DEVELOPING NEW TOOLS FOR DETECTING GERMLINE MUTATION INDUCTION IN MICE

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in Biology

University of Regina

By

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Regina, Saskatchewan

June, 2012

UNIVERSITY OF REGINA

FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

Identifying factors that influence germline mutation rate in animals is an important toxicological consideration. However, research in this area is limited by the lack of efficient and precise tools for characterizing germ cell mutagens in humans and model species such as mice. Thus, the purpose of my research was to develop new tools for quantifying germline mutation induction in mice. My first objective was to identify polymorphic microsatellites as a new tool for detecting changes in germline mutation frequency. Enrichment and DNA sequencing of microsatellites in closely related inbred mouse lines was used to identify polymorphic loci. The loci were used to screen family pedigrees of outbred Swiss-Webster mice in which the sires were irradiated. No mutation induction was detected using the microsatellites; however, the samples used had previously shown mutation induction at other tandem repeat loci. Failure to detect mutation induction using the microsatellites does not rule them out as a useful tool, but rather raises questions as to why induction was detected at other loci but not at microsatellites. After negative results with microsatellites, I determined if whole genome sequencing (WGS) using next-generation sequencing (NGS) technologies would be a suitable alternative for detecting germline mutation induction. WGS is more relevant than tandem repeat DNA markers to health because it examines all loci, both neutral and coding, simultaneously. However, NGS is still limited by sequencing error rates, which are high in comparison to the intergenerational mutation rate, and the high cost associated with sequencing multiple large genomes. Therefore, WGS is not yet suitable for large-scale germline mutation studies. In conclusion, WGS will be an optimal tool for germline mutation studies in the near future when costs and error rates decline. WGS will need to

be used to determine if there is a correlation between mutation induction at coding sequence and at neutral marker loci. If a correlation exists, then germline mutation studies using neutral markers, such as microsatellites, can continue to serve as primary tools for detecting germline mutation induction.

ACKNOWLEDGEMENTS

I would like to extend my gratitude to the following people whose indispensable efforts have led me to completing my thesis. I would first like to extend a special thank you to Dr. Chris Somers. As my supervisor, he has guided me to learn skills necessary to pursue a future in science, and as a friend he has taught me so much in life. I am also very grateful to Dr. John Stavrinides for his willingness to provide me with advice and support. I would like to thank Dr. Travis Glenn and Dr. Stacey Lance for all their work getting me started on my project. Many thanks are owed to Jessica Martino for teaching me necessary lab skills and Caitlin Hunter for her assistance in the lab. In addition, I would also like to thank all my labmates for sharing their office and their wisdom with me.

My research would not be possible without funding from the Canada Research Chairs program and the Natural Sciences and Engineering Research Council of Canada. I would like to also thank the Faculty of Graduate Studies and Research for all the funding they have provided me with through teaching assistantships, research awards, and the graduate centennial merit award.

DEDICATION

To my family for always being supportive of my dreams.

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Mutagens in the Environment

Environmental contamination with mutagens, agents that are capable of inducing DNA sequence alterations, is a major ecological and medical problem [Pollack et al. 2003; Wegrzyn and Czyz 2003]. Innumerable chemical compounds are released into the environment daily through human activities (traffic pollution, energy production, manufacturing etc.), and for some of the chemical agents there have been no studies done to determine if they possess genotoxic properties. It is estimated that more than 68,000 chemicals have been in use prior to any legislation that required pre-market toxicity assessment [Rovida and Hartung 2009]. Thus, there are numerous environmental factors that could potentially be mutagenic, and efforts are needed to identify and regulate these factors.

Identification of germ cell mutagens should be of concern because any mutations that occur in gametes can be directly inherited in the offspring. However, there is currently no regulatory body that declares agents as germ cell mutagens [DeMarini 2012]. There is a general belief in the field of toxicology that evaluations of somatic cell mutagenecity are proportional to gametic mutagenicity (eg. [Ashby and Tinwell 2001]); thus, germ cell assays are not included in routine genotoxicity tests. This viewpoint relies on two major assumptions: (1) all somatic mutagens are germ cell mutagens, and (2) agents that are not somatic mutagens are not germ cell mutagens. If germ cell mutagens are indeed different than somatic mutagens, then the second pernicious assumption could result in under-regulation of factors that increase the frequency of transmissible

mutations, thereby increasing the incidence of sporadic genetic disorders. Recent evaluations of N-(hydroxymethyl)acrylamide [Witt et al. 2003] and sidestream tobacco smoke [Marchetti et al. 2011] showed mutation induction in germ cells but not in somatic cells, suggesting that these two factors are preferential germ cell mutagens. Consequentially, a paradigm shift is needed to place more focus on the importance of germ cell mutagenesis when determining the mutagenicity of different environmental factors. Despite these recent advancements, the field of germ cell mutagenesis is still limited by a lack of precise and efficient tools for quantifying *in vivo* germline mutation induction [Wyrobek et al. 2007; Singer and Yauk 2010]. In order for there to be more focus on germline risk assessment, more suitable tools are needed.

1.2 Current Methods for Detecting Germline Mutation Induction

A major problem associated with germline mutation studies is the lack of appropriate tools for efficiently quantifying changes in mutation frequencies. There are several tools available for studying germline mutation induction, and a wealth of information has been learned by using these methods [reviewed in Singer et al. 2006]. However, all of the methods currently available for detecting germline mutation induction are limited by technical challenges or the health relevance of the endpoints are uncertain.

The earliest and most revolutionary method used to measure mutation rates was the specific locus (SL) or Russell 7-locus test (reviewed in [Russell 2004; Davis and Justice 1998]). In SL studies, researchers identify mutation events in the offspring as visible changes at 7 phenotypic markers: 6 loci control skin colour and 1 locus controls

ear morphology. These studies have been used to determine how exposure of males to radiation and roughly 30 chemicals influence germline mutation frequency [Russell 2004]. The major problem with the SL test was that the mutation rates of the loci used were low (10^{-5}) and therefore hundreds of thousands of mice had to be screened in order to detect mutation induction. For example, in the pilot SL study more than 85,875 offspring were examined [Russell 1951]. Hence, this method is seldom or no longer used because it is expensive, unwieldy, and perhaps unethical.

Another important and widely used *in vivo* method that has been used to quantify germline mutation induction is the dominant lethal (DL) test (reviewed in [Epstein 1973]). In these studies males are exposed to potential mutagenic agents and mated with females over the complete course of the spermatogenic cycle. After pregnancy, females are dissected and the mutagenic effects are assessed by comparing the proportion of fetal deaths versus total implantations. The genetic basis for the fetal deaths in DL studies is aneuploidy, chromosomal breaks, or reciprocal translocations. At least 140 chemicals have been evaluated using this method and at least 65 chemicals have produced positive results [Green et al. 1985]. Similar to the SL test, the DL assay is labour-intensive, time consuming, expensive, and requires large numbers of animals (although less than the SL test) [Singer et al. 2006; Singer and Yauk 2010].

The mouse heritable translocation (HL) assay has also been widely used for detecting germ cell damage, specifically translocations [Generoso et al. 1980; Singer 2006]. Embryos produced from unbalanced gametes, that have duplicated or missing segments due to unequal exchange of chromosomal material, have increased mortality. Accordingly, males that have a higher number of translocations in their gametes are more

likely to produce a smaller litter size when mated with females. Therefore researchers using the HT assay compare litter sizes produced from mating females with untreated and treated males. Researchers also carry out cytological analysis to confirm reciprocal translocations in spermatocytes. The HT is very sensitive, but it is time consuming, expensive, and because of the low spontaneous translocation rate, many animals (more than 300) are still required [Singer et al. 2006].

Transgenic rodent (TGR) assays are commercially available methods useful for determining the mutagenicity of different agents in several tissues simultaneously (reviewed in [Lambert et al. 2005]). The first report of a TGR assay was provided by Gossen et al. [1989], and there are several modifications of this assay. TGR assays involve exposing transgenic mice carrying a bacterial transgene such as *lac*Z, to an agent being tested for mutagenicity. After exposure, the transgene is isolated from the exposed tissue, packaged into phage heads, and then the phages are absorbed onto *Escherichia coli* cells. Mutations in the transgene are detected by the appearance of different coloured plaques or the presence of plaques that can only develop on special media. The benefits of TGR studies is that they require fewer animals (as low as 25), germ cell mutagens can be characterized without screening offspring, point mutations can be detected, and studies have high reproducibility [Lambert et al. 2005; Singer et al. 2006]. The limitations of TGR studies are that they have low sensitivity, are expensive, cannot be automated, and there is little evidence to indicate heritability of the mutations.

Large tandem repeat DNA loci, such as expanded simple tandem repeats (ESTRs) and minisatellites, are currently the most efficient markers for detecting changes in germline mutation frequency (reviewed in [Somers 2006]). Minisatellites are ubiquitous

in eukaryotic genomes while the few known ESTR loci have been studied exclusively in mice [Kelly et al. 1989; Gibbs et al. 1993; Bois et al. 1998]. Large tandem repeat DNA loci mutate through gains or losses of repeat units, and mutation induction is a result of an unknown, indirect mechanism rather than by direct damage to the loci [Yauk 2004]. Mutations are detected by comparing allele sizes of parents to progeny using Southern blotting or by comparing the allele sizes of males to their sperm using single molecule PCR [Yauk et al. 2002; Yauk 2004]. The high spontaneous mutation frequencies of large tandem repeat DNA (~1.7 – 7.0% per gamete) [Tamaki et al. 1999; Kelly et al. 1989; Gibbs et al. 1993; Bois et al. 1998] and their sensitivity to mutation induction make them useful tools for germ cell mutagenesis studies.

The high sensitivity of these loci allows for far fewer animals to be used in studies compared to the other methods described above. For this reason, several studies have used ESTRs and minisatellites to quantify mutation induction. ESTRs have been used to detect mutation induction following different exposures, including ionizing radiation (egs. [Dubrova et al. 1993; Dubrova et al. 1998; Somers et al. 2004]), chemical mutagens (egs. [Vilariño-Güell et al. 2003; Glen et al. 2008]), and air pollution (egs. [Yauk et al. 2000; Somers et al. 2002]). For example, ESTR loci *Ms6-hm* and *Hm-2* have shown 1.6-4.0-fold elevations in mutation frequency following exposure of sires to 1.0 Gy of ionizing radiation [Dubrova et al. 1993; Dubrova et al. 1998; Somers et al. 2004]. Importantly, these studies used only 50-100 offspring per treatment group, and showed statistically significant changes in mutation frequency. ESTR loci have also been used to investigate the transgenerational effects of different mutagen exposures (egs. [Dubrova et al. 2000; Glen and Dubrova 2012]). In addition, studies using these markers have

quantified changes in germline mutation rate when DNA repair genes are knocked out (egs. [Yamauchi et al. 2002; Barber et al. 2004]). Studies using ESTRs, have contributed greatly to our knowledge of germ cell mutagenesis. However, ESTRs are limited by several technical challenges. The large size of these sequences makes their mutation detection imprecise, unsuitable for high-throughput analysis, and difficult to standardize between labs [Somers 2006]. Also, the indirect nature of tandem repeat DNA mutation processes makes it difficult to generalize findings to other loci in the genome [Somers and Cooper 2009]. An ideal marker would have the high mutation rates and sensitivity of ESTRs, but would enable more standardized and high-throughput mutation detection.

1.3 Microsatellite Alternative for Germ Cell Mutagenesis Studies in Mice

Given the limitations of all the available methods for quantifying mutation induction, there is a need to develop new, more effective tools. Microsatellites, which are essentially very short ESTRs, could be a useful tool to supplement or replace other methods used to detect mutation induction. Although mutation rates of microsatellite loci tend to be lower than those of large tandem repeat loci (eg. [Farfán et al. 2003]), polymorphic microsatellites with mutation rates comparable to large ESTRs and minisatellites have been identified in various animal species (egs. [Ellegren et al. 1997; Tsyusko et al. 2007]). Also, several studies have already capitalized on the benefits of using polymorphic microsatellites to quantify mutation induction in different species [Satoh et al. 1996; Ellegren et al. 1997; Kovalchuk et al. 2000; Slebos et al. 2004; Furitsu et al. 2005; Tsyusko et al. 2007; da Cruz et al. 2008; Anmarkrud et al. 2011; Tsyusko et al. 2011]. For example, Ellegren et al. 1997 used 2 highly unstable barn swallow

microsatellite loci to compare germline mutation frequencies between swallows breeding in Chernobyl and an uncontaminated control area in Ukraine. They were able to detect a 2-fold germline mutation induction in swallows breeding in Chernobyl by screening as few as 269 meioses (approximately 70 offspring) overall. In addition, microsatellites have the potential to resolve all the technical issues associated with large tandem repeat DNA loci. The small size of microsatellites allows for accurate (single base pair resolution) and high-throughput genotyping using capillary electrophoresis. However, no unstable microsatellites have been used to quantify germline mutation induction in mice, the standard model species for these types of studies. Hence, developing a panel of polymorphic mouse microsatellites could serve as a useful tool for efficient identification of germ cell mutagens.

1.4 Whole Genome Sequencing Alternative for Germ Cell Mutagenesis Studies in Mice

All germline mutation assays used to date suffer from an underlying assumption that a change in frequency of one event type is representative of other types of changes in the genome; the only way to validate this assumption is to examine mutagenic effects at the whole genome level. The advent of next-generation sequencing (NGS) technology makes it possible for any lab to do whole genome sequencing (WGS), which can potentially characterize the types and locations of all mutation events. Therefore, WGS using NGS has the potential to be the most informative tool for germ cell mutagenesis studies. WGS of family triads have already been used to determine the spontaneous mutation frequency in humans [Roach et al. 2010; Conrad et al. 2011]. In addition, this

technology has been used to provide unprecedented insight into the mutation spectra of three common mutagens in *Caenorhabditis elegans* [Flibotte et al. 2010]. However, studies have never been performed that directly quantify mutation induction in vertebrates, which have large genomes on the scale of gigabases. Therefore, the feasibility of using commercially available NGS platforms to carry out large scale germ cell mutagenesis studies at the whole genome level in vertebrates is undetermined.

1.5 Thesis Objectives

The overall purpose of my research was to develop new tools for quantifying changes in germline mutation frequencies in mice. The field of germ cell mutagenesis is currently limited by the lack of precise and efficient methods capable of detecting mutation induction. Development of new tools, especially those that better represent the whole genome, will aid researchers in identifying factors that influence germline mutation rates.

The specific objectives were to:

(1) Develop a panel of polymorphic mouse microsatellites and determine if the panel could be used to detect germline mutation induction in families exposed to a known germ-cell mutagen. This research has been written as a manuscript and will be submitted to *Environmental and Molecular Mutagenesis*.

(2) Determine whether next-generation DNA sequencing is a practical tool for quantifying germline mutation frequencies in whole genomes of humans and

model organisms. This work was published as Beal et al. [2012] and has been re-published here with permission.

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CHAPTER TWO. CHARACTERIZATION OF UNSTABLE MICROSATELLITES IN MICE: NO EVIDENCE FOR GERMLINE MUTATION INDUCTION FOLLOWING GAMMA-RADIATION EXPOSURE

2.1 Abstract

Large tandem repeat DNA loci such as expanded simple repeats (ESTRs) and minisatellites are efficient markers for detecting germline mutations; however, mutation detection using these loci can be imprecise and difficult to standardize across labs. Short tandem repeats, such as microsatellites, offer more precise and high-throughput mutation detection, but germline mutation induction at these loci has not yet been studied in model organisms such as mice. In this study we used selective enrichment for microsatellites and large-scale DNA sequencing of several closely related inbred mouse lines to identify a panel of 19 polymorphic microsatellites with potentially high spontaneous mutation frequencies. We used this panel and 4 additional loci from other sources to quantify spontaneous mutation frequency in pedigrees of outbred Swiss-Webster mice. In addition, we also examined mutation induction in families in which sires were treated with acute doses of either 0.5 Gy or 1.0 Gy to spermatogonial stem cells. Per locus mutation frequencies ranged from 0 to 5.03×10^{-3}. Considering only the 11 loci with mutations, the mutation frequencies were: control 2.78×10^{-3}, 0.5 Gy 4.09×10^{-3}, and 1.0 Gy 1.82×10^{-3}. There were no statistically significant changes in mutation frequencies among treatment groups. Our study provides the first direct quantification of microsatellite mutation frequency in the mouse germline, but shows no evidence for mutation induction at pre-meiotic male germ cells following acute gamma-irradiation.

Further work using the panel is needed to examine mutation induction at different doses of radiation, exposure durations, and stages during spermatogenesis.

2.2 Introduction

Studying germ cell mutagenesis is important because it has implications for human health, wildlife conservation, and evolutionary potential [Crow 1997; Cotton and Wedekind 2010]. Although germline mutations are required for natural selection, they can also cause genetic diseases in offspring. Sporadic de novo genetic disorders, chromosomal abnormality syndromes, and other birth defects occur in 5% of all human live births [Wyrobek et al. 2007]. In addition, the accumulation of nearly neutral deleterious germline mutations in small populations, like those of endangered species, can become fixed and affect long-term viability [Lande 1994; Lande 1995]. Determining what factors influence germ cell mutation induction is therefore of interest to a wide variety of people, including health scientists, evolutionary and conservation biologists, and the general public. Advances in DNA sequencing technology may soon make genome-wide mutation screening in humans and model species commonplace [Beal et al. 2012]. However, the current lack of precise and efficient mutation markers amenable to high throughput detection is a significant limitation to studying germ cell mutagenesis in vertebrates, specifically mice, which are a standard model species.

Currently, Expanded Simple Tandem Repeats (ESTRs) are the most efficient markers for studying germline mutation induction in mice. The high spontaneous mutation frequencies of ESTRs (~1.7 – 3.6% per gamete) [Kelly et al. 1989; Gibbs et al. 1993; Bois et al. 1998] and their sensitivity to mutation induction make them useful tools

for germ cell mutagenesis studies. ESTR studies have demonstrated significant mutation induction in the mouse germline following a variety of different exposures, including ionizing radiation and chemical mutagens (egs. [Dubrova et al. 1993; Dubrova et al. 1998; Vilariño-Güell et al. 2003; Somers et al. 2004]). Detection of ESTR mutants has been most commonly done using a pedigree approach whereby allele sizes of progeny are compared to their parents using extended agarose electrophoresis and Southern blotting. ESTRs are highly efficient because experiments require orders of magnitude fewer animals than previous approaches using visible phenotypic markers (eg. [Russell et al. 1979]). Animal use has been further refined in more recent studies that have examined ESTR mutation frequencies directly in the sperm of male mice using single-molecule PCR (SM-PCR) [Yauk et al. 2002; Yauk et al. 2007; Yauk et al. 2008]. ESTRs are very effective markers and have played an important role in understanding germ cell mutagenesis; however, their mutations are still of uncertain relevance to human health and other areas of the genome.

Studies that use ESTRs can also be limited by several technical challenges. Variations in ESTR arrays among mouse lines, resolution limits of agarose electrophoresis, and imprecise quantification of ESTR mutation induction makes direct comparisons of studies performed in different labs difficult [Somers 2006]. The large size of different ESTR arrays makes it nearly impossible to reliably detect single repeat unit mutations using agarose electrophoresis; only arrays of a certain size allow for the detection of single repeat unit mutations. Consequently, different labs have used different scoring criteria to identify mutation events. In the published literature, the most conservative studies required that offspring alleles be more than 200 base pairs different

from the parental alleles to be considered mutants [Hedenskog et al. 1997], while some other studies only required alleles to be different by 15 base pairs [Yauk et al. 2002]. The lack of standardization may be responsible for some of the discrepancies between labs. For example, low LET radiation doubling dose estimates (radiation dose required to double spontaneous mutation frequency) range from 0.28-4.80 Gy [Somers 2006], and this lack of agreement between studies may be due to differences in scoring criteria between labs [Niwa 2003]. Furthermore, the few ESTR loci characterized [Kelly et al. 1989; Gibbs et al. 1993; Bois et al. 1998] are found exclusively in mice, making it difficult to use ESTR mutation induction as a proxy for other animals. Currently, no effective alternatives to ESTRs exist for quantifying mutation induction in the germline of mice.

Polymorphic microsatellites may be useful as an alternative tool for studying germline mutation frequencies in mice. Microsatellite mutation frequencies have already been measured in many different species and some of the loci used had high spontaneous mutation frequencies between 0.81% and 4.2% per gamete [Ellegren et al. 1997; Gardner et al. 2000; Steinberg et al. 2002; Hoekert et al. 2002; Slebos et al. 2004; Furitsu et al. 2005; Tsyusko et al. 2007]. Several studies have also used microsatellites to quantify mutation induction resulting from exogenous mutagen exposure [Satoh et al. 1996; Ellegren et al. 1997; Kovalchuk et al. 2000; Slebos et al. 2004; Furitsu et al. 2005; Tsyusko et al. 2007; da Cruz et al. 2008; Anmarkrud et al. 2011; Tsyusko et al. 2011], or endogenous factors such as genotype [Baker et al. 1995]. For example, Tsyusko et al. 2007 were able to detect a roughly 2-fold increase in germline mutation frequency of irradiated Japanese medaka fish using 9 unstable microsatellite loci and around 200

offspring per treatment group. In addition, microsatellites offer solutions to most of the technical problems associated with ESTRs. The small size of microsatellites makes them suitable for PCR amplification and precision fragment analysis with single base pair resolution using capillary electrophoresis. This technique is more precise than previous methods using ESTRs, amenable to high-throughput analysis, and is easily standardized across labs. However, spontaneous microsatellite mutation frequencies in mice have only been estimated indirectly using loci with low mutation frequencies (0.047% being the highest) [Dallas et al. 1992; Dietrich et al. 1992]. Baker et al. [1995] measured microsatellite mutation frequencies in mice directly but the loci had such low mutation frequencies that no spontaneous mutations were observed. Thus, spontaneous microsatellite mutation frequencies in the lab mouse are essentially uncharacterized, so no unstable loci suitable for studies of mutation induction have been identified.

Here we identify polymorphic microsatellites that may be unstable in the mouse germline. We used these microsatellites to directly quantify spontaneous mutation frequencies in mouse pedigrees, as well as examine germline mutation induction following paternal exposure to ionizing radiation. We took advantage of samples from mouse pedigrees derived for a previous study, which showed ESTR mutation induction in irradiated families [Somers et al. 2004]. Our study is the first that we are aware of to provide direct estimates of germline mutation frequencies at mouse microsatellites.

2.3 Materials and Methods

2.3.1 Development of Microsatellite Panel

Whole genomic DNA was extracted from C57Bl mice provided by different suppliers, including 4 mice from Charles river labs, 12 from Jackson labs, 4 from Taconic labs, and 3 progeny from sires mutagenized with ENU (from D. DeMarini at the US EPA, RTP, NC). The untreated mice were adults from highly inbred lines; polymorphisms among the different sources of mice may therefore have resulted from microsatellite mutations occurring after their separation, possibly due to high spontaneous mutation frequencies. The progeny of the ENU-treated sires (75 mg/kg in 0.5 mL of 0.85% saline via IP injection) were chosen in an attempt to identify loci that may be sensitive to chemical mutagens. Extracted mouse DNA was used to construct two enriched microsatellite libraries following the approach of Glenn and Schable [2005]. The first library was enriched for common microsatellites: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$, and the second library was enriched for rare microsatellites: $(AAG)_8$, $(AATG)_6$, $(AACC)_5$. The enriched libraries were sequenced using a Roche 454 Genome Sequencer FLX+ with GS FLX Titanium series reagents. The raw reads were trimmed so that bases with a quality score of less than 10 were replaced with an 'N'. The 3' ends were shortened until the last 25 bases only had one 'N'. The final output only contained reads that were at least 50 bases in length.

The trimmed reads were assembled using gsAssembly (Newbler, 454 Life Sciences, Branford, CT, USA). MSATCOMMANDER [Faircloth 2008] was used to locate microsatellites within all of the contigs produced by the assembly. The microsatellites were then visualized using Tablet [Milne et al. 2010]. For a locus to be considered potentially useful, it needed to meet the following criteria: (1) multiple alleles were apparent; (2) flanking sequence on both sides of the microsatellite repeat; and (3)

the overlap of the assembly was not within the repeat sequence. The flanking DNA for each indentified locus was used to search the reference C57Bl genome (build 37.2; NCBI) to identify the genomic location of the locus. Trimmed reads were also mapped to the C57Bl reference genome, one chromosome at a time, using gsMapper (Newbler, 454 Life Sciences, Branford, CT, USA). Primers based on the reference sequence were designed for potentially variable loci using Gene Runner V3.01 (Hastings Software Inc. Hastings, NY, USA). In each primer pair one of the primers had an engineered sequence (CAGTCGGGCGTCATCA) added to the 5' end so that a fluorescently labelled third primer (CAGTCGGGCGTCATCA) could be used in the PCR to enable fragment detection on a DNA sequencer (adapted from [Schuelke 2000]).

To distinguish real microsatellite allelic variability from 454 sequencing errors (false-positives), the original DNA samples from C57Bl mice were screened at potentially polymorphic loci. PCR amplifications were carried out in 18 μL reaction mixtures consisting of 10 μL of 2X PCR Master Mix (Norgen Biotech, Ontario, Canada), 0.28 μM unlabelled primer, 0.028 μM tag labelled primer (primer with engineered sequence added), 0.25 uM universal dye labelled primer (CAGTCGGGCGTCATCA), and 10 ng of DNA template. Amplification of products was done using a touchdown thermal cycling program [Don et al. 1991] as follows: 95°C for 150 seconds; 22 cycles of 95°C for 20 seconds, 55°C for 20 seconds (-0.5 degrees per cycle, ramp 3 degrees / second), 72°C for 30 seconds; 25 cycles of 95°C for 20 seconds, 45°C for 20 seconds, 72°C for 30 seconds; final extension at 72°C for 10 minutes. PCR products were analyzed with single base pair resolution using an internal size standard (400 bp or 600 bp) on a Beckman Coulter GenomeLab GeXP Genetic Analysis System (Beckman-

Coulter, Fullerton, CA, USA). Microsatellites that were confirmed to have multiple alleles, and were therefore truly variable among the C57Bl mice, were used in subsequent pedigree analysis.

2.3.2 Spontaneous and Induced Germline Mutations

Pedigrees of out-bred Swiss-Webster mice (Taconic Breeding Laboratories, Germantown, NY, USA) from Somers et al. [2004] were used to quantify microsatellite mutation frequencies; a detailed description of the radiation procedure is described therein. Briefly, adult male mice (7-9 weeks old) were irradiated with acute doses of 0, 0.5, or 1.0 Gy (0.33 Gy/min) whole-body radiation from a cesium-137 source. Males were bred to untreated females 9 weeks post-irradiation to produce litters of offspring that developed from irradiated spermatogonial stem cells.

Microsatellites with confirmed allelic variation among inbred lines were used to measure germline mutation frequencies in 10 families (98-100 offspring) from each of three treatment groups: (1) control, (2) 0.5 Gy, and (3) 1.0 Gy (from [Somers et al. 2004]). Parents and their offspring were genotyped using the PCR reaction mixture, thermal cycling program, and sequencer described above. Genotypes of parents and offspring were compared to identify novel alleles in offspring that were not present in the parents. Offspring alleles were considered to be mutations if they varied from parental alleles by one or more repeat units. We did not consider smaller changes (less than one repeat unit) in our analyses; these smaller changes were infrequent and in all cases represented a single base pair change, which was likely due to the resolution limit of the sequencer, not true mutation events. To confirm mutations, parental samples were re-

amplified and genotyped once, whereas offspring samples were re-amplified and genotyped two additional times (each mutant fragment genotyped 3 times). Initial mutation detection was completed by an observer that was not blind to treatment groups. To investigate potential biases introduced, a subset of the loci and samples was re-scored by a second, blind observer (agreement between observers = 100%).

2.3.3 Statistical Analysis

Mutation frequencies were calculated as the number of mutant alleles observed divided by the total number of alleles scored; 95% confidence intervals were calculated based on the Poisson distribution. We limited our statistical analyses among treatment groups to only those loci that showed at least one mutation event; those that had zero mutations in all treatment groups were not informative for examining mutation induction. We compared mutation frequencies among treatment groups using pair-wise comparisons of pooled data in two-tailed Fisher's Exact tests (SAS 9.2, SAS Institute Inc., Cary, NC, USA). Power analysis for the Fisher's Exact tests was conducted using G*Power 3.1.3 (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/). Mutation frequencies at the family level (arcsine-transformed) were analyzed to account for variance among sires in the out-bred strain of mice used; we used separate one-way ANOVA tests to examine the main effect of dose on mutation frequency (as in [Somers et al. 2004]). For all tests $\alpha = 0.05$ unless otherwise indicated.

2.4 Results

2.4.1 Development of Microsatellite Panel

DNA sequencing and trimming of the two different microsatellite enrichment libraries produced 236,200 reads from the Jackson, Charles River, Taconic, and ENU-treated mouse samples combined. MSATCOMMANDER [Faircloth 2008] identified 6067 microsatellites in the assembled sequences, 3127 (52%) of which showed potential allelic variation. Loci (n = 55) that had the highest number of alleles were selected for further analysis because they had greater potential to be unstable (Table 2.1). Out of the 55 selected loci, 25 (45%) were confirmed to have multiple alleles within the original group of mice used, 26 (47%) loci were monomorphic, and 4 (7%) loci were difficult to characterize due to the presence of extensive PCR artefacts. Thus, the false positive rate using the enrichment and 454 sequencing approach was approximately 47%.

In addition to the 25 loci confirmed to display allelic variation in our study, 2 previously published loci [Baker et al. 1995], and 2 loci from the Eukaryotic Microsatellite Database [Aishwarya et al. 2007] were selected to screen the Swiss-Webster family pedigrees (Table 2.1). The published loci were selected because they had elevated mutation frequencies following DNA mismatch repair knock out, and are the only loci that have been previously used to directly quantify microsatellite mutation frequency in mice [Baker et al. 1995]. The two loci from the Eukaryotic Microsatellite Database were selected based on core sequence and size. Following initial genotyping of the Swiss-Webster parents using the 29 loci (25 from our study, 2 published, and 2 from the database = 29), 6 loci were excluded from further analysis because they were monomorphic, the allele sizes were very short, or they did not amplify well (Table 2.1). In total 23 loci were used in subsequent pedigree analysis.

Table 2.1 Characteristics of the 55 mouse microsatellite loci screened for variation. Underlined bases indicate nucleotides that are shared between the CAGTCGGGCGTCATCA sequence and the locus specific primer sequence. The locus repeat motif is based on the reference (R) C57Bl/6J genome (build 37.2; NCBI). The Swiss-Webster (SW) repeat motif is based on the reference sequence and the size is adjusted based on the fragment size difference between reference and Swiss-Webster mice. H_o is the observed heterozygosity in 60 parents and *k* is the number of alleles in the population used. The loci D9Mit67 and D15Mit59 are from Baker et al. [1995], and the loci 6-9487 and 3-29434 are from the Eukaryotic Microsatellite Database [Aishwarya et al. 2007].

Category	Locus	Primer Sequences 5' -> 3'		Sequence	H_o	k
True	Mm1.1.1	CAGTCGGGCGTCATCAAGTGGGAGTGGGTGTGTAG	R:	$(ACAT)_{21}$	0.33	3
Variation,		CCTGTGTATGGGTGTAAC	SW:	$(ACAT)_{18\text{-}20}$		
Screened	Mm1.1.2	CAGTCGGGCGTCATCACTGCTAGACAAGGACTCTG	R:	$(ACAT)_{12}$	0.05	2
		CAGTCTTGGTTCACCATG	SW:	$(ACAT)_{10\text{-}11}$		
	Mm2.2.1	CAGTCGGGCGTCATCAGGCTACTGGGTATCAAACAGG	R:	$(AGAT)_{17}\ldots(AGAT)_{18}$	0.48	6
		TGAAGGGTATTGGAGTTCAGTC	SW:	$(AGAT)_{17.5\text{-}21.5}$		
	Mm2.3.2	CAGTCGGGCGTCATCAGGAGGGAAGGGTATCAATACAG	R:	$(AGAT)_{12}$	0.03	2
		CTCTTTCTGGGAACAGTCAGC	SW:	$(AGAT)_{12\text{-}13}$		
	Mm3.2.1	CAGTCGGGCGTCATCAGGTGACACTGGTCAGGGATC	R:	$(GATA)_4\ldots(CAGA)_4\ldots(GATA)_7\ldots(AGAT)_{11}\ldots(GATA)_{16}$	0.83	6
		GCTTGTCTGGAAGTAGCCAAC	SW:	$(GATA/CAGA)_{45\text{-}50}$		
	Mm3.5.1	CAGTCGGGCGTCATCAAGTCTGAGTGTATAATACCG	R:	$(AACC)_{11}\ldots(AGAT)_{11}\ldots(AGAT)_{17}$	0.52	5
		GTATAAGTAAGACCACAGGG	SW:	$(AACC)/(AGAT)_{35\text{-}39}$		
	Mm6.3.3	CAGTCGGGCGTCATCATCTTCAAGTAGGCTGACATTTG	R:	$(AACC)_4\ldots(AACC)_8$	0.56	2
		TGTAGATCCTGCAAGGTAG	SW:	$(AACC)_{11.5\text{-}12.5}$		
	Mm7.2.1	CAGTCGGGCGTCATCATCTATCCAATACATCCATC	R:	$(AGAT)_{10}\ldots(AGAT)_{12}\ldots(AGAT)_{12}$	0.56	5
		TCAATATCACTCAGCTATAGG	SW:	$(AGAT)_{37.5\text{-}41.5}$		
	Mm8.6.3	CAGTCGGGCGTCATCAGTGCCATATCATGTGTGCCTAC	R:	$(AGAT)_{18}$	0.35	3
		TATCCTCTGAGCCATATTGCC	SW:	$(AGAT)_{16\text{-}18}$		

Table 2.1 Continued.

Category	Locus	Primer Sequences 5' -> 3'		Sequence	H_o	k
True	Mm10.2.1	CAGTCGGGCGTCATCAACTGACATCCAAGTCACAC	R:	$(AGAT)_{8}...(AGAT)_{16}$	0.37	4
Variation,		GTTGAAATGGAGGGTAATC	SW:	$(AGAT)_{24-27}$		
Screened	Mm10.2.2	CAGTCGGGCGTCATCATGTTGCCAGTGAGCAGTGTG	R:	$(AGAT)_{17}$	0.48	3
		ACTTGCAGGCTTCCCACAG	SW:	$(AGAT)_{12-18}$		
	Mm10.3.2	CAGTCGGGCGTCATCAGCCTGTGTTCAGCGTTCAG	R:	$(AGAT)_{15}...(AGAT)_{14.5}$	0.84	8
		CTGTAAGCCAAGCTCATC	SW:	$(AGAT)_{21-27}$		
	Mm12.2.1	CAGTCGGGCGTCATCATTCTCTGTTTAGCCTGCTACCC	R:	$(AGAT)_{17}$	0.42	4
		TGAGGACACTGCCATTAGGAC	SW:	$(AGAT)_{15-20}$		
	Mm14.1.1	CAGTCGGGCGTCATCAACTGTGAGCACAAATGAGAGG	R:	$(AGAT)_{17}$	0.65	6
		TGGCTTTAAGTTCATGGATTCC	SW:	$(AGAT)_{14.5-19.5}$		
	Mm15.3.1	CAGTCGGGCGTCATCATAGCAATAACAAGATGATGG	R:	$(AGAT)_{14}...(AGAT)_{14}...(AGAT)_{12}$	0.78	8
		AGAGTGTTGGACTTTCTCAG	SW:	$(AGAT)_{29-46}$		
	Mm16.1.2	CAGTCGGGCGTCATCATTTCAATCCCTGGAACCTTTG	R:	$(AGAT)_{17}$	0.33	3
		GCTGGAAACAGAACTCTGGTC	SW:	$(AGAT)_{15-17}$		
	Mm16.1.3	CAGTCGGGCGTCATCAACACTCAGGAGGCAGAAGC	R:	$(AGAT)_{17}$	0.03	2
		TGCATGGTCTCAGGGAGAC	SW:	$(AGAT)_{15-16}$		
	Mm19.2.3	CAGTCGGGCGTCATCAAAGCACCTTTCAGTTCTTC	R:	$(GATA)_{11}(GACA)_{15}(GATA)_{17}...(GACA)_{3}$	0.65	9
		CTAGAAGACCTTTGGTCTCAC	SW:	$(GATA/GACA)_{16-45.5}$		
	Mm19.2.4	CAGTCGGGCGTCATCAGGAACTGCTTCTTGCTGG	R:	$(AGAT)_{13}...(GGAT)_{11}...(AGAT)_{4}$	0.05	2
		GAGAATGAGAACCCAAAGTGG	SW:	$(AGAT)/(GGAT)_{29-30}$		
True	Mm3.3.1	CAGTCGGGCGTCATCAACATTCCTGAAGCACAAGG	R:	$(TTAG)_{15}...(TTAG)_{15}$	-	-
Variation,		GGAAGGGAGTCCCTATTTCTGC	SW:	$(TTAG)_{7.5-30}$		
Not	Mm6.3.5	CAGTCGGGCGTCATCATGTCCCTCACACATCTCATTG	R:	$(TTCA)_{11}$	-	-
Screened		CCTTGCTGTCACCCAGA	SW:	$(TTCA)_{6}$		
	Mm8.6.1	CAGTCGGGCGTCATCAGCAGCCTCACATATC	R:	$(AGCT)_{4}...(AGAT)_{19}$	-	-
		GTGTAGGAGATAGATAGACGAG	SW:	$(AGCT/AGAT)_{17.5}$		
	Mm8.6.2	CAGTCGGGCGTCATCATTCACGACATTGGTGTGC	R:	$(GAAT)_{7}$	-	-
		TGTATGGCATGCAAGAGTC	SW:	$(GAAT)_{7}$		

Table 2.1 Continued.

Category	Locus	Primer Sequences 5' -> 3'		Sequence	H_o	k
True	Mm10.3.1	CAGTCGGGCGTCATCAGCGGTTAAGAGCACTGAC	R:	$(AGAT)_{18}$	-	-
Variation,		AGGATTAAAGGCATTCACCAC	SW:	$(AGAT)_{13\text{-}14}$		
Not	Mm13.5.2	CAGTCGGGCGTCATCATTTAGAAGTGGACCTCACC	R:	$(TTGG)_6\ldots(TTGG)_5\ldots(TTGG)_8$	-	-
Screened		TGTGAAACTCATACCATTG	SW:	$(TTGG)_{24}$		
False-Positives	Mm1.5.1	CAGTCGGGCGTCATCAGAGCAAGTTTCAGGACAG	R:	$(ATAG)_{12}$	-	-
		CCTATCTGGGACTCAATC				
	Mm2.1.1	CAGTCGGGCGTCATCAGGACAGTCTGAAGCAACC	R:	$(ATAC)_9\ldots (CATA)_6\ldots(AC)_7$	-	-
		ACTATGGCATTTGTTTCTC				
	Mm2.3.1	CAGTCGGGCGTCATCATAGAAGACCAGGTGATTGGG	R:	$(GATA)_9\ldots(TGGA)_4\ldots(TGGA)_9\ldots(TGGA)_{11}$	-	-
		CAATGTGCCAAATGCCAG				
	Mm3.3.2	CAGTCGGGCGTCATCATTCAAGTTTGTTGTTGGTAG	R:	$(GGTT)_6\ldots(GT)_{15}$	-	-
		GGAGTGATAATTAGAAAGGAAG				
	Mm3.5.2	CAGTCGGGCGTCATCACCAGAAGGTCAGTAAATGCTG	R:	$(CA)_{14}\ldots(AG)_7\ldots(ACAG)_4\ldots(CAGA)_4\ldots(GACA)_6\ldots(AG)_{17}$	-	-
		GGTTCCCTGCAAGATCCTG				
	Mm5.4.1	CAGTCGGGCGTCATCAGCTATGTCCTCTCACCTAC	R:	$(GT)_{12}$	-	-
		AAAGTGTTCCTGAGTCAAG				
	Mm5.16.2	CAGTCGGGCGTCATCAGCCAGCAGTTAGAGAGAAGGG	R:	$(AC)_{10}\ldots(AC)_6\ldots(AC)_{11}$	-	-
		AACCAATATCCTGTTCTAAG				
	Mm6.1.1	CAGTCGGGCGTCATCAGGAACACTCTCAGCCTC	R:	$(TGAA)_4\ldots(TGAA)_6$	-	-
		TGGATGGTCATAGAGTTAAAG				
	Mm6.3.2	CAGTCGGGCGTCATCAGGAAACTATTCGAGCTGGGTG	R:	$(AC)_{10}\ldots(GTAT)_{12}$	-	-
		TGTTCTTTGACCTCCATGTGAG				
	Mm6.3.4	CAGTCGGGCGTCATCAGTCCTCTCCTGCTGAAC	R:	$(ACAT)_{13}$	-	-
		CACATGGTAGCTCAGAATC				
	Mm7.1.1	CAGTCGGGCGTCATCAATAGCTCTCCCAGAGAATC	R:	$(CTT)_{32}\ldots(CCT)_4\ldots(TAC)_{13}$	-	-
		TATCCCACATAAAGGTGTACCC				
	Mm8.2.1	CAGTCGGGCGTCATCATGCTTCTATGAAAGTGCCTCAG	R:	$(AG)_{12}(AGAC)_7$	-	-
		ATAGGGATGTCTGGTTTCCTG				

Table 2.1 Continued.

Category	Locus	Primer Sequences 5' -> 3'		Sequence	H_o	k
False-Positives	Mm8.6.4	CAGTCGGGCGTCATCAGGACTCTGGCTGACTTAGG	R:	$(AACC)_9...(CAAA)_4...(TCCCCTC)_5$	-	-
		ATCCATGCTTGCCTCCAC				
	Mm9.2.1	CAGTCGGGCGTCATCAGGCTGTCTTCCATTCTCCAC	R:	$(AC)_{20}$	-	-
		GGGCTGCTGTAAGCAACAG				
	Mm10.2.3	CAGTCGGGCGTCATCATAAGAGGTCAGGCATG	R:	$(AGAT)_{11}...(GATA)_{14}$	-	-
		TCTAAGTGGAAATAGTTCTCAC				
	Mm11.1.1	CAGTCGGGCGTCATCACCTGAAACCACCAACTCCTG	R:	$(GGTT)_6$	-	-
		ATGGAAGTGAGATCAGCC				
	Mm11.3.1	CAGTCGGGCGTCATCACTGAAGAATTTGTGCCAACTG	R:	$(TGTA)_7...(TGTA)_4...(TATG)_6...(GTAT)_5...(TGTA)_9$	-	-
		ATTCCACAGCCAATGGAGAT				
	Mm12.3.2	CAGTCGGGCGTCATCACCAAATTATACCCACATTCCAG	R:	$(TG)_{13}...(TATG)_5...(ATCT)_6$	-	-
		GATCTTCCTACAGTGCCACCTC				
	Mm13.1.1	CAGTCGGGCGTCATCAGCATTCCATTTCTCCATG	R:	$(ATCT)_{16}$	-	-
		ATTACAAACTGATCAAACAGCC				
	Mm13.5.1	CAGTCGGGCGTCATCATCTAGTTGCTAAGTCCATAGG	R:	$(TATG)_5A(ATGT)_8$	-	-
		CACACTCTCACAAATAAATGG				
	Mm13.5.3	CAGTCGGGCGTCATCAGCACCTCCTCACAGTAAC	R:	$(TTGG)_8$	-	-
		CCAAACATTGGTAGTAGCCA				
	Mm16.1.1	CAGTCGGGCGTCATCAATGGGAGAGAAAGGCTGTG	R:	$(AC)_{14}..(AC)_7$	-	-
		TGAATGCCAAGACAACTAGCAC				
	Mm17.3.1	CAGTCGGGCGTCATCAGGAAATGGGTTCACACCAAAC	R:	$(ATAG)_9(ATAC)_{11}$	-	-
		TGGCTGAGCCTATACAAAC				
	Mm18.1.1	CAGTCGGGCGTCATCAGGGAGGGTAACAGTGTATGGTC	R:	$(TAGA)_9$	-	-
		GCTGAAGAAGTGTGCCACAG				
	Mm19.2.1	CAGTCGGGCGTCATCATACGTTACGTGTGCTTGGAGAG	R:	$(ATCT)_{17}$	-	-
		CCAGGGCTACACAGAGAAACTC				
	Mm19.2.2	CAGTCGGGCGTCATCATGGAAGTTTGCTAACCAG	R:	$(GTAT)_{13}$	-	-
		GAGTATATTATTGTTCACCACC				

Table 2.1 Continued.

Category	Locus	Primer Sequences 5' -> 3'		Sequence	H_o	k
PCR Artefacts	Mm5.16.1	CAGTCGGGCGTCATCACCAGACAACCTTGACCTCC	R:	$(CA)_{18}...(CA)_6...(AC)_{18}...(CA)_7$	-	-
		GGCTACCTGAGTCACCCAAG				
	Mm6.3.1	CAGTCGGGCGTCATCATCCATTCCAGCTCAGGAGTC	R:	$(TTGG)_{11}$	-	-
		GCTGTGTGCCACAGTTTATGC				
	Mm8.1.1	CAGTCGGGCGTCATCAGCTAAGTAGCCACGAGTAGAC	R:	$(TTAG)_6$	-	-
		CCTTCCCAGTGTCTCCTTTAGG				
	Mm12.3.1	CAGTCGGGCGTCATCATTATGTGGCTTCTGGAAG	R:	$(TG)_{24}...(GT)_6...(GT)_{21}$	-	-
		ATATGCGGTATGCTGCAC				
Additional	D9Mit67	CAGTCGGGCGTCATCAGAACTGCCTTTAACTTCAGTTGC	R:	$(AC)_{22}$	0.43	2
Loci		ATCTCCTCCCCGAACTGC	SW:	$(AC)_{26-30}$		
	D15Mit59	CAGTCGGGCGTCATCAGAATGGCTATGATTAAG	R:	$(AC)_{21}$	0.45	2
		ATCACACATACCATGAATGAG	SW:	$(AC)_{22-24}$		
	3-29434	CAGTCGGGCGTCATCAATTACCACTGTCTGGAAAG	R:	$(AAAG)_{28}$	0.55	6
		CAACAGAGAGCCAATGTC	SW:	$(AAAG)_{19-24}$		
	6-9487	CAGTCGGGCGTCATCAGCCACGGCAAATGTAAAGAG	R:	$(GAAA)_{39}...(GGGA)_6$	0.72	7
		GCTCCACTTTGTGCTGTCAC	SW:	$(GAAA/GGGA)_{25-31}$		

Allele size ranges in 13/29 loci (45%) were smaller in Swiss-Webster mice than the allele in the reference genome (Table 2.1). In the remaining 16 loci, the largest alleles in Swiss-Webster mice were comparable in size to those in the reference genome. However, other alleles at these loci were much shorter in the Swiss-Webster mice (reduced in size by as many as 30 repeat units); polymorphisms occurred typically in the larger alleles.

2.4.2 Spontaneous and Induced Germline Mutations

Considering all loci and treatment groups (30 pedigrees, 298 offspring), we detected 19 mutants out of 11,908 alleles scored, which yielded an overall microsatellite mutation frequency of 1.60×10^{-3} (95% CI = 0.96×10^{-3} to 2.50×10^{-3}). The mutation frequency including only the 11 loci that had mutations was 2.90×10^{-3} (95% CI = $1.74 \times 10^{-3} – 4.53 \times 10^{-3}$; 19 mutations/6556 alleles scored). Mutation frequencies from all treatment groups were combined to determine the spontaneous mutation frequency because treatment groups did not differ (see analyses below). Nine of the loci identified through our enrichment and sequencing efforts had locus-specific mutation frequencies ranging from $1.68 \times 10^{-3} – 5.03 \times 10^{-3}$ (Table 2.2). Both of the loci selected from the Eukaryotic Microsatellite Database had mutation frequencies ranging from $1.68 \times 10^{-3} – 3.36 \times 10^{-3}$ (Table 2.2). No mutations were observed in the published loci used in Baker et al. [1995]. The parental origin of the mutations were 4 paternal, 7 maternal, and 8 of undetermined parental origin. In the 30 families assessed, 10 families had one mutation each, 3 families had 2 mutations, and 1 family had 3 mutations. In the families that had multiple mutations, the mutations were all in separate pups and at different loci.

Table 2.2 Number and type of germline mutations at 11 loci in control, 0.5 Gy, and 1.0 Gy families. *N* number of offspring; *p* paternal mutation; *m* maternal mutation; *u* mutation of undetermined origin.

Locus	Mutations			Mutation Frequency (x 10^{-3})	95% CI (x 10^{-3})
	Control *N* = 98	0.5 Gγ *N* = 100	1.0 Gγ *N* = 100		
Mm19.2.3	0	2 u, 1 m	0	5.03	0.95 - 14.90
Mm15.3.1	1 u, 1 p	1 u	0	5.03	0.95 - 14.90
Mm2.2.1	1 m	2 m	0	5.03	0.95 - 14.90
6-9487	1 p	0	1 p	3.36	0.32 - 12.34
Mm14.1.1	0	1 u	1 m	3.36	0.32 - 12.34
Mm10.3.2	0	0	1 m	1.68	0 - 9.62
Mm3.2.1	1 u	0	0	1.68	0 - 9.62
3-29434	0	1 u	0	1.68	0 - 9.62
Mm7.2.1	1 p	0	0	1.68	0 - 9.62
Mm10.2.1	0	1 m	0	1.68	0 - 9.62
Mm16.1.2	0	0	1 u	1.68	0 - 9.62
	6	9	4		

All of the mutations observed were most likely single repeat changes. Overall 8 (42%) mutations appeared to be single repeat gains, 8 (42%) appeared to be single repeat losses, and in 3 (16%) of the mutations it could not be distinguished whether the mutations were single repeat gains or losses because the parental genotypes did not allow for the origin of the mutation to be determined. The mutation spectra for the different treatment groups were: control 3 gains, 2 losses, 1 unknown; 0.5 Gy 3 gains, 5 losses, 1 unknown; 1.0 Gy 2 gains, 1 loss, 1 unknown. The distribution of gains/losses did not change by treatment group (Fisher's exact test, $P = 0.6737$).

The panel of 23 loci was used to screen families of mice from an earlier experiment in which ESTR mutation frequencies increased significantly following radiation exposure [Somers et al. 2004]. Initially, the panel was used to screen control and 1.0 Gy families; 8 of the 23 loci used to screen control and 1.0 Gy families had mutations (Table 2.2; see Figure 2.1 for examples). Mutation frequencies were higher in the control than in the 1.0 Gγ treatment group; however, there were no significant changes in germline mutation frequencies between these two groups (Figure 2.2A+B; statistical summaries in Table 2.3). Specifically, paternal mutation frequencies (including mutations of undetermined parental origin) in the control (4.64×10^{-3}) and 1.0 Gy group (1.82×10^{-3}) were not significantly different.

After failing to detect microsatellite mutation induction at the highest dose (1.0 Gy), we used a reduced panel of only the most mutable loci to screen families of sires treated with the lower dose of 0.5 Gy. Nine loci that did not have mutations, had a heterozygosity below 0.40, or had less than 3 alleles in the Swiss-Webster parents (Table 2.1) were excluded from the revised panel. In the remaining 14 loci, 6 had a total of 9

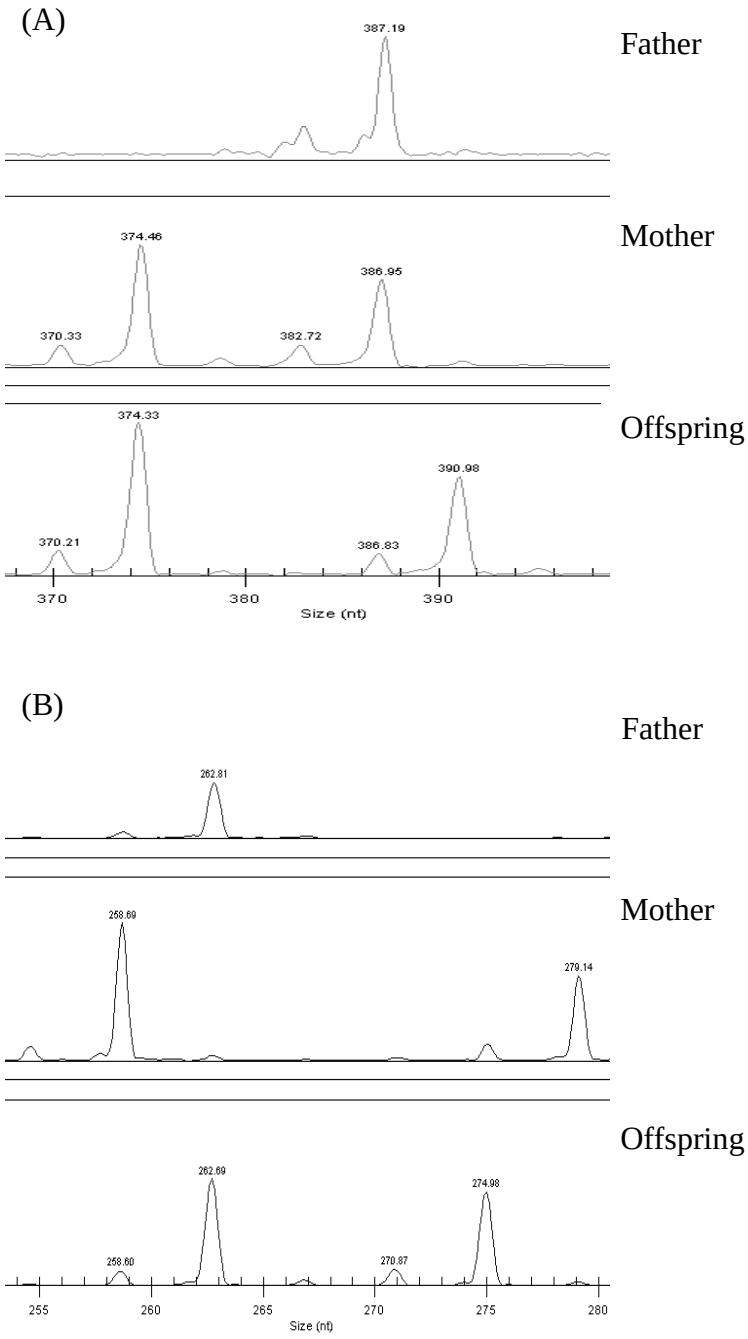
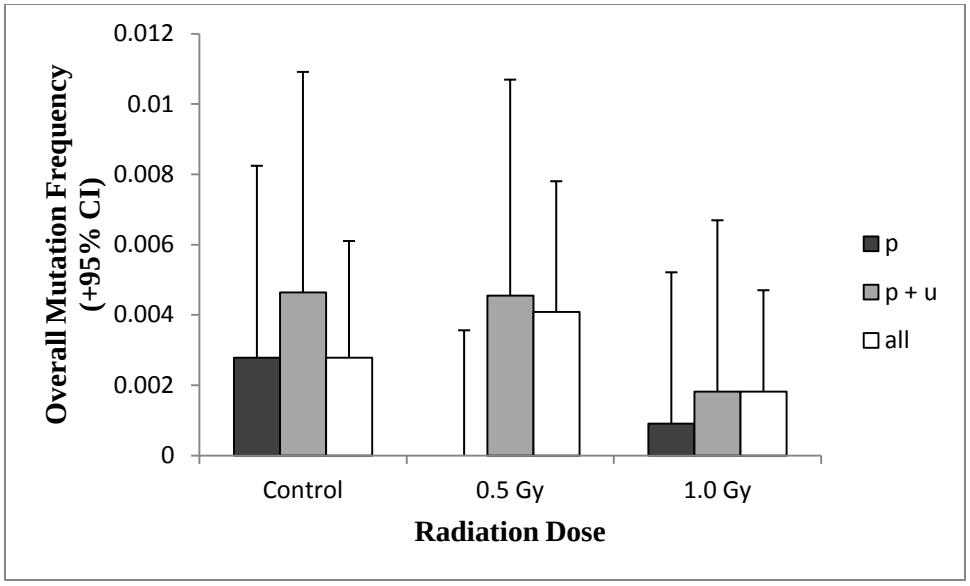
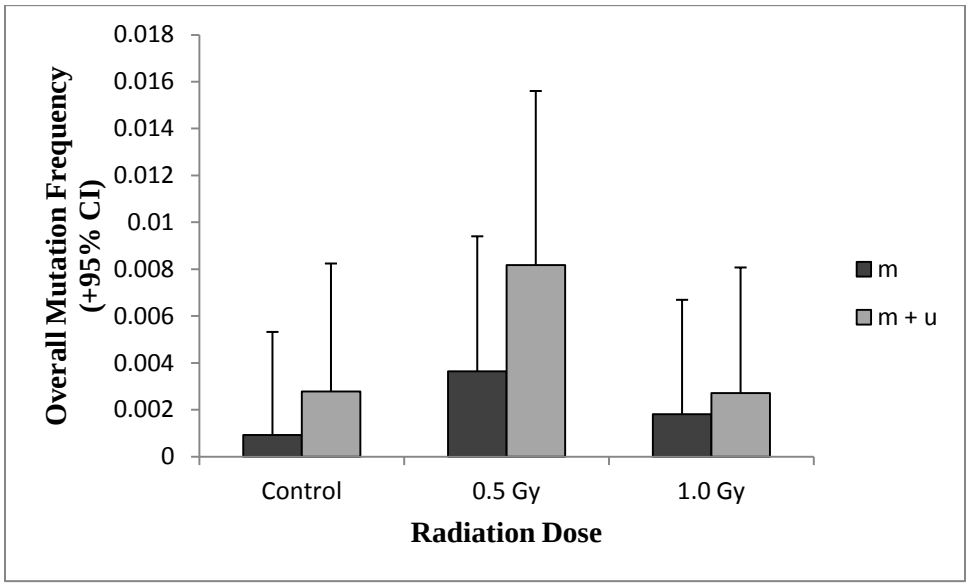


Figure 2.1 Examples of germline microsatellite mutations in the out-bred Swiss-Webster mice used. (A) The electropherogram shows an example of a paternal mutation at the locus Mm7.2.1 (core sequence AGAT). The offspring inherited a mutant paternal allele (4 nt gain from 387 nt to 391 nt) and the correct maternal allele (size = 374 nt). (B) The electropherogram shows an example of a maternal mutation at the locus Mm14.1.1 (core sequence AGAT). The offspring inherited the correct paternal allele (size = 263 nt) and a mutant maternal allele (4 nt deletion from 279 nt to 275 nt).

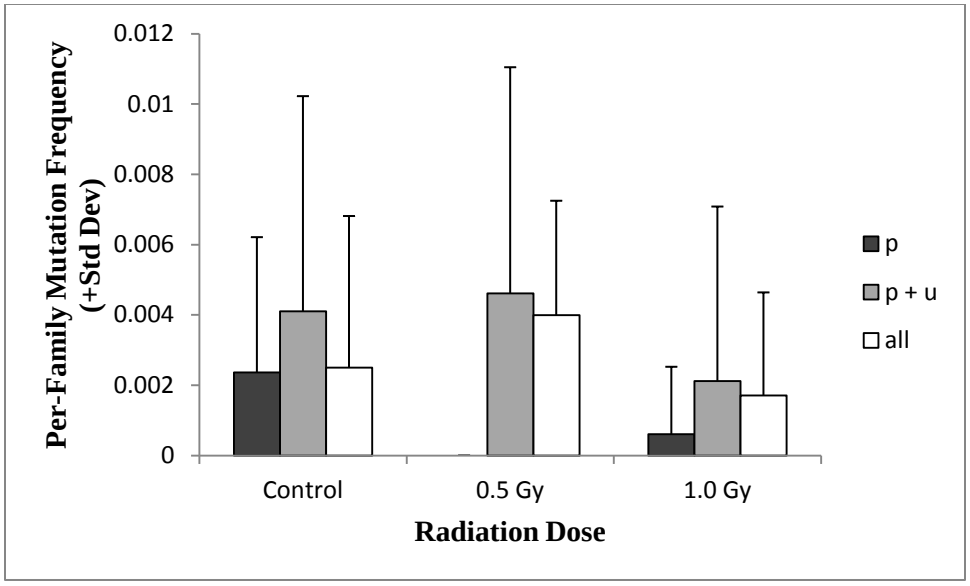
(A)


0.012
0.01
0.008
0.006
0.004
0.002
0
Overall Mutation Frequency (+95% CI)
Control
0.5 Gy
1.0 Gy
Radiation Dose
p
p + u
all

(B)


0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Overall Mutation Frequency (+95% CI)
Control
0.5 Gy
1.0 Gy
Radiation Dose
m
m + u

(C)



(D)

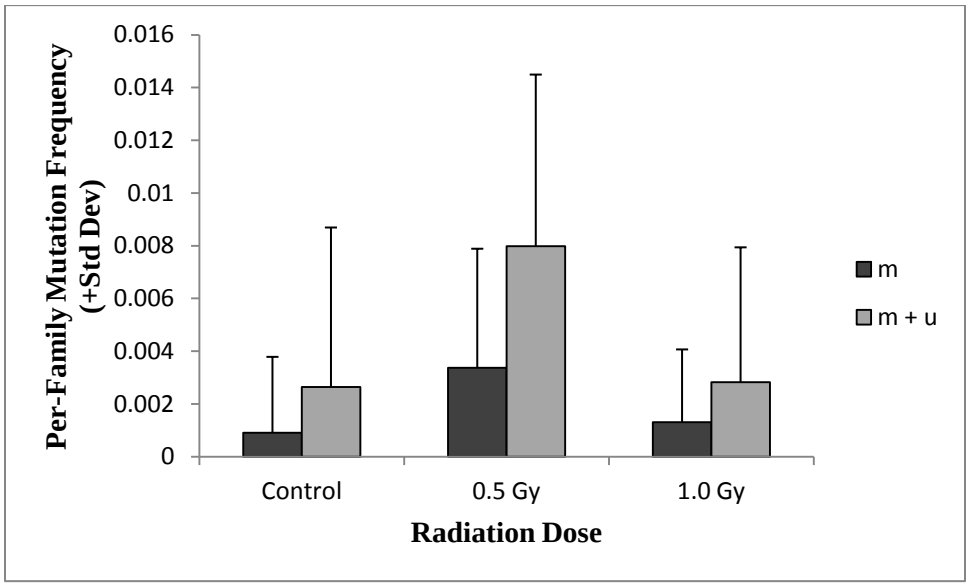


Figure 2.2 Germline microsatellite mutation frequencies at different radiation treatments and control. Comparisons were based on overall (A + B) and per-family (C + D) mutation frequencies. Mutation types are *p* paternal, *m* maternal, *p + u* / *m + u* paternal/maternal including all mutations of undetermined origin, or *all* mutations observed. Mutation frequencies were based on the number of mutant alleles versus the total number of alleles scored at the 11 loci with mutations in Table 2.2. No statistical differences were detected between treatments based on the different mutation types.

Table 2.3. Germline mutation data and statistical summaries for three groups of mice in which males received different acute doses of ionizing radiation from a cesium-137 source (mutation frequencies in Figure 2.2).

Treatment	Mutation Type[a]	Alleles Scored[b]	Mutants	Comparison[c]	*P-value* [d]
1. Control	p	1078	3	1 : 2	0.121
	m	1078	1	1 : 2	0.375
	p + u	1078	5	1 : 2	1.000
	m + u	1078	3	1 : 2	0.145
	all	2156	6	1 : 2	0.607
2. 0.5 Gy	p	1100	0	2 : 3	1.000
	m	1100	4	2 : 3	0.687
	p + u	1100	5	2 : 3	0.452
	m + u	1100	9	2 : 3	0.145
	all	2200	9	2 : 3	0.266
3. 1.0 Gy	p	1100	1	3 : 1	0.370
	m	1100	2	3 : 1	1.000
	p + u	1100	2	3 : 1	0.284
	m + u	1100	3	3 : 1	1.000
	all	2200	4	3 : 1	0.544

[a] Mutation types are *p* paternal, *m* maternal, *p + u / m + u* paternal/maternal including all mutations of undetermined origin, or *all* mutations observed.
[b] Based on the number of alleles scored at the 11 loci that had mutations
[c] Group comparisons correspond to order presented in first column.
[d] Probability value for two-tailed Fisher's exact test; no values were significant at $\alpha = 0.05$, so no Bonferroni Correction was applied.

mutations (4 maternal, 5 undetermined) in the 0.5 Gy families (Table 2.2). Considering only loci with mutations, there was no difference between the mutation frequency of the 0.5 Gy group and the other two treatment groups (Figure 2.2A+B; statistical summaries in Table 2.3). There was a non-significant 1.5-fold increase in overall mutation frequency in the 0.5 Gy group relative to control, but this was due to a non-significant 3.9-fold increase in maternal mutation frequency. Paternal mutation frequency (including mutations of undetermined parental origin) was 4.55×10^{-3} in the 0.5 Gy group, which is comparable to the mutation frequency in the control.

Power calculations were performed to determine the minimum sample sizes required to detect significant differences among treatment groups using Fisher's Exact test. Calculations were based on paternal/undetermined mutation frequencies at the loci that had observed mutations (Table 2.2). If the microsatellite panel had the same ratio of exposure to control mutation frequencies as ESTRs (~2.5-fold [Somers et al. 2004]), then at least 238 offspring would have been required in each treatment group for our study. Power calculations also showed that a 3.5-fold increase in paternal mutation frequency would be required in the 0.5 and 1.0 Gy treatment groups to achieve significance with the number of samples used here (100 offspring per group x 11 loci with mutations = 1100 paternal alleles scored per group).

Analyses were also conducted to compare mutation frequencies between control and irradiated families. Per-family paternal mutation frequencies (ANOVA, $F_2 = 2.29$, $P = 0.1204$), maternal mutation frequencies (ANOVA, $F_2 = 1.37$, $P = 0.2714$), paternal/undetermined mutation frequencies (ANOVA, $F_2 = 0.57$, $P = 0.5747$), maternal/undetermined mutation frequencies (ANOVA, $F_2 = 3.13$, $P = 0.0598$), and all

mutation frequencies (ANOVA, $F_2 = 1.56$, $P = 0.2289$) did not vary significantly among treatment groups (Figure 2.2C+D).

2.5 Discussion

This is the first reported study to identify unstable microsatellite loci for germline mutation studies and determine microsatellite mutation spectra in mice. All mutations observed were likely single repeat changes, which is consistent with the stepwise mutation model (reviewed in [Ellegren 2004]). This study was also the first to directly quantify microsatellite mutation frequencies in the germline of wild-type mice. The per-gamete mutation frequencies for the 11 polymorphic loci with mutations ranged from $1.68 \times 10^{-3} – 5.03 \times 10^{-3}$, while the overall microsatellite mutation frequency at all loci was between $1.60 \times 10^{-3} – 2.90 \times 10^{-3}$. The mutation frequencies of the mouse microsatellite loci are low relative to microsatellites with some of the highest reported mutation frequencies in other species ranging from $12.9 \times 10^{-3} – 42.3 \times 10^{-3}$ [Ellegren et al. 1997; Slebos et al. 2004; Furitsu et al. 2005; Tsyusko et al. 2007]. However, the locus-specific mutation frequencies of the mouse microsatellites identified here are high compared to other mouse microsatellites (0.05×10^{-3} to 0.5×10^{-3}) [Dallas et al. 1992; Dietrich et al. 1992] and to loci of other model species such as *Drosophila melanogaster* (0.3×10^{-3}) [Schlötterer et al. 1998]. The mouse microsatellites used in this study were also within the typical range of other animal species with mutation frequencies around 10^{-3} (egs. [Steinberg et al. 2002; Gow et al. 2005; Ortego et al. 2008]). Thus, the polymorphic microsatellite loci identified here have intermediate mutation frequencies relative to loci in other species.

In this study we used the developed panel of loci to investigate microsatellite mutation induction in mouse families from an experiment that showed elevated ESTR mutation frequencies following radiation exposure [Somers et al. 2004]. Unlike ESTRS, there was no significant mutation induction detected in the mouse families using the panel of microsatellites. The results of this study are unexpected because ESTRs are essentially extremely long microsatellites, and therefore it was predicted that microsatellites would show a similar response following irradiation. In the ESTR study, the mutation frequencies for the control, 0.5 Gy, and 1.0 Gy groups were 87.9×10^{-3}, 250×10^{-3}, and 266×10^{-3} respectively [Somers et al. 2004]; in this study, microsatellite mutation frequencies were 2.78×10^{-3}, 4.09×10^{-3}, and 1.82×10^{-3} respectively. As expected, elevated mutation frequencies (1.5-fold) were observed in the 0.5 Gy group, but the result was not statistically significant. ESTR studies with similar dose exposures have found significant differences between groups with a similar effect size around 1.5-fold (egs. [Dubrova et al. 1993; Yauk et al. 2002]). The major difference between this study and ESTR studies was that a non-significant *decrease* (1.5-fold) in microsatellite mutation frequency was detected in the 1.0 Gy group, which is surprising because several ESTR studies have shown significant mutation induction with a 1.0 Gy exposure. However, given that none of these differences were statistically significant, we can only conclude that there was no evidence for induction following irradiation.

It is possible that the discrepancy between microsatellite and ESTR results is due to the lower detection power of microsatellites. To increase the likelihood of detecting mutation induction, either more polymorphic microsatellites or families/offspring would be required. Based on the power calculations, if this panel were to have the same ratio of

exposure to control mutations as ESTRs, then at least 238 offspring would be required in each treatment group to achieve significance. So, it is clear that our sample sizes were not large enough; however, paternal mutation frequencies were actually lower (non-significant) in the treatment groups relative to the control, suggesting that adding more loci or samples would not likely change the results. Thus, the discrepancy between microsatellite and ESTR results does not appear to be due to detection power or sample size, but more conclusive evidence is needed.

There is a possibility that there was no mutation induction to be detected and that ESTRs and microsatellites respond differently to radiation-induced damage. ESTRs and microsatellites might have different sensitivities to mutation induction at different radiation doses, durations of exposure, and time points during spermatogenesis. Few studies have been published on radiation and germline microsatellite mutation induction, and until now there is no information available for rodents (Table 2.4). Some of the microsatellite studies did not detect significant mutation induction from irradiation [Satoh et al. 1996; Slebos et al. 2004; Furitsu et al. 2005]. Most of the studies that detected significant changes in mutation frequency had long durations of exposure ranging from 2 weeks to a lifelong exposure. Our study was different because males were only exposed to a single acute dose of radiation. Failure to detect a change in mutation induction here may suggest that chronic exposures may be more important for inducing microsatellite mutations in pre-meiotic spermatogonial stem cells. If this were the case, it would be different from ESTR results, which show no difference in mutation induction between acute and chronic exposures [Dubrova et al. 2000]. Further research is needed to address

Table 2.4. Comparison of studies that used microsatellites to detect germline mutation induction following radiation exposure.

Dose	Duration	Cell-type	Fold Difference	Significant	Species	Reference
0.5 - 1.0 Gy	Acute	Spermatogonia	(-1.5) - 1.5	No	Mouse	This study
Chernobyl (5 mGy)	Chronic	All	1.9 - 3.6	Yes	Barn Swallow	Ellegren et al. 1997
3 - 10.4 Gy Total	Chronic	Spermatogonia	1.9	Yes	Japanese Medaka	Tsyusko et al. 2007
0.1 - 5.0 Gy	Acute	Spermatogonia, Spermatocytes, Spermatids	1.6 - 2.0	Yes		Tsyusko et al. 2011
0 - 7.0 Gy	2 Weeks	Spermatogonia	52.9	Yes	Human	da Cruz et al. 2008
Atomic Bomb (> 10 mGy)	?	Spermatogonia	n/a	No		Satoh et al. 1996
Chernobyl (> 50 mGy)	?	Spermatogonia	1.6	No		Slebos et al. 2004
Chernobyl (39 mGy)	?	Spermatogonia	(-1.3)	No		Furitsu et al. 2005

differences in mutation induction resulting from acute and chronic irradiation using the panel of microsatellites developed here.

Another factor to consider is that stem cells may not be the most sensitive stage for microsatellite mutation induction. In this study, germ cells were only irradiated at one very specific time point (spermatogonial stem cells). These pre-meiotic germ cells may not be as radiosensitive at microsatellite loci compared to other germ cells. Evidence provided by other assays, such as the specific locus test, have shown that pre-meiotic cells are less sensitive than other germ cell types [Russell et al. 1998]. Most of the studies that used microsatellites to detect mutation induction had pre-meiotic spermatogonia exclusively exposed to radiation [Table 4], and the majority of these studies did not detect mutation induction. In contrast, Tsyusko et al. 2011 examined mutation induction in offspring that were the result of fertilizations involving irradiated spermatids, spermatocytes, differentiating spermatogonia, and spermatogonia, and they did detect a 1.6-2.0 fold mutation induction. However, there is no indication of whether the mutation induction occurred at pre-meiotic or post-meiotic male germ cells. It is possible that the different germ cells irradiated in Tsyusko et al. [2011] were more sensitive to acute radiation exposure at microsatellite loci than the spermatogonial stem cells irradiated in this study. If this explanation is true, then it would suggest that the timing of microsatellite mutation induction is different from ESTRs. Based on different studies (eg. [Dubrova et al. 1998]) it is generally accepted that pre-meiotic germ cells are the most sensitive to radiation at ESTR loci [Somers 2006]. However, a few studies have shown contradictory evidence in which spermatids are the most radiosensitive germ cell type [Sadamoto et al. 1994; Fan et al. 1995; Niwa et al. 1996]. The explanation for the

discrepancy is that the studies that showed no induction in spermatids examined early spermatids, which have functional DNA repair capabilities, while the other studies examined late spermatids, which are incapable of correcting DNA lesions that can be transmitted to the zygote [Niwa 2003]. Therefore, if the microsatellite mutation induction in Tsyusko et al. [2011] occurred in late spermatids, it would be due to failure to correct DNA lesions resulting in genomic instability in the zygote. In this study, it is possible that no mutation induction was detected in the offspring because functional DNA repair systems minimized microsatellite mutations in the irradiated spermatogonial stem cells. More work is needed to examine stage-specific mutation induction at microsatellite loci to see if the results are consistent with ESTR studies.

Differences in DNA repair efficiency between microsatellites and ESTRs could also have contributed to our findings. Specifically, DNA repair appears to play a role in reducing spontaneous ESTR mutation frequency, but it is much more effective at reducing spontaneous microsatellite mutations. The evidence for this conclusion comes from studies of genetically modified mice with deficiencies in DNA repair, which have shown elevated ESTR and microsatellite mutation frequencies relative to wild-type. Severe-combined-immuno-deficiency mutant mice with impaired double-strand break repair [Yamauchi et al. 2002; Barber et al. 2004], poly-ADP ribose-1 polymerase knockouts with impaired single-strand break repair [Barber et al. 2004], DNA polymerase kappa knockout mice deficient in translesion synthesis [Burr et al. 2006], and Msh2 knockouts deficient in mismatch repair [Burr et al. 2007] all had elevated paternal ESTR mutation frequencies that were 2.1 – 4.4 fold higher than wild-type. Radiation exposure failed to significantly elevate mutation frequencies in these mice suggesting that

mutation frequencies had reached saturation. In contrast, microsatellite mutation frequencies in the sperm of PMS2 (mismatch repair) deficient mice were at least 49 times higher than control [Baker et al. 1995]. This large difference suggests that DNA repair of microsatellite mutations may be more effective or important relative to ESTR mutations. Thus, it is possible that efficient DNA repair in spermatongonial stem cells simply negated mutation induction following irradiation in our study. Further research with transgenic mouse strains is needed to better understand differences between microsatellite and ESTR DNA repair.

For future studies we would not recommend using out-bred mice in conjunction with the microsatellite panel for measuring germline mutation induction. A major problem with the mice we used was that it was difficult to determine the parental origin of each mutation when both parents had similar or identical genotypes. The parental origin of 8/19 (42%) mutations was undetermined, which is problematic when paternal mutations are of special concern. Another issue is missed mutations (false-negatives) because the parents had similar genotypes. For example, if both parents had allele sizes of 262 bp and 266 bp, the number of detectable single gain/loss mutations is limited because gains from 262 bp to 266 bp, or losses from 266 bp to 262 bp would appear normal in the offspring. An additional concern using out-bred animals is the additional variance that can be introduced based on differences in parental genetic composition. Genetic variance among treated parents may lead to additional variance in mutation induction, which can lead to reduced statistical power. For example, Tsyusko et al. [2011] showed that familial mutation frequencies in out-bred Japanese medaka fish varied greatly within treatment groups, providing a possible explanation as to why the 2.5 Gy treatment in their

experiment did not show a significant increase in mutation frequency. To solve all of these issues it would be best to cross two different sources/strains of inbred mice with consistent allele size range differences so that the parental origin of mutations can be clearly determined. Before selecting different mouse strains to be used, it is necessary for researchers to screen the mice using the loci to determine that the allele size ranges are different and not assume different strains will have different allele size ranges. Furthermore, the source/strain of mice should be chosen based on the allele length of the different loci. Mice with longer allele sizes at the different loci would be ideal because mutation frequency increases with microsatellite length [Ellegren 2004]. Overall, before carrying out a germline mutation study using the microsatellites identified here, careful selection of mouse strain(s) is required.

In conclusion, we have identified unstable mouse microsatellite loci with relatively high mutation frequencies, but they are not as sensitive to germline radiation exposure as ESTRs. Further research with greater sample sizes will need to be conducted to determine why microsatellites did not display a similar magnitude of mutation induction as ESTRs following irradiation, especially when a larger number of loci were available for mutation detection. Additional work will also need to be done to investigate how microsatellite mutation frequencies are influenced by other exogenous mutagens, such as chemical mutagens, that have been shown to increase ESTR mutation frequencies (reviewed in [Somers 2006]). Given the superior detection power ESTRs offer for detecting mutation induction, these markers should still be used for mutation detection until other potential methods, such as next-generation DNA sequencing (reviewed in

[Beal et al. 2012]), are developed to the point where they can accurately and easily measure mutation frequencies.

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CHAPTER 3. WHOLE GENOME SEQUENCING FOR QUANTIFYING GERMLINE MUTATION FREQUENCY IN HUMANS AND MODEL SPECIES: CAUTIOUS OPTIMISM.

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3.1 Abstract

Factors affecting the type and frequency of germline mutations in animals are of significant interest from health and toxicology perspectives. However, studies in this field have been limited by the use of markers with low detection power or uncertain relevance to phenotype. Whole genome sequencing (WGS) is now a potential option to directly determine germline mutation type and frequency in family groups at all loci simultaneously. Medical studies have already capitalized on WGS to identify novel mutations in human families for clinical purposes, such as identifying candidate genes contributing to inherited conditions. However, WGS has not yet been used in any studies of vertebrates that aim to quantify changes in germline mutation frequency as a result of environmental factors. WGS is a promising tool for detecting mutation induction, but it is currently limited by several technical challenges. Perhaps the most pressing issue is sequencing error rates that are currently high in comparison to the intergenerational mutation frequency. Different platforms and depths of coverage currently result in a

range of $10–10^3$ false positives for every true mutation. In addition, the cost of WGS is still relatively high, particularly when comparing mutation frequencies among treatment groups with even moderate sample sizes. Despite these challenges, WGS offers the potential for unprecedented insight into germline mutation processes. Refinement of available tools and emergence of new technologies may be able to provide the improved accuracy and reduced costs necessary to make WGS viable in germline mutation studies in the very near future. To streamline studies, researchers may use multiple family triads per treatment group and sequence a targeted (reduced) portion of each genome with high (20–40x) depth of coverage. We are optimistic about the application of WGS for quantifying germline mutations, but caution researchers regarding the resource-intensive nature of the work using existing technology.

3.2 Introduction

Germline mutations are the raw material for natural selection, and can profoundly affect the health of individuals and populations (reviewed in Refs. [1–3]). Identification of factors that modulate the frequency and type of mutations in germ cells, particularly in humans or relevant model organisms such as mice, has been a significant scientific pursuit (reviewed in Refs. [4,5]). One of the main difficulties presented by germline mutation studies is the lack of appropriate tools for efficiently detecting mutation induction (i.e., changes in mutation frequency). Consequently, many studies have used enormous numbers of animals (e.g., [6,7]), or panels of select marker loci with uncertain relevance to mutation frequencies elsewhere in the genome (e.g., [8–13]). Despite these efforts, we still lack a broad perspective on how endogenous factors such as genetic

polymorphisms, and environmental factors such as contaminant exposure influence the type and frequency of mutations in the germline. Accordingly, examples of conclusive links between environmental exposures, elevated germline mutation frequencies, and disease in humans remain elusive [4]. Nevertheless, inherited disorders continue to represent a major cost for health care systems [14], and there is longstanding public concern over the genetic consequences of lifestyle choices and environmental exposures [15]. Thus, there is a need to expand our knowledge in the field of germ cell mutagenesis, and begin to take advantage of rapidly advancing technologies in the biosciences.

Next-generation DNA sequencing (NGS) technologies (for review see Ref. [16]) enable whole genome sequencing (WGS) applications in germline mutation studies. WGS data eliminate the need for marker loci and proxy measures of mutation frequency, providing a global view of trans-generational genomic changes. So far NGS and WGS have been used mainly to characterize individual genomes (e.g., [17–27]), assess changes to tumor cells (e.g., [28–34]), aid forward genetic screens [35], and discover novel mutations that result in inherited disease (e.g., [36–38]). NGS and WGS have been used to quantify germline mutation rates in mutation accumulation lines of several model organisms, including: *Drosophila melanogaster* [39,40], yeast [41], *Caenorhabditis elegans* [42], and *Arabidopsis thaliana* [43]. NGS and WGS have also been used to discover mutations within human families [37,44], but these approaches have not yet been used to quantify changes in germline mutation type and frequency in humans or model vertebrate species as a result of exogenous mutagen exposure. Here we review the potential of applying NGS/WGS technologies to directly quantify germline mutation frequency and type in humans and model species. Specifically, we are interested in the

capacity for NGS to quantify changes in the frequency of mutations that occur as a result of experimental treatments such as exposure to genotoxic environmental agents. Although different model organisms and natural populations of diverse taxa could be useful for monitoring the effects of genotoxic environments, the focus of this review will be on humans and mice because of their relevance to human health.

3.3 The Design of Germline Mutation Induction Studies

In genetic toxicology, by far the most common approach for experiments has been exposure of one parent to a mutagen, followed by breeding and examination of a select panel of phenotypes or genotypes in the resulting progeny to detect germline mutation events (Fig. 3.1a). This pedigree screening process is repeated in many families within treatment groups, enabling quantification of a mutation frequency that can be statistically compared to that of other experimental groups. Early studies applying this approach used phenotypic markers to measure mutation frequencies via visible mutations in the offspring of lab mice (reviewed in Refs. [45–48]). These early studies produced the first direct and reliable estimates of mutation frequencies in mammals. However, the low frequency of mutation events made it necessary to use high mutagen doses and screen many thousands of progeny to arrive at reliable and statistically comparable mutation frequencies (e.g., Russell et al. [7] used a total of 539,084 mice). Consequently, studies using visible phenotypic markers are now too expensive, unwieldy, and perhaps even ethically questionable (based on animal usage requirements) for routine application.

More recent genetic toxicology studies have used a class of neutral tandem repetitive DNA known as Expanded Simple Tandem Repeats (ESTRs) [49–51] to detect

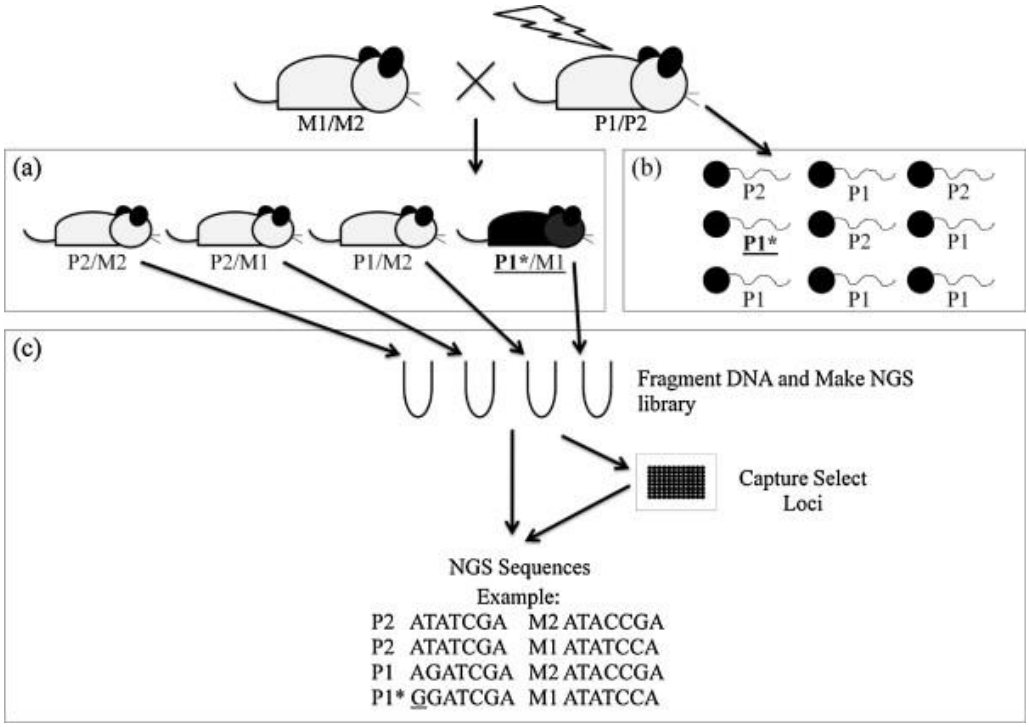

Fig. 3.1. Standard approaches for measuring germline mutation frequency. (A) The pedigree approach for measuring germline mutation induction. The paternal mouse is irradiated or exposed to a chemical mutagen and then mated with an unexposed female to produce a litter of progeny. The offspring are then screened for changes at phenotypic markers or a panel of variable loci. The phenotypic change shown in this example is a change in coat color from gray to ebony. The same individual has a mutant allele P1* that is paternal in origin. All other progeny are normal. The same general approach is used with molecular markers such as ESTR loci, where changes in allele size are detected in offspring DNA. (B) The single-molecule or small-pool PCR approach for detecting mutations in sperm. Again the father is irradiated or exposed to a chemical mutagen and then his sperm is analyzed for mutations in select loci. One sperm inherited a mutant allele P1* that could have potentially been passed onto the offspring had it fertilized the egg. This method is not yet suitable for WGS studies because current methods cannot sequence individual cell genomes reliably. (C) The NGS approach for measuring germline mutation frequencies in families. The male is mutagenized and then mated with a female to produce a litter of offspring. DNA is extracted from all mice and fragmented to make a NGS library. From there the DNA can be enriched prior to sequencing for specific loci or the whole genomes can be sequenced. Genome sequence comparisons can then be made between the offspring and parents in attempt to detect de novo mutation events. In this example one individual inherited a mutant allele P1* containing an A → G transition.

germline mutation events. ESTRs have short repeat units (4–6 base pairs) and are arranged in tandem arrays that can be as long as 20 kilobase-pairs. ESTR mutants are detected by comparing allele sizes between parents and progeny using the experimental design introduced above (Fig. 3.1a), but size changes in repeat arrays are the mutation marker, rather than an expressed phenotype (reviewed in Ref. [52]). ESTR mutations occur much more frequently than visible phenotypic changes and are very sensitive to mutagen exposure, thereby reducing sample size requirements for statistical analysis to only tens of parents and hundreds of progeny (e.g., [53–60]). ESTRs have been instrumental for examining germline mutation induction under low dose exposure conditions (e.g., [8,54,59,61]), and in response to chronic exposures to complex environmental mixtures [62–65]. However, interpretation of ESTR studies in a human health context has been problematic because of uncertainty regarding the relationship between mutation frequencies at ESTR loci and those elsewhere in the genome [66,67]. Alternatives to the family pedigree approach involve using small-pool (SP) or single-molecule (SM) PCR to detect mutations directly in sperm (Fig. 3.1b). SP-PCR involves amplifying DNA from small aliquots of sperm and has been used to compare minisatellite allele sizes between somatic cells and sperm in men to quantify germline mutation frequency (e.g., [68]). SP-PCR has been used to examine the effects of radiation on human minisatellite mutation frequencies in sperm, but there was no evidence for radiation induced instability at the loci used [69–71]. Importantly, minisatellite mutations in these studies were assessed in a large number of sperm pools for each male representing hundreds of individual cells. In SM-PCR, the DNA extracted from the sperm is diluted so that each PCR reaction contains one DNA molecule, facilitating the

amplification of one locus from many individual sperm collected from each male [72]. So far, SM-PCR has been used to detect elevated mutation frequencies in sperm in response to radiation [72], air pollution [64], and tobacco smoke exposure [73,74]. SP and SM-PCR approaches are beneficial because they minimize the number of adult mice needed for an experiment by increasing the number of gametes that are assessed, and thereby the precision of the mutation frequency estimate for each individual and treatment group. SM-PCR studies generally involve comparisons of genotypes from many germ cells per individual (at least 100 in total), and at least 5 individuals per treatment group (e.g., [72]).

Experiments using NGS/WGS to quantify and compare germline mutation frequencies are in essence no different than those that have preceded them; it is only the genomic coverage and resulting precision of the mutation measurements that is different. Thus, the approach for genetic toxicology experiments (or other modifying factors) is the same as summarized in Fig. 3.1a for other marker types: a parent or parents is exposed, and the resulting mutations inherited by offspring are detected using NGS (Fig. 3.1c). It is important to realize that although NGS produces a huge amount of information for each comparison of offspring to parents (triad), the resulting mutation frequency that is estimated for the triad is a single datum. Accordingly, mutation frequencies from at least several, and probably more, triads per treatment group will be required for statistical analyses comparing different treatments. Sample size per treatment group becomes even more important when comparing mutation frequencies in study species or populations with high levels of genetic variation [75]. For example, the recent ground-breaking WGS paper by Conrad et al. [44] examined only 2 human family triads, and found dramatically different contributions of mutations from the male and female parents in each case. A

hypothetical study using similar techniques, but designed to determine the influence of GSTM1 polymorphism on air pollution-induced mutation frequency (e.g., as might be prompted by Rubes et al. [76]), would need to have a sufficiently large sample size to account for variation in mutation frequency both within and between treatment groups (GSTM1 genotypes and pollution exposures). Deciding on appropriate sample sizes and statistical approaches for NGS studies on factors that modify mutation type and frequency will be a critical consideration as the field and technology advance. Unfortunately, data on background mutation frequencies from WGS studies on humans or model animals are currently unavailable, so detailed recommendations regarding sample size and statistical detection power cannot be made.

Unfortunately, there is no current viable NGS equivalent to the SP- and SM-PCR methods for quantifying mutation frequency directly in sperm. To measure the germline mutation frequency of treated males using NGS of sperm, whole-genome amplification or single molecule sequencing would be required to sequence the individual sperm cells. Whole-genome amplification protocols introduce errors that cannot be corrected, and they amplify genomic DNA in a biased manner [77]. Although single molecule sequencing technology is available and continues to improve (e.g., HeliScope [21] and PacBio RS [78]), read length (HeliScope) and raw accuracy (PacBio RS) remain a challenge. Additionally, construction of libraries is very inefficient, requiring the equivalent of thousands to millions of genomes. Thus, single molecule sequencing is not at a stage where it can accurately sequence the genome of a single cell. For these reasons NGS/WGS is best applied to the family pedigree approach.

3.4 Benefits of Using WGS

When comparing mutation frequencies between mutagen exposed families and controls, WGS can not only facilitate comparison of overall mutation frequency, but it also can be used to determine if there are changes in mutation location and type. Researchers can calculate the total number of potentially detrimental mutations (missense, nonsense, non-initiation, frameshifts, coding indels, splice acceptor/donor sites, non-coding RNA transcripts, and untranslated region mutations) and compare their relative frequencies. It has been estimated that 20–29% of missense mutations are effectively neutral while the rest are mildly to highly deleterious [79–81], which highlights the importance of finding out if these types of mutation events are increased as a result of mutagen exposure. In a simple family pedigree, many of these mutations would be recessive, regardless of whether they are indeed detrimental or not and would therefore not be detected through other means. For example, Flibotte et al. [82] generated mutant strains of the model nematode, *C. elegans*, using three different mutagens: ethyl methanesulfonate (EMS), ENU, and ultraviolet trimethylpsoralen (UV/TMP). Through WGS of the different strains they found that EMS had a bias for G/C to A/T transitions, ENU was biased towards A/T to T/A transversions, G/C to A/T transitions, and A/T to G/C transitions, whereas UV/TMP showed no biases in terms of mutation induction. They also compared the relative variant frequency of the genomic locations (exon, intron, or other) between the three mutagens. The authors determined that EMS caused a higher relative variant frequency in exonal DNA compared to the other mutagens. This quality of information is useful when assessing the potential genomic damage environmental

mutagens are capable of causing. Conrad et al. [44] published the results of sequencing two human family triads. They were able to distinguish between somatic and germline mutation events as well as filter out the false-positives. Examination of the germline mutations in the two triads showed that only one synonymous mutation occurred in an exon while the rest were located in non-coding regions, introns, or in intergenic DNA. The results from their study show the potential usefulness of using WGS in germline mutation studies.

With WGS, many of the limitations associated with previous germline mutation experiments will no longer be an issue. Studies that used phenotypic markers to quantify mutation frequencies needed enormous sample sizes (thousands of mice) and could only detect mutations that altered the phenotypes associated with the few loci used [45–48]. Importantly, the loci used could tolerate mutations because they did not disrupt viability. Hence, the mutation frequency for phenotypic markers provides no information on how mutation frequencies at loci more critical for fitness are altered by mutagen exposure. WGS will suffer from this same limitation to some degree; i.e., only mutations that produce viable embryos or offspring can be assessed for mutations. However, WGS could be applied to offspring much earlier in development (e.g., newborns, early embryos, or even partially resorbed embryos), thereby expanding capacity to determine mutation frequencies at functional loci. An additional problem with the phenotypic loci used in earlier experiments was the rare occurrence of phenotype altering mutation events (10^{-5}), which necessitated high doses of acute mutagen exposure to invoke a response. These studies were therefore unable to provide any information on how chronic low-dose exposure or mixtures of mutagens can affect germline mutation frequency. By monitoring

all changes in all loci, WGS may be able to overcome this problem and provide information on how different types of exposures affect the genomic integrity of germ cells.

WGS also overcomes some of the major problems associated with ESTR loci as well. ESTRs are highly mutable, and sensitive to exposure to a variety of agents, but their mutation detection can be imprecise, and is of uncertain relevance to other regions of the genome (reviewed in Ref. [66]). Also, the well-characterized ESTR loci are found exclusively in mice [49–51], making it difficult to use ESTR studies as a model for human mutation induction. In contrast, the general structure of the genome is similar in humans and mice, so WGS will produce similar and comparable data in each species. Mutation data from WGS are relatively straightforward to produce, and once quality checked, should be easy to interpret (unlike ESTRs, but see sections below on current challenges with WGS data). WGS data can also be produced in high-throughput facilities, and are amenable to a variety of quality checks and bioinformatics filters, which should be universally available. Thus, although studies measuring mutation frequency via ESTRs were fundamental for understanding germline mutation induction, using few loci in any mutation detection assay tells us little about the global damage caused by mutagen exposure. The only possible way to completely understand changes caused by mutagens is through WGS.

3.5 Challenges Associated with NGS for Germline Mutation Induction Studies

WGS offers tremendous promise for detecting germline mutation induction, but it also has some important drawbacks (see [83,84]). Theoretically, WGS allows detection of

all possible mutations in a genome, with the exception of some heterochromatic and other regions that have not yet been fully sequenced. Potential mutations detected using WGS would include single nucleotide polymorphisms (SNPs), insertions, deletions, and rearrangements in exons, introns, promoters, and intergenic regions. Unfortunately this expectation is only partially fulfilled with current NGS technologies. An overview of how WGS would be used to measure mutation frequencies is provided in Fig. 3.2 to aid the brief discussion here. WGS quality library preparation is nontrivial in time, expense or expertise. High quality WGS assemblies require multiple libraries with varying insert sizes, which requires relatively large amounts of high molecular weight DNA to construct. Many sequencing reads are needed to cover each piece of the genome to achieve a depth of coverage great enough to minimize the rate of sequencing errors. With the higher depth of coverage comes an associated increase in cost and data management. Following sequencing, quality control steps are required to trim or eliminate reads that have quality scores that fall below an assigned threshold, thus reducing overall coverage. After high-quality reads have been collected, each parental genome will need to be assembled using a reference and available bioinformatics tools. Following completion of the parental genomes, a laborious task on its own, offspring DNA sequences will need to be mapped to those genomes. This step may fail to incorporate certain mutant reads into the assembly and can also increase the number of putative mutants that need to be confirmed as a consequence of mis-mapping reads to the wrong genomic location. Efficient in-house scripts are then needed to find all putative mutants and design primers or oligonucleotide probes for these sequences so that the mutants can be confirmed by other means. Depending on the caliber of the quality control done up to this point, there

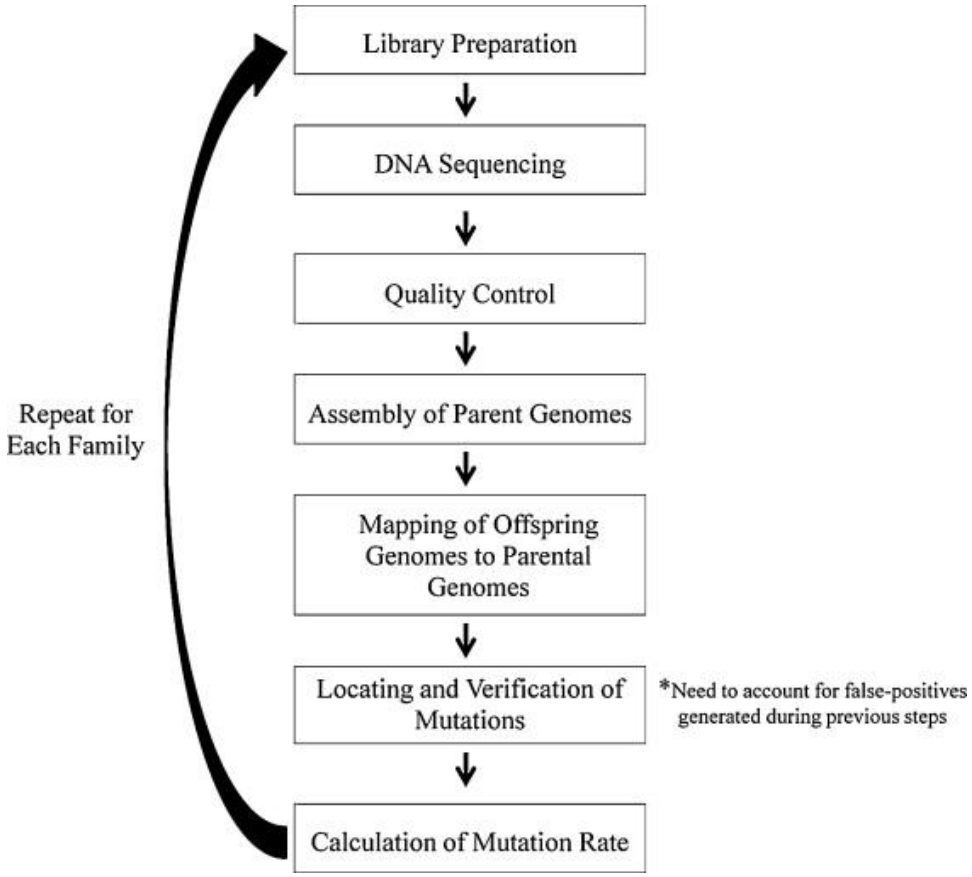

Fig. 3.2. Brief overview of how WGS can be used to measure germline mutation frequency.

could be tens to thousands of sequence errors for each true mutation that would need to be confirmed. The problems are further multiplied by the fact that all the steps mentioned would have to be repeated for all families in each treatment group in an experiment, dramatically increasing the time and cost. The following sections elaborate on these specific issues in more detail.

3.5.1 DNA Sequencing Errors and Detection Bias

NGS results in a high ratio of calling SNP false-positives versus true de novo mutations because of errors generated during template preparation, sequencing, and alignment/mapping (Table 3.1). Roach et al. [37] sequenced the genomes of a family of four humans and found a germline mutation frequency of approximately 1.1×10^{-8} per position per haploid genome, which corresponds to 30 SNPs per generation. This is comparable to previous estimations of $\sim 10^{-8}$ to 2.5×10^{-8} per nucleotide site per generation [85–87]. Even with a sequence accuracy of 99.999%, there will be a sequencing error every 1 in 100,000 nucleotides, resulting in at least 30,000 false-positives (Table 3.1) compared to the 30 real spontaneous mutations in a haploid genome of 3 GB (effectively at least 1000 sequencing errors per true mutation). In 1.83 GB of sequenced DNA where potential mutations were observed, Roach et al. [37] found that 28 of 33,937 potential mutations were true de novo mutation events (1212 errors per mutation). This was done by resequencing candidate polymorphisms while applying a stringent base-calling algorithm. Mass spectrometry was then used to confirm the 28 de novo mutations. Conrad et al. [44] more recently compared the sequences of the genomes in 2 family triads showing a very similar intergenerational mutation frequency of 0.97 x

Table 3.1 Comparison of the approximate false-positive SNP discovery rate between different genome sequencing studies.

Sequencing Scope	Sequencing Platform	Average Depth of Coverage	Approximate False-Positive Rate [g]	Estimated Number of False-Positive SNPs	References
Whole Genome (Watson)	Roche/454	7.4x	3×10^{-4}%	$10^{4,a}$	[17]
African (Yoruba) Genome	Illumina	41x	3×10^{-4}%	$10^{4,b}$	[18]
Asian (Han Chinese) Genome	Illumina	36x	3×10^{-5}%	$10^{3,b}$	[19]
African (Yoruba) Genome	ABI SOLiD	18x	3×10^{-5}%	$10^{3,c}$	[20]
Asian (Korean) Genome	Illumina	28.95x	3×10^{-4}%	$10^{4,b}$	[22]
Whole Genome (Quake)	Helicose	28x	3×10^{-5}%	$10^{3,b}$	[21]
Asian (Korean) Genome	Illumina	27.8x	3×10^{-4}%	$10^{4,b}$	[23]
3 Human Genomes	Complete Genomics[h]	45x to 87x	1×10^{-3}%	30,000/haploid genome[d]	[26]
African (Namibian) Genome	Roche/454	10.2x	3×10^{-5}%	$10^{3,d}$	[25]
Whole Genome (Family Quartet)	Complete Genomics	52x to 88x	1×10^{-3}%	33,909[e]	[37]
Whole Genome (Two Family Triads)	Roche/Illumina /SOLiD	22x	3×10^{-5}%	1,304/1,065	[44]
Whole Genome (Moore)	Ion Torrent	10.6x	3×10^{-5}%	$10^{3,f}$	[27]

[a] Number of false positives for known SNPs estimated conservatively by counting the number of known SNPs that did not match known SNPs in dbSNP.
[b] Number of estimated SNP false positives based on reported concordance between assembled genome and genotyping assay.
[c] Number of estimated SNP false positives based on reported sequencing accuracy.
[d] Number of estimated SNP false positives reported (False-Positive Rate).
[e] Determined by resequencing a portion of suspected SNPs.
[f] Number of estimated SNP false positives based on reported concordance between genome assembled using Ion Torrent and genome assembled using ABI SOLiD.
[g] Estimated based on the number of estimated false positives relative to the size of the haploid genome.
[h] Complete Genomics sequences Human genomes exclusively.

10^{-8} and 1.17 x 10^{-8} for the respective families. They found a total of 49/35 germline mutations relative to 1304/1065 sequencing errors and 952/634 somatic mutations (an improvement to around 50 false variants per germline mutation event). With the best estimated false positive frequencies in WGS studies ranging from 3 x 10^{-5}% to 1 x 10^{-3}%, there would be at the very least 10^{1}–10^{3} sequencing errors for every true mutation event (Table 3.1). Thus, even in the best studies, researchers will be forced to deal with reliable identification of false positives and verification of true mutation events. This could include using more stringent base calling filters, similar to how Wheeler et al. [17] filtered 14 million variant positions down to 3.32 million, genotyping the variant positions using a microarray, and/or re-sequencing the variant positions using an orthogonal sequencing approach. However, with the many genomes required for even a simple mutagen dose–response study, the number of variants to confirm via re-sequencing could begin to multiply into the hundreds of thousands with lower accuracy, and would quickly become most efficiently done by replicating the entire WGS with a second NGS technology.

One approach to minimize error rate, is to sequence all genomes with a high degree of coverage. Smith et al. [88] sequenced a mutant strain of yeast multiple times using 3 different sequencing technologies: Roche 454, Illumina, and AB SOLiD. After comparing the sequences to the reference to look for SNPs, they found that a minimum of 10–15x average genome coverage was required to minimize the number of false positive and false negative calls between the different sequencing platforms to less than 2 for the whole genome. However, other experiments that sequenced larger whole human genomes needed even higher average fold coverage (10.2x to 88x) to reduce sequencing errors

(Table 3.1). Wang et al. [19] found that increasing depth of coverage to greater than 10x using an Illumina sequencer only increased the total amount of the genome covered slightly, but it greatly reduced the error rate of the assembled sequence. Using paired-end reads rather than single-end reads also reduced errors in the assembly. Different platforms and their amplification methods may have different biases, inevitably resulting in an unequal distribution of sequence coverage. Using a combination of technologies could compensate for this, if this option is available. Skryabin et al. [24] sequenced a human individual using both Illumina and SOLiD sequencers and demonstrated that the addition of the SOLiD data set to the Illumina data set increased genomic coverage. Du et al. [89] also demonstrated that using a combination of sequencing platforms allows for the identification of structural variants at reduced sequencing coverage. Conrad et al. [44] used three different sequencing platforms to reduce the number of false-positives close to a thousand at only 22x coverage. Overall, by increasing the depth of sequencing coverage throughout the genome, sequencing errors will be easier to recognize and fewer putative mutations will need to be screened in the confirmation step.

Depending on the sequencing technology used, certain types of indels, which are capable of causing frameshift mutations, may be difficult to detect. It is well known that Roche 454 sequencing has difficulty with homopolymeric regions [90], which can result in miscalled or missing mutations. Kim et al. [23] found that indels detected using an Illumina sequencer were 100% accurate, but this technology failed to detect more than 20% of indels that were actually present. These detection errors (false negatives) were mostly at repetitive DNA sequences containing indels at or near the end of reads. The significance of these repeat DNA mutation events may not be immediately apparent, but

they can cause genetic disorders when found within the untranslated, intronic, or coding regions of a gene (reviewed in Ref. [91]) and are capable of influencing gene expression when found outside genes [92]. One option to increase the sensitivity of indel detection is to exclude these repetitive DNA sequences from the analysis. However, by doing this a potentially important source of information on germline mutations would be lost. At the very least, special attention to indel detection will be required for WGS studies using particular platforms.

Copy Number Variants (CNVs), which can be associated with neuropsychiatric disorders [93], mental retardation [94], and cancers (reviewed in Ref. [95]), can also be difficult to detect using NGS alone. Using NGS, Wheeler et al. [17] and McKernan et al. [20] successfully detected 78% and 65% of CNVs respectively that were detected using comparative genomic hybridization (CGH) microarray analysis. Pushkarev et al. [21] were able to validate 93% of tested CNV regions using digital PCR. Paired-end mapping strategies have been used to successfully identify CNVs and other structural variants (reviewed in Ref. [96]), however these methods have difficulty distinguishing paralogous variation from allelic because of failure to unambiguously align end sequences in the duplicated regions [97]. A good approach to detect CNVs in the offspring may be to first assemble the parental genomes and then map the offspring to the parents, using mate-paired ends with different insert sizes. There are also different strategies (reviewed in Ref. [98]) and algorithms for detecting CNVs, such as MrFast [97] and CNVer [99], that should be taken advantage of. NGS methods are also available for detecting other types of Structural Variants (SVs) such as insertions, inversions, or translocations. For example, Hillmer et al. [100] used an optimized paired-end-tag sequencing approach,

using 10 kb genomic DNA inserts, to detect SVs in humans. After filtering out technical noise (chimeric ligation products formed during library construction) they validated 72.6% of SVs using PCR and sequencing. The remaining SVs could not be validated because no PCR products were obtained or the PCR resulted in many bands due to short sequence homologies throughout the genome. In summary, this section highlighted how detection biases and sequencing errors could influence the precision of germline mutation measurements. For WGS to be useful in measuring germline mutation frequency, considerable effort will be needed to reduce these biases and minimize sequencing errors.

3.5.2 Data Management and Bioinformatics

A second major hurdle to overcome in WGS experiments is the bioinformatics bottleneck. The assembly and mapping of several genomes can take hundreds to thousands of CPU hours, requires hundreds of gigabytes of RAM, needs efficient computer algorithms and skilled bioinformaticians to analyze the enormous data sets, and appropriate storage hardware. Analysis platforms such as Galaxy [101,102] are allowing researchers with limited bioinformatics skills to access sophisticated analysis tools. Researchers can minimize the amount of local infrastructure necessary by using publicly available instances of Galaxy or implementing cloud computing resources (e.g., Amazon EC2, Google AppEngine, or Rackspace). There is a plethora of sequence alignment software available and depending on which algorithm is used, the number of putative mutations that are detected can be amazingly different. It is also important to realize that some of the available tools are only capable of detecting SNPs. Different algorithms also have difficulty mapping reads with more than 3 mutations in them or may map the reads

to alternative sequences (D. Chen, personal communication). With more accurate mapping algorithms comes the trade-off of significantly longer CPU hours. These problems can be further extended by the reality that essentially all mutations are going to result in heterozygous loci that can sometimes be difficult to characterize. Heterozygotes can be undercalled during alignment/mapping if depth of coverage is low. For example, when Watson's genome was sequenced, the Roche 454 sequencer only detected 75.8% of heterozygous loci that were detected by genotyping using an Affymetrix 500 K microarray [17]. This problem can be overcome by using higher coverage; for example, Bentley et al. [18] found an average coverage of 33x was required for accurate detection of heterozygous variants and appropriate alignment criteria. The best reported concordance between NGS and high-density SNP array genotypes, used here to validate X chromosome SNPs, is 99.52% or 99.99% using the ELAND [18] or Maq [103] alignment tools respectively [18]. Moreover, comparison of the SNP detection in an engineered series of heterozygotes using RFLP methods versus DNA sequencing with an ABI Prism 377 automated DNA sequencer, which used first-generation fluorescence based Sanger sequencing, showed that the sequencing method failed to detect some of the heterozygotes [104]. Reliable detection of heterozygous loci can be problematic if traditional Sanger-type sequencing is going to be used to confirm putative mutations. It is important to realize that if WGS is used for a germline mutation study, the informatics work will be the most challenging and time consuming portion of the study.

3.5.3 Sample Size and Cost

The major benefit of WGS in germline mutation studies is that it uses essentially all loci in analysis as opposed to just a panel of markers. If we can deal with the challenges outlined above, WGS and its genome-wide assessment of mutations could substantially improve our insight into factors that modify mutation induction. However, for genetic toxicology studies, or others that attempt to examine changes in mutation type and frequency, experiments will still require appropriate sample sizes of families per treatment group; i.e., although each triad or family group will yield a much more detailed assessment of germline mutations using WGS, it is still a single measurement of frequency. The obvious problem with using multiple families per treatment group, and the previously discussed high sequence coverage, is the increased cost of experiments. With the current cheapest reagent cost/MB of around $0.04, the minimal cost of reagents needed to sequence a lab mouse's genome with over 30-fold coverage would be about $3000 (reviewed in Ref. [105]). It is estimated that the cost of sequencing each genome in Table 3.1 ranged from $48,000 to $100,000 [16], excluding the genomes sequenced by Complete Genomics estimated to have a reagent cost of less than $4400 [26]. To put the cost into perspective using the smallest sample sizes reported from the ESTR studies (e.g., [72]), a minimum of 5 triads (15 individuals) would need to be sequenced per treatment group. With two treatment groups (single dose versus control), this simple experiment requires 30 whole genomes to be sequenced, which would cost $90,000 in reagents alone for the initial sequencing. Then, the WGS would need to be done again with the next lowest sequencing technology ($0.07/MB; or ~$5000 per sample, or $150,000 total). If the lowest cost NGS platforms are not available in-house, then costs are often about double to obtain such data from outside sequencing providers. Testing

stage-specificity of mutation induction, examining transgenerational effects of mutagen exposure, or adding more dose groups would further increase the cost of the experiment. We fully expect cost to become less and less of a barrier as sequencing technology becomes cheaper; however, it remains a substantial concern at the time of this review.

3.5.4 Other Considerations

Caution should also be exercised when choosing which somatic cells should be used for comparative WGS of parental and offspring genomes. Sequencing of white blood cells may further increase the number of false-positive mutations because of how they reprogram their genomes to aid in antigen recognition. Specifically, white blood cells undergo somatic recombination and random nucleotide additions within T-cell receptor and immunoglobulin genes to produce a population of cells that have different genotypes at these loci [106]. Mutations in propagating cells that have accumulated with age or as a result of the mutagen exposure can also result in false-positives; i.e., parents may appear different from offspring due to acquired somatic mutations, rather than true germline mutation events. Thus, blood samples commonly used in human studies because they are relatively non-invasive and easy to collect may provide extra challenges in terms of verifying mutations. For example, Conrad et al. [44] had to filter out 1586 somatic mutations found in the lymphoblastoid cell lines they used to sequence two family triads. For lab animal studies, it may be worth consideration to collect DNA samples from the parents prior to exposure so that the wild-type somatic DNA of the parents is compared to the mutant somatic DNA of the offspring. Another option may include sequencing slowly reproducing, long-lived cells that have not undergone many rounds of division,

and therefore may not have accumulated as many somatic mutations (e.g., brain or liver). In human studies, the options for cell-types are quite limited for ethical reasons. To compensate for this a combination of available cell types may need to be used for sequencing to tease out somatic mutations accumulating with age. Regardless, researchers need to be aware of the potential implications of cell type used when designing experiments.

3.6 Alternatives to WGS of Humans and Mice

3.6.1 Large-scale Next Generation Sequencing of Select Genomic Targets

3.6.1.1 Reduced Representation Libraries

One option to reduce cost and the number of mutations to validate is to sequence a select portion of the genome with deep coverage (e.g., greater than 40x). There are different methods for accomplishing this, but each involves narrowing the targets included in the sequencing effort. Reduced representation libraries (RRL), created by digesting the genomes with a restriction enzyme followed by selecting fragments of a certain size range, reduce the amount of DNA to be sequenced. Altshuler et al. [107] first employed this strategy to generate a SNP map of the human genome, identifying SNPs using Sanger-based sequencing methods. This method has also been modified (often called restriction-site associated DNA [RAD] tags) and used with NGS to identify thousands of SNPs in fish [108], cattle [109], pigs [110,111], and birds [112,113]. To determine the most optimal restriction enzyme for generating their RRL, Van Tassell et al. [109] did in silico work to find the enzyme that captured the lowest repetitive content, captured the most unique fragments for the desired size range, and allowed for the best

genomic distribution. Whether or not the size of the library is sufficient to cover a large enough portion of the genome necessary for mutation analysis depends entirely on the restriction enzyme. Procedurally, one or two enzyme digests can be performed. A relatively rare-cutting enzyme is used to reduce the number of DNA fragments assayed, then a second, frequent-cutting enzyme or random shearing is used for the other end. Because the human and mouse genomes are available, many enzymes can be tested in silico to find the optimal enzyme(s). Using offspring and parental RRLs for germline mutation detection can reduce the cost, number of errors, and size of resulting data sets. The RRL/RAD-tag libraries are also efficient to construct for large numbers of samples. The major disadvantage, however, is that the loci are not a strictly random subset of the genome. Thus RRL/RAD-tag sequences represent a good option to reduce costs very significantly (down to <$10/sample for library preps, and sequencing ≥ dozens of samples per Illumina lane), but how well any particular set of sequences represents the genome overall will require additional investigation.

3.6.1.2 Sequence Capture

An alternative option is to use a targeted sequence capture strategy that focuses subsequent sequencing efforts on particular selected DNA regions. Currently, researchers can capture and sequence roughly 10–20 exomes (all exons) for the same price as one whole genome [114], which would reduce the cost of germline mutation studies dramatically. The human exome, for example, comprises approximately 1% of the entire genome and is divided into 180,000 exons, accounting for around 30–45 MB of DNA compared to 3 GB in the whole genome. The benefit of sequencing exomes in germline

mutation studies would be that any detected mutation has the potential to affect phenotype or health (even synonymous mutations [115]), thereby limiting the assay to only the most relevant endpoints. Not all diseases or changes in phenotype are a result of mutations in coding regions; it is estimated that over 60% of disease-causing mutations affect gene splicing rather than amino acid sequence [116]. For this reason it would be logical to sequence the introme (all introns) in addition to the exome. The only drawback to this is that information would be lost for other potential disease-causing mutations occurring throughout the 'functionome' (a term coined by Cooper et al. [117] that refers to any sequences that have some sort of function).

The most seemingly popular method for targeted sequencing uses microarray-based genomic selection (MGS) [118–124]. MGS uses oligonucleotide probes that hybridize to the sequences of interest while other DNA fragments are washed away, allowing for sequencing efforts to be more focused. Most exome probe pools are designed for human exome enrichment, however probe pools are available for other species such as mice [125]. If this strategy were chosen for the mutation analysis, the MGS chips can be used at least more than once without any signs of contamination [119], or tagged samples from different individuals could be used to cut down on costs. The Nimblegen resequencing array used in most of these studies is intended to be used with Roche 454 technologies but has been adapted for use with an Illumina sequencer [122]. There are other microarray platforms available and their performances have recently been compared [126–129]. Other methods for target capture that have been reported use some form of multiplex amplification of the target sequences [130–132], but these methods

were used for smaller scale enrichments and may not be as efficient for whole exome sequencing, which involves enrichment of close to 30,000 loci.

Another method called Solution Hybrid Selection (SHS) uses biotinylated RNA baits transcribed from oligonucleotides synthesized on a microarray for target capture [133] and it has recently been automated [114]. Two companies now offer SHS kits, Agilent (SureSelect) and Mycroarray (MYselect) with up to 55,000 or 60,000 independent 120-mer probes. The probes are readily designed to regions of interest. In general, it is optimal to tile probes at 2x across regions of interest. Thus, thousands of independent loci can be assayed simultaneously. By using a randomized block design where a type of genomic DNA (exons, introns, promoters, ultra-conserved elements, etc.) is the block, researchers can obtain information from up to hundreds of replicated loci per block. If less than the full complement of independent probes are needed (e.g., 5500 independent probes), then the probes can be replicated within the total pool to give a more concentrated bait solution (i.e., a ten sample kit of 55,000 probes at 1x, can be made into a ten sample kit of 5500 probes at 10x = 100 samples at 5500 probes at 1x). In this example the kit would be used for 100 enrichments instead of 10. Finally, because it is possible to use sequence tags within the sequencing adapters and thus pool the DNA prior to target enrichment, many samples can be enriched simultaneously (e.g., by pooling 12 indexed samples into a single pool, and eight sample enrichment kit can be used to enrich 8 x 12 = 96 samples). In practice, it is currently possible to sequence about 100 samples at 2000 independent loci at 100x coverage within a single Illumina HiSeq lane using paired-end 100 base reads (~$3000 full commercial price), the lowest cost MYselect kit can be obtained for <$2000, and library preps can range from $10 to $100/sample.

The effectiveness of some of the different experiments, including MGS and non-MGS based, are reviewed in-depth in Ref. [134]. It is important to note here that the use of smaller DNA fragments in MGS sequence enrichment reduces the specificity of the capture, but greatly increases the sequencing efficiency for the region of interest [120]. As useful as these methods would be for mutation analysis, they too would have their drawbacks. The major problem is that allelic competition in the enrichment pool could result in reduced capture of mutant alleles. For example, in MGS the wild-type allele may hybridize to the capture probe with higher affinity than the mutant allele, limiting the amount of mutant DNA for subsequent sequencing. When measuring mutation frequency, this problem will result in an underrepresentation of mutations. The magnitude of allelic competition will depend on the platform used and the type of allelic variation. For SureSelect/MYselect and assays that use similar sized probes, allelic variance will be much less of an issue because changes of a few bases should not result in under-calling mutations; however, large rearrangements will certainly be an issue because they will likely be captured, but the informatics/analyses will have difficulties identifying them. Whichever method is chosen for germline mutation studies needs to capture enough of the exome with limited bias so that mutation events are observed in all treatment groups, and captured regions are consistent across all individuals.

Recently, exome sequencing studies have been performed in human family triads comparing the frequency of de novo mutation events between controls, and families where the second generation individuals are probands for mental health disorders. For example, Xu et al. [135] sequenced the exomes of 53 schizophrenic probands, 22 unaffected individuals, and their parents. In the affected trios they observed 33 SNPs, 1

dinucleotide substitution, and 4 indels compared to 7 SNPs in the control group. Although there was a higher proportion of probands harboring at least one de novo mutation relative to the control individuals, no statistically significant difference was detected. A similar study performed by Girard et al. [136] found 73 putative variants passed onto 14 schizophrenic probands from their parents. Sanger sequencing confirmed 15 of the variants to be true de novo mutations, while the remaining 58 variants turned out to be false-positives. The number of false-positives obtained from sequencing 14 exomes offers a marked improvement over the number of false-positives that would be obtained from sequencing one whole genome. These studies are just two examples demonstrating the potential sequence capture has for comparing germline mutation frequencies between study groups.

3.6.2 Alternative Model Organisms – Smaller Genomes

An alternative to the RRL or sequence capture strategies is to sequence the whole genome of a model organism with a smaller genome size. The standard species for germline mutation studies is lab mice, which have a genome size comparable to humans. Instead of using a portion of the mouse's genome for analysis, it may be worth considering using the whole genomes of other model organisms such as the nematode *Pratylenchus coffeae*, that has the smallest known animal genome size estimated at 20 MB [137], the most commonly used nematode for research *C. elegans*, with a genome size of 97 MB [138], the fruit fly *D. melanogaster*, that has a genome containing 120 MB of euchromatin [139], Japanese medaka with a genome size of 700 MB [140], or the puffer fish *Tetraodon nigroviridis*, that has the smallest reported vertebrate genome size

around 340 MB [137,141]. Complete reference genomes and a suite of bioinformatics tools are available for these model species, with the exception of *Pratylenchus coffeae*. Few genomes have been sequenced de novo using NGS but this is changing rapidly (reviewed in Ref. [142]). A study that may be of considerable interest is to use WGS mutation analysis in multiple families of different species to characterize the mutation frequencies and types in the different model and non-model organisms under a variety of conditions.

3.7 Future Directions and Recommendations

NGS technologies have made WGS of several individuals feasible, but at the moment the technology is not practical for very large-scale germline mutation studies in humans and mice. Within the near future (few years) the technology will be at the level necessary to perform a study of this magnitude, but until then researchers will be better served by conducting smaller, proof-of-principle type experiments with WGS, or collecting the animal and human samples and conducting some initial sequence capture experiments to begin investigating mutation frequency changes in exomes or other parts of the genome. When sequencing speed, cost, accuracy, and software improves, those same samples could be analyzed at the WGS level.

3.8 Conclusions

Knowing what environmental factors influence germline mutation frequencies is of significant importance because of the associated health implications. Studies attempting to identify these factors continue to be limited by the lack of available tools

that can quickly and accurately measure genomic changes in the offspring of exposed parents. The major benefit of using WGS is that nearly all mutations can theoretically be uncovered, allowing for a representative assessment of how mutagenic a condition is. The challenges associated with WGS involve error rates that are much higher than mutation frequencies, sequencing biases that could potentially miss certain mutations, a series of bioinformatics hurdles, and a large experimental price tag. The recommendations made here can resolve some of these problems, but the rest will continue to persist until NGS platforms and software are improved. The challenges addressed are not meant to discourage researchers from trying WGS in a germline mutation study, but rather to make them aware of the resources, skills and time that will be needed to perform studies of this magnitude.

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CHAPTER 4. GENERAL DISCUSSION

4.1 Conclusions

The objectives of my thesis were to (1) Identify polymorphic mouse microsatellites and determine if the loci could be used to detect germline mutation induction in families exposed to a known germ cell mutagen. (2) Determine if NGS is a practical tool for quantifying changes in germline mutation frequencies in humans and model organisms by reviewing the available literature. The objectives of my thesis were addressed in chapters 2 and 3 sequentially.

To achieve the first objective of my thesis, I used targeted capture of microsatellite loci and DNA sequencing of different inbred mouse lines of the same strain in order to identify polymorphic loci (Chapter 2). Using this method I was able to identify a final panel of 11 polymorphic microsatellite loci that could be used for quantifying changes in germline mutation frequency in mice. The microsatellites were used to screen irradiated mouse families that had previously shown elevated ESTR mutation frequencies [Somers et al. 2004]. There was no evidence for mutation induction at the microsatellite loci. Failure to detect mutation induction does not mean the panel is not a valuable tool, but rather raises several questions regarding why mutation induction was detected in ESTR loci but not in microsatellite loci. Further research is needed to evaluate how effective the microsatellites are at detecting mutation induction. If the microsatellites are sensitive enough to detect mutation induction, then more work will be needed to better understand microsatellite mutation induction in relation to acute versus chronic radiation exposure, the importance of stage-specificity, whether DNA repair is more efficient in

microsatellites than in ESTRs, and how other types of exposures influence microsatellite mutation induction.

For my second objective, I assessed the potential of WGS using NGS for detecting germline mutation induction in animals exposed to environmental factors (Chapter 3). The major benefit of WGS is that it allows for direct determination of mutation type and frequency at all loci simultaneously. Therefore, germline mutation studies using WGS are more predictive of the health consequences of germ-cell mutagens because non-neutral loci are evaluated. However, WGS is very resource-intensive and is currently limited by several challenges. Even though different platforms offer 99.999% accuracy, sequencing of large genomes that are on the scale of gigabases can still result in thousands of sequencing errors per genome. In addition, sequencing genomes with a high enough coverage to minimize sequencing errors (20x – 40x) can be expensive. In conclusion, sequencing accuracy needs to improve and costs need to decline before large-scale germline mutation studies using WGS are feasible. Until DNA sequencing technologies advance further, I recommend that researchers using WGS to quantify mutation induction use model organisms with smaller genomes or scale down the portion of the genome being analyzed (using RRL or sequence capture).

4.2 Future Directions with Tandem Repeat DNA

There are different possible explanations why mutation induction following irradiation was detected using ESTRs but not with the panel of microsatellites I developed. As discussed in chapter 2, the magnitude and causes of microsatellite mutation induction may be different from that of ESTRs. Microsatellites and ESTRs may

have different responses to similar doses of radiation, durations of exposure, and stages during spermatogenesis. Before these explanations can be evaluated, we need to first know if the panel of microsatellites are sensitive enough to detect mutation induction. The microsatellite study I did for this thesis was limited by the number of available samples. Further studies should be conducted with more samples (preferably using inbred mice with large allele sizes) to determine if the discrepancies between ESTRs and microsatellites was due to a lack of detection power in microsatellites. For more direct comparisons between markers, the panel should also be used to screen other samples that showed ESTR mutation induction (DNA repair knockouts and mice exposed to chemicals, air pollution, and other types of radiation). For example, the sensitivity of the panel of microsatellites could be evaluated by screening family pedigrees in which sires were exposed to ENU, one of the most potent germ cell mutagens in mice [Russell et al. 1979; Vilariño-Güell et al. 2003]. Overall, more work is needed to focus on the utility of microsatellites as a mutation marker in mice.

Based on the results of chapter 2 it appears as though microsatellite and ESTR mutation induction may be different; if this difference is real, should researchers rethink the validity of ESTR studies? I would suggest that the answer to this question is no. Even though some discrepancies already exist between ESTR results and the results of other assays [Singer 2006], the validity of these markers has never been questioned before [Bridges 2001; Armour 2006]. ESTRs are still used because they are indicators of global genomic instability, and mutation induction is possibly due to damage elsewhere in the genome or in other sensor molecules [Bridges 2001]. For example, it has been demonstrated using ESTRs that radiation exposure in sperm can elevate maternal

mutation frequencies [Niwa and Kominami 2001], suggesting that gametic instability is passed onto the zygote. Furthermore, ESTR loci have illustrated that exposures to radiation or chemicals can cause instability that is passed through the germline over multiple generations [Dubrova 2000; Barber 2002; Dubrova et al. 2008; Glen and Dubrova 2012]. In order for there to be elevated ESTR mutation frequencies over multiple generations, some sort of cellular instability or signal would be required. The exact processes causing this ESTR instability are unknown; however, it has been suggested that epigenetic changes may be responsible [Dubrova 2000]. A correlation between germline ESTR mutation induction and DNA hypermethylation has been observed following air pollution exposure [Yauk et al. 2008], providing indirect evidence to support the hypothesis regarding epigenetic changes. A better understanding of the mechanisms leading to genomic instability and ESTR mutations is still needed to determine the health consequences associated with ESTR mutation induction. However, based on the studies discussed here, it should be clear that the results of ESTR studies are still important because of the implications for instability in the germline.

It is still unknown why some tandem repeat loci are more mutable than others. Factors such as the number of repeats [Wierdl et al. 1997], repeat unit size [Vigouroux et al. 2002], core repeat sequence [Bachtrog et al. 2000], flanking GC content [Glenn et al. 1996], and location in the genome (proximity to coding sequences) may influence the instability of loci. In chapter 2 I found evidence to support the theory that length is an important factor because mutations occurred in the longer length alleles. In addition, certain core sequences were more mutable than others. Microsatellites that had observed mutations either had the repeat sequence AGAT or AAAG. Both of the AAAG loci,

selected essentially at random from the Eukaryotic Microsatellite Database [Aishwarya et al. 2007], had mutations. These results suggest that these two core sequences may be more unstable than others in mice. Previous studies in other species have found that loci with these same core sequences had high mutation frequencies [Gardner et al. 2000; Ellegren et al. 1997; Tsyusko et al. 2007], indicating that these core sequences may be generally polymorphic in all species. Another factor that may influence the mutability of a locus is whether it is a pure (no interruptions) or compound (multiple repeats separated by interruptions) microsatellite. The majority of loci that showed polymorphisms in the inbred mice or showed mutations in the out-bred mice were compound microsatellites, suggesting that compound microsatellites may be more unstable. Knowing what other inherent factors increase mutability in tandem repeat DNA sequences will aid researchers in narrowing down polymorphic microsatellite loci in other species, which can be used for population genetics studies or germline mutation analysis. Therefore more work needs to be done to determine the attributes of microsatellites that make them unstable.

4.3 Future Directions with Next Generation Sequencing

WGS will no doubt be the most accurate/relevant method for quantifying germline mutation induction, but current NGS, or second generation sequencing (SGS), is not optimal for these purposes. However, third generation sequencing (TGS) technologies (reviewed in [Schadt et al. 2010]), capable of sequencing single molecules, may offer the improvements needed to make germline mutation studies at the genome level feasible. TGS technologies were not discussed in chapter 3 because they are currently not commercially available (with the exception of the HeliScope [Pushkarev et al. 2009] and

the PacBio RS [Eid et al. 2009]), and some of the technologies lack proof of concept. However, since the publication of chapter 3, Oxford Nanopore Technologies has announced that their GridION and MinION platforms, that use "strand sequencing" [Branton et al. 2008], will be made available within the year [Hayden 2012]. Other TGS platforms may soon follow suit depending on their stage of development. TGS platforms have the potential to fix problems that currently exist with SGS by offering longer reads, shorter run times, and reduced costs (the human genome can be sequenced for a cost as low as $100 theoretically). Moreover, TGS methods that don't use sequencing by synthesis will have fewer biases, resulting in improved consensus accuracy. However, TGS technologies may introduce new problems and bioinformatic hurdles. TGS may be the future for germline mutation studies, but until these platforms become available and are optimized, pilot studies should be conducted using current SGS technologies.

Currently available NGS technologies can be used to better understand the importance of tandem repeat DNA mutations and the processes associated with their mutation induction. The major issue associated with using tandem repeat DNA as markers for mutation induction is the health relevance of these mutations are still for the most part uncertain [Somers 2006]. Vigorous studies that use exome sequencing/WGS and tandem repeats in parallel are needed to determine if there is a correlation between mutation induction using neutral markers and mutation induction in coding sequences. If neutral loci reflect the whole genome, then the use of expensive and time-consuming WGS will not be necessary. In conclusion, even though WGS using NGS will be the future focus of germline mutation studies, tandem repeat DNA markers may still serve as useful tools.

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