replated under selective conditions to insure isolation and purification of each cII mutant plaque. DNA polymerase chain reaction (PCR) was performed using primers specific for the  $\lambda$ cII target vector: 5'-'AAAAAGGGCATCAAATTAAACC-3' and 5'-CCGAAGTTGAGTATTTTGTGT-3'. The PCR product was cleaned using a QIAquick<sup>®</sup> PCR Purification Kit from Qiagen<sup>®</sup>. The cleaned product was analyzed on a Beckman DU<sup>®</sup> 530 Life Science UV/VIS Spectrophotometer to determine its concentration, diluted to approximately 0.01  $\mu\text{g}/\mu\text{l}$ , and used in cycle sequencing with Big Dye<sup>®</sup> 1.0 or Big Dye v.3.1<sup>®</sup> (Applied Biosystems). The amplified product was cleaned of excess dye using a Qiagen<sup>®</sup> DyeEx 2.0 Spin Kit and dried overnight. Template suppressant reagent (TSR) was added (Applied Biosystems) to DNA samples followed by a series of mixing, heating and cooling using instructions provided



by Applied Biosystems. Samples were sequenced on an ABI Prism 310 Genetic Analyzer and analyzed for mutations using Sequencher 3.1.1 (Gene Codes Corporation). Sequences were excluded if they contained more than one mutation because it is highly unlikely that this would occur in a single cII gene.

### Statistical Analyses

Analysis of cII mutant frequencies was conducted using several different statistical approaches. Differences in cII mutant frequencies in offspring collected before and after ENU treatment were analyzed using SAS (v. 8.1) (SAS Institute, Cary, NC, 2001) (32). A Kruskal-Wallis test ( $p < 0.05$ ) was performed to determine if there were significant differences between offspring from untreated and treated males. We attempted several transformations of data, including log-normal, exponential, and arcsine, and tested for normality by the Kolmogorov-Smirnov test ( $p > 0.05$ ). However, we were unable to transform data so it was normally distributed. Mutations are rare events and can be characterized as counts, and thus their distribution is best approximated by the Poisson distribution (33). We used the generalized model procedure (PROC GENMOD) of SAS (v. 8.1) to test for differences between the mutation counts in pre- and post-treatment offspring. Pearson transformation was applied to the counts to adjust for the overdispersion, and counts were weighted by the number of gene targets per cell.

Untreated offspring, those collected before males were exposed to ENU, were grouped by male parent, and treated offspring, those collected after ENU exposure of males were further categorized as non-elevated and elevated for each male parent. We defined non-elevated offspring as those with cII mutant frequencies that were equal to or below the upper 95% confidence interval of the mutant frequency for the untreated offspring. We defined elevated

offspring as those with cII mutant frequencies that were above the 95% confidence interval of the mutant frequency for the untreated offspring.

## RESULTS

### cII Mutant Frequency

Total PFU recovered from offspring of untreated males ranged from 4,780,000 to 12,400,000. Average PFU recovered from offspring of untreated males ranged from 434,545 to 1,240,000. Total PFU recovered from offspring of treated males ranged from 6,842,500 to 17,637,500 (Table 2.1). Average PFU recovered from offspring of treated males ranged from 342,125 to 568,952. Average untreated cII mutant frequencies exhibited by offspring from the six males prior to ENU-treatment ranged from  $2.27 \times 10^{-5}$  to  $3.52 \times 10^{-5}$ , with a mean frequency of  $2.80 \pm 0.41 \times 10^{-5}$  for offspring of all males. Average cII mutant frequencies exhibited by offspring from the six males after ENU-treatment ranged from  $4.87 \times 10^{-5}$  to  $1.60 \times 10^{-3}$ , with a mean frequency of  $5.99 \pm 5.76 \times 10^{-4}$ . The upper 95% confidence interval for untreated offspring ranged from  $4.38 \times 10^{-5}$  to  $6.05 \times 10^{-5}$  across males, and the mean was  $5.18 \times 10^{-5}$ . Average cII mutant frequencies for mutant offspring ranged from  $7.23 \times 10^{-5}$  to  $3.67 \times 10^{-3}$ , and the mean was  $1.97 \times 10^{-3}$  (Table 2.2 and Figure 2.1).

If an offspring inherited a single mutation, the expected elevated cII mutant frequency was as much as one mutant per 75 copies of the cII gene per cell, which is an equivalent to  $1.33 \times 10^{-2}$ . The highest mutant frequency of the cII gene in a mutant offspring was  $2.62 \times 10^{-2}$ , approximately one mutant in 50 genes. The lowest cII mutant frequency in a mutant offspring was  $4.88 \times 10^{-5}$  approximately one mutant in 20,000 genes.

Both statistical approaches indicated significant differences between mutation counts in offspring of untreated and treated males. Offspring of untreated and treated males were

significantly different by the Kruskal-Wallis test ( $X^2 = 25.51$ ,  $p = 0.0001$ ). The Kolmogorov - Smirnov test indicated that the transformed data differed from the normal expectation ( $p < 0.05$ ). Therefore, the generalized model, assuming the Poisson distribution with overdispersion was probably the most realistic approach to test differences between distributions of mutation counts, and it showed highly significant results ( $F = 9.86$ ,  $p = 0.002$ ) (Table 2.3). Percentages of mutant offspring per male were calculated by dividing the number of mutant offspring by the number of offspring analyzed for each male. Percentages for each male ranged from 11% to 48%.

### Sequencing

Each plaque represented a separate cII gene recovered from each fish. Sequencing of cII genes from 13 offspring of treated males with elevated cII mutant frequencies revealed that 18% to 100% of genes from an individual contained identical mutations. DNA sequences from three offspring fathered by two different males contained a group of identical transitions, and a separate group of identical transversions at the same base that was unique for each of those offspring. The most frequently observed mutations in offspring collected after male treatment appear to be located at bases 179 to 182. Two separate frameshift mutations, an insertion or a deletion, were detected in multiple untreated and treated offspring. Such mutations have been previously reported in DNA from somatic cells of adult Japanese medaka from various tissues following ENU exposure, so this region represents a hotspot within the cII gene (34). Furthermore, these were found in offspring of untreated males, and so are not solely due to ENU exposure. Sequencing of DNA from offspring of the untreated males and whose cII mutant frequency was classified as non-elevated showed mostly non-repeating and few identical mutations. In contrast, sequencing of DNA from offspring classified as having elevated mutant frequencies showed high numbers of identical mutations.

## DISCUSSION

This study represents the first use of the  $\lambda$ cII mutation detection assay to measure germ-line mutations that occurred in spermatozoa and proliferated in whole medaka offspring. We found that offspring produced by male fish exposed to ENU, had mutant frequencies much higher than previously reported in the literature (Appendix 2). Several factors may account for the exceptionally high cII mutant frequencies observed in this study. A major factor is the large number of mutations in cII target genes recovered in each animal. This can be attributed to the high number of target cII gene copies present in each animal (75 copies per cell), which increases the chances of a mutation occurring in a target gene and being replicated in subsequent cells of the developing embryo. The high number of mutations in cII target genes, combined with the efficiency of the cII mutation detection assay, which allows for the recovery of many target genes recovered per animal, results in a very sensitive and efficient approach detecting germ-line mutations.

Detecting visible morphological changes resulting from mutations is the basis for the specific locus test (SLT), a widely used method of assessing heritable mutations. Most specific locus tests examine mutations in one to seven loci, and are usually limited to one mutation per animal. In mice, typical marker genes include the *vermillion* gene, an eye color determinant, and genes that control coat pigment, hair structure or morphology of the external ear (9, 16). Other methods used to study germ-line mutations, include the dominant lethal test, which examines numbers of dead embryos in offspring of an exposed parent, and the sex-linked recessive lethal test, which examines numbers of lethal mutations in offspring of an exposed parent (27, 35).

These are also limited because each individual offspring represents either a lethal or non-lethal mutation.

A significant difference between these morphologically based assays and ours is that the former employ genes or loci that are expressed in the species, while our approach utilizes an unexpressed gene. The *cII* gene has been permanently integrated into the genome of the medaka, so it is subjected to endogenous repair during replication, but it is not transcribed and therefore does not undergo transcriptional repair. A disadvantage of methods using expressed genes is that each animal expresses a single copy of the gene, whether mutant or normal. Because mutations are rare, hundreds to thousands of animals have to be screened to document a mutation. An advantage of the *cII* mutation detection assay is that hundreds of thousands to over a million genes per animal are targets for potential mutations. This feature increases the sensitivity and the overall the statistical power for detecting germ-line mutation rates. Furthermore, unlike the SLT, where data from all male parents are combined, our study was not only able to separate data for male parents, but we also calculated mutation rates separately for each offspring.

A recent study measured mutant frequencies in offspring of transgenic Big Blue<sup>®</sup> mice to detect and quantify germ-line mutations. Male mice were exposed to weekly regimes of 100 mgENU/kg body weight for three weeks and observed mutant frequencies as high as  $4.88 \times 10^{-2}$  were observed in the testes of offspring that were collected at least 70 days after exposure (36). Recovery of *lacI* genes in selected offspring of male ENU-treated mice ranged from approximately 30,000 to 50,000 copies per individual, and the number of mutations observed in these offspring ranged from approximately 1,000 to 2,000 per individual in some tissues (36). In our study, recovery of *cII* genes collected after ENU treatment ranged from 100,000 to 1,880,000 copies per offspring, and the number of mutations per offspring ranged from 2 to 27,032 (data

not shown). Recovery of genes in our study was from a 3-fold to 100-fold higher than the *lacI* study. In addition, mutations observed in the *lacI* study stemmed from lesions present in spermatogonial cells, and our study measured lesions in spermatozoa that were present at the time of exposure.

Germ-line mutation data has been collected on germ cells from each spermatogenic stage in previous studies (Appendix 2). Pre-meiotic germ cells (spermatogonia and spermatogonium) are subjected to DNA repair until they become post-meiotic germ cells (spermatocytes, spermatids and spermatozoa), and DNA in these cells is not repaired until after fertilization. If a lesion occurred in a pre-meiotic cell and was replicated in subsequent spermatozoon, separate offspring can contain copies of the same mutation that stemmed from a lesion in the pre-meiotic cell. The time until fertilization following male treatment will determine if offspring are harboring mutations that stemmed from pre-meiotic or post-meiotic germ cells. Time to fertilization can also affect the number of offspring carrying mutations, and the number of separate offspring carrying copies of the same mutation that occurred in an earlier germ cell and was replicated to subsequent spermatozoon. This leads to the question, would there be more offspring that contained mutations from the pre-meiotic cells or post-meiotic germ cells of a treated male parent? Pre-meiotic cells are subjected to DNA repair, and therefore the number of offspring carrying mutations stemming from pre-meiotic cells could be similar to the number of offspring carrying mutations stemming from post-meiotic cells. However, if lesions that occurred in pre-meiotic cells are not repaired then there could be a higher number of spermatozoon carrying lesions. This would result in more mutant offspring, compared to offspring fertilized by mutagen-exposed post-meiotic germ cells.

It is important to note that the mutant frequency for the SLT is derived from dividing the number of offspring screened by the number of offspring displaying a phenotypic mutation. The cII mutant frequency for our study is calculated by dividing the total number of recovered plaques by the number of mutant plaques. The percentage of mutant offspring for each male is calculated by dividing the total number of offspring screened by the number of mutant offspring, or offspring classified as harboring an elevated mutant frequency. Mutant frequency for the SLT is therefore similar to the percentage of mutant offspring for our study.

Results from SLT studies that compared mutant frequencies in cells from different spermatogenic stages did not find that the numbers of mutant offspring stemming from one spermatogenic stage were consistently higher than those from another stage. Shima and Shimada (37) compared mutant frequencies in offspring produced for a period after exposure where offspring would inherit DNA from several germ cell types present in the father at the time of exposure. Using the SLT in medaka, they found that spermatozoa and spermatids were more sensitive to mutagens than spermatogonia. They found that mutant frequencies in offspring that stemmed from spermatozoa and spermatids exposed to a mutagen were higher than those in offspring that stemmed from spermatogonia exposed to the mutagen. Tosal et al. (16) also used the SLT in offspring to determine that spermatozoa in *Drosophila* were more sensitive than spermatogonial cells after exposure with 1 mM ENU. However, other researchers found spermatogonia to be more sensitive than spermatozoa by isolating germ cells in different spermatogenic stages from ENU exposed male transgenic mice (38-41). These inconsistent results probably not only reflect changes in mutant frequency in different germ cells due to DNA repair or replication, but also the method and species used.

We sequenced the cII genes from several offspring of pre- and post-treatment males. We characterized the mutational spectra and classified offspring as having mosaic or non-mosaic mutations. Several offspring classified as having elevated mutant frequencies exhibited “cluster” or “jackpot” mutations. A “jackpot” is an observation of a high frequency of identical mutations in an offspring that results from the replication of a single mutated cell (36, 42). A “clonal” expansion stems from a mutation that occurred in a pre-meiotic cell (spermatids or spermatocytes), and replicated to spermatozoon. A “cluster” stems from a mutation that occurred in a pre-meiotic cell and led to many identical mutants in numerous spermatozoa.

In our study we observed propagation of mutations that arose through replication after fertilization by spermatozoa carrying a lesion in as many as 100% of the plaques sequenced per individual. However, these identical mutations would not be considered jackpot or cluster mutations because the original mutations in our study occurred in post-meiotic cells and not pre-meiotic cells. Offspring carrying 100% identical mutations indicated probable offspring with non-mosaic mutations or a single, whole-body mutation. This is a mutation that occurs at or before the one cell stage in the embryo and is replicated in every subsequent cell. In contrast, offspring that harbor identical mutations in multiple (but not all) sequences contained a mutation that would have had to occur at the two-cell stage or later. If the mutation occurred at the two-cell stage or later, 50% or less of the subsequent cells would carry that same mutation.

Offspring containing DNA that stemmed from a pre-meiotic cell can be non-mosaic, where 100% of cells contain the same mutation (36). Five out of the 13 offspring sequenced were non-mosaic, or whole-body mutations. The remaining eight offspring sequenced were mosaic. A mosaic individual has been defined as an individual harboring two separate mutations in which a further mutation occurred in an already existing gene (43). In mosaic individuals, the



same mutation occurs in <100% of cells, or some cells carry one mutation and other cells carry a different mutation. We observed three mutant offspring harboring two separate mutations, and each occurred more than once at the same base unique to each individual.

The occurrence of a mosaic individual may reflect genetic instability, defined as two or more mutations appearing at the same base in one individual. If a mutation occurred and the DNA was repaired incorrectly at the two-cell stage, or a further mutation occurred at that base position, that second mutation would be replicated and detected in up to 50% of subsequent cells. In three separate offspring in this study one mutation was detected in approximately 80% of cells, and another was detected in approximately 20% of cells at the same location in the target cII gene. This indicates that the second mutation occurred after the two-cell stage. Further sequencing could reinforce these observations, but time and cost considerations prevent all mutant plaques from being sequenced for each offspring.

We attempted to predict whether an individual was mosaic or harbored a single mutation based on the cII mutant frequency of an individual. Figure 2.1, shows two horizontal dashed lines; the first separates offspring with a non-elevated cII mutant frequency from offspring that we classified having an elevated cII mutant frequency. The second dashed line separates those predicted to be mosaic from those predicted to be non-mosaic. Offspring to the left of the second dotted line have what we classified as an elevated mutant frequency and are expected to contain mosaic mutations. Offspring to the right of the second line have a cII mutant frequency above the expected,  $1.33 \times 10^{-2}$ , and are expected to contain non-mosaic mutations, or whole-body mutations. Bars that are not labeled represent offspring identified as mosaic mutants by sequencing, and bars that are labeled as non-mosaic contain offspring with non-mosaic

mutations. We concluded the cII mutant frequency of the individual was inadequate to predict mosaicism, however, more data would be needed to confirm this observation.

## CONCLUSIONS

We developed a sensitive, efficient, and economical approach that improved detection limits for studying germ-line mutations compared to other currently applied techniques. Each offspring used in our study increased the statistical power of the analysis, because the cII mutation detection assay allows recovery of a number of different mutations along with a high total target gene recovery. Our current approach decreased the need for a higher sample size and increased the sensitivity of germ-line mutation detection. Studies screening lower numbers of genes are likely to underestimate mutation rates, or at the very least yield much more variable estimates of mutation rates. It appears that this approach may exceed the sensitivity of other phenotypic-based approaches by 1-2 orders of magnitude.

Our approach needs to be replicated and extended to other mutagens, species, genes, different tissue types and stages in the germ-line to fully evaluate the utility of the novel methods we employed. We predict that timing of reproduction relative to mutagen exposure will influence both the number of mutant offspring and the diversity of the mutations they carry (i.e. mutations arising from spermatozoa, to 0-6 days after treatment vs. mutations arising from spermatogonia at > 30 days after treatment). Sequencing of the mutations was instrumental in characterizing the specific mutations carried by the mutant offspring, and illustrated differences between non-mosaic and mosaic mutants.

The unprecedented cII mutant frequencies in offspring as well as the number of mutant offspring per male parent found in our study may have important implications for risk assessment models, both for humans and for natural populations. This will allow future studies

to efficiently and routinely detect mutations with high specificity and sensitivity, and further the conceptual development of mutation theory. The framework is now established for using this approach to assess germ-line mutations in fish exposed to other mutagens.

**Table 2.1.** cII Mutant frequencies in offspring of  $\lambda$ cII transgenic male medaka (M1-M6) collected before and after ENU treatment. Columns represent number of offspring, total mutants, total PFU, mean PFU, and mean mutants  $\times 10^{-5}$  PFU  $\pm$  standard deviation.

Exposure to ENU	Male Medaka	# Obs.	Total Mutants	Total PFU	Mean PFU	Mean mutants $\times 10^{-5}$ PFU $\pm$ SD
Un-treated						
	M1	14	316	12,320,000	880,000	2.84 (0.98)
	M2	12	168	6,895,000	574,583	2.76 (1.28)
	M3	11	123	4,780,000	434,545	2.72 (1.33)
	M4	10	256	12,400,000	1,240,000	2.27 (1.05)
	M5	10	164	6,410,000	641,000	2.67 (1.22)
	M6	16	255	8,580,000	536,250	3.52 (1.25)
Treated						
	M1	20	8,092	6,842,500	342,125	160 (486)
	M2	28	610	14,550,000	519,643	4.87 (2.47)
	M3	21	541	7,740,000	368,571	12.2 (31.7)
	M4	20	6,844	11,139,835	556,992	52.1 (147)
	M5	31	27,679	17,637,500	568,952	89 (471)
	M6	19	2,708	8,599,000	452,579	41.3 (157)

**Table 2.2.** Number of hemizygous offspring characterized for cII mutations from breeding six medaka before and after treatment of ENU with wild-type un-treated female medaka (M1-M6, homozygous for  $\lambda$ LIZ). Offspring were collected from the six males before and after ENU treatment. Mean cII mutant frequency was calculated for offspring grouped according to father for un-treated and treated group with elevated mutant frequencies. The upper 95% confidence interval was calculated for each mean mutant frequency in the un-treated group and all offspring harboring a mutant frequency above this interval were considered elevated. Averages were calculated from data in mean cII mutant frequency, upper 95%, and elevated offspring from the treated group.

<b>Male Medaka</b>	<b>Un-Treated Offspring</b>	<b>Mean cII Mutant Frequency</b>	<b>Upper 95%</b>	<b>Treated Offspring</b>	<b>Elevated Treated Offspring</b>
M1	14	2.84E-05	4.81E-05	20	3.52E-03
M2	12	2.76E-05	5.33E-05	28	7.23E-05
M3	11	2.72E-05	5.40E-05	21	2.20E-04
M4	10	2.27E-05	4.38E-05	20	1.67E-03
M5	10	2.67E-05	5.09E-05	31	2.67E-03
M6	16	3.52E-05	6.05E-05	19	3.67E-03
Average		2.80E-05	5.18E-05		1.97E-03

**Table 2.3.** Results of type III general model analysis of variance of cII mutant frequencies between the offspring of the un-treated and treated group. The un-treated group corresponds to offspring collected before ENU treatment of male parent  $\lambda$ cII transgenic Japanese medaka. The treated group corresponds to offspring collected from the same males after ENU treatment. Effects of the model are the following: “Treated” represents the difference between un-treated and treated mutant frequencies; “Individuals” represents the differences between the individual offspring; “Treated\*Individuals” represents the interaction term.

Source	Num df <sup>a</sup>	Den df <sup>b</sup>	<i>F</i> -value	<i>p</i> -value <sup>c</sup>
<b>Treated</b>	1	199	9.86	0.002
<b>Individuals</b>	5	199	2.35	0.042
<b>Treated*Individuals</b>	5	199	1.05	0.390

<sup>a</sup>Numerator degrees of freedom;

<sup>b</sup>Denominator degrees of freedom;

<sup>c</sup> $p < 0.05$ -value is significant.

**Fig. 2.1.** Distributions of cII mutant frequencies in offspring of six pre-ENU treated (white bars) and post-ENU treated (black bars)  $\lambda$ cII transgenic male parents. A dashed vertical line is at the upper 95% confidence interval for offspring collected before treatment. Mutant frequencies for offspring to the left of the first vertical line are offspring from the un-treated and treated group with non-elevated cII mutant frequencies. A second dashed vertical line separates expected mosaic and non-mosaic offspring. Offspring to the left of this second dashed line have an elevated mutant frequency that does not exceed one mutant per 75 copies of the cII gene ( $1.33 \times 10^{-2}$ ). Bars to the right of the second dashed vertical line are offspring with a mutant frequency above  $1.33 \times 10^{-2}$  and are expected to be non-mosaic. Mosaic is used to refer to offspring that contain different and/or multiple identical mutations. Bars that are labeled non-mosaic are offspring that contained 100% identical mutations.

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## APPENDIX

**Appendix 1.** cII mutant frequencies in  $\lambda$ cII transgenic Japanese medaka offspring of six males collected before and after ENU treatment of males (M1-M6). Means, standard deviations, minimum and maximum of cII mutant frequency distributions were calculated.

<b>Exposure to ENU</b>	<b>Individual Medaka</b>	<b># Obs*</b>	<b>Mean</b>	<b>Standard Deviation</b>	<b>Minimum</b>	<b>Maximum</b>
Un-Treated	M1	14	2.84E-05	9.80E-06	1.70E-05	4.52E-05
	M2	12	2.76E-05	1.28E-05	1.37E-05	5.26E-05
	M3	11	2.72E-05	1.33E-05	1.11E-05	4.67E-05
	M4	10	2.27E-05	1.05E-05	1.05E-05	4.00E-05
	M5	10	2.67E-05	1.22E-05	1.16E-05	4.51E-05
	M6	16	3.52E-05	1.25E-05	1.17E-05	5.45E-05
Treated	M1	20	1.60E-03	4.86E-03	1.58E-05	1.86E-02
	M2	28	4.87E-05	2.47E-05	1.90E-05	1.36E-04
	M3	21	1.22E-04	3.17E-04	1.33E-05	1.50E-03
	M4	20	5.21E-04	1.47E-03	1.37E-05	6.42E-03
	M5	31	8.90E-04	4.71E-03	1.47E-05	2.62E-02
	M6	19	4.13E-04	1.57E-03	1.04E-05	6.90E-03

\*Numbers of offspring analyzed for cII mutant frequencies using the  $\lambda$ cII mutation detection assay.

**Appendix 2.a.** Literature review of germ-line mutagenesis studies. This is a general overview of some observed mutant frequencies after ENU exposure. For a more complete description of the study and findings refer to the manuscript.

Species <sup>a</sup>	Technique <sup>b</sup>	ENU dose <sup>c</sup>	Germ stage <sup>d</sup>	Collection <sup>e</sup>	Spontaneous <sup>f</sup>	Induced <sup>g</sup>	Author <sup>h</sup>
mouse	LacI	10mg/kg	spermatogonia	offspring	1.396 X 10 <sup>-3</sup>	4.17 X 10 <sup>-2</sup> kidney 4.75 X 10 <sup>-2</sup> testis	(Barnett, 2002)
mouse	SLT	150mg/kg	spermatogonia	parent	8 X 10 <sup>-6</sup> to 1 X 10 <sup>-4</sup>	5 X 10 <sup>-4</sup> high 100d post treatment	(van Delft, 1995)
mouse	LacZ	150mg/kg	post stem and stem	parent		2.8 ± 1.1 X 10 <sup>-5</sup> post stem 3.7 ± 1.25 X 10 <sup>-4</sup> stem	(van Delft, 1997)
mouse	LacZ	50mg/kg	semi tubules/diff days	parent		5.8 X 10 <sup>-5</sup> after 5 d 10, 15, 35, 45 d (4.56 X 10 <sup>-4</sup> ), 55 d after exposure	(Douglas, 1995)
mouse	LacZ	150mg/kg	semi tub and epidid	parent		25 d (1.86 ± 0.1 X 10 <sup>-4</sup> ) semi 91 d (2.57 ± .18 X 10 <sup>-4</sup> ) semi 25 d (3.8 ± 0.5 X 10 <sup>-5</sup> ) epi 91 d (2.56 ± .14 X 10 <sup>-4</sup> ) epi	(Douglas, 1997)
mouse	SLT	160mg/kg	spermatogonia	offspring	1.6 X 10 <sup>-5</sup>	2.6 to 7.7 X 10 <sup>-5</sup>	(Favor, 1998)
mouse	LacI	150mg/kg	semi tub/3 days	parent		2.5 to 10.5 X 10 <sup>-5</sup> sperm 9 to 18.1 X 10 <sup>-5</sup> semi tub	(Gorelick, 1997)
mouse	Big Blue <sup>®</sup> Muta <sup>®</sup>	150mg/kg	sperm/diff days	parent	1.57 to 4.28 X 10 <sup>-5</sup>	3 d 2-3.83 X 10 <sup>-5</sup> sperm 14 d, 22 d, 93 d (1.7962.441X 10 <sup>-4</sup> )	(Katoh, 1997)
mouse	LacZ	150mg/kg	separated	parent	9.5 ± 5.3 X 10 <sup>-5</sup>	6.53 ± 2.98 X 10 <sup>-4</sup> 1.01 X 10 <sup>-3</sup> (highest)	(Liegibel, 1997)
mouse	LacI	250mg/kg/day 100mg/kg/week	semi tubules/90 days	parent	1.0 ± .4 X 10 <sup>-5</sup>	3 d (3.2 ± 0.8 X 10 <sup>-5</sup> ) 3 weeks (20.5 ± 7.5 X 10 <sup>-5</sup> )	(Provost, 1994)

<sup>a</sup>Study species;

<sup>b</sup>Technique used in the study;

<sup>c</sup>ENU dose administered in study;

<sup>d</sup>Type of germ cell analyzed for mutant frequency;

<sup>e</sup>Offspring or parent used to measure mutant frequency;

<sup>f</sup>Spontaneous mutant frequency;

<sup>g</sup>Summary of ENU-induced mutant frequencies, includes and/or range of mutant frequency observed (limited), tissue(s) analyzed type of cell(s) analyzed, and time (days = d) of collection after exposure;

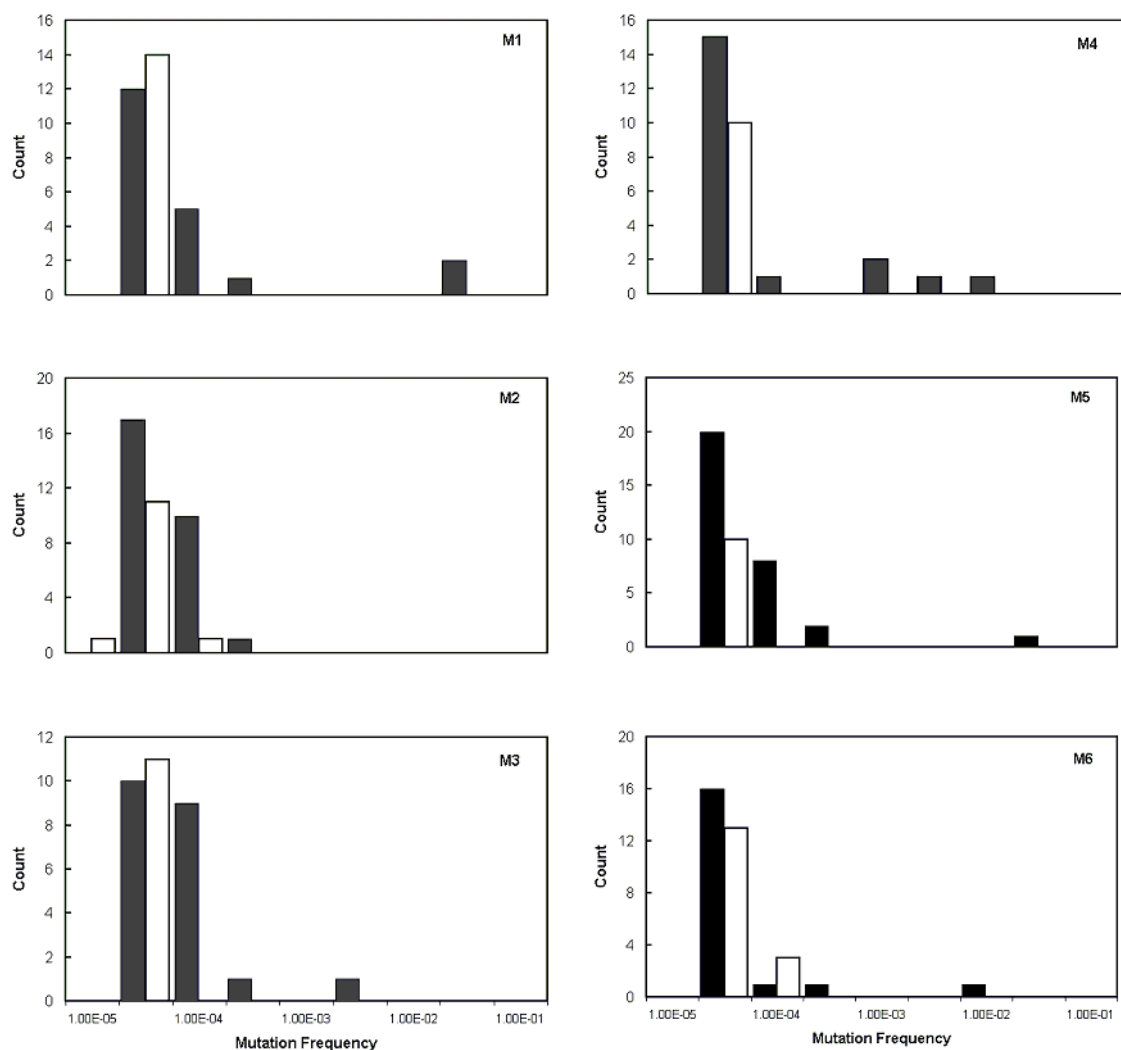
<sup>h</sup>Reference for study.



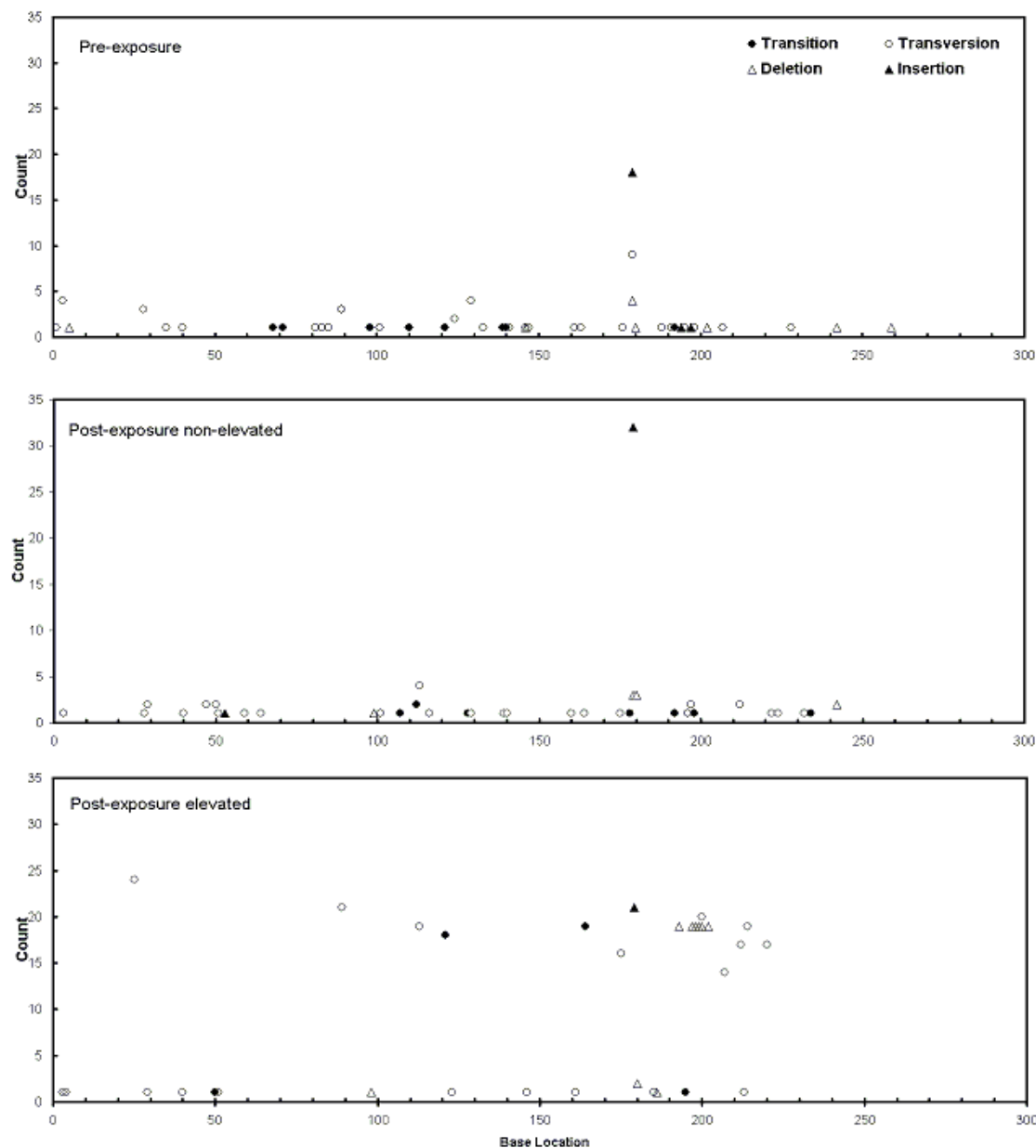
**Appendix 2.b.** (continued) Literature review of germ-line mutagenesis studies.

Species	Technique	ENU dose	Germ stage	Collection	Spontaneous	Induced	Author
mouse	<i>LacI</i>	150mg/kg	semi tub separated	parent	1.1 X 10 <sup>-5</sup>	3.05 X 10 <sup>-5</sup> pre semi tub 1.47 X 10 <sup>-5</sup> post semi tub	(Provost, 1997)
mouse	<i>LacZ</i>	150mg/kg			1.5 X 10 <sup>-5</sup>	3 d (3.64 ± 1.29 X 10 <sup>-5</sup> ) 14 d, 25 d, 50 d (1.675 ± 2.67 X 10 <sup>-4</sup> )	(Renault, 1997)
mouse	SLT	250mg/kg	spermatogonia	offspring	5.4 X 10 <sup>-5</sup> gamete 7.7 X 10 <sup>-6</sup> /locus	35/7584	(Russell, 1981)
mouse	SLT	250mg/kg	spermatogonia		8 X 10 <sup>-6</sup>	6.59 X 10 <sup>-4</sup>	(Russell, 1979)
medaka	SLT	1mM			StotMF 3.8 X 10 <sup>-5</sup> /locus SVM 4.7 X 10 <sup>-6</sup> /locus	1.4 X 10 <sup>-3</sup> /locus	(Shima, 1994)
zebrafish	SLT					1st 2 weeks (3-44 X 10 <sup>-3</sup> )	(Solnica-Krezel, 1994)
<i>Drosophila</i> <i>vermilion</i>	SLT	1mM	spermatogonia	offspring		3rd and 4th wks (0.4-10 X 10 <sup>-3</sup> ) >month (0.6 X 10 <sup>-3</sup> ) 1.63 X 10 <sup>-4</sup>	(Tosal, 1998)
mouse	Big Blue <sup>®</sup>			offspring	1 X 10 <sup>-5</sup>		Lyon et al. 1972 *
mouse	SLT	160mg/kg	Post-spermatogonia	offspring		7.2 X 10 <sup>-5</sup>	Favor et al. 1990 *
mouse	Big Blue <sup>®</sup>	150mg/kg	spermatogonia	offspring		6 X 10 <sup>-4</sup>	Murota et al. 1983 *
mouse	SLT	160mg/kg	spermatogonia	offspring		5.78 X 10 <sup>-4</sup>	Favor et al. 1986 *
mouse	SLT	250mg/kg	spermatogonia	offspring		9.36 X 10 <sup>-4</sup>	Favor et al. 1983 *

\* Articles reviewed in Katoh, 1997



**Appendix 3.** Distributions of cII mutant frequencies in offspring of six pre-ENU treatment (white bars) and post-ENU treatment (black bars)  $\lambda$ cII transgenic male parents.



**Appendix 4.** Sequencing results categorized into transition, transversion, deletion, and insertion mutations for offspring of five pre-ENU and post-ENU treated  $\lambda$ cII transgenic male parents. Offspring from males were combined into three groups: un-treated (84 sequences), treated non-elevated (85 sequences), and treated elevated (240 sequences). Five offspring were sequenced from the un-treated group, six offspring were sequenced from the treated non-elevated group, and thirteen offspring were sequenced from the treated elevated group. The un-treated group consisted of offspring having a spontaneous cII mutant frequency. The treated non-elevated group consisted of offspring collected after treatment that had a cII mutant frequency that was not significantly different from the un-treated group. The treated elevated group consisted of offspring that had a cII mutant frequency that was significantly above the un-treated group.

## APPENDIX 3

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