Radiation-Induced Instability at Mouse Expanded Simple Tandem Repeat (ESTR) Loci

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Contents

Abstract

Expanded Simple Tandem Repeat (ESTR) loci provide a useful system to assess the effect of exposure to ionising radiation on the germline of male mice; however, little is known about the mutation mechanism(s) at these loci. Information about mutation processes at these loci may provide important clues concerning the damaging effects of irradiation at the DNA level. A number of approaches have been used to investigate possible mutation mechanisms. No correlation was observed between the levels of meiotic recombination and ESTR mutation rate in the germline of exposed male mice, ruling out the possibility that radiation induced mutation at ESTR loci resulted from an increase in meiotic crossing-over. The analysis of the murine *scid* mutation on ESTR mutation rate demonstrated that the process of non-homologous end-joining (NHEJ) is important in the stability of ESTR loci in the germline of non-exposed mice, but was unable to ascertain whether the activation of NHEJ could provide a plausible explanation for radiation-induced increases in ESTR mutation rate. A transgenerational study of the descendants of directly exposed male mice provided evidence for a long-term effect of ionising radiation on ESTR stability in the mouse germline. ESTR instability was observed in the germline of the offspring and grandoffspring of the initially irradiated males, with no evidence for a decrease in mutation rate. This analysis also provided additional information about the inheritance of ESTR instability in the mouse germline, demonstrating the possibility that the transmission of instability was epigenetic in nature, and showing that the effect could be observed after exposure to both high- or low-LET sources of irradiation. The data also showed transgenerational effects in three different mouse strains, and that there were no differences in the inheritance of ESTR instability between the male and female germlines. The work presented here provides the basis for a number of new and exciting directions to further analyse radiation-induced mutation at ESTR loci.

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Abbreviations

ATM – Mutated in ataxia telangiectasia

bp – Base pairs

dNTP – Di-nucleotide tri-phosphates

DNA – Deoxyribonucleic Acid

DSB – DNA double-strand break

CEPH – Centre d'Etude du Polymorphisme Humain/National Institutes of Health consortium

ENU – Ethylnitrosourea

ESTR – Expanded simple tandem repeat

$\mathbf{F_{(0,1,2,3\ etc)}}$ – Filial generation, F_0 parental, F_1 first generation, F_2 second generation , F_3 third generation

FAN – Fanconi anaemia

FISH – Fluorescence *in situ* hybridisation

Gy – Gray

HNPCC – Hereditary non-polyposis colon cancer

HPRT - Hypoxanthine guanine phosphoribosyl transferase

HR – Homologous recombination

kb – Kilo-base pairs

LOH – Loss of heterozygosity

Mb – Mega-base pairs

pM, μM, mM – Pico-, micro- , milli-Molar

MGD – Mouse genome database

MSI – Microsatellite instability

MMR – Mismatch repair

MNU – Methylnitrosourea

NBS – Nijmegen breakage syndrome

NHEJ – Non-homologous end joining

NER – Nucleotide-excision repair

ORFs – Open reading frames

PCR – Polymerase chain reaction

PPI – Pre-conception paternal irradiation

RFLP – Restriction fragment length polymorphism

RPA – Replication protein A

SCE – Sister chromatid exchange

SLT – Specific locus test

SI – The international system of units

SM-PCR – Single molecule polymerase chain reaction

SNP – Single nucleotide polymorphism

SP-PCR – Small pool polymerase chain reaction

SSA – Single-strand annealing

SSB – DNA single-strand break

SSR – Simple sequence repeats

STR – Simple tandem repeats

TPA – 12-*O*-tetradecanoylphorbol-13-actetate

***T* stock** – Recessive test stock used for the Specific Locus Test

UNSCEAR – United Nations scientific committee on the effects of atomic radiation

VNTR – Variable number tandem repeat

Glossary of terms

Absorbed dose (D)	The energy imparted per unit mass by ionising radiation to matter at a specific point.
Accumulated dose	The total dose received taking into account dose fractionation.
Alpha particle (α)	A positively charged particle emitted by radioactive material.
Cosmic rays	Radiation of many sorts, originating outside the earth's atmosphere, forms a part of the natural background radiation.
Decay, radioactive	The spontaneous transformation of one nuclide into a different nuclide or to a different energy state for the same nuclide, follows a reactivity series.
Dose	General term for the quality of radiation
Dose rate	The radiation dose delivered per unit time which is measured, e.g. Grays per hour.
Effective dose	The radiation dose taking into account the fact that some types of radiation are more damaging than others, and that different parts of the body are more sensitive to the damaging effects of radiation. Given as the sum over specified tissues of the equivalent dose in a tissue and the weighting factor for that tissue.
Equivalent dose	A quantity used for radiation-protection purposes. Takes into account the different probabilities, which occur with the same absorbed dose, delivered by radiations with different weighting factors. The dose is given in Grays and the equivalent dose is in Sieverts.
Functionally relevant polymorphism	A non-conservative change in the DNA sequence of a gene coding for a protein, leading to an alteration in the resultant protein which alters the protein structure or function.
Gamma rays (γ)	High energy, short-wavelength electromagnetic radiation.
Gray (Gy)	The SI unit of absorbed dose. The amount of radiation that deposits one joule of energy per kilogram of tissue. One Gray equals 100 rad (previously used term).
Ionising radiation	Any radiation displacing electrons from atoms or molecules, leading to the production of ions.

Isotope	Different forms of a chemical element that has the same number of protons and electrons but differ in the number of neutrons within the atomic nucleus.
Kilovolt (kV)	A unit of electrical potential difference equal to 1,000 V.
Linear Energy Transfer (LET)	The rate of energy loss along the track of an ionising particle. Usually expressed in keV/mm.
Mutation rate	The frequency with which mutations take place at a given locus or in a population.
Nuclide	A general term applicable to all forms of the elements. Nuclides are distinguished by their atomic number, atomic mass and energy state.
Occupational exposure	Exposure to radiation as a direct result of employment.
Radiation dosimetry	The measurement of the radiation delivered to a specific place or the amount of radiation that was observed there.
Radiation protection	Legislation and regulations to protect the public and radiation workers against radiation exposure. Also used to explain measures to reduce exposure.
Radioactivity	A property of all unstable elements that regularly decay to an altered state by releasing energy in the form of photons (X- and γ-rays) or particles (electrons, neutrons and α-particles).
Relative Biological Effectiveness (RBE)	A value which is used to compare the effectiveness of different radiations, using X-ray damage as a standard. The value of 1 is used for β, γ, and X-rays, 10 for neutrons, and 20 for α-particles.
Relative risk	The expression of the risk of a disease resulting from some injury expressed as a percentage increase over the normal rate of occurrence of that disease.
Roentgen (R)	A unit of exposure to ionising radiation, the amount of X- or γ-rays required to produce one electrostatic unit of electric charges in 1 cm^3 of dry air under standard conditions.
Roentgen absorbed dose (Rad)	The old unit of absorbed dose, superseded by the Gray.
Roentgen equivalent man (rem)	Old unit of equivalent or effective dose. One rem is one hundredth of a Sievert.
Sievert (Sv)	Unit of equivalent or effective dose. One Sievert is 100 rem.

X-ray	A penetrating form of electromagnetic radiation emitted from the inner orbital of an excited atom returning to their normal state (characteristic X-rays) also produced when metal is bombarded with high-speed electrons.

Chapter 1

Introduction

1.1 Why is the study of germline mutation induction important?

Human endeavours over the last century have increased the levels of mutagenic agents to which we may be exposed during our lifetime via occupational, accidental and medical exposure. It is therefore important to understand which agents found within the environment possess mutagenic properties. Over the years, many different approaches have been taken to assess the large number of potential mutagenic agents and how these may affect organisms at the level of general health and at the DNA level. Studies have shown that one of the most important types of mutagenic exposure for human population is that of ionising radiation.

1.1.1 The importance of ionising radiation as an environmental mutagen

The effect of ionising radiation on human populations has become increasingly important over the years due to the increased use of nuclear facilities for the production of electricity. This has lead to increased numbers of people being occupationally exposed and concerns for the health of populations living near such facilities due to the possibility of an increase in environmental exposure to ionising radiation. The atomic bombs dropped on Hiroshima and Nagasaki, plus nuclear bomb testing in the USA, USSR and other countries, together with major technological accidents, such as the Chernobyl disaster, have lead to a dramatic increase in exposure to ionising radiation at the population level. The use of radiotherapy as a cancer treatment has also increased greatly over the last hundred years. These changes in human activity have precipitated the need to analyse and understand the effect of ionising irradiation on living organisms.

1.1.2 Health implications of exposure to ionising radiation for human populations

Traditionally epidemiological studies have been undertaken to assess the effect of exposure to mutagenic agents in the work place, after accidental exposure, and after medical treatment. Epidemiological studies have analysed the effects of direct exposure to ionising radiation mainly in somatic tissues by assessing the cancer frequency (solid tumours and leukaemia) in exposed populations (Cardis *et al.*, 1995; Kellerer, 2000). Other studies have addressed the possibility that exposure to ionising radiation may also effect the germline; by analysing the rate of congenital malformations (Otake *et al.*, 1990; Doyle *et al.*, 2000), and the assessment of cancer risk in the offspring of exposed parents (Gardner *et al.*, 1990). Such studies showed evidence of increased health risks for the offspring of exposed parents, but were unable to unequivocally correlate parental radiation exposure with an increased risk of congenital malformations or cancer in the subsequent generation.

The findings from epidemiological studies, assessing the impact of exposure to ionising radiation on human health have lead to changes in work practice and legislation, for example, the Management of Health and Safety at Work Regulations 1999. The Ionising Radiations Regulations 1999 (IRR99) provides important information on such topics as effective dose limits, areas of key risk including exposure from industrial radiography, radon and naturally occurring radioactive material, and information on approved dosimetry services (The Ionising Radiations Regulations, 1999). Advisory committees such as the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), established in 1955, have been appointed to address widespread concerns regarding the effects of radiation, from all sources (natural and manmade), on human health and the environment. The committee's mandate within the United Nations is to assess and report on the levels and effects of exposure to ionising radiation.

Governments and organisations throughout the world rely on the committee's estimates as a basis for the evaluation of radiation risk, for the establishment of radiation protection and safety standards, and for the regulation of radiation sources. UNSCEAR provide annual reports outlining important issues relating to the long-term health implications of radiation exposure. The UNSCEAR reports constantly update and evaluate approaches for the monitoring of mutation induction and the calculation of risk estimates for human health. Previous UNSCEAR reports have assessed such issues as the sources and effects of ionising radiation, and the genetic and somatic effects of ionising radiation; the most recent 2001 report covers the issue of hereditary effects of exposure to ionising radiation. This report outlines the current information assessing the adverse genetic effects of ionising radiation that can be transmitted to future generations (UNSCEAR, 2001).

1.1.3 The limitations of epidemiological studies

Although a wealth of data is available from epidemiological surveys of the effect of ionising radiation on human populations, a number of problems are associated with this approach. Firstly, there is the issue of the assessment of the doses and dose rates that individuals have been exposed to. Detailed records are often available for the occupational exposure of nuclear industry workers, but little information is available for individuals that have been exposed from the environment or after accidental exposure. Another confounding factor, with respect to environmental radiation exposure, derives from the fact that individuals may receive both external dose from the air and internal doses from contaminated food and water sources; such differences in the route of exposure may have profoundly different biological effects.

Epidemiological studies depend upon well-matched control and exposed groups; if this is not achieved any observed differences may be accounted for by stochastic variations between the control and exposed populations, and may not be due to exposure. A number

of factors need to be considered when selecting an appropriate control group; these include parental age, smoking habit, occupation and ethnic background.

The limitations of an epidemiological approach for the assessment of the health implications of exposure to ionising radiation can be illustrated by the conflicting results produced by a number of studies. Studies that have assessed the rates of congenital malformations in the offspring of parents exposed by the Chernobyl accident have shown that data obtained from different cohorts from the same exposed population produced quite different results (Little, 1993). Although epidemiological studies provide important information on the effects of ionising radiation on human health, alternative methods of analysis are required to clarify the data obtained. A common solution to the problems associated with the complexity of human populations is to analyse the impact of possible mutagens using an appropriate model system.

1.1.4 The use of model systems

The analysis of the effects of ionising radiation using model systems eliminates a number of variables that lead to complications in epidemiological studies. Model systems can be exposed to known doses of mutagenic agents, enabling the establishment of dose response curves and the estimation of the doubling dose. The doubling dose is the level of exposure required to double the spontaneous mutation rate of a particular test system; this value can then be used to compare the sensitivity of mutation detection systems and to compare the mutagenicity of different agents. Model systems also have the advantage that the control and exposed populations will be genetically homogeneous and can be kept under identical environmental conditions. Many studies have been performed to assess the mutagenicity of agents as diverse as ionising radiation and food additives.

Early work analysing the mutagenic effects of ionising radiation showed that X-rays were mutagenic in *Drosophila* (Muller, 1927). Later the mutagenic properties of X-rays

were demonstrated in mice; these results have been verified over the years by numerous studies using laboratory rodents, and have provided clear evidence for an increase in germline mutations after the exposure of mice to ionising radiation (reviewed by Searle, 1974). From these initial observations it became clear that the passage of radiation-induced damage through the germline needed to be further investigated due to long-term implications for exposure to environmental mutagens.

1.2 Assays for the detection of germline mutations in model systems

Since the initial discovery of germline mutation induction in mice a number of different systems have been used to further verify and characterise the effects of ionising radiation on the germline. It should be stressed that the efficiency of the numerous systems for monitoring germline mutation induction clearly differs. The following sections outline the most popular methods used to date, highlight some problems associated with these test systems, and outline the requirements that a new test system would need to fulfil.

1.2.1 Assays for germline mutation induction in mice

1.2.1.1 Dominant visible mutations

The first evidence demonstrating that exposure to ionising radiation could lead directly to mutations affecting the phenotype came from a study by Charles (1950) assessing the induction of dominant mutations leading to clearly visible phenotypic changes in the F_1 offspring of irradiated parents. It is likely that the number of dominant mutations that lead to clear-cut heterozygous effects will be small in comparison to the total number of dominant mutations; therefore, systems have been developed to assess the other types of dominant mutations including metabolic effects, embryonic lethal mutation and skeletal malformations (Searle, 1974). To improve the efficiency and scope of phenotypic analysis and to allow the detection of recessive mutations the specific locus test was developed.

1.2.1.2 The Specific Locus Test (SLT)

The specific locus test provides the most reliable system for the analysis of germline mutation induction in mice used to date, and is based on the work of William Russell (Russell, 1951). The system has been designed to allow the detection of mutations (both recessive and dominant) at a number of clearly defined loci, where distinctive phenotypic changes are observed in offspring that carry mutations at the loci studied. The design of this test requires a specific tester strain of mice, which is recessive for the chosen loci, which are then mated with a wild-type strain. As the wild type mice have dominant alleles for the same loci as the recessive specific locus stock, this allows the detection of mutations within the germline of the wild type parent only. The wild-type parental mice are either non-treated (control), or irradiated, to assess spontaneous and induced mutation rates respectively.

The most commonly used specific locus stock is the *T* stock (test stock), created in 1948 by W. L. Russell, which are homozygous recessive for seven loci. The extensive use of these seven loci has lead to the specific locus method using the *T* stock being called the Russell Seven Locus Test; however, a second specific locus stock was developed and used at Harwell (Lyon and Morris, 1966).

Locus	Locus abbreviation	Phenotype	Chromosome
Agouti	*a*	*non-agouti*	2
Tyrosine-related protein	*b*	*brown*	4
Pink-eyed dilution	*p*	*pink-eyed*	7
Tyrosinase	c^{ch}	*chinchilla*	7, 14 cM distal to *p*
Myosin Va	*d*	*dilute*	9
Morphogenic protein 5	*se*	*short-ear*	9, 0.16 cM distal to *d*
Endothelin receptor type B	*s*	*piebald spotting*	14

Table 1.1- Specific-test loci

Data adapted from Searle (1974) with additional information from the Mouse Genome Database (MGD) (Blake *et al.*, 1999) (http://www.informatics.jax.org/).

The analysis of forward mutations using the specific locus test is performed by scoring the phenotypes of offspring obtained from a cross between the specific locus and wild-type strains. Mutant individuals appear as deviations from the expected wild-type phenotype, providing a non-subjective and reproducible method for mutation detection. Such a breeding scheme would allow the detection of recessive mutations, in addition to any dominant phenotypic mutations in wild-type parents.

It should be noted that a number of studies have utilised the analysis of a single specific locus (Russell *et al.*, 1995; O'Brien *et al.*, 1996) or multiple specific (but not all seven) loci to analyse the effects of mutagenic agents on the mouse germline (Russell, 1971).

The analysis of germline mutation induction using the specific locus test provides the best model system to date; however, the system is not ideal due to the large numbers of individuals required, the requirement for high-dose exposure to ionising radiation, and the lack of application for the assessment of mutation induction in human populations.

1.2.1.3 Dominant Lethality

To analyse dominant lethality in mice, males are exposed to ionising radiation and mated with untreated females. Pregnant females are then sacrificed, approximately 17 days after conception, and the uterine contents are assessed. The numbers of potential offspring, shown by the number of corpus lutea, are compared to the number of viable offspring. A number of studies have found that the number of inter-uterine deaths (both pre and post-implantation) increase with increasing dose after paternal exposure to ionising radiation (Luning and Searle, 1971; Searle and Beechey, 1981; Kirk and Lyon, 1984). This analysis of dominant lethality is able to indicate mutation events in the germline of irradiated parents, but is unable to provide any information on the induction of non-lethal mutations, and therefore does not produce reliable estimates of spontaneous or induced mutation rate. Additionally the analysis of dominant lethality is not a robust test as the *in utero* loss of embryos is also affected by maternal health and other environmental factors which may confound the effect of paternal exposure to ionising radiation on mutation induction.

1.2.1.4 Skeletal malformations

The analysis of skeletal malformations provides an additional test for the assessment of dominant mutations, either as part of a dominant visible test where skeletal mutations have a clear external phenotypic effect, or by the internal analysis of the skeletal malformations. As skeletal malformations have been shown to increase in the offspring of irradiated parents (Bartsch-Sandhoff, 1974; Selby, 1979; Ehling, 1991), it has been proposed that skeletal malformations may provide a good system to analyse the effects of ionising radiation. The skeleton is formed over a long period of embryonic development and is subject to modification by a large number of genes, and therefore could provide a good representation of the total amount of damage caused to the genome by radiation exposure (Ehling, 1991). The analysis of skeletal malformations, however, requires the

analysis of large numbers of offspring (several thousand) due to the low spontaneous frequency of these types of malformation (0.06%) (Searle, 1974). Additionally, the detailed analysis of internal malformations requires a number of specialised techniques and is therefore difficult to perform (Searle, 1974).

1.2.1.5 Dominant cataracts

The development of dominant cataracts was also been assessed in the offspring of mice exposed to ionising radiation (Kratochvilova, 1981; Ehling, 1985; Graw *et al.*, 1986; Favor *et al.*, 1987; Kratochvilova and Favor, 1988; Pretsch *et al.*, 1994). The analysis of the development of lens opacity in mice has been undertaken, as such radiation-induced effects have also been observed in human populations. It has therefore been suggested that the scoring of dominant cataract induction may provide a useful comparison for the rates of mutation induction in both man and mouse (Schull, 1983; Ehling, 1988; Favor, 1989; Hejtmancik, 1998). The analysis of dominant cataracts to detect radiation-induced germline mutation requires specialised techniques, and is therefore quite difficult to perform.

1.2.1.6 Recessive mutations

Recessive mutations can be observed in the offspring of irradiated parents (Luning and Eiche, 1976; Graw *et al.*, 1986; Searle and Edwards, 1986; Pretsch *et al.*, 1994) Few studies have been directed to analyse recessive mutations as they result in less clearly defined changes than those produced by dominant mutations and require further breeding procedures such as the analysis of the effect in the grand-offspring of irradiated parents. Recessive visible mutations can however be detected using the specific locus approach (Graw *et al.*, 1986; Searle and Edwards, 1986; Pretsch *et al.*, 1994).

1.2.1.7 Enzyme activity

It has been suggested that screening for enzyme activity mutants offers a number of advantages over other commonly used systems to assess germline mutation induction by ionising radiation. The assessment of enzyme mutants has a similar sensitivity to that of the specific locus test, but is not dependent upon the use of precisely matched strains or species allowing comparisons between different strains and species (Pretsch *et al.*, 2000). The analysis of enzyme activity has been performed in the offspring of irradiated mice, showing increases in the alterations of enzyme activity after paternal exposure (Thorndike *et al.*, 1973; Pretsch *et al.*, 1994; Pretsch *et al.*, 2000). A number of enzyme systems have been investigated, of which the most commonly used is the alteration of erythrocyte enzyme function, which assesses 10 enzymes encoded by at least 14 loci. The approach for scoring enzyme activity mutants has been described in detail by Charles and Pretsch (1986 and 1987). The analysis of enzyme activity provides a model system to assess alterations in the overall biochemical 'fitness' of offspring conceived after parental exposure to ionising radiation (Pretsch *et al.*, 1994; Pretsch *et al.*, 2000). However, all the enzyme-based analyses present a number of drawbacks; these include the low mutation rate observed at the loci studied (which leads to a requirement for the analysis of large numbers of offspring, see Table 1.2 for details), and additionally the high cost of experimental procedures involved to assay enzyme activity.

1.2.1.8 Semi-sterility

The analysis of heritable semi-sterility in mice has been used to assess the effect of paternal exposure to ionising radiation on the fertility of male mice in the subsequent generation. Irradiated male mice are mated with non-exposed females from a different inbred strain and their male offspring are assessed for fertility by mating with out-bred female mice. Males showing semi-sterility are then out-crossed to females from a multiple recessive stock in order to demonstrate the possibility of heritability of the semi-sterile phenotype, and to perform linkage tests. Acute paternal irradiation (12 Gy) results in significant increases in the number of F_1 male offspring of irradiated fathers showing semi-sterility (Lyon *et al.*, 1964). Lyon *et al.* (1964) were able to demonstrate that all the male mice that they had deemed to be semi-sterile carried heterozygous reciprocal translocations when assessing meiotic chromosomes. Chronic exposure to γ-rays (a cumulative dose of 12 Gy) has been unable to demonstrate induction of semi-sterility in the F_1 male offspring of exposed male mice (Phillips and Searle, 1964). It is thought that the difference between these two studies may be due to difference in the efficiency of chronic and acute exposure at inducing reciprocal translocations. The analysis of semi-sterility provides evidence that DNA damage, in the form of reciprocal translocations, can be passed through the germline of irradiated males to subsequent generations. The use of this test system is limited by the number of mice required, (427 pairs of control and exposed mice, used by Lyon *et al.*, 1964), and the complex breeding requirements. Additionally the analysis of semi-sterility may not be sensitive enough to measure DNA damage after chronic exposure to ionising radiation, which the most common form of exposure within human populations.

1.2.1.9 Chromosomal aberrations

A significant proportion of the genetic damage caused by ionising radiation results in the alteration of chromosomal structure; therefore the analysis of the levels of such aberrations induced in germ cells after exposure to radiation may provide useful information on the germline effects of ionising radiation (van Buul, 1983). The scoring of chromosome aberrations in the mouse germline is often performed on the self-renewing population of stem cell spermatogonia, as these cells are capable of producing aberrant cells for the whole reproductive life span of an individual. The most relevant aberrations are stable reciprocal translocations that can be efficiently transmitted to the offspring of irradiated males. During meiosis reciprocal translocations can give rise to imbalances that are known to cause severe mental retardation and other adverse health effects in humans (van Buul, 1983). Chromosomal aberrations are normally caused during the repair of DNA double strand breaks (DSBs) initiated in the G_0-G_1 phase of the cell cycle; these aberrations can then be scored visually using either conventional cytogenetic techniques or FISH during the subsequent metaphase.

To date the majority of studies aiming to analyse the frequency of chromosome aberrations in the germline have been performed on laboratory mice. Such studies have identified a number of key components affecting the frequency and spectrum of chromosome aberrations in the mouse germline; these include sampling time after exposure (Leonard and Deknudt, 1970), genetic differences in DNA repair capacity of different mouse strains (Lee and Suzuki, 1981), and the sources and dose-rate of exposure to ionising radiation (van Buul 1983).

The assessment of chromosomal aberrations has been conducted in the F_1 offspring conceived from exposed spermatogonia and has shown that the rate of chromosomal aberrations increases in a linear fashion with dose (Leonard and Deknudt, 1967). More recently this work has been extended by the measurement of chromosome aberrations in

directly exposed germ cells (Generoso *et al.*, 1984; Griffin and Tease, 1988; Tease and Fisher, 1996).

The analysis of chromosomal aberrations in the male germline after exposure to ionising radiation, via direct analysis of sperm DNA or by the assessment of aberration frequencies in the offspring of exposed individuals, has provided evidence for the induction of chromosomal damage after irradiation. Although the data from human studies are limited it has been suggested that extrapolation from the mouse may be appropriate taking into account a number of correction factors (van Buul, 1983). However, due to the complexity of the process leading to the formation of chromosomal aberrations it had been suggested that more data should be obtained from systems more closely related to man (*i.e.*, higher primates) in order to generate a firmer basis for the estimate of radiation-induced genetic damage in man (van Buul, 1983).

1.2.1.10 Micronucleus assay

The micronucleus assay was introduced to provide a fast simple assay system to ascertain radiation-induced DNA damage. Micronuclei are formed during cell division and can be detected in the cytoplasm, often near the nucleus, as small nucleus-like particles. Micronuclei are formed from damaged DNA, resulting from the repair of acentric fragments, multi-centric chromosomes, or from damaged kinetochores, or due to spindle fibre defects. The assay is based upon the detection of micronuclei in hundreds to thousands of cells. The analysis of radiation-induced damage using the micronucleus assay has concentrated on the assessment of DNA damage in the nuclei of bone marrow cells after exposure; however a number of studies have been performed assessing germline effects after relatively low doses of ionising radiation. Pampfer *et al.* (1989) assayed the levels of micronucleus formation in the 2-cell stage embryos derived from spermatozoa of male mice exposed to different doses of X-ray irradiation, and found that the dose-response

relationship for micronucleus formation per embryo was linear-quadratic, where the number of embryos with micronuclei increased linearly with dose. Direct analysis of micronuclei formation in sperm was performed by Collins *et al.* (1992) who observed a 10-fold increase in micronuclei in spermatids exposed to 0.4 Gy of X-rays during meiotic prophase, compared to non-exposed controls. The micronucleus assay provides *in vivo* evidence for the physical damage of DNA but is unable to provide any information on the consequence of such damage with respect to the viability of the cell or the types of mutations that such cells may contain.

1.2.1.11 Comet assay

The comet assay is based on the gel electrophoresis of a single cell and is capable of detecting the amount of DNA damage. This system was developed by Ostling and Johanson (1984) and is based on microscopic measurements of the amount of degraded DNA in each cell. During electrophoresis, the cell takes on the shape of a 'comet', with the nucleus appearing as fluorescent head and a tail; where the length and breadth of the tail increases proportionally with the amount of DNA damage. A number of studies have shown the amount of DNA damage in individual sperm increases after exposure to ionising radiation and other mutagens (Haines *et al.*, 1998; Schindewolf *et al.*, 2000; Haines *et al.*, 2001). Haines *et al.* (1998) were also able to demonstrate that the comet assay could detect the amount of DNA damage increasing with increases in radiation dose, and that this occurred in a dose dependent manner. The comet assay is able to detect *in vivo* the relative amounts of physical damage caused to the DNA within the nuclei of exposed cells; however it is unable to provide any information about the types of mutations or the viability of the cells.

1.2.1.12 Transgenic mouse constructs

Transgenic mice containing reporter systems, such as *lacI*, were developed to assess mutation induction by chemical mutagens (Kohler *et al.*, 1991), and have recently been used to analyse mutation induction by ionising radiation (Luke *et al.*, 1997; Hoyes *et al.*, 1998). Mice were created using a λ shuttle vector containing the *lacI* target gene, which encodes the repressor for the *lacZ* gene (which encodes the β-galactosidase enzyme); such mice are now commercially available as Big Blue mice. Mice are treated with mutagenic agents and the vector is then recovered from mouse genomic DNA; the resultant extracts are plated on bacterial lawns on agar containing the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Phage carrying *lacI* mutations will form blue plaques (β-galactosidase induced) and those with a non-mutated gene will form colourless plaques (β-galactosidase repressed). The assay compares the number of blue plaques in control (non-exposed) individuals with those observed for exposed individuals. The effect of preconception paternal irradiation was assessed in transgenic mice containing a *lacI* reporter system; this showed a significant increase in the number of mutations in the bone marrow of the F_1 offspring of male mice exposed to 4 Gy of γ-rays (Luke *et al.*, 1997). Additionally the analysis of testis tissue, by Hoyes *et al.* (1998), demonstrated a slight but not statistically significant increase in mutation rate after exposure to 1 Gy of γ-rays. The data obtained using the *lacI* reporter system has been shown to be limited and it has been proposed that this is because the *lacI* transgenic mouse may be relatively inefficient at detecting mutations induced by exposure to ionising radiation or other agents which produce a large spectrum of mutations, ranging from point mutations to deletions which are larger than the *lacI* transgene (Hoyes *et al.*, 1998).

1.2.1.13 Summary of mutation detection systems

The phenotypic based systems described in this section provide valuable *in vivo* tools for the analysis of germline mutation induction by ionising radiation. Using such systems, mutation induction in the germline of male mice exposed to various sources of ionising radiation has been proven beyond any doubt (Searle, 1974). However, due to the relatively low resolution of phenotypic approaches for monitoring germline mutation, little is known to date about the mutagenicity of low-dose radiation exposure in mice. Additionally phenotypic based systems are unable to provide useful information about the total amount of DNA damage within the nucleus after irradiation. Germ cells containing extensive DNA damage may not be viable and could be lost from the germ cell population via apoptosis or cell cycle checkpoint arrest. The loss of germ cells is manifested as a period of infertility after exposure to higher doses of radiation, which is associated with spermatogonial killing (Preston and Brewen, 1976). New molecular techniques could potentially provide additional methods for the detection of DNA damage in the germ cells of directly exposed individuals. Ionising radiation is known to induce gene mutations (point mutations, deletions and insertions) in addition to chromosomal damage in mammalian cells. A number of molecular techniques have been developed to assess the damage caused directly to the DNA. Given the large number of test-systems specifically designed to evaluate mutation induction in the germline, the important issues of their sensitivity and ability to detect radiation-induced increases in mutation rate need to be further addressed. The following section provides comparisons of a number of characteristics of the most commonly used systems for monitoring germline mutation in mice.

1.2.2 Comparison of assays of germline mutation induction in model systems

Table 1.2 summarises a number of features of the most commonly used systems for monitoring phenotypic mutations in the mouse germline.

Detection system	Spontaneous mutation rate	Sample size†	Dose (Gy)	Doubling dose (Gy) ‡	Application to humans
Specific locus test	7.95×10^{-6}	1,051,869	3, 6, 6.7	0.34 (0.22, 0.50)	no
Dominant visibles	8.11×10^{-6}	225,017	6, 12	0.17 (0.00, 0.59)	yes
Dominant cataract	7.38×10^{-7}	107,369	1.5 - 6	0.56 (-0.14, 3.75)	yes
Skeletal mutations	2.89×10^{-4}	83,472	6	0.27 (-0.07, 1.67)	yes
Enzyme activity	2.85×10^{-6}	36,422	6	0.44 (-0.09, 2.68)	yes
Semi-sterility	1.04×10^{-3}	2,124	12	0.31 (0.03, 0.95)	no
Mean for 6 systems	-	-	-	0.35 (0.20 0.95)	-

Table 1.2 - Comparison of experimental systems for germline mutation induction in mice Data adapted from (Dubrova *et al.*, 1998a). † Minimal sample size for detection a two-fold increase in the mutation rate, including offspring from the control and irradiated parents. ‡ The lower and upper 95% confidence limits calculated from the Poisson distribution are given in parentheses.

The assessment of doubling dose provides a useful tool for comparing the efficiency of different mutation detection systems; this value equates to the dose of radiation required to increase the spontaneous mutation rate by two fold. The doubling dose estimates obtained for a number of the phenotypic systems are given in Table 1.2. The estimates of

doubling dose range from 0.17 Gy, for the dominant visible system, to 0.56 Gy, for dominant cataract mutations; these values do not differ greatly from the average value of 0.35 Gy. The mean value is also close to the estimate provided for the specific locus test (0.34 Gy), the most reliable system used to date.

The systems currently used for the analysis of mutation induction in the mouse germline are able to provide valuable information about the mutagenicity of ionising radiation, although they still have a number of drawbacks, including the necessity to analyse large numbers of offspring of parents exposed to high doses of ionising radiation. Additionally high doses (3-12 Gy) are required for the analysis of induction of phenotypic mutations in the mouse model due to the low spontaneous mutation rates at protein coding genes. The use of such high doses does not reflect the dose range to which human populations are most commonly exposed; i.e. they are way in excess of most environmental, occupational and medical levels of exposure. For humans the worldwide average annual exposure can be set at about 3.5 mSv, with the legal 'safe' limit for occupational exposure being 50 mSv/year, (Masse, 2000) and a value of 0.02 mSv being the average dose from a single chest X-ray (National Radiological Protection Board, 1994). The high doses required for mutation induction in the current mouse models suggest that the extrapolation of data obtained from these systems to human exposure may be problematic.

Although the specific locus test provides the most reliable mutation detection system used to date because of the requirements for specific mouse strains and breeding schemes it is uncertain how directly applicable the data obtained are to the situation within human populations. A number of phenotypic tests are directly applicable to human populations (see Table 1.2 for details), however it would be difficult to assess the mutation component of such changes within a human population (Sankaranarayanan, 1998). Indeed, the failure of large-scale studies analysing congenital malformations, which have a very low mutation

component, conducted on the offspring of parents exposed to irradiation from the atomic bombs in Hiroshima and Nagasaki (Neel *et al.*, 1989) and populations exposed after the Chernobyl accident (Dolk and Nichols, 1999), clearly illustrates the low resolution of such approaches.

The test systems that are currently used provide important information about germline mutation induction in mice; however, a number of problems remain concerning the extrapolation of the data obtained from these systems for the evaluation of the risk to human populations from exposure to ionising radiation (Bridges, 2001). As the direct analysis of human populations for the effects of radiation exposure is also fraught with difficulties (see Section 1.1.3 for details), there remains a need for additional methods to be developed and evaluated to monitor DNA damage.

1.2.3 Requirements for a new test system

A number of assumptions are commonly made when estimating genetic risk after exposure to ionising radiation; these include the supposition that the analysis of mutation induction at a small number of specified loci (those included in the specific locus test and others) is suitable for extrapolation to the genome as a whole. The development of a new test system to analyse radiation-induced mutation in the mammalian germline should take into account the requirement for the extrapolation of mutation rates from the loci studied to the genome as a whole. The system would also need to fulfil a number of other requirements; it must be applicable to the analysis of model systems, such as the mouse, and must also be applicable to the analysis of the effects of mutagenic agents within human populations. A new test system must also be quick and easy to assay, with a high degree of reproducibility. It would also be preferable to choose loci that possess high spontaneous mutation rates, in order to reduce the numbers of individuals required to achieve statistically significant differences between control and exposed groups. Finally, the

mutation rates at the loci chosen must show an increase after parental exposure to known mutagenic agents, such as ionising radiation.

Judging from these criteria, the most obvious system for monitoring radiation-induced mutations is an analysis of mutation rates at the DNA level. Given the complexity of mammalian genome, it appears essential to identify a set of DNA loci that are suitable for the analysis of mutation induction in the mammalian germline. As previously discussed in Section 1.2.2, mutation detection at protein-coding genes would require the profiling of a very large number of offspring and would not therefore provide a realistic alternative to the Specific locus test. On the other hand, repetitive loci within the mammalian genome could potentially provide a useful system for the analysis of mutation induction at the DNA level. The repeat families found within mammalian genomes fit all of our criteria for a system to test the effects of mutagenic agents in the germline; e.g. they have high spontaneous mutation rates and they are found within the genomes of most organisms, including mice and humans. Furthermore, the mutations at repeat loci are predominantly changes in the repeat number, which are easily detectable by standard molecular techniques such as Southern blot hybridisation of genomic DNA or by PCR based analysis and therefore should be readily reproducible. The following sections provide a summary of the major characteristics of the repetitive sequences found within mammalian genomes.

1.3 Review of repetitive regions of DNA

Repetitive DNA forms a large part of many genomes. It has been estimated that repeat sequences constitute greater than 50% of the human genome and that less than 2.5% of the genome relates to coding sequences i.e. open reading frames (ORFs) (Lander *et al.*, 2001); therefore, the analysis of mutation induction at repetitive regions may be more representative of the damage that ionising radiation causes to the genome as a whole. Repetitive DNA can be roughly divided into five major classes; transposon-derived repeats or interspersed repeats, retroposed copies of genes (processed pseudogenes), simple sequence repeats (SSRs), segmental duplications, and tandemly repeated sequences.

The following section will review the members of the family of tandemly repeated sequences. Tandem repeat sequences can roughly be divided into three main classes, satellite, minisatellite and microsatellite DNA; these distinctions are made mainly with respect to the size of repeat units and the overall size of the repeat array. Another class of tandemly repeated DNA can be found within the mouse genome which does not fall neatly into any of the three major classes, but possess some of characteristics of both micro- and mini-satellite loci; these loci are known as Expanded Simple Tandem Repeat loci or ESTRs. The final class of tandemly repeated DNA found in the genome are telomere repeats. Telomere repeats are found at the end of chromosomes and are associated with the prevention of end to end fusion of chromosomes and the problems associated with the replication of the end of linear chromosomes. The arrangement of telomere repeats differs greatly between different genomes, and they are known to be very different between the mouse and human genomes (Wright and Shay, 2000); therefore as they are unlikely to be good candidates to compare radiation-induced mutation in the mouse and in humans they will not be considered further in this section.

1.3.1 Satellite DNA

Major satellite arrays are known to account for several percent of eukaryotic genomes and are comprised of single repeat sequence repeated many times over with individual array lengths of up to about 5Mb. Satellite DNA sequences, especially those in the alphoid family, are often associated with centromeres. Satellites are infrequently used for genotyping purposes, as they are difficult to analyse due to their large size, but have provided useful anchor points for genome mapping close to centromeres.

1.3.2 Minisatellites

1.3.2.1 General characteristics

According to some estimates, hundreds to thousands of different minisatellite loci are observed within most eukaryotic genomes (Armour, 1999). The array sizes range from 0.5 to 30 kb, and are comprised of tandemly repeated units ranging from 8 to 90 bp, showing sequence variants within a repeat array. Minisatellites are highly polymorphic due to allelic variation in repeat copy number. The majority of minisatellite loci are located in non-coding regions of the genome.

The mutation rate at minisatellites is locus specific, i.e. a large number of stable minisatellite loci with relatively low mutation rates (less than 0.5%) are found in the human genome; however, mutation rates at some minisatellite loci exceed 0.5% and these are known as hypervariable or hyper-mutable minisatellites. To date these hypervariable loci constitute the best-characterized group of human minisatellite loci (Table 1.3).

Minisatellite loci (location)	Consensus repeat unit length	Germline mutation rate (%)[†]		
		Maternal	Paternal	Combined
CEB1 (*D2S90*)	39	0.3	16	8.2
CEB15 (1p36.33)	18	1.8[‡]	0[‡]	1.8[‡]
CEB25 (*D10S180*)	52	1.9	3.5	2.7
CEB36 (*D10S473*)	42	1.8	1.8	1.8
CEB42 (8q24.3)	41	NA	NA	0.5
CEB72 (17q25)	21	NA	NA	1.8
MS1 (*D1S7*)	9	4.9	5.5	5.2
MS31 (*D7S21*)	20	0.3[*]	1.2[*]	0.8[*]
B6.7 (20q13)	34	1.2	7.6	4.4

Table 1.3 - Hypervariable minisatellite loci in the human genome
Data from [‡]Dubrova *et al.*, (1997), [*]Jeffreys *et al.* (1988a) and [†]Vergnaud and Denoeud (2000). NA, not available.

The "core" repeat units found in a subset of GC rich minisatellites is similar to the generalized recombination signal (chi) of *Escherichia coli* (GCTGTGG). Alternative "cores" have also been reported which are associated with other sets of minisatellite loci, such as AT rich minisatellites (Jarman *et al.*, 1986; Nakamura *et al.*, 1987a). The overview provided here will outline the properties of GC rich hypervariable minisatellite loci only, as these loci have the most extensively characterized mutation mechanisms of all of the classes of repetitive DNA, and the characteristics of this subset of minisatellites may be the most relevant for the analysis of mutation induction at repeat loci.

1.3.2.2 The distribution of minisatellites within different genomes

Minisatellites (or variable number of tandem repeats, VNTRs) have been detected in the genomes of most higher eukaryotes including mice and humans and a number of other mammalian genomes (Jeffreys *et al.*, 1985a; Vergnaud *et al.*, 1993; Jeffreys and Morton, 1987; Burke and Bruford, 1987; Schonian *et al.*, 1993; Andersen and Nilsson-Tillgren, 1997; Dallas, 1988). To date, detailed analyses of the distribution and general characteristics of minisatellite loci have been performed in humans (Jeffreys *et al.*, 1985b; Jeffreys *et al.*, 1987b; Vergnaud *et al.*, 1993; Jeffreys *et al.*, 1999), rats (Pravenec *et al.*, 1996), mice (Jeffreys *et al.*, 1987a; Bois *et al.*, 1998a) and pigs (Archibald *et al.*, 1995). These studies have shown that GC-rich minisatellite loci prevalent in the genomes of these species have a number of consistent features; these include the size of the repeat units (between10 to 60 bp), and variant repeat types found within a single array.

The major difference noted between human minisatellites and those of other mammalian genomes was in the chromosomal location of the repeat arrays (Amarger *et al.*, 1998). Human minisatellites are found predominantly in sub-teleomeric regions (~ 90%), however in the pig, rat and mouse genomes they show less teleomeric clustering (66%, 30% and 15% respectively); however, synteny comparisons show that the chromosomal positions correspond to terminal regions within the human genome. In addition, germline mutation rate at human minisatellite loci substantially exceeds those in the pig, rat and mouse genomes (Vergnaud *et al.*, 1991).

1.3.2.3 The proposed functions of minisatellite loci

Some minisatellites have been identified in coding regions of the human genome where variations in the number of minisatellite repeats ultimately gives rise to proteins of different lengths and therefore slightly different functions. These include members of the apolipoprotein gene family (Mahley *et al.*, 1984), human epithelial mucin (Lancaster *et al.*,

1990), involucrin (Eckert and Green, 1986), loricrin and the SPRR genes (Gibbs *et al.*, 1993b).

Most minisatellite loci however are located in non-coding regions of the genome and whilst their function remains unclear, a number of possible functions have been proposed. Minisatellites may possess a regulatory role; it has been proposed that the large amount of size polymorphism at minisatellite loci in non-coding DNA in gene rich areas of the genome may disturb the expression patterns of neighboring genes (Bois and Jeffreys, 1999). VNTR variants may influence transcription by an as yet unidentified mechanism; this was found for the size variants of a VNTR in the vicinity of the H-ras (HRAS1) gene, with some variants potentially predisposing to heritable forms of cancer (Phelan *et al.*, 1996). Also the presence of a VNTR within the human immunoglobulin heavy chain (IGH) locus, which contains a core sequence similar to that of the binding site for the adenovirus major late promoter (MLP), may be associated with the suppression of transcriptional activity (Trepicchio and Krontiris, 1993). Minisatellites within intronic regions of genes have been shown to interfere with exon splicing; this may be due to the similarities of the consensus repeat of the minisatellite with splice donor repeat sequences, as is seen for the human interferon-inducible gene 6-16 (Turri *et al.*, 1995). It has been proposed that minisatellites may play a role in the control of imprinting (Chaillet *et al.*, 1995; Neumann *et al.*, 1995). It has also been proposed that minisatellite loci may have a role in the initiation of chromosomal pairing in a number of eukaryotic genomes (Ashley, 1994; Sybenga, 1999); this could possibly be related to the proposed recombinogenic properties of minisatellite loci (Boan *et al.*, 1998; Wahls, 1998). Furthermore, minisatellite loci have been found in the vicinity of a number of recurrent translocation breakpoints and may actually provide chromosomal fragile sites (as reviewed by Sutherland *et al.*, 1998).

Although at present the role of minisatellite loci remains unclear, the large number of loci, their persistence in the genome and the fact that they are found within the genomes of most eukaryotic organisms would suggest that they possess some functional role.

1.3.2.4 Mutation detection at minisatellite loci

Pedigree analysis

The use of pedigree-based analysis has been the traditional approach for the detection of germline mutations at human minisatellite loci and has been used to detect the rates at which new length mutant alleles are observed (Jeffreys *et al.*, 1988a). The use of pedigree analysis is limited due to the inefficiency of mutation detection, even for highly variable loci, and it is also unable to provide any detailed information about the processes acting at minisatellite loci (Jeffreys *et al.*, 1997). Mutation rates and processes have been characterised in detail for the male germline using the analysis of sperm DNA by PCR however, the pedigree-based analysis of minisatellite loci provides the only information available to date on the mutation rate in the female germline (Jeffreys *et al.*, 1997).

PCR based analysis

The development of a number of PCR-based techniques has circumvented the limitations of pedigree and cell line analyses. PCR analysis is used to detect rare mutation events from genomic DNA and can be easily applied to the analysis of DNA from somatic tissues, such as peripheral lymphocytes or the analysis of the germline via sperm DNA. The analysis of sperm DNA enables almost unlimited access to paternal mutations, but due to the lack of oocytes for analysis, a pedigree approach remains the only method for detection of maternal mutants (Jeffreys *et al.*, 1997).

The PCR amplification of a single molecule of DNA provides the basis of the mutation systems for minisatellite loci (Jeffreys *et al.*, 1990). Single molecule PCR (SM-

PCR) relies on the dilution of genomic DNA to the equivalent of a single diploid genome per reaction followed by the amplification of multiple aliquots, each containing a single molecule equivalent (~ 7.2 pg for the human and mouse genome, taking into account 80% PCR efficiency). The PCR reactions are then used to ascertain the size of the progenitor allele and to assess any size changes, representing mutant alleles (Jeffreys *et al.*, 1988b).

Mutations at minisatellite loci can also be detected by small-pool PCR (SP-PCR), which relies on the amplification of a limited number of DNA molecules (100-200); the DNA input is estimated from the knowledge of the quantity of DNA which represents a single molecule (Jeffreys *et al.*, 1994). This technique has been used to assess the mutation rate at a number of hypervariable minisatellite loci, and has been shown to be efficient at detecting mutant molecules for loci with mutation frequencies as low as 10^{-3} per progenitor allele; however, below this level PCR artefacts become problematic (Jeffreys *et al.*, 1994; May *et al.*, 1996). Other PCR approaches incorporating size enrichment have also been utilised to reduce the number of PCR artefacts and allow the detection of mutant alleles with rates of between 10^{-5} and 10^{-7} events per cell; however, the estimates from these approaches are only semi-quantitative due to problems with estimating the levels of enrichment (Jeffreys *et al.*, 1990; Jeffreys and Neumann, 1997). A limitation of mutation detection by Southern blotting of genomic DNA or PCR products is that this only allows the detection of mutation events that result in an alteration in length of the repeat array; however, the development of minisatellite variant repeat analysis by PCR (MVR-PCR) has allowed the analysis of the internal structure of minisatellite loci and has provided additional information on the processes taking place to create new mutant alleles (Jeffreys *et al.*, 1991a; Jeffreys *et al.*, 1994; May *et al.*, 1996).

1.3.2.5 Applications of minisatellite loci

The discovery of minisatellite loci has led to the development of 'DNA fingerprinting' for individual identification. Multilocus fingerprinting probes contain a 'core' repeat sequence (see Section 1.3.2.1 for details) which can be used to detect large numbers of highly variable loci simultaneously and which can provide individual-specific DNA 'fingerprints' of general use in human genetic analysis (Jeffreys *et al.*, 1985b; Jeffreys *et al.*, 1985c). Multilocus fingerprinting was used for forensic applications, such as individual identification (Gill *et al.*, 1985), and relatedness testing, such as paternity testing in the late 1980's (Jeffreys *et al.*, 1985a; Smith *et al.*, 1990). Single locus fingerprinting utilises probes that are specific for a unique minisatellite within a known genome (Wong *et al.*, 1986); the use of such probes for fingerprinting superseded multi-locus fingerprinting in the early 1990's and they were used for similar applications (reviewed by Jeffreys *et al.*, 1991b). Additionally single locus minisatellite probes have been utilised in population studies (Balazs *et al.*, 1989) and have provided information on human evolutionary histories through the analysis of chromosomal lineages (Jobling *et al.*, 1998). Minisatellite loci have been used for the analysis of mutation induction after exposure to environmental mutagens, e.g. after exposure to ionising radiation in human populations (Dubrova *et al.*, 1996; Dubrova *et al.*, 1997; Dubrova *et al.*, 2002), and after exposure to complex mixtures of urban and industrial chemicals from steel mills in Herring gull populations (Yauk and Quinn, 1996; Yauk, 1998; Yauk *et al.*, 2000). Minisatellites also provided the first highly polymorphic, multi-allelic markers for linkage studies (Nakamura *et al.*, 1987b), i.e. sets of minisatellite loci were used extensively by the Centre d'Etude du Polymorphisme Humain/National Institutes of Health consortium to produce some of the first human linkage maps (NIH/CEPH Collaborative Mapping Group, 1992).

1.3.3 Microsatellites

1.3.3.1 General characteristics

Historically the term microsatellite was used to refer to $(CA)_n$ or $(GT)_n$ repeats only (Litt and Luty, 1989; Weber and May, 1989); however it is now used for all simple sequence repeats (SSRs), all short tandem repeats (STRs) (Edwards *et al.*, 1991) and all simple repeat sequences (Tautz and Renz, 1984), including mono-nucleotide repeats such as poly(A)/poly(T) sequences. Microsatellites comprise one of the major classes of repetitive DNA and are ubiquitous in both prokaryotic and eukaryotic genomes (Hancock, 1996; Field and Wills, 1996). Polymorphic microsatellite loci consist of tandemly repeated units of DNA, with a unit (motif) length of 1 - 6 bp and with array sizes between 10 bp - 1 kb in length, containing mainly identical repeat units.

The mutation rates at microsatellite loci have been shown to be much greater than those observed in non-repetitive genomic sequences. The mutation rate at protein coding genes has been estimated to be approximately 10^{-6} per locus per generation (Lewin, 2000), in contrast, the estimated rates for microsatellite loci range from 10^{-4} to 10^{-3} per locus per generation in eukaryotic genomes (Weber and Wong, 1993).

1.3.3.2 The distribution of microsatellites within different genomes

Microsatellite loci are found in relatively large numbers within the genomes of most species in both coding and non-coding regions although the distribution and relative abundance vary between species. The abundance of microsatellite loci has been estimated experimentally using hybridisation techniques (Tautz and Renz, 1984) and via database surveys (Katti *et al.*, 2001). Large differences have been observed in the abundance of repeats within different genomes, e.g. the genomes of rodents have been shown to contain large numbers of repeat loci of may different types, whereas *Caenorhabditis elegans* has

the fewest microsatellite loci per megabase, even less than the levels observed in yeast and fungi (Toth *et al.*, 2000). Differences in the maximum length of microsatellite repeats have also been observed, with a tendency for prokaryotic genomes to contain shorter repeat arrays than those observed in eukaryotes (Katti *et al.*, 2001). Toth *et al.* (2000) reviewed the abundance and types of microsatellites in a number of different genomes, including primates, rodents, and other mammals, by analysing the number of perfect repeats arrays greater than 12 bp. In primates the most abundant type of repeat are mononucleotide repeats, which account for 42% of all repeat types in the genome. Rodent genomes however contain the highest proportion of di-nucleotide repeats, accounting for 39% of all repeats and also contain more microsatellite sequences than any of the other taxa examined.

1.3.3.3 Mutation detection at microsatellite loci

The main method of mutation detection at microsatellite loci relies on a simple PCR approach. Microsatellite loci are PCR amplified and separated by electrophoresis; the mutant alleles are designated as those with a different mobility, and therefore allele length, to the progenitor allele. A number of different methods of electrophoresis have been employed to provide resolution for the small size changes (sometimes as little as one repeat unit) observed at microsatellite loci. The type of electrophoresis used is dependent on the size of the repeat arrays and repeat units. Di-nucleotide repeats are often analysed using polyacrylamide gel electrophoresis, either with radioactively or fluorescent labelled primers to assist visualisation. The development of fluorescent technologies has allowed the use of multiplex-PCR (Wang *et al.*, 1996) and the analysis of up to 20 microsatellite markers per lane of a gel to maximise the throughput of large numbers of samples. For the analysis of large repeat arrays (those greater than 500 bp in length) agarose gel

electrophoresis is used, with DNA visualisation by ethidium bromide or by Southern blot hybridisation.

Pedigree analysis

Microsatellite mutations are most commonly detected by pedigree analysis with a mutant individual identified as possessing an allele of a different size to the parental alleles. This approach has been used for a number of different applications, such as linkage mapping of the human genome and for the analysis of human evolution (see Section 1.3.3.4, for details). DNA samples derived from tissue culture systems have also been used in the analysis of mutation processes at microsatellite loci, e.g. the analysis of CEPH family cells in culture has shown that somatic mutation also occurs at microsatellite loci, although at a low level (Banchs *et al.*, 1994).

Small Pool PCR analysis

Small pool PCR approaches, similar to those described for the analysis of minisatellite mutation (Section 1.3.2.4), have been used to assess germline mutation rates of poly A/T tracts in the human genome (Bacon *et al.*, 2001). SP-PCR has also been used in the characterisation of mutation spectra for a number of repeat expansion diseases (Monckton *et al.*, 1995; Mornet *et al.*, 1996; Crawford *et al.*, 2000).

***In vitro* detection of microsatellite mutations**

The study of mammalian cell lines has provided some information on the stability of different repeat types; by comparing the mutation rates at microsatellite arrays with different sizes of repeat unit it has been shown that di- nucleotide repeats are less stable than tetra-nucleotide repeats (Lee *et al.*, 1999). Additional analyses have demonstrated that

longer repeat arrays are less stable (Farber *et al.*, 1994; Lee *et al.*, 1999). A study by Twerdi *et al.* (1999) assessed the rates of expansions and contractions of repeat arrays in mouse cell lines with mismatch repair deficiencies, and human colorectal cancer cells (H6), and demonstrated significantly higher rates of insertions than deletions in constructs containing $(CA)_{17}$ repeats.

In vivo detection, using model systems

A number of studies have utilised *in vivo* systems to analyse mutation processes at microsatellite loci in yeast or *Escherichia coli* (Levinson and Gutman, 1987; Bichara *et al.*, 1995; Hancock, 1995; Hancock, 1996; Wierdl *et al.*, 1997; Field and Wills, 1998). Additionally mouse models have been established to analyse mutation mechanisms, and disease progression for human triplet repeat expansion disorders such as for Huntington's Disease (HD) (Kennedy and Shelbourne, 2000) and myotonic dystrophy (Lia *et al.*, 1998; Zhang *et al.*, 2002).

The analysis of unstable expanded repeats

Unstable expanded repeats are simple sequences which show variable copy number within 'normal' individuals, but which when expanded beyond a critical level become pathogenic. It has been shown in familial studies that only one allele is amplified (Mirkin and Smirnova, 2002). The repeat units themselves, and the size threshold for disease manifestation, are unique for each disorder; therefore it was initially thought that the mutation processes acting at these loci may have been under the influence of an uncommon mechanism and unique to the associated conditions. However, the increasing number of repeat expansion disorders associated with human fragile sites has indicated that the

dynamic mutation processes at these loci are important for the genome as a whole (reviewed by Richards, 2001).

1.3.3.4 Applications of microsatellite loci

The very high mutation rates exhibited at microsatellite loci (10^{-4} to 10^{-3} per locus per generation) and the simplicity of the detection methods as outlined in Sections 1.3.3.1 and 1.3.3.3 have led to the widespread use of microsatellite loci as DNA markers for a number of diverse applications.

Microsatellite loci have been used extensively for forensic applications, including the identification of individuals (Hagelberg *et al.*, 1991; Jeffreys *et al.*, 1992), and for paternity testing (Papiha and Sertedaki, 1995; Ingvarsson *et al.*, 2000). A multiplex Short Tandem Repeat (STR) system has also been developed for use in forensic analysis (Urquhart *et al.*, 1994; Budowle *et al.*, 1997; Caglia *et al.*, 1998).

Microsatellite loci have facilitated the production of linkage maps for the human genome (NIH/CEPH Collaborative Mapping Group, 1992; Weissenbach, 1993) and for many other genomes, including the mouse (Hearne *et al.*, 1991). Genome wide panels of microsatellite markers (Dib *et al.*, 1996) have also been used for mapping the genes involved in simple (Hastbacka *et al.*, 1992; Coyle *et al.*, 1996) and complex diseases (Bergthorsson *et al.*, 1995; Todd, 1995; Trembath *et al.*, 1997), by utilising the patterns of linkage disequilibrium between microsatellite markers (in association with) and disease causing genes.

The high mutation rates and relative abundance of microsatellite markers has allowed the study of population evolution by the assessment of variation at a number of microsatellite loci from different populations (Ellegren, 2000). Studies have also been performed analysing human population history using clonally transmitted sequences such as the Y chromosome (Kasyer *et al.*, 2001). Studies of population histories have used

microsatellite loci as useful markers of diversity and they have been used for a number of different of organisms, ranging from man (Dios *et al.*, 2001), through primates (Perelygin *et al.*, 1996), other mammals (Menotti-Raymond and O'Brien, 1995) and also for model organisms such as yeast (Pupko and Graur, 1999).

Increases in microsatellite instability (MSI) have been observed in the genomes of individuals with a number of different cancers (especially in association with gastric tumours and hereditary nonpolyposis colorectal cancer (HNPCC); Miyoshi *et al.*, 2001; Ross and Cohen, 2001; Starostik and Muller-Hermelink, 2001), and it has been suggested that microsatellite instability could be used as a biomarker for the early diagnosis of cancer (Srinivas *et al.*, 2001). It is not currently known if MSI predisposes an individual to cancer or whether it is a genomic consequence of carcinogenesis, therefore further investigation of the link of MSI with cancer would be required.

1.3.3.5 Microsatellite expansions and human disease

Many studies have been performed on a subset of microsatellites, tri-nucleotide repeats, which have been discovered in association with human disease. During the 1990's it became apparent that stretches of apparently 'benign' tri-nucleotides could expand and cause human disease; currently 14 tri-nucleotide repeat diseases have been discovered in the human genome and there may be yet more (reviewed by Cummings and Zoghbi, 2000). Tri-nucleotide repeat disorders can be divided in the two main categories; those where the repeats are in non-coding sequences, and those containing exonic $(CAG)_n$ repeats that are known to code for polyglutamine tracts.

The expansion of other classes of microsatellite loci can also result in human disease; these include a large intronic expansion within the spinocerebellar ataxia type 10 gene (*SCA 10*) (Matsuura *et al.*, 2000), and the causative expansion for Myotonic Dystrophy Type 2 (*DM2*) (Liquori *et al.*, 2001). Spinocerebellar ataxia type 10 is characterised by

cerebellar ataxia and seizures, and is caused by an expansion of a penta-nucleotide repeat (ATTTCT) in the 9th intron of the *SCA 10* gene. The normal alleles contain 10 to 20 repeats (50-100 bp) in intron 9 and the disease causing alleles are those with greater than 20 repeats, with the largest reported allele containing 4500 repeats (22.5 kb) (Matsuura *et al.*, 2000). It was thought that such a large expansion near the 3′ end of a large intron might affect the transcription or post-translational processing of the *SCA 10* gene; however no obvious changes in the levels of *SCA 10* mRNA were observed. It is however possible that the ATTCT expansion may affect other genes, in *cis* or in *trans* in a manner similar to that observed for *DM1* (Matsuura *et al.*, 2000).

Myotonic dystrophy is a multi-systemic disease showing a large range of clinical features including, myotonia, muscular dystrophy, cardiac defects, cataracts and endocrine disorders. The first causative locus for *DM* (*DM1*) is characterised by a tri-nucleotide repeat expansion (CUG) in the 3′ untranslated region of the dystrophia myotonica-protein kinase (*DMPK*) gene. The second locus *DM2* is also associated with an untranslated repeat expansion, a tetra-nucleotide (CCTG) in the first intron of the zinc finger protein 9 gene (*ZNF9*). The patients with this expansion show the same phenotype as those with *DM1* expansions, although the *DM2* expansion is usually more benign. The disease association of the *DM* loci, and others (SCA8 and SCA10), may be due to a pathogenic role of the repeat expansion within the RNA, which disrupts RNA splicing and ultimately cellular metabolism (Liquori *et al.*, 2001). Normal individuals have CCTG repeat tracts at the *DM2* locus interrupted by variant repeats (GCTG and TCTG), but in *DM2* patients the CCTG repeats are greatly expanded and no variant repeats can be detected. The expanded alleles range from 75 to 11,000 repeats (300 bp to 44 kb) but the smallest pathogenic repeat size is unknown (Liquori *et al.*, 2001). *DM2* also shows high levels of somatic mosaicism, with approximately 25% of affected individuals showing two to four alleles at the *DM2* locus in DNA from peripheral lymphocytes. The high levels of somatic mosaicism are also

demonstrated by monozygotic twins that possess alleles of very different sizes, e.g. 13 and 24 kb. Additionally repeat expansions in DNA from peripheral lymphocytes were also seen to increase in size over a three-year period increasing by approximately 2 kb per year (Liquori *et al.*, 2001).

The two loci *SCA10* and *DM2* are interesting examples of expanded repeat disorders as they show a number of characteristics in common with the Expanded Simple Tandem Repeat (ESTR) loci seen in the mouse genome; e.g. the repeat sizes are very similar and greatly expanded repeat arrays are common to both groups; it is therefore possible that the mutation mechanisms acting at greatly expanded microsatellite loci would be similar to those observed at ESTR loci. A more detailed discussion of the potential mutation mechanisms at microsatellites, tri-nucleotide repeats and ESTRs can be found in Section 1.6.1.2.

1.3.4 Expanded simple tandem repeat loci (ESTRs)

1.3.4.1 General characteristics

ESTR loci are tandemly repeated DNA sequences containing repeat units of 4 to 9 bp, with mainly homogenous repeat arrays. The array sizes are between 1 and 22 kb in length, which is substantially larger than those of microsatellite loci. ESTR loci have high germline and somatic mutation rates. Germline mutation rates range from 1.7 to 3.6% per gamete, which represents a range similar to that observed at hypervariable minisatellite loci (see Section 1.3.2.1, for details). The high somatic mutation rate at ESTR loci is characterised by high levels of mosaicism, i.e. between 2.8% and 20% of adult mice possess more than two alleles at the mouse ESTR loci *Ms6-hm* and *Hm-2,* respectively (Kelly *et al.*, 1989; Kelly *et al.*, 1991; Gibbs *et al.*, 1993a).

ESTRs were originally classified as hypervariable mouse minisatellites (Jeffreys *et al.*, 1987a) but were reclassified due to a number of differences from 'true' human hypervariable minisatellites (Bois *et al.*, 1998a). The most notable difference is the complexity of the repeat array; ESTRs contain only a single repeat unit per array unlike the large number of variant repeats shown in the arrays of minisatellite loci. The size of the repeat units also tends to be less for ESTR loci (4 to 9 bp) making them more similar to those seen at microsatellite loci (1 to 6 bp) than at minisatellite loci (10 to 60 bp). The size of the repeat arrays at ESTR loci (1 to 22 kb) is however comparable to those for minisatellites (0.5 to 30 kb). The mouse genome does also contain 'true minisatellite loci'; however these loci cannot be considered hypervariable as the germline mutation rates are well below 10^{-3} per generation (Bois *et al.*, 1998a).

The mouse ESTRs characterised to date include three single locus repeats, *Ms6-hm* (also called *Pc-1*), *Hm-2* and *Pc-2*, plus a repeat family, MMS10 (containing 1000 to 3000 dispersed members), the characteristics of which are detailed in Table 1.4.

Loci	Chromosomal location	Repeat sequence	Array size (no. of repeats)	Mutation rate*
Ms6-hm (or *Pc-1*)	4	GGCAG	400 - 3000	2.3 %
Hm-2	9	GGCA	300 - 5300	3.6 %
MMS10 repeat family	across the genome	GGCAGA (80%) & GGCAGAGGA (20%)	15 - 3000†	< 1.7 %
MMS10-E†	11	GGCGGA	250-420	2.9%
MMS10-F	ND	GGCAGGA	85 - 150	0.05 %
MMS10-O‡	14	GGCGGA	2 - 60	ND
*Pc-2*ℓ	6	GGCAGGA	14 - 2800	3.3 %

Table 1.4 – Characteristics of ESTR loci identified to date.

Data from ℓ Suzuki *et al.* (1993), * Bois *et al.* (1998a), ‡ Bois *et al.* (2001) and † Unpublished data. ND, not determined.

MMS10-E, *MMS10-F* and *MMS10-O* are members of the MMS10 family which are detected by the hybridisation of genomic DNA with the MMS10 multi-locus probe, the mutation rates of these loci can also be analysed individually following PCR amplification, then hybridisation of the PCR product with the MMS10 probe.

1.3.4.2 The distribution of ESTRs within different genomes

Currently ESTRs have only been identified in the mouse genome, but are thought to be present in other genomes. It has been shown that ESTR loci are derived from highly expanded interspersed repeat elements in the mouse genome. Interestingly the ESTR loci

that are members of the MMS10 family, and the *Pc-2* locus are expanded from the rodent specific interspersed repeats in the SINE B1 family (Mahtani and Willard, 1993; Bois *et al.*, 1998b). The other characterised ESTR loci in the mouse genome have expanded from the members of a subset of the mouse transcript (MT) family, specifically the mammalian retroposon-like superfamilies (MaLR) that include *Ms6-hm* (Heinlein *et al.*, 1986) and *Hm-2* (Kelly, 1994). It still remains possible that related sequences within other genomes could also undergo such massive expansions, e.g. the human specific *Alu* retroposons, which are closely related to the rodent specific SINE B1 elements (Quentin, 1994).

1.3.4.3 Mutation detection at ESTR loci

Pedigree analysis

Southern blot analysis of mouse pedigrees has been used to assess both somatic and germline ESTR mutation rates. High levels of spontaneous somatic mutation were observed at ESTR loci with levels up to 2.8% for *Ms6-hm* and 20% for *Hm-2* (Kelly *et al.*, 1989; Gibbs *et al.*, 1993a). Further analyses of somatic tissues during different stages of development were able to demonstrate that the high levels of somatic mutation at *Ms6-hm* and *Hm-2* take place during the early stages of embryogenesis (Kelly *et al.*, 1989; Gibbs *et al.*, 1993a). Spontaneous germline mutation rates have been assessed for *Ms6-hm*, *Hm-2* and MMS10; these were also found to be high, i.e. 2.5%, >3.6% and 1.7% respectively (Bois *et al.*, 1998b). Increases in ESTR mutation rate have also been observed in the germline after exposure to ionising radiation (Dubrova *et al.*, 1993; Sadamoto *et al.*, 1994; Fan *et al.*, 1995; Niwa *et al.*, 1996; Dubrova *et al.*, 1998a; Dubrova and Plumb, 2002).

Single Molecule-PCR

A more sensitive single molecule PCR based approach has been developed by Yauk *et al.* (2002) to further analyse the spectrum of mutations at ESTR loci. This analysis is based upon the system used for analysis of germline mutation at human minisatellite loci; see section 1.3.2.4 for details (Jeffreys *et al.*, 1994). The SM-PCR approach has so far allowed detailed analysis of the spectrum of spontaneous and induced mutations at a single ESTR locus, *Ms6-hm* (see Figure 1.1) (Yauk *et al.*, 2002). The analysis of the other known ESTR loci is not currently possible due to problems with the resolution of small mutations at the MMS10 and *Hm-2* loci.

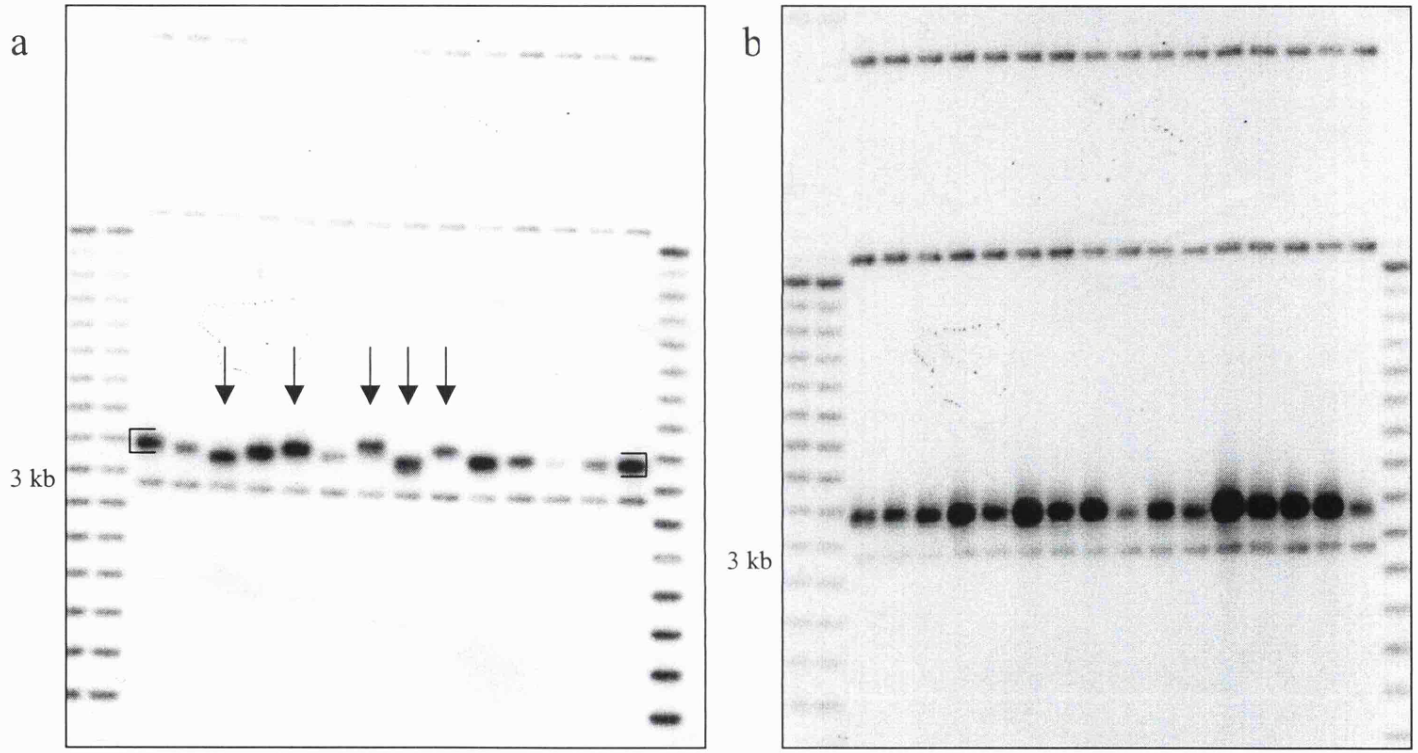


Figure 1.1 – Single-molecule PCR analysis of *Ms6-hm*

Southern blot of PCR reactions containing approximately one amplifiable DNA molecule per reaction from a single male mouse plate a) Shows sperm DNA, progenitor allele size indicated in brackets, b) DNA extracted from brain. Arrows indicate mutant alleles. 1 kb ladder in lane size standard (Invitrogen), 100 bp side lane marker (Promega). Adapted from Yauk *et al.*, 2002.

1.3.5 Which repeat loci could provide a suitable system for germline mutation detection?

The importance of the analysis of germline mutation induction by environmental mutagens was outlined in Sections 1.1.1 and 1.1.2, and the need for a reliable and easy monitoring system in Section 1.2.3 where it was stated that repeat loci may possibly provide a useful monitoring system to assess genome wide levels of DNA damage. In Sections 1.3.1 to 1.3.4 the characteristics of the major groups of tandemly repeated DNA were outlined and now it is important to decide which particular group or groups of repeat loci may provide the most useful system to assess the effects of environmental mutagens on the germline.

To date, ESTR loci have been used to analyse mutation induction in the germline of male mice exposed to ionising radiation (Dubrova *et al.*, 1993; Sadamoto *et al.*, 1994; Fan *et al.*, 1995; Niwa *et al.*, 1996; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Dubrova and Plumb, 2002; Yauk *et al.*, 2002). Additionally, hypervariable minisatellite loci have been utilised to analyse the effect of environmental exposure to ionising radiation in human populations (Kodaira *et al.*, 1995; Dubrova *et al.*, 1996; Satoh *et al.*, 1996; Dubrova *et al.*, 1997; May *et al.*, 2000; Livshits *et al.*, 2001; Dubrova *et al.*, 2002).

Hypervariable human minisatellites have high spontaneous mutation rates, between 0.5 and 7 %, and ESTRs also show high spontaneous mutation rates, between 1.5 and 4%. The most ideal situation would have been to assay the same repeat types in the human and mouse genomes; however, the mouse minisatellites identified to date have low spontaneous mutation rates which complicates the analysis of mutation induction at these loci. Both minisatellite loci and ESTRs are detected using standard molecular biology techniques, Southern blot hybridisation of genomic DNA or PCR products. The

reproducibility of the system can be tested by the comparison of spontaneous mutation rates obtained over a number of different studies. The spontaneous mutation rates for the CBA/H mice have been analysed four times and have been found to be very similar (Dubrova *et al.*, 1998a; Barber *et al.*, 2000; Dubrova *et al.*, 2000a; Barber *et al.*, 2002). Therefore, it is possible to conclude that human hypervariable minisatellite loci and ESTR loci in mice may provide useful systems to analyse mutation induction after exposure to ionising radiation.

1.4 Germline mutation induction at repetitive DNA sequences by ionising radiation

The systems used for the monitoring of mutation induction, to date, have been dependent upon the analysis of clear phenotypic changes, the incidence of deleterious health effects or the direct measurement of the level of DNA damage; they are known to have a number of drawbacks and therefore the development and assessment of a new monitoring system was required. The analysis of repetitive sequences would appear to provide a good system for the analysis of germline mutations; however, one question remains unanswered – do the mutation rates at these loci increase after exposure to environmental mutagens?

1.4.1 Mouse studies

The analysis of germline mutation rates in human populations can be confounded by a number of issues, e.g. the difficulty in obtaining suitable subjects and controls, controlling for ethnicity, and other factors such as smoking and parental age; therefore the initial studies to assess mutation induction at repeat regions after exposure to ionising radiation were conducted in mice. Dubrova *et al.* (1993) demonstrated that the germline mutation rate at repetitive regions in the mouse genome could be increased after exposure to ionising radiation. The analysis of approximately 200 offspring of male mice exposed to either 0.5 or 1 Gy of γ-radiation using two different human multilocus minisatellite probes (33.6 and 33.15) and two single locus mouse ESTR probes (Ms6-hm and Hm-2) demonstrated a two fold increase in the mutation rate at these loci after exposure. Further studies have also shown that ESTR loci show mutation induction after paternal exposure to

ionising radiation (Dubrova *et al.*, 1993; Sadamoto *et al.*, 1994; Fan *et al.*, 1995; Niwa *et al.*, 1996; Dubrova *et al.*, 1998a; Dubrova and Plumb, 2002).

1.4.2 Human studies

Studies assessing the effect of mutagenic agents which utilised highly mutable minisatellite markers have shown that exposure to certain mutagenic agents can induce germline mutations at these loci. Initial studies using the human multilocus probe 33.15 and four hypervariable single locus probes have shown that mutation rates at minisatellite loci increased, by two-fold in the germline of individuals who were exposed to ionising radiation after the Chernobyl disaster (Dubrova *et al.*, 1996). Further studies, using three independent sets of minisatellites (detected separately by multi-locus probes 33.15, 33.6 and six single-locus probes), also showed the two-fold increase observed in the initial cohort; this further indicates a generalised increase in minisatellite germline mutation rate in exposed families from the Belarus area (Dubrova *et al.*, 1997). Human populations exposed after nuclear bomb testing in Kazakhstan, were analysed using eight hypervariable single locus minisatellite probes which produced a significant 1.8 fold increase in mutation rate for the germline of highly exposed F_0 individuals and a less marked 1.5 fold elevation of mutation rate in the less exposed F_1 generation (Dubrova *et al.*, 2002).

A number of studies have however shown no evidence of increases in mutation rate at human minisatellites after environmental exposure to ionising radiation. Such studies have assessed the germline mutation rates of the atomic bomb survivors (Kodaira *et al.*, 1995; Satoh *et al.*, 1996), the families of clean up workers after the Chernobyl accident (Livshits *et al.*, 2001) and PCR based sperm analysis of the hypervariable loci CEB1 and B6.7 in seminoma patients before and after radiotherapy (May *et al.*, 2000).

The discrepancy between the Chernobyl studies by Dubrova *et al.* (1996 and 1997) and those from the atomic bomb studies could result from the totally different types of

radiation to which individuals were exposed. The atomic bomb survivors from Hiroshima and Nagasaki studied by Kodaira *et al.* (1995) were externally exposed to a single high dose of radiation (acute exposure), whereas chronic internal and external exposure was the main source of radiation hazard after the Chernobyl disaster. Evidence from a number of recent publications has suggested that chronic low-dose exposure to radiation may be more mutagenic than previously thought (Kovalchuk *et al.*, 2000a;Vilenchik and Knudson, 2000). The majority of the children in the Japanese study were born greater than ten years after parental irradiation, and it is therefore possible that some of the radiation-induced DNA alterations could have been repaired. In contrast, the cohort examined from the Chernobyl affected areas have been constantly irradiated and the children were conceived shortly after the accident. Finally, the data from the Japanese studies are derived from families where it was mainly only a single parent who received A-bomb radiation; the data for the Chernobyl families were obtained by profiling individuals where both parents were exposed to chronic irradiation.

The study performed by Livshits *et al.* (2001) on the families of the clean up workers may show differences to the studies of other Chernobyl exposed families due to high levels of heterogeneity within the group of clean up workers in terms of the types of exposure and the doses received. Most of the participants involved in the decontamination work around the Chernobyl nuclear power plant, the sarcophagus construction and in other clean-up operations received doses less than 0.25 Gy (Pitkevitch *et al.*, 1997). The main exposure to this cohort was due to external, relatively uniform γ-irradiation, with a relatively minor contribution from the intake of radionuclides. Most importantly, this group was exposed to repeated small daily doses of ionising radiation. Studies in male mice have clearly shown that the yield of germline mutations after such an acute external fractionated exposure was less than when the same dose was given in a single exposure (Lyon *et al.*, 1972b). Given the fact that the maximum reported dose to the Chernobyl clean-up workers was of 0.25

Gy, which is below any known estimates of the doubling dose for mice (Searle, 1974; Dubrova *et al.*, 1998a), and assuming the dose-fractionation effects, the expected increase in mutation rate in this group may be quite small (less than 25%) and therefore may not be statistically detected within the exposed group.

A study of sperm DNA from three seminoma patients using a single-molecule PCR approach before and after radiotherapy, also failed to detect an increase in mutation rates at the hypervariable minisatellite loci B6.7 and CEB1 (May *et al.*, 2000). Although the total testicular doses received were close to the estimated doubling dose for male mice (0.4 to 0.8 Gy, Searle, 1974; Dubrova *et al.*, 1998a) it is possible that dose fractionation may lead to a decreased yield in germline mutations (Lyon *et al.*, 1972b; Lyon *et al.*, 1972c). Additionally there are differences between the types of exposure and doses between the seminoma patients (acute external exposure) and the exposure for the Chernobyl families (chronic internal and external).

1.4.3 Other organisms

Ellegren *et al.* (1997) used two highly expanded tetra-nucleotide microsatellite loci in order to assess alterations in the germline mutation rates of barn swallows (*Hirundo rustica*) after environmental exposure to ionising radiation from the Chernobyl accident. A highly elevated mutation rate was observed at the *HrU6* locus in the exposed populations; however there was no evidence of an increase in the *HrU9* locus mutation rate compared to a population from an uncontaminated area of the Ukraine. However, both loci showed evidence of increased mutation rates when compared to a similar barn swallow population from Italy, suggesting the possibility that microsatellite loci could be used for the analysis of mutation induction after exposure to environmental mutagens. It should be noted however, that the loci used were atypical microsatellites due to the large repeat expansions and the fact that the repeat units were of a relatively large size (tetra-nucleotide repeats).

These loci may in fact be more similar to the mouse ESTRs that have been used for similar analyses in mice than other microsatellite loci.

Microsatellite loci have also been used to assess the mutation rate in wheat plants after the Chernobyl accident (Kovalchuk *et al.*, 2000b). Plants grown in a heavily contaminated plot near the power plant showed a significant increase in the number of heterozygous mutations (gains and losses of repeat units) at 13 single copy monomorphic microsatellite loci compared to a control population grown in a clean area with similar agrochemical properties. These data further suggest that the analysis of repetitive DNA may be a useful tool to assess mutation induction by ionising radiation.

1.5 Further analysis of mutation induction at ESTR loci in the mouse germline

1.5.1 Evidence for non-directed targeting of DNA damage

The level of mutation induction observed at ESTR loci after exposure to ionising radiation suggests that ESTR loci themselves are not the direct target of DNA damage, but that the effects may be attributed to some as yet unknown events elsewhere in the genome. These uncharacterised events may lead to genomic instability, which in turn increases the rate of mutation at ESTR loci themselves, via an unknown process (Sadamoto *et al.*, 1994; Niwa *et al.*, 1996; Dubrova *et al.*, 1998a).

Dubrova *et al.* (1998a) calculated that a 4 fold increase in paternal mutation rate, detected in male mice after pre-meiotic irradiation of 1 Gy would require 45,000 extra points of damage per haploid genome than expected if mutations at ESTR loci were produced by direct DNA damage. The calculation assumed the approximate size of the ESTR loci *Ms6-hm* and *Hm-2* as 1.6×10^4 bp, the mouse genome as 3×10^9 bp, and the estimates of DNA damage caused per cell per Gy of ionising radiation at 70 DNA double-strand breaks, 1,000 single-strand breaks and 2,000 damaged bases (Frankenberg-Schwager, 1990; Ward, 1990). As the levels of radiation-induced damage observed are not consistent with the levels of direct damage expected the mutation at ESTR loci must be due to non-targeted events.

Several mechanisms have been proposed to account for the highly elevated mutation rates observed at ESTR loci; these include, damage to DNA repair genes leading to inefficient repair of radiation-induced damage (Dubrova *et al.*, 1998a; Shiraishi *et al.*, 2002), or epigenetic alterations (Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000b; Dubrova and Plumb, 2002; Shiraishi *et al.*, 2002), both of which could lead to genomic instability. A transgenerational approach was used by Dubrova *et al.* (2000b) to investigate whether the

high levels of mutation induction at ESTR loci were due to a defect in DNA repair or by the alteration of the epigenetic control of the mouse genome. A uniform elevation of ESTR mutation rates was observed in the germline of the F_1 offspring of exposed males; this suggests that the elevation in ESTR mutation rates was not due to the inactivation of a DNA repair gene as it would be highly unlikely that all the offspring from ten irradiated males would contain a mutation in the same gene. Therefore Dubrova *et al.* (2000b) proposed that the effect that ionising radiation has on the mouse genome is epigenetic, with the most likely explanation being an alteration in methylation patterns. The transgenerational analysis was limited to only one mouse strain, only one source of ionising radiation, only pre-meiotic exposure was assessed and the experiment assayed only in the F_0 and F_1 germlines; therefore additional analysis of the phenomena of transgenerational germline instability was required; details of this additional work can be found in Chapter 5.

1.5.2 Dose response

A number of studies have analysed the dose response of ESTR loci to radiation-induced germline mutations. Dubrova *et al.* (1993) were the first to show that mutation induction at ESTR loci increased with dose. Fan *et al.* (1995) demonstrated that exposure of the spermatid (post-meiotic) stages of spermatogenesis led to a linear dose response pattern for the induction of ESTR mutations at the *Ms6-hm* locus after paternal exposure to γ irradiation of 1, 2 and 3 Gy. Niwa *et al.* (1996) demonstrated a linear dose response by the analysis of mutation induction at *Ms6-hm*, at the spermatid stage, after paternal exposure to γ radiation (^{252}Cf) 0.35, 0.7 and 1.02 Gy. Dubrova *et al.* (1998a) analysed the dose response of mutation induction at two single locus hypervariable ESTRs, *Ms6-hm* and *Hm-2*, after paternal exposure to X-rays at either 0.5 Gy or 1 Gy. Again a linear dose-response curve was observed for paternal mutation induction at pre-meiotic stages of exposure alone (spermatogonia and stem cells). Additional analysis using paternal

exposure to chronic γ irradiation (0.5 and 1 Gy) and also fission neutrons (0.125, 0.25 and 0.5 Gy) for the loci *Ms6-hm* and *Hm-2* also demonstrated a linear response to increasing doses of ionising radiation (Dubrova *et al.*, 2000a). The results of these two studies are summarised on Figure 1.2.

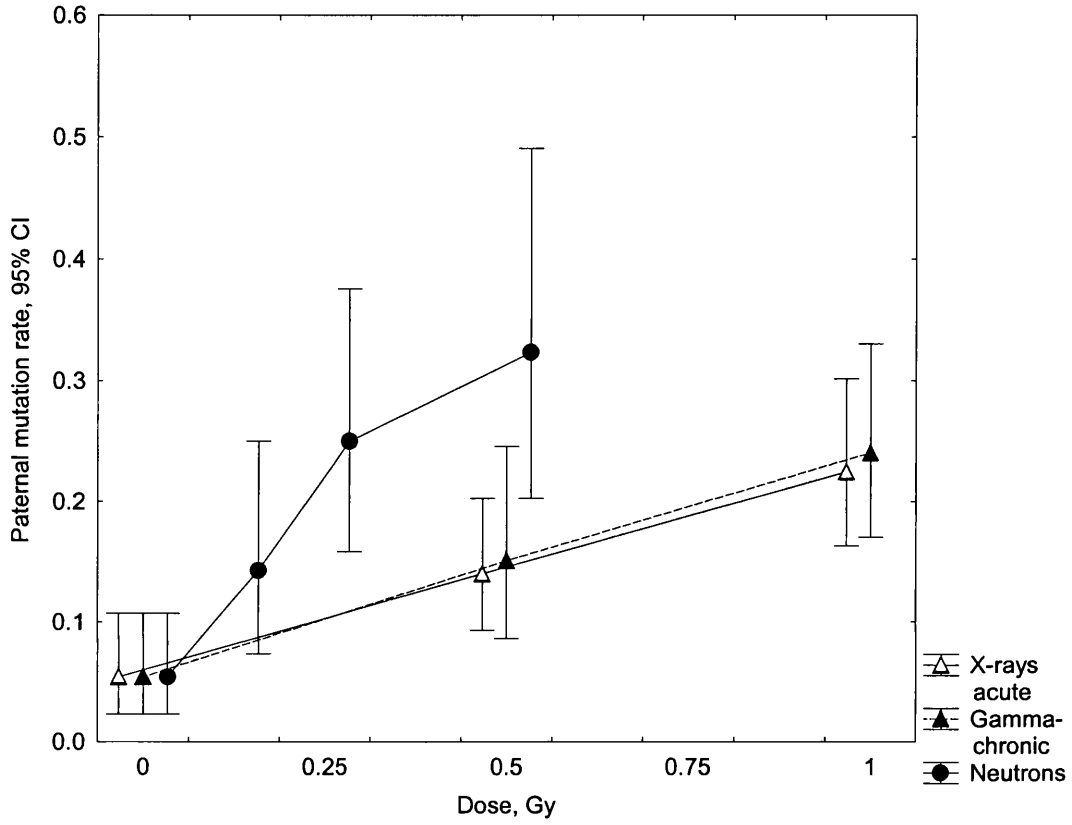


Figure 1.2 – Dose response curve for paternal ESTR mutation induction in mice after exposure to different sources of ionising radiation. Figure adapted from Dubrova and Plumb (2002), data taken from Dubrova *et al.* (1998a and 2000a).

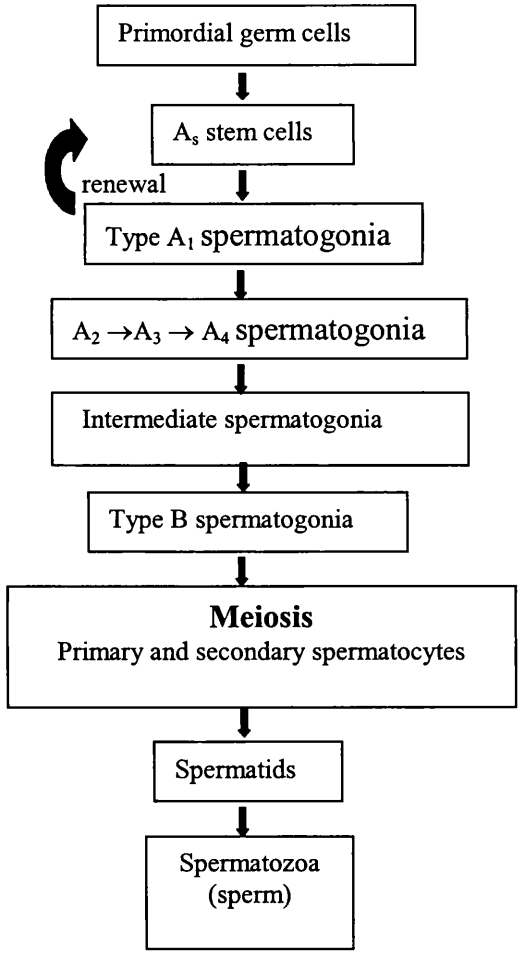
1.5.3 Stage specific responses

It is well recognised that different stages of gametogenesis, with spermatogenesis being the most well characterised, show differing sensitivities to the DNA damaging and cell killing effects of ionising radiation (reviewed by Searle, 1974). To analyse the possibility of stage-specific responses to ionising radiation an understanding of the

dynamics of a number of biologically important mechanisms is required, especially the processes of gametogenesis and meiosis (see Figures 1.3 and 1.4, for the details of spermatogenesis and meiosis respectively). Detailed information about the duration of the different stages of gametogenesis has been important in the analysis of mutation induction, and has enabled the analysis of differing levels of susceptibility of maturing germ cells to the effects of ionising irradiation. The details of the duration of different stages of spermatogenesis is well characterised for the mouse (see Table 1.5 and Figure 1.3).

Germ cell type	Days taken to reach ejaculate	Mating scheme
Type A spermatogonia	Over 42	>6 weeks
Intermediate spermatogonia	35-37	5 weeks
Type B spermatogonia	34-36	
Primary spermatocytes		
Preleptotene	33-35	
Leptotene	32-33	
Zygotene	30-32	4 weeks
Pachytene	23-30	
Diplotene	22-23	
Diakinesis – Metaphase I	21-22	3 weeks
Secondary spermatocytes	21-22	
Spermatids	7-21	
Spermatozoa	0-7	< 1 week

Table 1.5 - Stages of spermatogenesis. Adapted from Searle (1974).



Stage of development	Life span	Days to reach ejaculate	Sensitivity to killing
Primordial germ cells	Not known	Not known	Intermediate
A_s stem cells	≅ 240 hrs	> 42 days	Low
Type A_1 spermatogonia	28-31 hrs		High
$A_2 \rightarrow A_3 \rightarrow A_4$ spermatogonia	26-31 hrs each		High
Intermediate spermatogonia	26-28 hrs	35-37 days	Very high
Type B spermatogonia	29-30 hrs	34-36 days	Very high
Meiosis Primary and secondary spermatocytes	≅ 320 hrs in total	35-22 days	Intermediate/Low
Spermatids	229 hrs	21-7 days	Low
Spermatozoa (sperm)		7-0 days	Very low

Figure 1.3 - Stages of spermatogenesis.Adapted from Searle (1974).

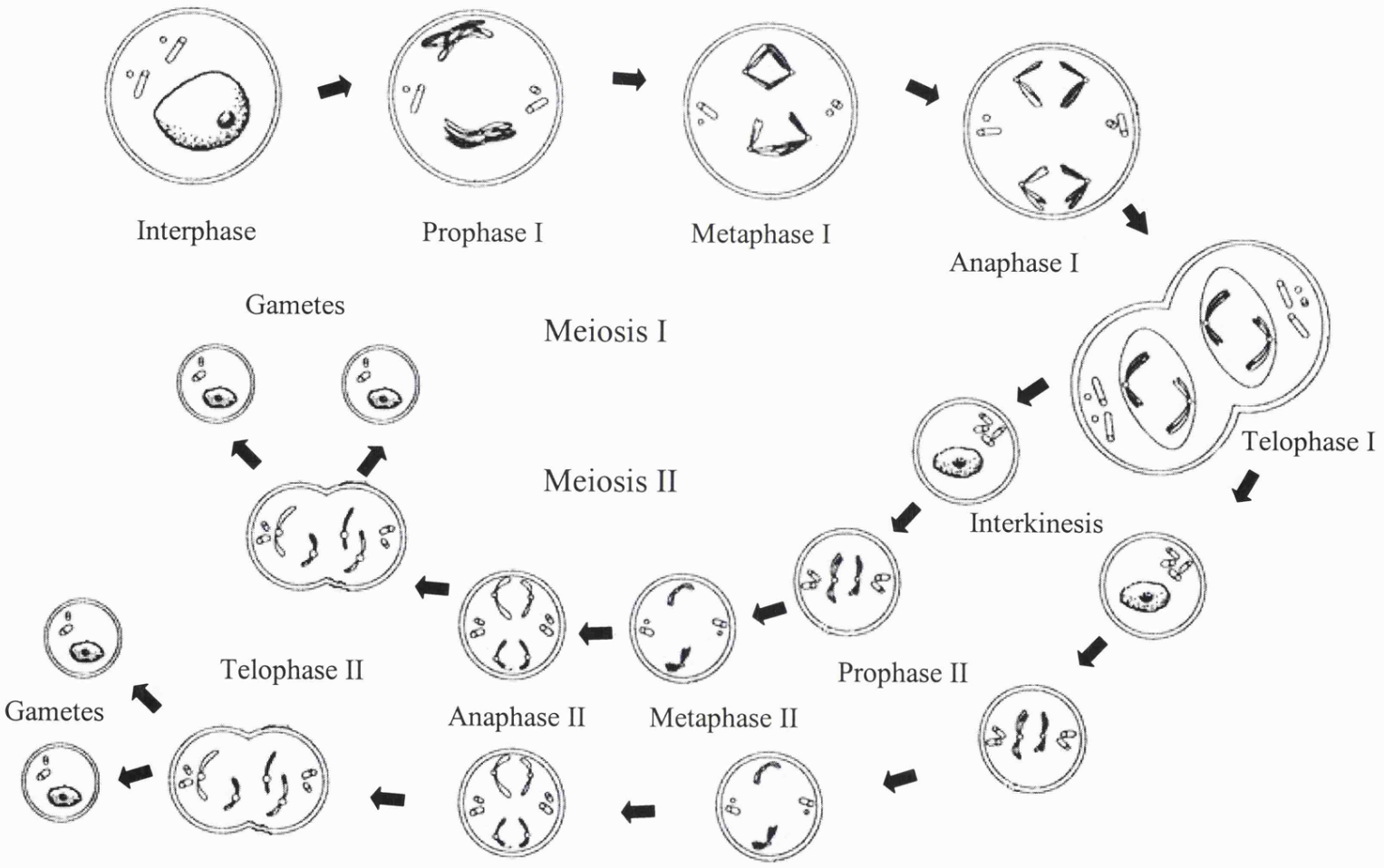


Figure 1.4 - Schematic diagram of the meiotic division.

Many studies have addressed the progression of germ cells during spermatogenesis and the effect that ionising radiation has upon the different stages of spermatogenesis (reviewed by Searle, 1974, see Figure 1.3). Using traditional systems for the analysis of germline mutation in mice it has been shown that irradiation of post-meiotic spermatids results in the highest yield of germline mutations, most probably due to the decreased activity of DNA repair at these stages of spermatogenesis (Favor, 1999). The high sensitivity of spermatids has been detected in a number of different systems including the specific locus test (Searle, 1974), dominant lethal mutations (Ehling, 1971), and in association with malformations and tumour formations (Nomura, 1982).

The analysis of stage specific mutation induction at ESTR loci has produced a number of studies showing conflicting data. The analysis of *Ms6-hm* has shown that although radiation exposure at both pre- and post-meiotic stages of spermatogenesis leads to mutation induction it is the post-meiotic spermatids that are the most sensitive (Fan *et al.*, 1995; Niwa *et al.*, 1996, Sadamoto *et al.*, 1994). Fan *et al.* (1995) proposes an indirect mechanism of mutation induction at ESTR loci where the initial radiation-induced damage to DNA in the germline later results in ESTR mutations in the fertilised embryo. Other studies have been unable to detect any mutation induction in the post-meiotic stage of spermatogenesis (spermatids), but show significant increases in ESTR mutation rates after paternal irradiation at the pre-meiotic stages of spermatogenesis (spermatogonia and stem cells) (Dubrova *et al.*, 1998a). It may be possible to explain the apparent discrepancy between the different studies by addressing the issue of mutation scoring. Sadamoto *et al.* (1994) describe high levels of mosaicism in the offspring of irradiated sperm for *Ms6-hm*. The problem with scoring mosaics as mutations at ESTR loci is due to the high degree of somatic mosaicism that takes place during early embryogenesis for *Ms6-hm* (Kelly *et al.*, 1989; Kelly *et al.*, 1991). Therefore, mosaic bands in the offspring of irradiated males most probably represent mutation events that are somatic not germline in origin. Some studies have also demonstrated increases

in mutation rates from the maternal as well as paternally derived ESTR alleles, after paternal exposure (Sadamoto *et al.*, 1994; Fan *et al.*, 1995). Such a phenomenon has not been observed in other studies of ESTR loci (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Dubrova *et al.*, 2000b; Dubrova and Plumb, 2002). The differences observed in the maternal mutation rates between these studies may also possibly be explained by increased somatic instability in the developing embryo.

Additionally the work by Yauk *et al.* (2002) provides compelling evidence for ESTR mutation induction in pre-meiotic diploid cells. Using a novel single-molecule PCR approach, the authors have analysed the frequency of ESTR mutation in sperm DNA samples from non-exposed and irradiated male mice. The results of this study clearly show an elevated ESTR mutation rate in the sperm of male mice exposed at pre-meiotic stages and therefore provides conflicting evidence for the previously proposed mechanism of the resolution of ESTR pre-lesions into full mutations in the fertilised egg or during embryogenesis.

The observation that post-spermatogonial cells show a higher sensitivity to the mutagenic effects of ionising radiation at protein-coding genes (7 locus test) has been explained by the lack of DNA repair (Russell *et al.*, 1958; Adler, 1996). It is known that the DNA repair ability of cells during spermatogenesis reduces with time after the meiotic division and is associated with the morphological and biochemical changes required in the formation of mature sperm. During the later stages of spermatogenesis the chromatin of the sperm head becomes more condensed and biochemically inactive, thus reducing the effectiveness of the DNA repair machinery (Wright, 1999). This in turn may lead to damaged DNA being passed onto the next generation and may induce a variety of damage responses in the zygote; however, this hypothesis fails to explain the lack of ESTR mutation induction in post-meiotic cells and the high yield of radiation-induced mutations following exposure of pre-meiotic spermatogonia and stem cells.

The decrease in repair function during spermatogenesis may also be used to explain the high levels of mutation induction at ESTR loci in spermatogonial stages, and the lack of induction in post-meiotic stages. It is possible that the mutations observed at ESTR loci are a result of the repair of DNA damage; therefore, an intact repair system would be required to produce mutations at these loci. The analysis of ESTR mutation induction by ionising radiation performed by Dubrova *et al.* (1998a) indicates that diploid cells are required for mutation induction at ESTR loci. This information has led to the suggestion that the process(es) of mutation induction at ESTR, may involve alterations in the levels of crossing over, and the cells may therefore need to go through the meiotic division to observe mutation induction at ESTR loci. The possibility that the process of recombination may be involved with mutation induction at ESTR loci requires further analysis; the work presented in Chapter 3 outlines the experimental analysis of this hypothesis.

1.5.4 Analysis of exposure to different sources of ionising radiation

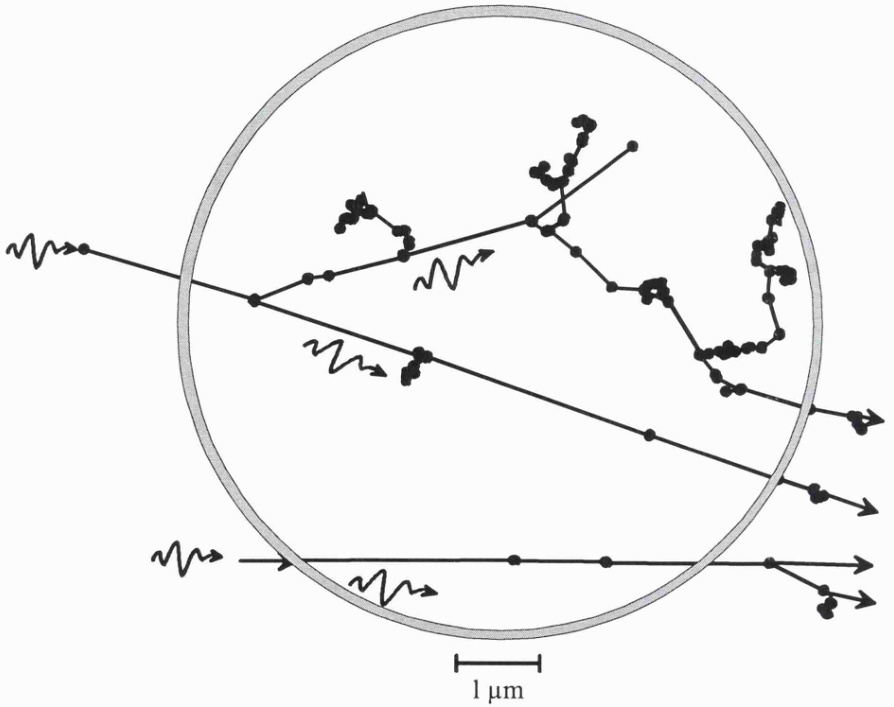
1.5.4.1 Different sources of ionising radiation

Ionising radiation can be classified as electromagnetic or particulate sources. The most common forms of electromagnetic radiation are X- and γ-rays. The essential properties of these X- and γ-rays are similar; they can be both considered as streams of photons or "packets of energy", and when they are absorbed by living material the energy is deposited in the cells. Other naturally occurring types of radiation are particulate in nature and contain electrons, protons, α-particles, neutrons, negative π-mesons or heavy charged ions. Those of importance in radiobiology are mainly the α-particles and the neutrons. α-particles are emitted during the decay of naturally occurring radionuclides, such as uranium and radon, and form the major source of natural background radiation for the general public. Radon gas seeps from the soil, especially in certain geographical areas such as the parts of Devon and Cornwall, and can build up in houses where it is breathed in and irradiates the lining of the lungs. It has been estimated that half of the lung cancer cases in the United States may result from radon inhalation (Hall, 2000). Neutrons are produced as a by-product of the fission of heavy radionuclides and are present in large quantities in nuclear reactors; they are also an important constituent of cosmic radiation and can contribute significantly to exposure during high altitude air travel.

Ionising radiation acts both directly and indirectly to cause damage in biological systems. When radiation is absorbed the atoms of the target cells may become excited; this represents the direct action of ionising radiation, which is though to be the main effect of high-Linear Energy Transfer (LET) sources such as neutrons and α-particles. Radiation may also lead to indirect effects though the production of free radicals, such as hydroxyl radicals, which are highly reactive species that may lead to processes such as DNA damage. It is thought that approximately two-thirds of the biological effects of X- and γ-rays are produced

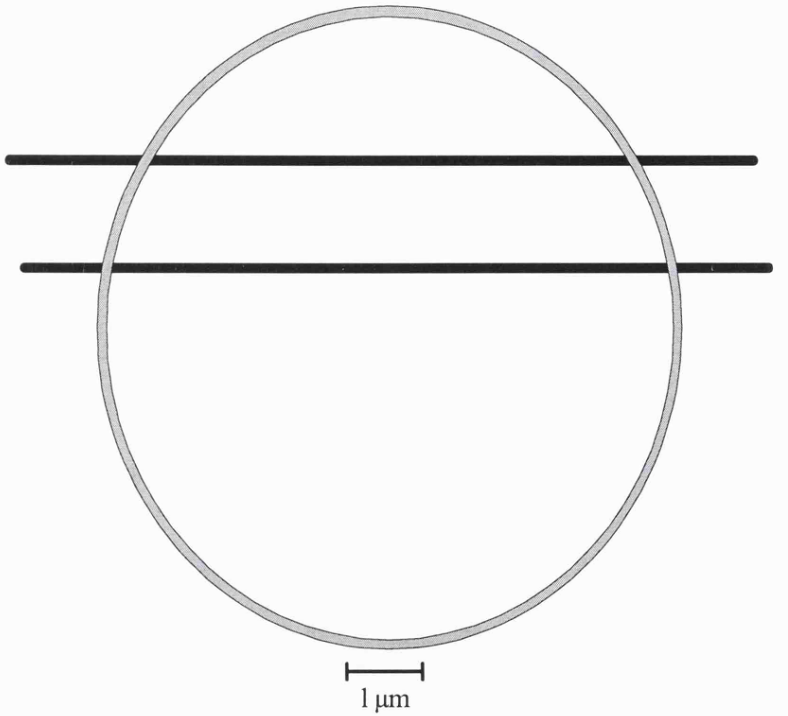
via indirect action. Exposure to high-LET neutrons also leads to indirect as well as direct effects and the deposition of the damage is much more concentrated, leading to larger biological effects than are observed for the same exposure of low-LET irradiation such as the X- and γ-rays (Goodhead, 1988). X- and γ-rays are 'sparsely ionising' with the damage being distributed in space, while neutrons and α-particles are considered to be 'densely ionising, due to the damage being more concentrated (see Figure 1.5). A number of other factors are also important in determining the relative biological effectiveness (RBE) of exposure to different sources of ionising radiation. These include, the radiation quality (LET), the sources of exposure (internal or external), the accumulated dose, and the dose rate.

Small traces of radioactive particles are naturally found within the body; including trace values of thorium, radium and lead can be detected, which produce very low doses usually quoted as < 10 μSv/y. Radioactive potassium-40 is the only radionuclide that has an appreciable effect by ingestion and this is thought to be approximately 0.2 mSv/y. As mentioned previously the most important natural source of internal irradiation is from exposure to radon gas, where the decay series leads to the production of α-particles in the lung. Internal radiation may also occur by the ingestion of contaminated food and water sources, for example the situation after the Chernobyl accident, where radionuclides were deposited in the soil and contaminated food and water sources in areas of Belarus and Ukraine. The exposure of populations after the Chernobyl accident was initially due to high levels of the short-lived isotope ^{131}I (8 day half life) from both internal and external sources. After decay of ^{131}I more stable isotopes, such as ^{137}Cs, and ^{90}St, became more important (UNSCEAR, 2000).



Low-LET tracks from γ rays.

1 Gy of irradiation corresponds to approximately 1000 tracks.



High-LET tracks from α-particles.

1 Gy of irradiation corresponds to approximately 4 tracks.

Figure 1.5 – Differences in energy distribution between high and low-LET irradiations.

The figure represents a cell nucleus irradiated with two electron tracks from γ rays (low-LET) and two α-particle tracks (high-LET).

Adapted from Goodhead *et al.* (1988).

A number of experiments to assess the effects of ionising radiation on the germline have been performed in the mouse model using high dose, low-LET sources of ionising radiation (3-6 Gy) mainly from acute external exposure to γ and X-rays (Lyon *et al.*, 1972a; Russell and Kelly, 1982a; Russell and Kelly, 1982b). High-LET sources have also been studied and found to be more effective at mutation induction in the germline of exposed male mice (Russell, 1965; Batchelor *et al.*, 1966; Batchelor *et al.*, 1967; Niwa *et al.*, 1996). The data obtained from high-LET studies provide important information about the passage of radiation-induced damage via the germline; however, the use of the data from such experiments is limited for extrapolating to the situation within human populations because acute high-LET exposure is uncommon in the environment. With the notable exception of the atomic bomb survivors of Hiroshima and Nagasaki, low-LET chronic irradiation, often with an internal component, is the most common type of human exposure whether occupational, therapeutic or environmental. As we are ultimately interested in the estimation of the risks of radiation exposure in relation to the human germline, it is important to use a monitoring system that will be able to detect levels of mutation induction after exposure to chronic low-LET sources of ionising radiation.

The mutation rates at ESTR loci in the offspring of male mice exposed to varying doses of high-LET fission neutrons and low-LET X- and γ-rays have been analysed (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a; Dubrova *et al.*, 2000b). The data from these studies show that both high and low-LET sources of ionising radiation are able to increase mutation rates at ESTR loci in the germline of male mice exposed to relatively low doses of ionising radiation.

1.5.4.2 Chronic and acute exposure

Traditional systems used to analyse radiation-induced mutation in the mouse germline, such as the specific locus test, suggest that acute low-LET exposure is more efficient in causing mutation induction than chronic exposure (Lyon *et al.*, 1972a; Russell and Kelly, 1982a; Russell and Kelly, 1982b). Data from the analysis of spermatogonia irradiated at relatively high accumulated doses (3 Gy), suggest that acute doses above 0.24 Gy min^{-1} lead to high levels of mutation induction, whereas doses of 0.008 Gy min^{-1} and below result in lower levels of mutation induction. Additionally analyses of the intermediate dose range have suggested a sharp transition point from low to high levels of mutation induction (Lyon *et al.*, 1972a; Russell and Kelly, 1982a; Russell and Kelly, 1982b). It has been suggested that these differences may be explained by the fact that acute exposure leads to saturation of the DNA repair system resulting in a number of damaged sites remaining un-repaired and hence higher mutation rate in the subsequent offspring (Russell *et al.*, 1958; Adler, 1996). The analysis of post-meiotic germ cells (mainly spermatids and spermatozoa) further supports this hypothesis, i.e. in post-meiotic germ cells, where the levels of DNA repair are reduced, similar levels of mutation induction are observed for both acute and chronic low-LET exposure (Russell *et al.*, 1958).

The efficiency of chronic low-LET exposure on mutation induction at ESTR loci in the germline of irradiated mice has recently been studied (Dubrova *et al.*, 2000a). The results of this study show that at low doses of ionising radiation (0.5 to 1 Gy) there are no differences in the efficiency of mutation induction at ESTR loci after exposure to low-LET chronic ($1.66x10^{-4}$ Gy min^{-1}) or acute irradiation (0.003 Gy min^{-1}) during the spermatogonial stages of mouse spermatogenesis. Previous analyses have compared high doses of acute and chronic exposure (3-6 Gy) (Lyon *et al.*, 1972a; Russell and Kelly, 1982a; Russell and Kelly, 1982b), which correspond to the part of the dose response curve where the effect of chronic exposure is non-linear (see Figure 1.2), in contrast, the data

presented by Dubrova *et al.* (2000a) were obtained at much lower doses. The results of this study suggest that the estimates produced from the extrapolation of data obtained from high dose exposure to ionising radiation may not hold true for exposure at lower doses; this is in contrast to the previous estimates which have assumed that chronic radiation exposure induces only a third of the number of germline mutations as the same dose given acutely (UNSCEAR, 1993). The fact that the data presented by Dubrova *et al.* (2000a) show no differences in the germline mutation induction at ESTR loci and the fact that most human exposure is derived from low-dose chronic sources of radiation may have potential implications for estimating the risk of radiation in the human germline.

1.5.5 Mutation processes at ESTR loci

Many studies have demonstrated the use of ESTR loci as a monitoring system for the analysis of germline mutation induction in male mice exposed to ionising radiation; however little is known about the processes leading to spontaneous and induced mutations at these loci. The lack of information about the mutation processes at ESTR loci limits the use of these loci for the analysis of mutation induction in the germline and hinders the extrapolation of data obtained from this system to the levels of DNA damage occurring in the genome as a whole. Additionally information about mutation mechanisms at ESTR loci may lead to a better understanding of the action of mutagenic agents on the mammalian germline and may provide information to enable the prediction of which chemicals mutagens which lead to mutation induction at ESTR loci.

The assays developed to analyse mutation mechanisms at minisatellite loci utilise the complexity of the internal structure of these loci which contain variant repeats within a single repeat array; however the simple internal structure of ESTR loci, containing a single repeat, means that the such systems are uninformative. The little information that is known about the mutation processes at ESTRs has been gained from the analysis of mutation rates at these loci.

1.5.5.1 Analysis of mutation spectra at ESTR loci

The analysis of the mutation spectra at *Ms6-hm*, using Southern blot analysis, showed that the size changes were similar in the germline and in somatic tissues with the mean change in repeat copy number being 10% and 13% respectively (Kelly *et al.*, 1989). Kelly *et al.* (1989) also noted that the changes in repeat length were small, less than 200 repeat units, that the mutation events were sporadic, and that there was no evidence of mutation clustering. Further analysis of the mutation spectra at *Ms6-hm* has shown that spontaneous

and induced mutations in both somatic tissue and the male germline were similar (Yauk *et al.*, 2002). Yauk *et al.* (2002) were able to show that the mutation rate at *Ms6-hm* appears to correlate with the proliferation rates of different tissue types. DNA extracted from tissues with low rates of cell division showed the lowest mutation rates; i.e. brain has a mutation rate of 3% per progenitor molecule, followed by a 7% mutation rate in spleen DNA (medium turnover rates), and sperm DNA, which corresponds to the tissue with the highest rate of cell division, showed the highest spontaneous mutation rate of 12%. No evidence for mutation induction at *Ms6-hm* after exposure to ionising radiation was observed in the somatic tissues analysed; however, further analysis would be required to completely rule out the possibility of somatic mutation induction after irradiation, as the ESTR mutation rate is lower in somatic tissue and therefore alterations in somatic mutation rate may not be detected in this experiment. Sperm DNA showed a statistically significant 1.7 fold increase in mutation rates in individuals that had been acutely exposed to 1 Gy of X-rays. The majority of the mutations scored (76%) resulted from small gains or losses of 3 to 5 repeat units. A significant bias towards gains was also observed, with 69% of all somatic mutants showing gain mutations. Due to limitations in the resolution of the system the analysis of very small changes in repeat length, 1 to 2 repeats, was not possible and this in turn may abolish the apparent gain bias and greatly increase the actual mutation rate (Yauk *et al.*, 2002).

1.5.5.2 Possible mutation mechanisms at ESTR loci in mice

The evidence gained so far regarding the mutation processes at ESTR loci in the mouse genome is limited; however, it is plausible to suggest that the mutation mechanisms at these loci may be similar to those observed for other repetitive regions within mammalian genomes. It was initially thought that ESTR loci were hypervariable mouse minisatellite loci and therefore that the mutation mechanisms would be very similar.

Subsequent analysis has revealed that ESTRs are less similar to minisatellite loci than previously thought, i.e. they lack variant repeats within repeat arrays, and show high levels of somatic instability observed at *Ms6-hm* and *Hm-2* during early embryogenesis. These discrepancies have been used to suggest possible differences in mutation mechanism(s) between ESTR loci and minisatellites (Kelly *et al.*, 1989; Kelly *et al.*, 1991; Gibbs *et al.*, 1993a; Bois *et al.*, 1998b); however, it remains possible that the mutation mechanisms at ESTR loci may still be similar to those characterised at human minisatellite loci, or alternatively, they may be more like the mechanisms attributed to microsatellite loci (Bois *et al.*, 2001; Yauk *et al.*, 2002)

The analysis of the levels of instability for members of the MMS10 ESTR family shows that instability is influenced by the length of the uninterrupted repeats (Bois *et al.*, 2001). Members of the MMS10 family fall into two classes, those with an uninterrupted array of GGCAGA repeats and those that have longer repeat arrays with occasional variant repeats of GGCAGAGGA. Eleven members of the two classes were analysed in a number of inbred mouse strains and in BXD recombinant mice; the mutation rates showed a clear and significant correlation between the level of instability and the length of uninterrupted repeats similar to that observed for microsatellite loci (Rolfsmeier and Lahue, 2000). In the analysis of the BXD mice mutant alleles were only observed in individuals with greater than 20 uninterrupted repeats (>120 bp), which is similar to the threshold repeat sizes observed for a number of repeat expansion diseases. These data further suggest that the mutation mechanisms at ESTR loci may be very similar to those at microsatellite loci, especially disease associated microsatellites with large repeat expansions.

As the mutation mechanisms at ESTR loci remain unknown, it may be useful to assess the possibility that the processes acting at these loci may be similar to those at other repeat regions; therefore, knowledge of mutation mechanisms at repeat loci would be useful. The following Sections outline mutation processes at micro- and minisatellite loci.

1.6 Germline mutation mechanisms in repetitive DNA

1.6.1 Minisatellites

Minisatellites have the most extensively characterised mutation processes of all the classes of tandem repeat loci, and clear differences have been shown between these processes in somatic and germline tissues.

1.6.1.1 Differences between somatic and germline mutation processes

Somatic processes

Analyses of minisatellite loci have shown that the mutation processes in somatic tissue and the germline are different. The analysis of MS32 somatic mutants, by size-enrichment small-pool-PCR (SESP-PCR), has provided an estimate of the frequency of somatic events in peripheral lymphocytes of 0.9-2.0 x 10^{-5} per molecule and indicated that there was a degree of somatic mosaicism at minisatellite loci (Jeffreys *et al.*, 1997). MVR-PCR analysis showed that the mutation processes in somatic tissue were simple. The types of mutations recovered were intra-allelic duplications/deletions of blocks of repeats, with evidence for a slight bias towards losses. Additionally the rearrangements were not clustered but were found to be randomly distributed along the repeat array. These data and additional evidence suggests a simple mode of mutation induction probably by a recombination-based process (Jeffreys *et al.*, 1997). Recombination appears to be more likely than strand slippage as a mechanism as the rates of minisatellite mutation remain the same in mismatch repair (MMR) deficient cells; in contrast microsatellite mutation rates have been shown to increase in such cell lines and are thought to mutate via replication slippage (Aaltonen *et al.*, 1993; Parsons *et al.*, 1993; Thibodeau *et al.*, 1993). Additionally minisatellite mutations are much larger than those observed at microsatellite loci and are

not dependent on homogeneous arrays, i.e. mutants are also found in heterogeneous parts of the array which further suggests that the mutation processes involve mitotic recombination or unequal sister chromatid exchange, rather than replication slippage (Jeffreys, 1997; Jeffreys *et al.*, 1999).

Germline processes

The mutation processes at minisatellite loci appear to be much more complicated in the germline than those observed in somatic tissue, probably as a result of the action of different processes (reviewed by Jeffreys *et al.*, 1999). The use of MVR-PCR analysis has enabled the demonstration of a number of different features of germline mutation at human minisatellite loci. The repeat arrays at MS31, MS32 and MS205 show mutation polarity, where mutation events are only observed at one end of the repeat array (Jeffreys *et al.*, 1994; Jeffreys *et al.*, 1998b). The sperm mutation rates obtained from SP-PCR analysis of a number of different loci have shown a high degree of rate variation between different minisatellite loci, MS205, MS32, B6.7 and CEB1, shown mutation rates of 0.4, 0.8, 5 and 13 % per sperm respectively (reviewed by Jeffreys, 1997). In contrast to the situation observed for disease associated repeat expansions there is no evidence for the requirement of a threshold size for instability at minisatellite repeat arrays. Analysis of mutation rates and allele length has demonstrated a positive correlation of larger allele sizes and higher mutation rates for the loci CEB1 and B6.7; however MS32 and MS205 show no correlation between mutation rate and allele size. Most importantly the germline mutation rates obtained from SP-PCR analysis of sperm DNA are similar to the rates obtained from pedigree analysis; this suggests that SP-PCR analysis is not biased towards assessing mutations in sperm cells that would be non-viable.

The analysis of mutation rates in sperm DNA has demonstrated that they are up to 250 fold higher than those in somatic tissue (reviewed by Jeffreys *et al.*, 1994). The

predominant mutation mechanism is the gain or loss of a small number of repeats. The size distribution of sperm mutants remains constant between different minisatellite loci and is not dependent on allele size. Evidence exists showing that most alleles have a bias towards gains of repeat units and only very large alleles are prone to deletions, for example large CEB1 alleles. The types of rearrangements seen in sperm mutants are complex in nature and unlike the simple changes observed in somatic tissue; these rearrangements observed include target site duplications or deletions in the recipient alleles and multiple imperfect reduplications of donor and /or recipient alleles at the mutation junction.

1.6.1.2 Germline mutation processes

Inter and intra-allelic processes

The germline mutation processes at minisatellite loci are complex and involve both intra-allelic events (transfer of information within the repeat array of a single allele) and inter-allelic events (transfer of information between repeat arrays). Both inter- and intra-allelic events are observed for the five minisatellite loci which have been studied in depth (MS31, MS32, MS205, CEB1 and B6.7), however, variations are seen in the details of the mutation process between minisatellite loci (Buard and Vergnaud, 1994; Jeffreys *et al.*, 1994; May *et al.*, 1996; Buard *et al.*, 1998; Tamaki *et al.*, 1999; Buard *et al.*, 2000a; Buard *et al.*, 2000b). The processes involving interactions between alleles have been reviewed by Jeffreys *et al.* (1999). The acquisition of blocks of repeats from one allele into the array of another was frequently observed but the phase of markers in the DNA flanking the repeat array was mostly unaffected by the transfer event; this suggests that such inter-allelic events occur by gene conversion rather than crossover events. Indirect evidence from studies of MS32 shows that the transfer events are conservative (the gain of repeat units in one allele is not accompanied by an equivalent loss in repeats at the other); this suggests

that the transferred segments must be copied, again implying gene conversion like events (Jeffreys *et al.*, 1994). Because the processes of gene conversion and crossing over are recombination-biased and the fact that complex rearrangements at minisatellite loci are germline specific it has been suggested that the processes may take place during meiosis (Jeffreys *et al.*, 1997). The lack of germline mosaics at minisatellite loci also point to the process being meiosis specific (Jeffreys *et al.*, 1997).

Differences between loci

The mutation processes at a number of different minisatellite loci were described by Jeffreys *et al.* (1999). The processes acting at MS31, MS32 and MS205 in sperm are intensely polar; mutations are observed mainly at the 3′ end of the repeat array and these events are characterised by inter-allelic exchanges; in contrast intra-allelic processes are dominant at CEB1 (Buard and Vergnaud, 1994; Buard *et al.*, 2000a; Buard *et al.*, 2000b). The B6.7 locus shows the most complex rearrangements observed at any of the minisatellite loci studied to date, with evidence of both inter- and intra-allelic transfer. The mechanism of mutation at B6.7 suggests a pathway involving multiple rounds of copying information from one or both alleles which results in mutant alleles that are a patchwork of the repeat units observed in both of the progenitor alleles (Tamaki *et al.*, 1999).

1.6.1.3 Proposed mutation mechanism at minisatellite loci

No single model of recombinational instability has yet been developed to account for all of the different types of rearrangements observed at minisatellite loci (Jeffreys *et al.*, 1999). Jeffreys *et al.* (1999) suggests that mutations may be initiated by DNA double-strand breaks (DSBs) or by staggered nicks that extend into the repeat array, and that this would be followed by single-strand DNA invasion by the broken DNA strand into the other minisatellite allele (inter-allelic exchange) or into a sister chromatid (intra-allelic exchange). These events could be followed by DNA synthesis, DNA strand extrusion from the donor strand and then gap bridging in the recipient allele. This process is conservative, since there is no alteration to the length or repeat composition of the donor allele. Multiple rounds of strand invasion could account for the patchwork insertions seen at B6.7 and other loci. This type of model would be able to account for the large duplications and deletions; however, it is not able to explain the imperfect repeats seen in some loci e.g. MS32 (see Figure 1.6 for details). This type of model may explain the different types of mutations observed at minisatellite loci and was first proposed by Buard and Vergnaud (1994), then further developed by Jeffreys *et al.* (1994) and Buard and Jeffreys (1997).

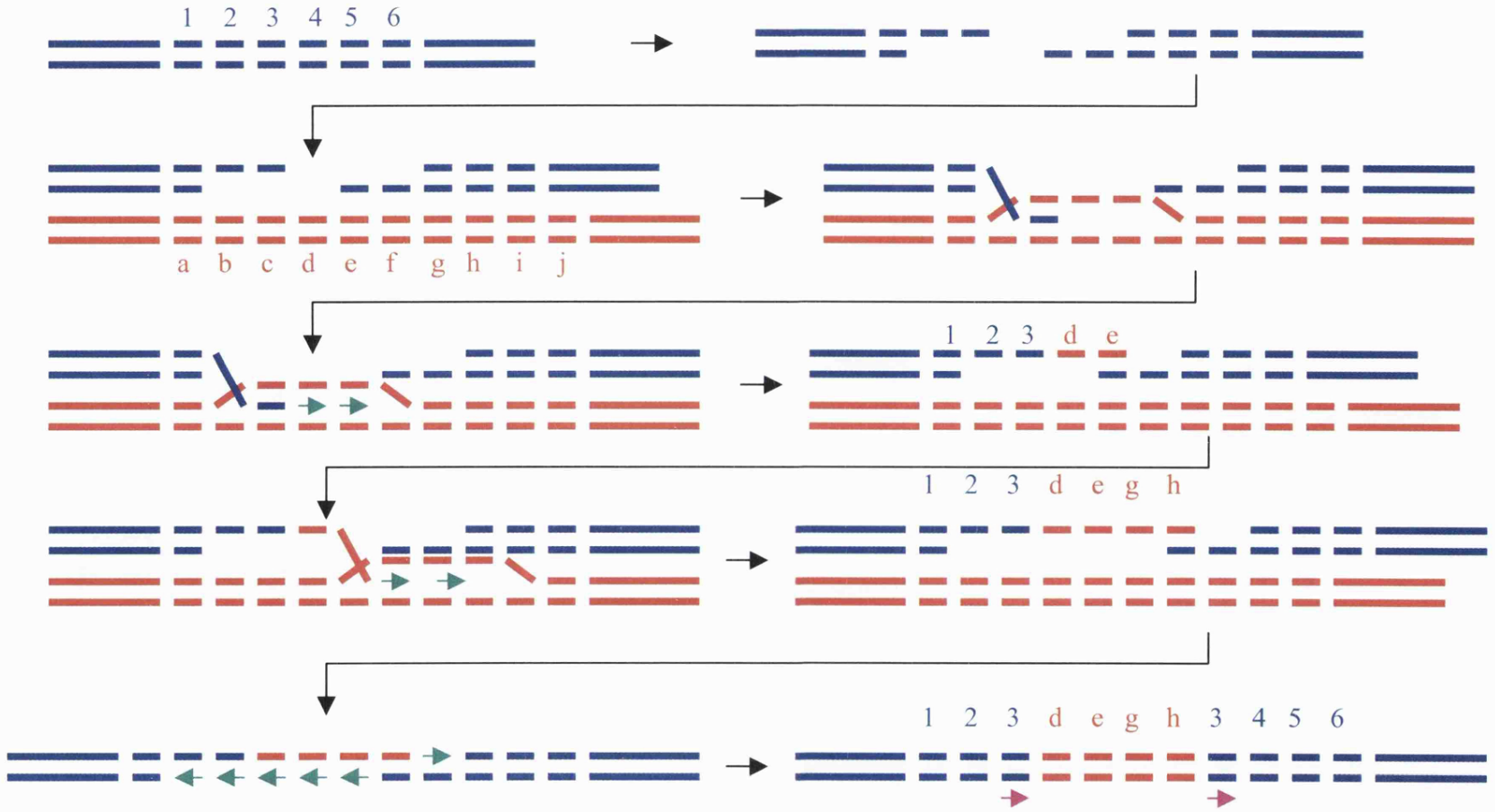


Figure 1.6 - Proposed model of minisatellite mutation.

Blue lines represent the initial (recipient) allele containing a double-strand break, red lines represent the donor allele, green arrows represent DNA synthesis, purple arrows represent duplicated segments in the mutant allele. Adapted from Jeffreys *et al.* (1999).

Minisatellite polarity and meiotic hotspots

In addition to the model of minisatellite mutation processes in the human germline studies have revealed another possible mechanism that may drive the instability process at minisatellite loci that show a tendency for polar mutations (MS31, MS32 and MS205). The study of MS32 has led to the characterisation of a recombination hotspot in the 3′ flanking region of the MS32 minisatellite which extends into the beginning of the repeat array (Jeffreys *et al.*, 1998a; Jeffreys *et al.*, 1998b). It has been suggested that the hotspot may drive the high levels of repeat instability and that it may also explain the polarity observed at the 3′ end of the MS32 repeat array (Jeffreys *et al.*, 1998a; Jeffreys *et al.*, 1999).

Evidence exists suggesting that the minisatellite loci MS31 and CEB1 are active during meiotic recombination, however, it has not been established whether hotspots are associated with these loci (Jeffreys *et al.*, 1998b). The analysis of DNA flanking the CEB1 minisatellite has shown a high density of single-nucleotide polymorphisms (SNPs) and shows extensive haplotype diversity which is consistent with elevated recombinational activity near the minisatellite. The analysis of mutant molecules at CEB1 has identified unequal crossovers, which are often associated with the transfer of DNA immediately flanking the repeat array, in the 5' end of the array suggesting that this region may be very active during meiosis. This analysis suggests that distinct recombination-processing pathways may produce both the simple crossovers and also the complex conversions that can extend into flanking DNA observed at CEB1 (Buard *et al.*, 2000b).

The analysis of DNA flanking the minisatellite arrays and other known recombination hotspots shows no obvious sequence determinants or secondary structure that may affect crossover efficiency (Jeffreys *et al.*, 1998b; Jeffreys *et al.*, 1999; Jeffreys *et al.*, 2000; Jeffreys *et al.*, 2001). It has however, been proposed that hotspot activity may be associated with open chromatin structure, as it is in yeast (Ohta *et al.*, 1994; Wu and Lichten, 1994). Recombination processes are known to be important in the production of

mutant alleles at minisatellite loci but the mechanisms are not yet fully understood; however, the current mutation model (Figure 1.6) may explain the majority of the common features of the mutation process observed at minisatellite loci. The analysis of recombination frequency in male mice exposed to ionising radiation or the anti-cancer drug cisplatin which is known to induce recombination in the germline of male mice (Hanneman *et al.*, 1997a), was initiated to assess the possibility that an elevation in meiotic crossing over events could explain mutation induction at ESTR loci (see Chapter 3 for details).

1.6.2 Microsatellites

The mutation processes at microsatellite loci are not as well characterised as those for minisatellite loci. Microsatellites usually contain only a single repeat type within a repeat array, unlike the variant repeats of minisatellite loci; therefore this means that the analysis of the internal structure of the repeat is not possible. A number of different systems have been utilised to assess mutation rates and processes at microsatellite loci.

1.6.2.1 Microsatellite mutation processes

The analysis of mutation mechanisms at microsatellite loci has concentrated mainly on the repeat types that are known to be associated with unstable repeat expansion disorders in the human genome. The analysis of the mechanisms at these loci have utilised familial studies, and *in vivo* and *in vitro* model systems. Whilst the mutation mechanism(s) at microsatellite loci remain unknown a number of common features have been associated with such studies and form the basis of the models proposed for mutation at both pathogenic unstable repeat expansions and other microsatellite repeats in the genome.

Mutation processes at unstable expanded repeats

The process of dynamic mutation is known to be affected by a number of factors, including, *cis*-acting factors (such as the repeat copy number) and whether the repeat array is interrupted with imperfect repeats.

The effects of repeat copy number have been analysed using many different systems and it has been shown that the length of the array of uninterrupted repeat units is one of the most important factors in the regulation of mutation rates at microsatellite loci. The *in vitro* analysis of the di-nucleotide locus *D2S123*, using small-pool PCR (SP-PCR) to investigate mutation rates in cell culture of non-tumour cells, showed that the mutation rate decreased with an increase in the number of variant repeats within the array (Bacon *et al.*, 2000). High levels of somatic and germline mosaicism have been noted in the analysis of Huntington's-affected individuals, with a reduction in the age of onset of the condition being positively associated with the inheritance of larger alleles through the parental germline (Telenius *et al.*, 1994; Aronin *et al.*, 1995). The PCR based analysis of single-sperm from Huntington's-affected individuals was able to demonstrate that the previously reported somatic mutation rates at the $(CAG)_n$ repeat were far less than those observed in the germline and that the most important factor for germline repeat instability was the size of the progenitor alleles (Leeflang *et al.*, 1995). The analysis of patents with adult onset myotonic dystrophy (DM) demonstrates that the levels of somatic heterogeneity increase with age and are dependent on the size of the progenitor allele (Monckton *et al.*, 1995; Wong *et al.*, 1995). Additional work by Leeflang *et al.* (1999) suggested that as no *cis* acting factors could be identified in the DNA surrounding the repeat array, which could account for the differences in mutation rate between individuals, then *trans*-acting factors must be important; this suggests that the molecular mechanism for repeat expansion at microsatellite loci is complex. Leeflang *et al.* (1999) suggested that the Okazaki model of

mutation could provide a satisfactory explanation for the expansions observed at microsatellite loci.

Trans-acting factors have been shown to affect mutation rates at expanded repeat loci, e.g. the congenital form of myotonic dystrophy shows a parental sex bias as it is often associated with maternal inheritance (Richards, 2001). Another possible *trans*-acting factor is the association of Okazaki fragment processing with repeat expansions, which occurs via the inhibition of Okazaki flap endonuclease (Gordenin *et al.*, 1997; Freudenreich *et al.*, 1998).

The analysis of Huntington's-affected individuals show high levels of somatic instability in the $(CAG)_n$ repeat, which is most pronounced in areas of the brain known to be affected during the pathogenic stages of Huntington disease (HD) repeat expansion; this suggests that there may be cell type specific factors which also affect repeat instability (Telenius *et al.*, 1994; Aronin *et al.*, 1995).

Microsatellite mutation in model systems

The analysis of microsatellite repeat instability in a number of model systems, especially those studies performed in yeast and *Escherichia coli*, supports the general findings seen in human studies and provide additional information on mutation mechanisms.

The study of Huntington disease in transgenic mice has been able to demonstrate cell-specific amplification of the CAG repeat, similar to that seen in human studies, further supporting the idea of cell specific triggers for microsatellite instability (Kennedy and Shelbourne, 2000). The analysis of tissue culture cells obtained from different organs of mice carrying the CNG tri-nucleotide repeat, associated with myotonic dystrophy (DM), shows high levels of germline and somatic instability, and tissue specific expansions (Gomes-Pereira *et al.*, 2001). Gomes-Pereira *et al.* (2001) however, reject the possibility that the expansions are due to mitotic index as no correlation was observed with the rates

of cell division; instead they suggest that the levels of repeat expansion may be governed by additional cell-type specific factors.

The use of yeast (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli*) as models has shown that the general processes of microsatellite mutation in these organisms are similar to those observed at loci associated with human unstable repeat disorders; for example the relationship of increased mutation rates with longer and uninterrupted repeat arrays holds true (Levinson and Gutman, 1987; Bichara *et al.*, 1995; Petes *et al.*, 1997; Wierdl *et al.*, 1997). It has also been observed in yeast that very large alleles are more prone to deletions; this may provide an explanation for the reductions in size observed at some highly expanded unstable repeats in the human genome (Wierdl *et al.*, 1997).

It has been proposed by Sturzeneker *et al.* (2000) that the analysis of the instability of microsatellite loci within tumour cells may provide a useful model system in which to analyse the mutation processes at these loci. Sturzeneker *et al.* (2000) have been able to demonstrate that the instability observed in tumour cells is positively correlated with the average levels of heterozygosity of a given microsatellite loci, and that the mutation rate also correlates with array length; therefore the mechanism acting within tumour cells should be representative of the situation within the germline. The data also provide support for a stepwise model of mutation at microsatellite loci.

1.6.2.2 Models of microsatellite mutation

The mutation mechanisms at microsatellite loci remain unknown. The loci do not lend themselves to the analysis of the internal structure of the repeat array because the repeat units within the array are identical and the insertion of variant repeats causes the mutation rate to decrease dramatically (Bois *et al.*, 2001). Therefore, a number of hypotheses have been developed which are based upon evidence obtained from the analysis of unstable repeat expansions within the human genome and the experiments

performed within model systems. The following section outlines the possible mechanisms and highlights the evidence in support of them.

The initial model which was proposed to explain the mode of mutation at microsatellites used a previous model developed by Ohta (1973) to explain the mode of mutation at protein coding genes. The model proposed a simple stepwise mode of mutation where each mutation event occurred independently and involved the gain or loss of a number of repeats, where the likelihood of gains and losses were the same. Over the years a number of studies have shown that the dominant mode of mutation involves the gain or loss of a small number of repeats (2 to 5 repeats being the most common range), however, other more complex events also appear to take place (Shriver *et al.*, 1993; Valdes *et al.*, 1993; Deka *et al.*, 1995). This resulted in the proposal of a process where most mutation events are small but in addition, rare large mutational events can also take place. This model can be used to explain the large expansions and contraction sometimes observed at microsatellite loci, especially those associated with human disease (Di Rienzo *et al.*, 1994).

The mathematical modelling of mutation processes at microsatellite loci in yeast have shown that the distribution and abundance of such loci in the genome can be explained by a balance between the processes of strand slippage and the accumulation of point mutations (Kruglyak *et al.*, 2000). This model is able to explain the 'birth' and 'death' of microsatellite loci by proposing initial expansion by slippage events followed by the introduction of point mutations which then decreases the expansion rate; this is then followed by an accumulation of point mutations which leads to the homogenisation of the structure of the repeat array.

The models proposed to explain mutations at microsatellite loci depend upon the action of a number of underlying biological processes which may act upon repeat loci. The potential processes acting to cause mutation at repeat arrays are discussed in the following section.

1.6.2.3 Potential biological processes acting at microsatellite loci

Slipped strand synthesis

The most commonly accepted process of mutation at microsatellite loci is that of slipped strand synthesis or replication slippage (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992; Strand *et al.*, 1993). Although no direct evidence is available to prove that this mechanism exists within biological systems a number of circumstantial pieces of evidence suggest that it is a likely process. The mechanism of slipped strand synthesis is outlined in Figure 1.7. Slipped strand synthesis refers to the out of register alignment of two strands of DNA containing a repeat element after the dissociation of the two stands for DNA replication. After dissociation most of the 3′ end rehybridises but a small section may form a loop structure which leads to an alteration in the final length of the repeat array. If the loop is formed on the nascent strand then the subsequent array will be longer; however, if the loop forms upstream (5') of the polymerase on the template strand the newly synthesised strand will be shorter than the progenitor allele.

Evidence supporting the slipped strand synthesis mechanism includes the observation that most mutant alleles are not associated with the transfer of flanking markers; this suggests that recombination events (unequal crossing over or gene conversion) are not a predominant mode of mutation and that the events are intra-allelic (Levinson and Gutman, 1987; Morral *et al.*, 1991). Additionally the rates of mutation for the Y chromosome appear to be similar to those of autosomes (Mahtani and Willard, 1993) which suggests that homologous chromosomes are not a pre-requisite for the major mutation mechanism acting at microsatellite loci (Kayser *et al.*, 2000). Furthermore mutations in the mismatch repair genes in yeast lead to an increase in the rates of instability at microsatellite loci, therefore suggesting that the repair of slipped strand mis-pairing is involved in the mutation process (Strand *et al.*, 1993). Finally *in vitro* studies have demonstrated that

repeat DNA sequences are able to undergo slippage like events (Schlotterer and Tautz, 1992).

Hairpin formation

The formation of hairpin structures during the replication of repeat arrays has been demonstrated *in vitro* by Mariappan *et al.* (1996). It has also been shown that such secondary structures inhibit the action of DNA polymerases (McMurray, 1999), and could therefore lead to slippage-like events being initiated due to polymerase stalling.

Okazaki fragment hypothesis

The other most favourable biological process to explain mutations at microsatellite loci, especially with reference to large repeat expansions, is the Okazaki fragment hypothesis, which was initially suggested as a possible mechanism by Richards and Sutherland (1994) (see Figure 1.8). Mutations in the yeast flap-endonuclease gene (FEN1) have been shown to increase the rates of tri-nucleotide repeat expansion (Freudenreich *et al.*, 1998). Further analysis has demonstrated that flap-endonuclease, which is responsible for the removal of the RNA primers from Okazaki fragments during replication may be affected by the formation of hairpin structures at tri-nucleotide repeats (McMurray, 1999). If the RNA primer is not removed but is displaced to the 3′ end of the next Okazaki fragment this will lead to an increase in the size of the subsequent repeat array. It has been suggested that the bimodal distribution of instability observed between small and large changes in copy number corresponds to the size of an Okazaki fragment (Richards, 2001).

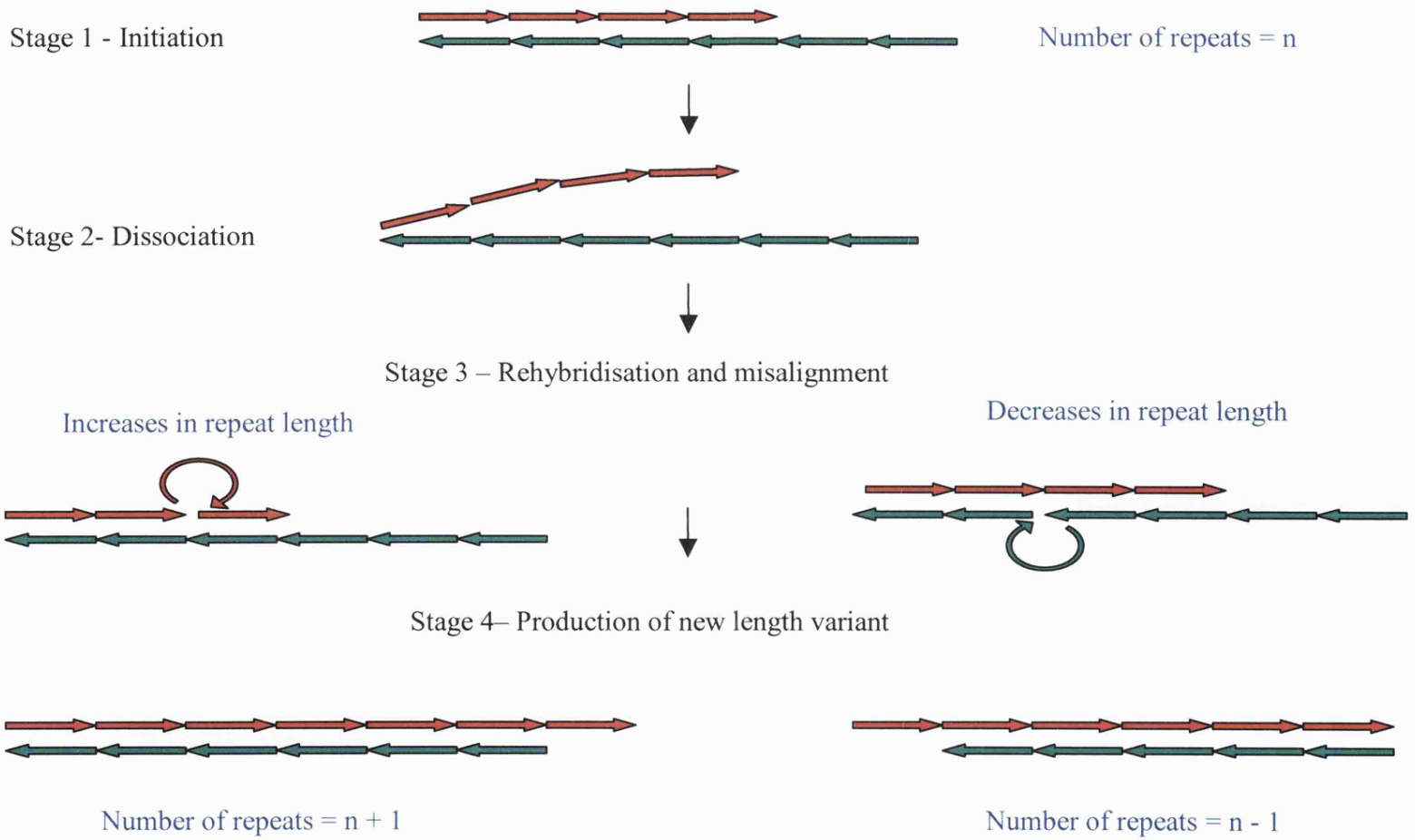


Figure 1.7 - Slipped-strand mispairing model for microsatellite mutation. Adapted from Ellegren, H. (2000).

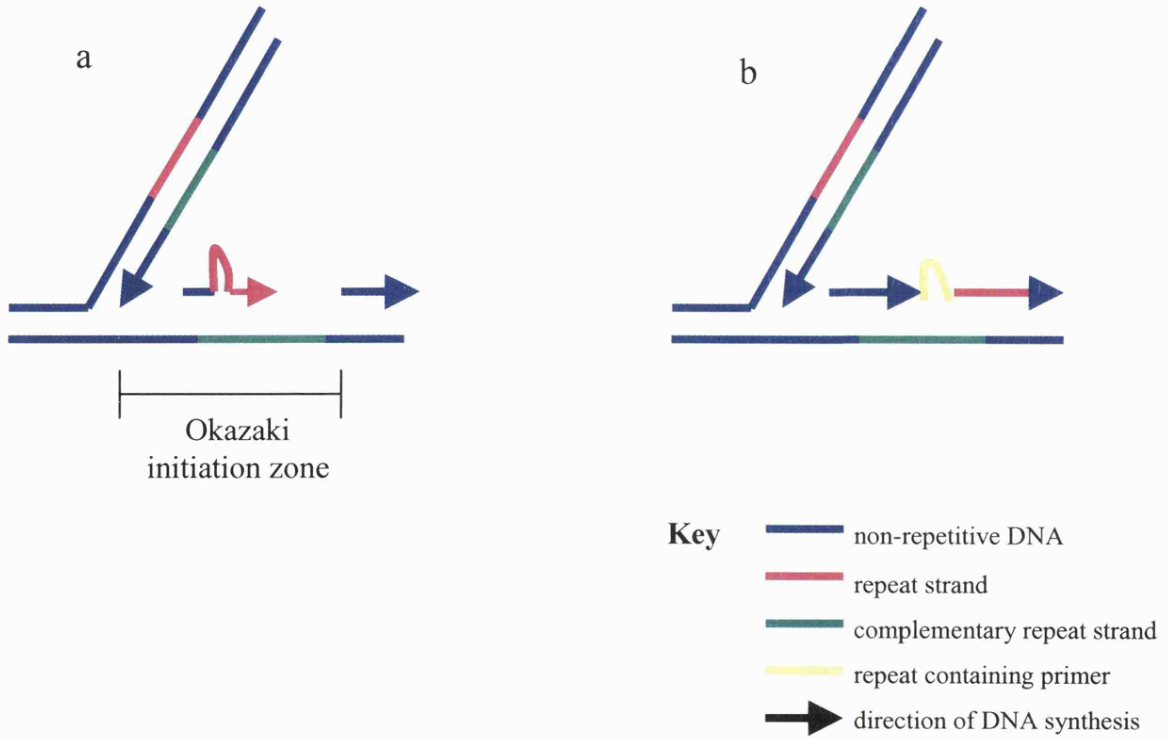


Figure 1.8 - Okazaki fragment hypothesis.

a) Hairpin formation. Expansion may be caused by the formation of a hairpin in the lagging strand and followed by misalignment of the lagging stand and its template.

b) RNA displacement. The RNA primer is not removed but ligated to the 3` end of the next Okazaki fragment leading to expansion

Adapted from Mirkin and Smirnova (2002).

Recombination based processes

The possibility that recombination processes may also act at microsatellites remains unresolved (as reviewed by Richard and Paques, 2000). Evidence from Paques *et al.* (1998), in the yeast *Saccharomyces cerevisiae* shows that the induction of DNA double-strand breaks in the yeast genome may lead to the repair of repeat sequences by the interaction with a donor molecule in a manner which is consistent with the synthesis-dependent strand annealing hypothesis (Paques and Haber, 1999). In the synthesis-dependent strand-annealing model two newly synthesised DNA strands unwind and anneal to each other; this may then cause size changes at repeat regions by out-of-frame re-annealing leading to reciprocal exchanges of DNA fragments. Richard and Paques (2000) suggested that mitotic and meiotic double-stand breaks could initiate such a mechanism. Further evidence from Jankowski *et al.* (2000) demonstrates that the level of meiotic instability in yeast at long CAG tracts correlates with double-stand break formation in the DNA flanking the repeat array. Evidence has also been obtained showing that mutation rates at microsatellite loci are higher at meiosis than mitosis (Cohen *et al.*, 1999); however, other studies fail to show such phenomena (Moore *et al.*, 1999). It may be possible to explain large expansion events at microsatellite loci by successive rounds of strand invasion and DNA synthesis, similar to that seen for complex re-arrangements at minisatellite loci (Paques *et al.*, 1998) (see Figure 1.6). However, recombination processes cannot account for the most predominant type of mutations observed at microsatellite loci and further analysis in yeast shows that microsatellite instability is not affected by mutations in genes involved in recombination (Henderson and Petes, 1992).

Position effects

It has been suggested that position effects or *cis* acting elements may have a role in the mutation processes at microsatellite loci (Richards, 2001). A study by Cleary *et al.* (2002) analysing $(CAG)_n$•$(CTG)_n$ repeat arrays within COS cells in culture was able to demonstrate the importance of the position of the repeat with respect to the origin of replication. If the distance of the repeat array from the origin of replication was altered then the mutation processes at the repeat array also changed, and the mode of mutation switched from gains to losses of repeats. Cleary *et al.* (2002) suggest that the position effect is due to the fact that the repeat has been moved within the 'Okazaki initiation zone'; they also propose in their model that that tri-nucleotide repeats expand when they are positioned at the 3′ end of the Okazaki initiation zone and contract when at the 5′ end.

Additional support for the influence of position effects on mutation rates at unstable expanded repeats derives from the fact that all chromosomes which carry the expanded $(CTG)_n$ repeat at the myotonic dystrophy 1 locus also contain an *Alu* element 5 kb from the repeat (Neville *et al.*, 1994); this suggests that a change in the distance from the origin of replication to the repeat array may be able to account for the bias towards gains of repeats in the disease associated allele, this has been termed an 'ori-shift' (Mirkin and Smirnova, 2002). Where no insertion/deletion polymorphisms have been identified it has been suggested that either an inactivation of the normal origin of replication may have occurred, or that a cryptic origin of replication may have been activated; such effects have been termed an 'ori-switch'. Mirkin and Smirnova (2002) suggest that the 'ori-shift/switch' hypothesis may explain the underlying processes of mutation at unstable repeat expansions associated with human disease as 'ori-shift/switch' events may lead to the activation or inactivation of instability at such repeat loci.

Relevance to mutation processes at ESTR loci

Although the exact mechanism(s) of mutation at microsatellite loci remain unknown, due to the similarities between the mutation spectra and rates at microsatellite and ESTR loci it may be possible that the mechanisms hypothesised to account for mutations at microsatellites may also be responsible for mutation processes at ESTR loci. The repeat expansions identified at *DM2* and *SCA 10* show a high level of similarity with ESTR loci in the mouse genome, e.g. the size of the repeat units is similar, with *DM2* and *Hm-2* having tetra-nucleotide repeat units, and *SCA 10* and *Ms6-hm* being penta-nucleotide repeats. Additionally the repeat units of the *DM2* and *Hm-2* loci are remarkably similar, CCTG and GGCA (CCTG as a reverse complement). Furthermore the sizes of the repeat expansions are comparable, *Ms6-hm* having repeat arrays up to 15 kb, *Hm-2* up to 22 kb, *SCA 10* up to 22 kb and finally *DM2* with repeat expansions of up to 44 kb in length. The somatic instability reported for the *DM2* locus is also reminiscent of the high levels observed at mouse ESTR loci. These similarities between the mouse ESTR loci and the expanded repeats in the human genome further suggest that these loci may mutate via similar mechanisms.

1.7 Approaches to assess mutation mechanisms at ESTR loci in the mouse germline

The work presented in this thesis aims to investigate the processes of mutation induction at expanded simple tandem repeat (ESTR) loci in the mouse germline. The possibility that mutation induction at ESTR loci may occur as a by-product of meiotic recombination was investigated by analysing a possible correlation of ESTR mutation rate with crossover frequencies in the male germline after exposure to mutagenic agents (Chapter 3).

I have also addressed the possibility that mutation process at ESTR loci may be controlled by DNA repair genes by the analysis of mice with defects in DNA repair. The role of DNA double-strand break repair via non-homologous end joining was investigated by the analysis of mutation induction after exposure to ionising radiation in mice carrying the naturally occurring severe combined immunodeficient (scid) mutation (Chapter 4). An assessment of a number of functionally relevant single nucleotide polymorphisms in the *Prkdc* gene (coding for the DNA-PKcs protein) and *p16* gene products may also provide evidence for the role of DNA repair in the mutation processes at ESTR loci (Chapter 4).

The long-term effects of exposure to ionising radiation are also addressed by the analysis of ESTR mutation rates in the germline of subsequent generations of mice after initial paternal exposure (Chapter 5). The information from this type of analysis may provide evidence for the mode of inheritance through which radiation-induced instability is passed onto subsequent generations. The overall aim of the work is to further the understanding of the mechanism(s) of mutation induction at ESTR loci, in order that we may be able to validate their use as a method of monitoring mutation induction in the mammalian germline.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Chemical reagents

Chemicals were obtained from Fisher Scientific (Loughborough, UK), Flowgen (Ashby de la Zouch, UK), BioWittaker Molecular Applications (BMA) (Rockland, USA), and Sigma-Aldrich Company Ltd. (Poole, UK).

Molecular biology reagents were obtained from ABgene (Epsom, UK), Ambion, Inc. (Austin, USA), Amersham Biosciences (Little Chalfont, UK), Applied Biosystems (Warrington, UK), Bio-Rad (Hemel Hemstead, UK), BioWittaker Molecular Applications (BMA) (Rockland, USA), Invitrogen UK (Paisley, UK), Millipore (Watford, UK), New England Nuclear (NEN) Life Sciences (Division of Perkin-Elmer Life Sciences Ltd, Zaventem, Belgium), New England Biolabs (NEB) (Hitchin, UK), Promega (Southampton, UK), Qiagen Ltd. (Crawley, UK), ResGen (Division of Invitrogen Ltd, Paisley, UK), Sigma-Aldrich Company Ltd (Poole, UK), Stratagene (Amsterdam, The Netherlands) and United States Biochemical Corp (USB) (Cleveland, USA).

Specialised equipment was obtained from Bio-Rad (Hemel Hempstead, UK), Cecil Instruments (Cambridge, UK), Eppendorf (Hamburg, Germany) Fisher Scientific (Loughborough, UK), Genetic Research Instrumentation (GRI) (Braintree, UK), Helena Biosciences (Sunderland, UK), Heraeus Instruments (Hanau, Germany), ThermoHybaid (Ashford, UK), MJ Research (Waltham, USA), Applied Biosystems (Warrington, UK), Thermo Shandon (Pittsburgh, USA), Turner Designs (Sunnyvale, USA), and Ultra Violet Products (UVP) Life Sciences (Cambridge, UK).

2.1.2 Enzymes

Proteinase K for DNA extraction was supplied by Sigma-Aldrich. Restriction enzymes were supplied by Invitrogen, using the REact™ buffer series or New England Biolabs, using buffers 1 to 4. A cloned version of the Klenow fragment of DNA polymerase I of *Escherichia coli* produced by USB and supplied by Amersham Biosciences was used. The *Taq* DNA polymerase and cloned *Pfu* polymerase were obtained from Abgene and Stratagene respectively.

2.1.3 Molecular weight markers

1 kb ladder was supplied by Invitrogen. ΦX174 DNA digested with *Hae*III and λ DNA digested with *Hin*dIII were obtained from ABgene.

2.1.4 Oligonucleotides

Oligonucleotides for the PCR amplification of ESTR probes and for the RFLP analysis of allelic variation in mice strains were synthesised in-house (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, UK). Oligonucleotides used for the PCR analysis of microsatellite loci were obtained from ResGen or Applied Biosystems. The hexadeoxyribonucleotides (or Pd6 random hexamers) were obtained from Amersham Biosciences.

2.1.5 Mice

F_1 hybrid males (C57BL/6J x CBA/Ca) and CBA/Ca females were purchased from Harlan-Olac Ltd. (Bicester, UK). CBA/H, C57BL/6H and BALB/c inbred mice strains and the F_1 hybrid C57BL/6H x CBA/H males were obtained from The Medical Research Council Radiation and Genome Stability Unit (Harwell, UK). The C.B17 and scid mice were received from MCG-Department of Radiation Genetics and Chemical Mutagenesis (Leiden University, The Netherlands) and originated from the colony of Dr Phillips (Toronto, Canada).

2.1.6 Standard solutions

Southern blot solutions (depurinating, denaturing and neutralising), 20 x Sodium Chloride Sodium-Citrate (SSC) buffer and 10 x Tris-borate/EDTA (TBE) electrophoresis buffer, were made as described by Sambrook (Sambrook and Russell, 2001) and were supplied by the Media Kitchen (Department of Genetics, University of Leicester).

2.1.7 Computers

This thesis was produced using a Pentium III PC, an Epsom Perfection 1240U scanner and was printed on an HP4000 6MP LaserJet printer. DNA sequences were analysed using an Silicon Graphics mainframe computer, operating the Genetics Computer Group (GCG) Sequence Analysis Software Package version 10.0 programs, developed at the University of Wisconsin (Devereux *et al.*, 1984). Data were stored, analysed, and presented using a number of versions of the software packages Adobe Acrobat, Adobe Photoshop, Autoassembler, EndNote, Factura, Freehand, Microsoft Word, Microsoft Excel, and Microsoft PowerPoint for Macintosh computers or PC's, as appropriate. Internet

searches were performed using Microsoft Internet Explorer, the Sherlock search engine, and Netscape Navigator.

2.2 Methods

2.2.1 Mice

2.2.1.1 Housing

Leicester University

Mice for the cisplatin section of the recombination study, both control and treated, were maintained in isolators by the Biomedical Services staff at the Medical Research Council Centre for Mechanisms of Human Toxicity, Lancaster Rd, Leicester, LE1 6YT.

Harwell

Mice used in the radiation exposure experiments for the recombination and transgeneration studies were bred and maintained under standard conditions in the animal facilities at the MRC Radiation and Genome Stability Unit, Harwell, Oxon, OX11 ORD.

Leiden

Scid and the isogenic C.B17 strains were bred and maintained in the facilities at MCG-Department of Radiation Genetics and Chemical Mutagenesis, Sylvius Laboratory Animal House, Leiden University, Wassenaareseweg 72,2333 AL Leiden, The Netherlands. The scid colony is maintained under semi-sterile conditions, and the C.B17 mice under standard conditions.

All animal procedures based in the UK were carried out under guidance issued by the MRC in 'Responsibility in the use of animals for medical research' (July 1993). Animal procedures preformed in Leiden were carried out under the direction of The Netherlands 'National regulations for animal studies'.

2.2.2 Treatment of mice

2.2.2.1 Recombination study

Cisplatin (PPL 80/1353)

F_1 hybrid males (C57BL/6J x CBA/Ca) were given a single intraperitoneal injection of cisplatin (Sigma-Aldrich) at 10mg/kg, dissolved at 1 mg/ml in 0.9% (w/v) NaCl.

A separate study was performed (by A. Smith) where males were injected with 15mg/kg dose of cisplatin then culled a week later for the histological analysis of testis tissue.

Radiation (PPL 30/689 & 30/1272)

F_1 hybrid males (C57BL/6H x CBA/H) were exposed to whole body irradiation of 1 Gy X-rays delivered at 0.5 Gy/min (250 kV constant potential, HLV 1.2 mm Cu).

All male mice used for the recombination study were bred with untreated females to produce offspring that were profiled at 25 microsatellite loci, and three ESTR probes (for details see Chapter 3 Section 3.2).

2.2.2.2 Analysis of *scid* mutation

Male C.B17 and scid mice were anaesthetised with 0.06 mg Nembutal/g of body weight and immobilized in the sapine position with the anterior part of the body shielded with 6 mm of lead to allow only testicular irradiation. 1 Gy of X-rays were delivered at a dose rate of 0.6 Gy min^{-1} (200kV and 4 mA resulting in an HVL of 1.0 mm Cu) using an Andrex SMART 225 machine.

All males, both C.B17 and scid were mated with untreated C57BL/6 females and the offspring were profiled with ESTR probes (see Chapter 4 Section 4.2 for details).

2.2.2.3 Transgeneration study

Neutron exposure

Male CBA/H and C57BL/6 mice were exposed to 0.4 Gy of fission neutrons (^{252}Cf source, 0.03 Gy/min).

X-ray exposure

Additional CBA/H males were exposed to whole body irradiation of 2 Gy of X-rays and male BALB/c mice were exposed to 1 Gy of X-rays, both delivered at 0.5 Gy min^{-1} (250 kV constant potential, HLV 1.2 mm Cu).

Breeding scheme

Male mice exposed to fission neutrons or X-rays were mated with untreated females of the same inbred strain at either 3 or 6 weeks after irradiation and the offspring were profiled with ESTR probes (see Chapter 5, Section 5.2 for details).

2.2.3 Preparation of mouse DNA

Tissue samples were obtained from mice killed by a Home Office Schedule One approved method (Appropriate methods of humane killing (Appendix 1 – Schedule 1), 1990). Tail samples provide the preferred tissue for DNA extraction, but spleen was used if the initial extraction failed. The details of DNA extraction are given in Section 2.3.

Tissue samples were provided for the mice treated in Leicester or Harwell, but the samples from Leiden were received as DNA.

2.3 Methods for DNA extraction

2.3.1 Proteinase K digestion of tissue samples

Approximately half of the chosen tissue was finely chopped with a scalpel and suspended with 1ml of Lysis Buffer A (0.1 M NaCl, 25 mM EDTA pH 8.0, 20 mM TrisHCl pH 8.0), 1ml of Lysis Buffer B (1% (w/v) SDS, 12.5 mM EDTA pH 8.0, 10 mM TrisHCl pH 8.0) and 30 µl of Proteinase K (25 mg/ml) in a 15 ml Eppendorf Phase Lock Gel ^{TM} Light tube. The contents were mixed by inversion and incubated at 55°C for 5 hrs or overnight with occasional mixing. DNA was separated from proteins and bone by phenol/chloroform and chloroform extractions (see Section 2.3.2). DNA was precipitated from the aqueous layer and resuspended in ultrapure water (see Section 2.3.3 for details).

2.3.2 Phenol/Chloroform extraction

Phenol/chloroform and chloroform were used to purify genomic DNA away from proteins and bone after an initial digestion with Proteinase K (see Section 2.2.3). Half the volume of phenol/chloroform (Phenol: Chloroform:Isoamyl alcohol in the ratio 25:24:1, equilibrated with Tris-HCl pH 8.0) was added to the each sample and the mixture was emulsified by repeated inversion. The samples were separated using Phase Lock Gel^{TM} tubes, which rely on the physical separation of the aqueous layer from the contaminating solvent and protein phases. Additional phenol/chloroform extractions were occasionally required to obtain a clear aqueous layer. A final extraction with chloroform was used to remove traces of phenol, the DNA was then ethanol precipitated (see Section 2.3.2).

2.3.3 Ethanol precipitation

In order to recover DNA following phenol/chloroform extractions, ethanol precipitation was used. The aqueous layer was transferred into a 15 ml polypropylene tube containing two volumes of 100% (v/v) ethanol and 1/10 volume of 3 M NaAc (pH 5.5). The solution was gently mixed by inversion to precipitate the DNA, which was removed into a 1.5 ml eppendorf containing 80% (v/v) ethanol. DNA was pelleted by centrifugation in a bench top microcentrifuge for 1 min at 13,000 rpm. The ethanol was removed with a pipette and the pellet left to air dry. Finally, the DNA was resuspended in ultrapure water to achieve a stock DNA concentration of between 200 and 1000 ng/µl.

2.4 PCR

2.4.1 PCR buffers

2.4.1.1 10 x reaction buffer

Microsatellite and RFLP analyses were performed using a 10 x reaction buffer (750 mM Tris-HCl (pH8.8), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween20) with an appropriate quantity of $MgCl_2$ (25 mM), both supplied with the *Taq* polymerase (ABgene).

2.4.1.2 11.1x PCR buffer (Jeffreys *et al.*, 1990)

This buffer was produced in the laboratory by R. Neumann using the components outlined in Table 2.1. The dNTP solutions (as sodium salts) were supplied by Promega and the ultra pure (non-acetylated) BSA was supplied by Ambion. This buffer was used for the PCR amplification of the synthetic oligonucleotide probes used for Southern hybridisation (as explained in Section 2.4.2.3).

Component	Concentration of stock solutions	Final concentration in the PCR reaction
Tris-HCl pH 8.8	1 M	45 mM
Ammonium Sulphate	1 M	11 mM
$MgCl_2$	1 M	4.5 mM
2-mercaptoethanol	100%	0.045 %
EDTA pH 8.0	10 mM	4.4 µM
dATP	100 mM	1 mM
dCTP	100 mM	1 mM
dGTP	100 mM	1 mM
dTTP	100 mM	1 mM
BSA	50 mg/ml	13 µg/ml

Table 2.1- Components of 11.1x PCR Buffer (see text for details).

2.4.2 PCR protocols

DNA was amplified using the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988) Reactions were reformed using a PTC-220 DNA Engine Peltier thermal cycler (MJ Research).

2.4.2.1 PCR conditions used for microsatellite crossover analysis

PCR reactions were amplified in a 15 µl reaction using 0.2 mM of dNTPs, 0.2 µM of primers, 25 and 75 ng of template DNA, 0.5 units of *Taq* polymerase (ABgene), 1 x PCR buffer (ABgene) and 1-3 mM of $MgCl_2$.

Amplification was performed in thin-walled 96 well-plates (ABgene), after denaturing at 96°C for 5 min, PCR reactions were cycled at 96°C for 30 sec, 52-60°C for 30 sec (primer dependent, see Table 2.2 for details), and 72°C for 1 min for 28 cycles, ending with a 10-min incubation at 72°C.

PCR-primers for 25 mouse microsatellites were obtained from Research Genetics and Applied Biosystems. Sequences for the PCR primers were obtained from the MIT database at the Jackson Laboratory web page (http://www.jax.org/) and in Appendix 1. The chromosomal assignment of microsatellite loci, sizes and individual PCR conditions are given in Table 2.2.

2.4.1.2 PCR conditions for RFLP analysis

PCR reactions were set up in 15 µl containing 0.2 mM of dNTPs, 1 or 2 µl of unquantified genomic DNA (diluted at 1 µl stock DNA in 100 µl of water), 0.25 units of Taq polymerase (ABgene), 1 x PCR buffer (ABgene) and 1.5 mM of $MgCl_2$. Different concentrations of primers were added due to problems with primer-dimer formation interfering with the discrimination of allelic variants after restriction digesting (see Table 2.3 for details).

Amplification was performed in thin-walled 96 well-plates (ABgene), after denaturing at 94°C for 5 min, PCRs were cycled at 94°C for 30 sec, 58 or 60°C for 30 sec and 72°C for 1 min for 28 cycles, ending with a 10-min incubation at 72°C. See Table 2.3 for details of conditions of the different loci studied.

RFLP	Expected Size (bp)	Primer concentration	Annealing temperature
p16 A134C	176	1 μM	60°C
p16 G232A	330	1 μM	60°C
Prkdc M3844V	139	1 μM	60°C
Prkdc R2140C	514	1 μM	58°C
scid	69	0.2 μM	60°C

Table 2.3 - PCR conditions for RFLP analysis

2.4.1.3 PCR conditions for production of synthetic oligonucleotide probes

PCR reactions for Ms6-hm were performed in 20 μl reactions with 2 μl of 11.1 x PCR buffer as described above, plus 1 μM of each primer, and 2.5 U *Taq* polymerase. The reactions used to produce the Hm-2 and MMS10 were performed in a total volume of 7 μl containing 0.63 μl of 11.1xPCR buffer, 0.4 μM of each primer and 4.5 U *Taq* polymerase.

Note that no input DNA was required as the repeat specific primers also act as a template, producing various lengths of pure repeat sequence.

Primer pair	Allele size C57BL (bp)	Allele size CBA (bp)	Annealing temperature	$MgCl_2$ conc^n
D1Mit15	160	183	60°C	3 mM
D1Mit17	170	183	60°C	1 mM
D1Mit156	143	112	60°C,	3 mM
D1Mit187	147	163	58°C	2 mM
D1Mit231	267	219	55°C	2.5 mM
D2Mit265	103	146	58°C	3 mM
D2Mit412	128	104	60°C	3 mM
D2Mit420*	126	90	52°C	1.5 mM
D2Mit433	176	162	60°C	3 mM
D3Mit49	128	110	58°C	3 mM
D3Mit93	164	174	58°C	3 mM
D3Mit116	263	275	60°C	1.5 mM
D3Mit127	176	188	58°C	1.5 mM
D4Mit54	150	174	58°C	1.5 mM
D4Mit149	114	130	58°C	2 mM
D4Mit286	96	76	58°C	2 mM
D4Mit308	88	116	60°C	1.5 mM
D8Mit95	154	144	60°C	2 mM
D8Mit208	146	170	58°C	1 mM
D8Mit292	68	98	52°C	2 mM
D8Mit320	124	110	60°C	1 mM
D10Mit10	180	128	55°C	2.5 mM
D10Mit80	158	144	58°C	3 mM
D10Mit194	70	84	58°C	3 mM
D10Mit271	116	104	58°C	3 mM

Table 2.2 – PCR conditions for microsatellite markers.

*The number of PCR cycles was increased from 30 to 35.

2.4.3 PCR purification

2.4.3.1 Purification for sequence analysis

PCR products were cleaned up prior to performing sequencing reactions with an ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. This was performed by electroelution – the band was excised from the agarose gel and transferred to a slot cut within a second gel, slightly wider than the excised fragment. A piece of dialysis membrane was prepared by soaking in distilled water for at least ten minutes. The membrane was then inserted into the gel slot curled under, and folded over the excised band. The gel was run at 4 V/cm allowing the DNA to electroelute onto the membrane. Electroelution was monitored using a DarkReader hand-held wand. With continuous application of the current, the membrane was smoothly removed from the gel and placed into a microcentrifuge tube with a corner of the membrane trapped in the lid. Droplets of buffer containing the DNA fragment of interest were collected from the dialysis membrane by centrifugation at 15 000 rpm for 30 seconds in an Eppendorf centrifuge 5415 D, followed by rinsing the membrane with 20 µl of distilled water. DNA was recovered from solution by ethanol precipitation.

2.4.3.2 Purification for RFLP analysis

The *Prkdc* M3844V PCR product required purification prior to restriction digestion with *Hph*I, as the PCR buffer was incompatible with the conditions required by the *Hph*I enzyme. The PCR buffer was however, compatible with the conditions required for the other enzymes used. The clean-up procedure was performed using a QIAquick PCR Purification Kits (Qiagen) as per the manufacturer's instructions.

2.4.3.3 Purification for probe purification

PCRs used as ESTR probes were also purified using a QIAquick PCR Purification Kits (Qiagen) using the manufacturer's instructions.

2.5 DNA Sequencing

Sequencing was carried out using an Applied Biosystems Model 377 DNA Sequencing System, with the ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, in accordance with the manufacturer's instructions. Sequencing reactions were cycled at 96° for 10s, 60° for 4 min for 25 cycles using ~20 ng/kb of DNA template. Reactions were purified by ethanol precipitation and dissolved in 2 µl 83% (v/v) de-ionised formamide, 8.3 mM EDTA, prior to loading onto the sequencing gel. Gel running was performed by the Protein and Nucleic Acid Laboratory (PNACL), University of Leicester. Sequence data was edited and analysed using Factura™ Release 1.2.0 (Applied Biosystems) and Autoassembler Release 1.4.0 on Apple Macintosh computers.

Sequence data generated on the *Mus musculus* partial *Prkdc* gene sequences for the DNA dependent protein kinase catalytic subunit, intron 80 and partial exon 81 sequences were deposited with the GenBank/EMBL Data Libraries under Accession Nos. AJ315658, AJ315659, AJ315660 and AJ315661, the mouse strains BALB/c, C.B17, CBA/H and C57BL6/H respectively. The other sequences analysed did not differ from the database sequences available and were therefore not submitted.

2.6 Manipulation of DNA

2.6.1 Restriction endonuclease digest

2.6.1.1 RFLP analysis

15 µl of PCR product for each RFLP was digested with 5 U of the appropriate diagnostic enzyme (New England Biolabs or Invitrogen) using temperatures and reaction buffers according to manufacturer's recommendations, see Table 2.4 for details.

2.6.1.2 Southern blot analysis

Digests were performed on a standard volume of DNA (15.5 µl of unquantified DNA) using 18 U of *Alu*I (Invitrogen) using the appropriate REact™ buffer (REact I). Incubations were performed at 37°C for 5 hours or overnight to ensure complete digestion.

2.6.2.3 Estimation of DNA concentration

DNA concentration was assayed by the measurement of fluorescence at a wavelength of 365 nm in a fluorometer (TD-360 Mini-Fluorometer, Turner Designs, CA, USA) using 2 µl of digested genomic DNA in a Hoechst (H 33258)/TNE solution, 1µg/ml Hoechst (Sigma-Aldrich) in 1 x TNE buffer (0.2M NaCl, 10 mM Tris-HCl, 1mM EDTA pH 7.4).

Polymorphism	Restriction enzyme	Supplier	Reaction Buffer	Digest temp.	Normal allele size (bp)	Variant allele size (bp)	% Gel for resolution (w/v)
p16 A134C	*Nla*III	NEB	NEBuffer 4	37° C	76 + 52 + 48	128 + 48	4 %
p16 G232A	*Bsa*AI	NEB	NEBuffer 3	37° C	307 + 23	307	2 %
Prkdc M3844V	*Hph*I	NEB	NEBuffer 4	37° C	91 + 48	139	4 %
Prkdc R2140C	*Bsm*BI	NEB	NEBuffer 3	55° C	319 + 195	514	2 %
scid	*Alu*I	Invitrogen	REact 1	37° C	69	41 + 28	4 %

Table 2.4- Digest information for RFLP analysis

The data provided show the diagnostic restriction fragment length polymorphisms that led to functionally relevant single nucleotide changes (details of the data are presented in Chapter 5, Section 5.7) and for the diagnostic restriction fragment length polymorphism for the *scid* mutation, a single nucleotide polymorphism that results in the loss of the terminal 83 amino acids of the *Prkdc* gene (see Chapter 4 for details).

2.7 Agarose gel electrophoresis

2.7.1 Microsatellite crossover analysis

10 µl of the PCR products were electrophoresed through 4% (w/v) agarose gel (MetaPhor agarose, BMA) in 0.5 x TBE, containing 0.5 µg/ml ethidium bromide for 1- 3 hrs at 200V using Bio-Rad power-packs, to allow separation of the two-allele sizes (for details of allele size see Table 2.2). The marker ΦX174 DNA digested with *Hae*III was used which provides an adequate range of standard bands for assigning the size of the microsatellite alleles. Electrophoresis tanks were produced in-house (Bio/Medical Joint Workshop, University of Leicester, University Rd, Leicester, LE1 7RH), to a design that allows gel loading with a multichannel pipette and 96 products, plus size standards to be analysed per gel.

2.7.2 RFLP analysis

The *p16* single-nucleotide-polymorphisms (SNPs) A134C and G232A, *Prkdc* functional polymorphisms M3844V and R2140C and *scid* determining digests were visualised by electrophoresis of 15µl of PCR product, after restriction digestion with the appropriate diagnostic enzymes, on 2% (w/v) LE agarose (SeaKem™, BMA) or 4% (x/v) agarose gel (MetaPhor agarose, BMA) depending on expected size of restricted products, (see Table 2.4 for details). Electrophoresis was performed in 0.5 x TBE, containing 0.5 µg/ml ethidium bromide for 1- 3 hrs (dependent on product size and allele size difference) at 200V, to allow separation of the two-allele sizes. The marker ΦX174 DNA digested with *Hae*III was used as this provides adequate size standards for assessing the size of the restricted fragments. The information concerning electrophoresis tanks and power packs are given in Section 2.7.1.

2.7.3 Southern blotting

Gel electrophoresis was carried out using 0.8% (w/v) LE (SeaKem™, BMA) agarose gels in a 40 cm horizontal submarine format with 1 x TBE (44.5 mM Tris-borate pH 8.3, 1 mM EDTA) buffer containing 0.5 μg/ml ethidium bromide. Electrophoresis tanks were manufactured in-house (as before). Loading dye (5x TAE, 12.5% (w/v) Ficoll 400, 0.1% (w/v) Bromophenol Blue) was added to 5 μg of DNA samples to make a total volume of 20 μl. DNA samples were run alongside a 1 kb DNA ladder. DNA samples were run at 120 V using power packs supplied by Bio-Rad and Shandon Southern. DNA was visualised using a UV wand (Chromato-vue UVM-57, UVP Life Sciences). DNA samples were run until the 2 kb band of the 1 kb ladder was at the end of the gel (approximately 48 hr).

2.8 Southern blotting procedure

Following electrophoresis, the bottom 30 cm region of agarose gel was excised and inverted into distilled water. The gel was depurinated in 0.25 M HCl for 2x10 min (depurinated DNA is cleaved more readily by NaOH), then alkali-denatured in 0.5 M NaOH, 1M NaCl for 2x20 min (to denature and cleave the DNA into smaller fragments), and neutralised in 0.5M Tris-HCl pH 7.5, 3M NaCl for 2x10 min. DNA was transferred to MAGNA nylon membrane (MSI, Osmonics Laboratory Products) (pre-soaked in 5 x SSC) by the capillary transfer method using 20 x SSC as the transfer buffer (Southern, 1975). Blotting was continued for 5 hrs or overnight. The membrane was rinsed in 2 x SSC, dried at 80°C for 15 min, and the DNA covalently linked to the membrane by exposure to $7x10^4$ J/cm^2 of UV light in the RPN 2500 ultraviolet crosslinker (Amersham Biosciences).

2.9 Synthesis and purification of synthetic repeat probes

2.9.1 Probe synthesis

The synthetic repeat probes for the ESTR loci were MMS10, Ms6-hm and Hm-2 were produced by PCR amplification of synthetic primers containing the appropriate repeat unit (details in Section 2.4.2.3).

2.9.2 Probe purification

As described in Section 2.4.3.3.

2.10 DNA hybridisation

2.10.1 Random oligonucleotide labelling of DNA fragments

Double stranded DNA (10 ng) was generated by PCR amplification of the pure repeat for each locus of interest and was labelled by the random primed labelling reaction (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) which involves the use of randomly generated hexamers and the *E. coli* DNA polymerase Klenow fragment to incorporate α-^{32}P-dCTP (1000 Ci/mmol, NEN, Belgium) into newly synthesised DNA. The labelling reactions were performed in 30 µl reaction volume, and incubated at 37°C for 1-18 hr. The probe was recovered from unincorporated deoxyribonucleotides by ethanol precipitation using 100 µg high molecular weight salmon sperm DNA (Sigma-Aldrich) as a carrier. Probes were re-dissolved in 600 µl of distilled water, and were boiled for 3 minutes prior to use.

2.10.2 Hybridisation

Membranes were pre-hybridised for at least 15 min at 65°C in 7% (w/v) SDS, 0.5 M Na_2PO_4 pH 7.2, 1 mM EDTA, modified from Church and Gilbert (1984). Hybridisation was carried out at 65°C for 5 hours or overnight in Maxi 14, or Mini 10 hybridisation ovens (ThermoHybaid).

2.10.3 Post-hybridisation washing

After hybridisation the membrane was washed at 65°C once in phosphate wash solution (40 mM $NaHPO_4$, 0.5% (w/v) SDS) for 10 min and then in 2-5 changes of high stringency wash solution (0.1 x SSC, 0.01% (w/v) SDS), for 10 min each, until the wash solution recovered after washing was less than 5 counts/second.

2.10.4 Autoradiography

Filters were wrapped in Saran Wrap and placed in autoradiographic cassettes (GRI). The pattern of hybridisation was visualised by autoradiography using Fuji RX100 X-ray film (GRI) at -80°C with an intensifying screen for overnight to one week depending on the strength of the signal.

2.10.5Scoring

All autoradiographs were scored over the well-resolved region between 1.5 and 20 kb. The scoring criteria were that a band had to show a greater than 1 mm shift from the size of the parental allele to be considered a mutant; mosaic bands (those with greater than 2 alleles per loci) were not included as mutations (see Figures 2.1 and 2.2 for details). Details are provided, in results sections, for the total number of mutants scored for each locus and the number of independent mutations. The scoring of independent mutants takes into account apparently identical mutations that are shared by more than one offspring in a litter. These 'mutation clusters' can be seen to represent a single mutational event and can be a scored as such. Subsequent data presented in this thesis use the total number of mutant alleles observed; no significant difference was observed when comparing mutation rates for the total number of mutants or the number of independent mutations. The inclusion of all mutations scored is supported by the convention for calculation of mutation rates, where the total number of mutations observed is used for analysis. Autoradiographs were scored twice independently by two individuals and then a consensus of both individuals was used for the subsequent statistical analysis. (See Section 2.11 for details).

2.10.6Stripping filters for rehybridisation

Nylon filters required for hybridisation with a different probe were stripped by immersing in boiling 0.1% (w/v) SDS, and shaken for 15 min, and repeated if necessary until the counts detected were less than 5/second.

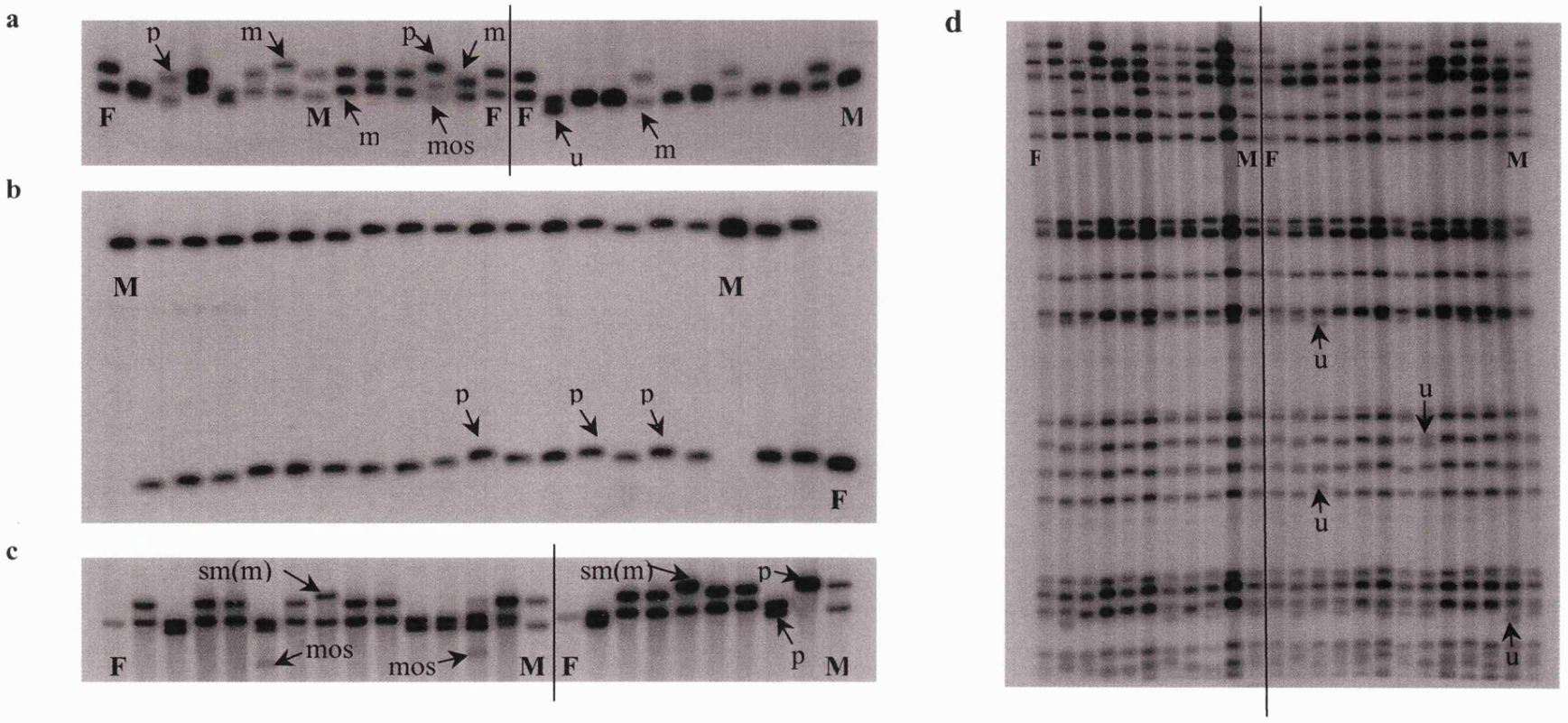


Figure 2.1 – Examples of ESTR mutations in mouse pedigrees

a) *Ms6-hm* for CBA/H mice, b) *Ms6-hm* for a cross between C.B17 and C57BL mice, c) *Hm-2* for C57BL6/H mice and d) MMS10 repeat family for C57BL6/H mice. Fathers are represented by F and mothers by M. Note the presence of paternal (p) and maternal (m) mutants, shared mutations (sm), mosaic bands (mos), mutations without clear parental origin (u), and individuals containing multiple mutations for the MMS10 loci.

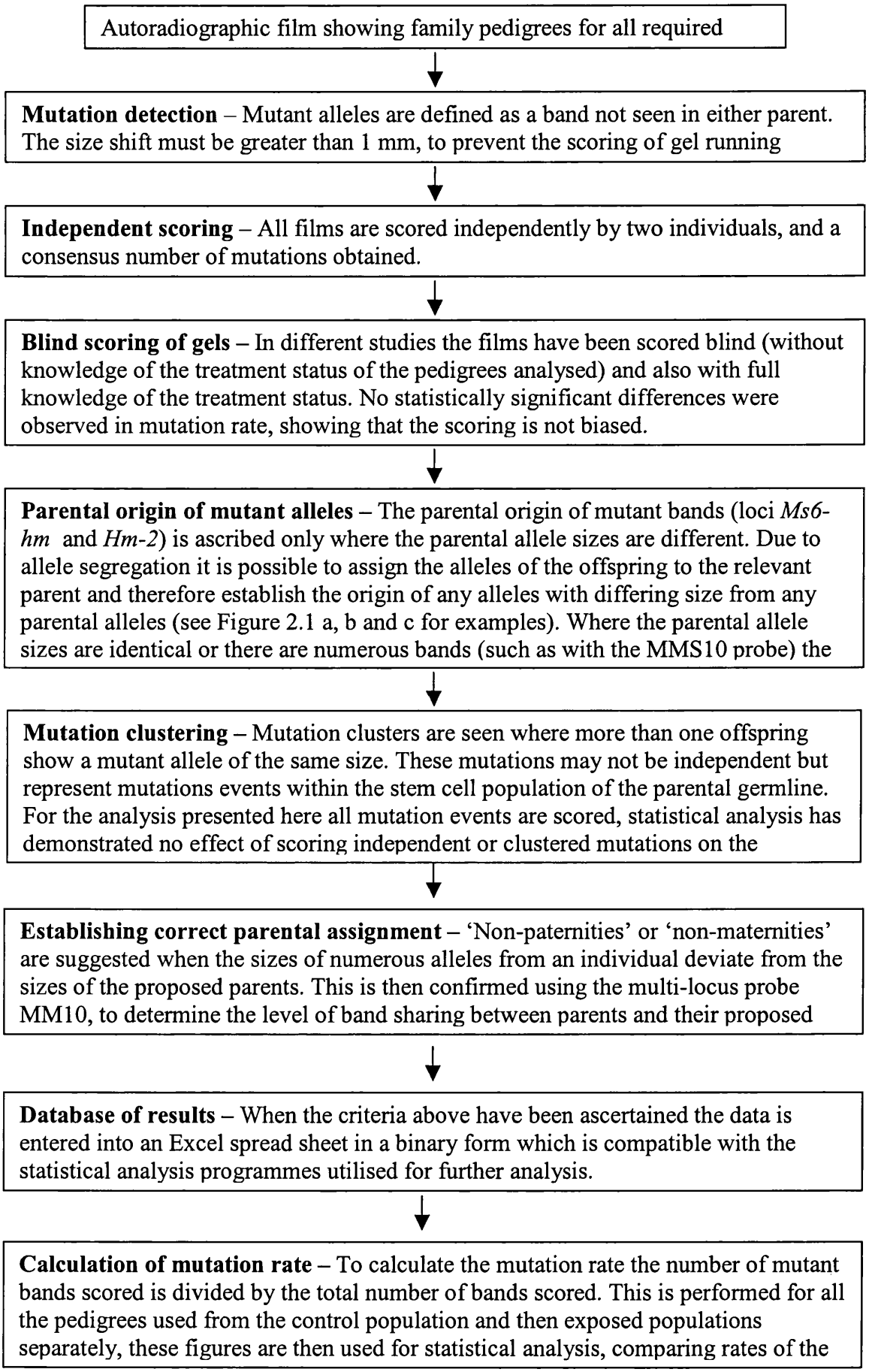


Figure 2.2 – Mutation scoring procedure.

2.11 Statistical Analysis

Most statistical procedures are described by Sokal and Rohlf (1995). The 95% confidence limits for mutation rate were derived from the Poisson distribution (Sachs, 1982).

A number of computer programmes were used for data analysis; results were compiled as a database using Microsoft EXCEL 2000, and statistically analysed using software written in Microsoft Basic by Y. E. Dubrova and the commercially available software packages SYSTAT, Version 10 (2000), SPSS Inc and STATISTICA, Data analysis software system, Version 6 (2001), Statsoft Inc.

2.12 Histology

An additional study was performed (by A. Smith) where F_1 hybrid males (C57BL/6J x CBA/Ca) were given a single intraperitoneal injection of cisplatin (Sigma-Aldrich) at 15mg/kg, at 1 mg/ml in 0.9% (w/v) NaCl. These males were then culled a week later and the testis tissue was used for histological analysis. The weight of the treated mice and their testicular weight were also taken, and analysed later.

The tissues were fixed in Bouin's fluid (a mixture of picric acid in formalin and glacial acetic acid) and were embedded in paraffin wax. Then 5 μm sections were cut in the Histology Laboratory of the MRC Toxicology Unit. Sections were de-paraffinised by immersion in xylene before re-hydration in industrial methylated spirits (IMS) and aqueous alcohol mixtures. Sections were then stained with hemotoxylin and eosin (this work was carried out by Mrs J Edwards, Histology Laboratory of the MRC Toxicology Unit). The slides were then examined and a reported upon by Dr. Mary Tucker (ex-pathologist at Zeneca Pharmaceuticals Safety of Medicines, Cheshire-an expert in reproductive toxicity).

Chapter 3

Analysis of recombination as a possible mechanism of mutation induction at Expanded Simple Tandem Repeat loci in mice

3.1 Introduction

3.1.1 General introduction

Expanded Simple Tandem Repeat loci (ESTRs) have provided a very reliable system to analyse germline mutation induction in male mice exposed to different sources of ionising radiation (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a); however, the mechanism(s) of mutation induction at these loci remain unknown. This lack of information may potentially complicate the further use of ESTR loci for monitoring germline mutation induction in mice.

It is known that exposure to ionising radiation leads to the accumulation of DNA double-strand breaks and other types of DNA damage (Frankenberg-Schwager, 1990). The direct targeting of damage to ESTR loci may potentially explain increases in mutation rates in the germline of male mice exposed to ionising radiation. To assess the possibility that increases in ESTR rate result from the direct effect of DNA damage at these loci a number of factors need to be taken into account. Firstly the size of the haploid mouse genome, which is approximately 3×10^9 base pairs, and secondly the size of the ESTR probes Ms6-hm and Hm-2 which cover a region of approximately 1.6×10^4 base pairs. From these data it can be calculated that a 4-fold increase in paternal mutation rate would require more than 45,000 extra points of DNA damage per haploid genome per Gy of irradiation, assuming that ESTRs are randomly targeted (Dubrova *et al.*, 1998a). The total amount of DNA damage caused to a nucleus after exposure to ionising radiation was estimated by Frankenberg-Schwager (1990) to be approximately 70 DNA double-strand breaks, 1000 single-strand breaks and 2000 damaged bases per cell, per Gy of ionising radiation. As

these levels of DNA damage per Gy of irradiation are less than 10, 000 times those calculated to explain the levels of mutation induction observed at ESTR it would appear that direct damage to ESTR loci cannot explain the radiation-induced increases in ESTR mutation rate observed in the germline of exposed mice (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a).

A number of mechanisms have been proposed to account for mutation processes in regions of repetitive DNA (see Chapter 1, Section 1.3 for details). A body of evidence exists that shows the association of meiotic recombination with germline mutation processes at human minisatellite loci (Jeffreys *et al.*, 1999) and with mutation induction in somatic tissues after exposure to mutagenic agents (Hoffmann, 1994). This evidence suggests that mutation rates at ESTR loci could be associated with the levels of meiotic recombination, and that the radiation-induced mutations observed at these loci may result from an increase in the genome wide frequency of meiotic recombination.

To investigate this hypothesis ESTR mutation rates and crossover rates were analysed in the germline of the same male mice before and after exposure to either ionising radiation or the anticancer drug cisplatin. A number of studies have shown that ionising radiation can induce mutations at mouse ESTR loci (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a). Cisplatin provided a good candidate for the first analysis of mutation induction at ESTR loci by chemicals, because evidence shows a link between recombination rates and cisplatin treatment. An active recombination system is required for the survival of cisplatin-treated somatic cells (Beck and Brubaker, 1973) and exposure of male mice to cisplatin has been shown to increase crossover rate in the germline (Hanneman *et al.*, 1997a). A combined approach was taken to examine meiotic crossover rates; using microsatellite markers in conjunction with a pedigree-based approach for the analysis of ESTR mutation rates for the offspring of the same exposed males. The analysis

of crossover frequency and the rates of ESTR mutation in the offspring of exposed mice are used to assess the effects of both processes in the germline of directly exposed males.

The subsequent sections cover the important characteristics of the anti-cancer drug cisplatin with respect to its mutagenic properties within *in vivo* systems.

3.1.2 Introduction to Cisplatin

3.1.2.1 Discovery of cisplatin

The biological activity of cis-diamminedichloroplatinum (II) or cisplatin was discovered by Rosenberg in 1965 during studies assessing the effects of electrical current on the growth of *Escherichia coli* (Rosenberg *et al.*, 1965). These studies showed that the platinum salts produced during electrolysis could lead to abnormal growth of bacteria (Rosenberg *et al.*, 1967). A number of the platinum complexes were tested for their biological activity, one of which was cisplatin. Because cisplatin was able to induce filamentous growth in bacteria, a known an indicator of DNA damage, further investigation of the properties of cisplatin were performed. Howle and Gale, (1970) speculated that cisplatin would be able to kill tumour cells, and experiments performed on the growth of sarcoma 180 cells and leukaemia L1210 cells showed that cisplatin is indeed a potent killer of tumour cells (Rosenberg *et al.*, 1969). By 1972, cisplatin was introduced into clinical trials as an effective treatment for may cancer types, including cancers of the testis, ovary, head, neck and lung (Loehrer and Einhorn, 1984). Cisplatin is commonly used today as a component of combination cancer therapy, in conjunction with ionising radiation or other anti-cancer drugs, such as bleomycin. Such treatments prove particularly effective for testicular tumours, with a greater than 95% success rate for tumour ablation. Many studies have been performed using cisplatin and its derived compounds, such as carboplatin, to investigate the biological effectiveness of platinum based compounds, their

side effects (Pratt, 1994; Zamble and Lippard, 1995; Murry, 1997) and the phenomena of intrinsic and acquired resistance (Reviewed by (Kartalou and Essigmann, 2001a).

3.1.2.2 Chemical structure

Cisplatin (*cis*-diamminedichloroplatinum (II)) is a square planar complex of platinum surrounded by two ammonia molecules and two chloride ions in the *cis* position; many experiments have shown that this is the active form and that the alternative isomer, *trans*-diamminedichloroplatinum (II) or (*trans*-DDP) where the chloride ions are in the *trans* position, shows little biological effect (see Figure 3.1).

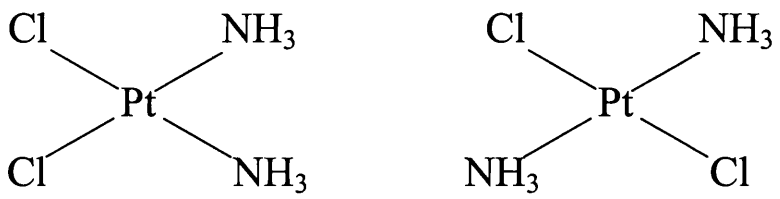
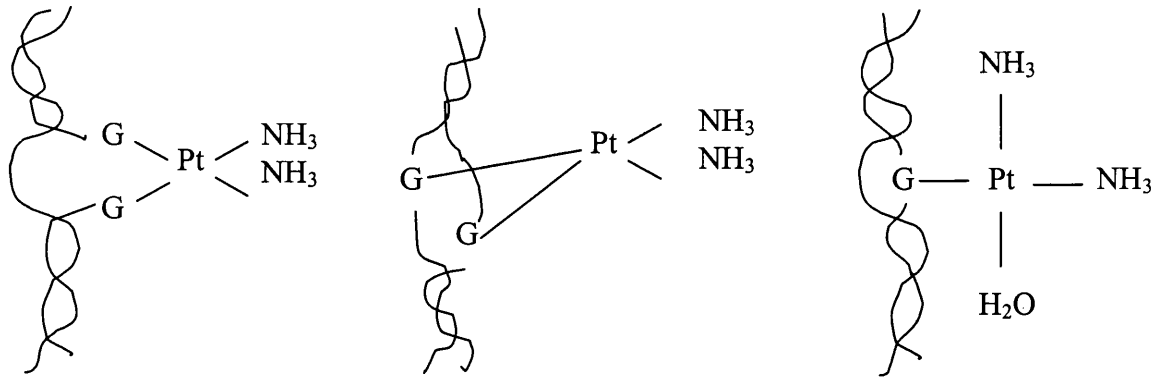


Figure 3.1 - Chemical structures of cisplatin (left) and *trans*-DDP (right).

3.1.2.3 Mode of action

It appears that the biological effectiveness of platinated compounds for anti-cancer treatments depends upon the positioning of the reactive chloride ions (Pinto and Lippard, 1985). The chloride ions undergo aquation, where they are replaced by water molecules to form a positively charged compound which is able to react with nucleophilic residues on intracellular molecules to form adducts with proteins or DNA molecules. The type of adduct depends upon the structure of the platinum compound and the level of aquation. Mono-aquo species react to form mono-functional adducts, where the platinum compound is attached to the DNA at only one site. If another potentially active site is nearby the mono-adduct can further react with DNA to form a number of different di-adducts: intra-1,2-d(GpG), intra-1,2-d(ApG), intra-1,3-d(GpNpG), inter-d(G*pC)/ d(G*pC) and inter-d(G*pC)/ d(GpC*) (Kartalou and Essigmann, 2001b).



a. Intra-strand adduct b. Inter-strand adduct c. Mono-adduct

Figure 3.2 - Cisplatin-DNA adducts

The bulky adducts that these platinum based compounds produce lead to significant problems for the cell that are associated with distortion of the DNA helix, which affects the stability and replication of the DNA molecule. The inter-strand adducts lead to the largest distortion of the DNA backbone and although they represent only a small percentage (between 2 and 10%) of the total adducts number formed they are thought to provide the

basis of cisplatin cytotoxicity. The cisplatin isomer is able to form inter-strand adducts whereas *trans*-DDP is unable to do so due to the configuration of the molecule, which in turn reflects the respective biological activity of these compounds.

Inter-strand adducts have been shown to be very toxic to cells, i.e. just 40 inter-strand cross-links have been shown to be able to kill repair-deficient mammalian cells (Lawley and Phillips, 1996). Inter-strand cross-links are also able to induce mutations, DNA rearrangements, and may play a role in uncontrolled cell growth; therefore recognition and repair of these cross-links is important for cellular survival.

3.1.2.4 Repair pathways

The work available to date postulating the repair pathways for the removal of cisplatin adducts in mammalian cells is limited, although repair deficiencies in mammalian cells have provided a useful tool in elucidating the mechanisms involved. Yeast models have provided most of the current information. The repair pathways of yeast are well characterized and are mostly homologous to those within mammalian systems. A number of pathways are known to be involved in the repair of DNA adducts including homologous recombination, non-homologous end joining (NHEJ) and nucleotide-excision repair.

Break-induced replication during homologous recombination is frequently used to repair DNA adducts in yeast, but no direct evidence for double-strand break formation during adduct repair has been observed within mammalian systems. However, homologous recombination would appear to play a role in the repair of DNA inter-strand cross-links because knockouts of Rad51 and Rad54 (recombinationally important proteins) in tissue culture cells lead to severe cytotoxicity following the cellular exposure to cross-linking agents (Kanaar *et al.*, 1998). Also many cross-linking agents, including cisplatin, have been shown to effect recombination rates; for example, treatment with cisplatin has been shown to increase the number of sister chromatid exchanges (Wiencke *et al.*, 1979; Adler and El-Tarras, 1989; Adler and el Tarras, 1990), and also to increase crossover frequency

in the germline of male mice (Hanneman *et al.*, 1997a). BRAC1 and BRAC2 proteins may play important roles in DNA-adduct removal by coupling the control of the DNA damage responses of DNA repair and cell cycle checkpoint. Cells deficient in BRACA1 and BRACA2 have been shown to be deficient in homologous recombination (Snouwaert *et al.*, 1999; Moynahan *et al.*, 2001). Non-homologous end joining (NHEJ) does not appear to play a large role in the removal of DNA cross-links in mammalian systems, as mutations in the key components of this pathway do not result in increased sensitivity to cross-linking agents (Biedermann *et al.*, 1991b).

Nucleotide-excision repair may also be involved in the repair of DNA adducts, but must play a less important role than recombination-based repair, as mutations in this pathway only lead to moderate increases in cellular sensitivity to cross-linking agents (Wang *et al.*, 2001). The proteins ERCC1 and XPF may be important in nucleotide-excision repair, but their role is unclear as they may also play an essential role in a recombinational sub-pathway, possibly involving single-strand annealing to resolve recombinational intermediates (Dronkert and Kanaar, 2001).

A number of other proteins may be important in the repair of DNA cross-links as mutations in these genes lead to a number of syndromes which are characterized by increases in genomic instability, sensitivity to mutagenic agents, and cancer predisposition (Dronkert and Kanaar, 2001). These genes include ATM (mutated in ataxia telangiectasia), NBS1 (Nijmegen breakage syndrome), and the FANC gene products (currently six of which have been mapped and are associated with Fanconi anaemia).

3.1.2.5 Mutagenicity of cisplatin

Over the years many systems have been used to analyse the mutagenic properties of cisplatin. The first evidence suggesting cisplatin had mutagenic properties came from the studies performed by Rosenberg in the 1960's. Studies of the bacteria *Escherichia coli* grown in the presence of cisplatin were performed, and showed that growth and division of

the treated bacteria were altered in a manner that suggested that DNA damage had occurred (Rosenberg *et al.*, 1965; Rosenberg *et al.*, 1967). Later another *in vitro* study tested cisplatin and its degradation products, using the Ames microbial mutagenicity assay in a *Salmonella* tester strain, and showed that platinum degradation complexes were weak mutagens capable of producing base-pair and frame-shift mutations (Peer and Litz, 1981).

Studies of somatic tissue have provided additional evidence of the mutagenic effects of cisplatin. Wiencke *et al.* (1979) investigated the ability of cisplatin to induce sister chromatid exchanges (SCE) and chromosomal aberrations within *in vitro* systems (human lymphocytes in cell culture) and *in vivo* systems (mouse bone marrow). Significant dose-dependent induction of both SCE and chromosomal aberrations was observed in the tissue culture cells, and significant increases were observed in the bone marrow cells of mice exposed to an inter-peritoneal (i.p.) dose of 13.85 mg/kg of cisplatin. Additional evidence showing that cisplatin can induce chromosomal aberrations in somatic tissue was provided by analysis of the bone marrow of rats (Levine *et al.*, 1980) and mice (Adler and El-Tarras, 1989). Evidence was also obtained for the mutagenic properties of cisplatin in somatic tissue using micronucleus tests on the bone marrow of treated mice (Kliesch and Adler, 1987).

The analysis of somatic tissue within both *in vitro* and *in vivo* systems is consistent and points clearly to the fact that cisplatin is capable of inducing DNA damage; however, a number of studies using different end points to analyse the effects on germ cells show conflicting evidence for mutation induction by cisplatin. The analysis of mice exposed to doses ranging from 1.25 to 10 mg/kg of cisplatin failed to show any dominant lethal mutations (Levine *et al.*, 1980; Katoh *et al.*, 1990). Analyses using the male-specific locus test for spermatogonial stem cells and post-spermatogonial stem cells also failed to reveal any evidence of mutation induction in the germline after exposure to cisplatin (Russell, 1990). Paradoxically the analysis of chromosomal aberrations in differentiating

spermatogonia produced a dose-dependent significant induction of chromosomal aberration with a dose range of 1 to 5 mg/kg (Adler and El-Tarras, 1989). Although Adler and El-Tarras (1989) were able to demonstrate significant increases in chromosomal aberrations, the differentiating spermatogonia showed approximately 50% less aberrations than bone marrow cells treated with the same dose of cisplatin suggesting that germ cells are less sensitive to DNA damage by cisplatin.

The fact that differentiating spermatogonia are less sensitive to the chromosome damaging (clastogenic) effects of cisplatin has lead to speculation about stage-specific induction of DNA damage by cisplatin during spermatogenesis, an effect which has also been shown after exposure to other chemical mutagens (Ehling *et al.*, 1972; Ehling, 1974). Meistrich *et al.* (1982) demonstrated cell-killing effects of cisplatin treatment on differentiated spermatogonia (spermatocytes and spermatids) but were unable to observe an effect on stem cell populations. Additional work by Adler and el Tarras (1990) analysing the response of different stages of spermatogenesis to the effects of cisplatin (within the dose range of 5 to 10 mg/kg), also demonstrated a stage-specific response. Toxic effects were observed in cells treated at the zygotene and preleptotene stages of the meiotic division, demonstrated by reduced testicular weight. Increased chromosomal aberrations were observed for the most sensitive stages of the meiotic division (leptotene and preleptotene), but no significant increases in chromosomal aberrations were observed in treated stem cells. These studies support stage specificity in the DNA-damaging effects of cisplatin during spermatogenesis and indicate that cells are most responsive during the meiotic stages.

To further analyse the effect of cisplatin on meiotic cells, direct measurements of DNA damage caused by cisplatin have been performed by studying the formation of synaptonemal complexes in germ cells and the levels of gene conversion in spermatids. Various patterns of structural damage at synaptonemal complexs and synaptic irregularities

were observed after mice were exposed to cisplatin, thereby providing a direct measure of chemical-specific alterations to meiotic chromosomes (Allen *et al.*, 1988). Cisplatin has also been shown to be able to increase gene conversion events during the meiotic stages of spermatogenesis in transgenic mice carrying *lacZ* fusions (Hanneman *et al.*, 1997b). Stage specificity may provide an explanation for the fact that a number of studies have been unable to shown that cisplatin induced germline mutations (Levine *et al.*, 1980; Katoh *et al.*, 1990; Russell, 1990). A very narrow treatment window during the meiotic phase of spermatogenesis may be required to result in cisplatin-induced DNA damage. The data presented by Hanneman *et al.* (1997a) also provided evidence that the effect of cisplatin (10 mg/kg) on the germline of male mice was restricted to meiotic division, because the elevation in crossover frequency was only observed in cells that were meiotic at the time of exposure.

The information available from the studies performed to date shows that the mutagenic capabilities of cisplatin remain unclear. Data from somatic systems, both *in vivo* and *in vitro* studies suggest that cisplatin exposure results in DNA damage and mutation induction; however the evidence from germ cell studies suggests that stage-specific exposure to cisplatin is required to elevate mutation rates. The mutagenic effects of cisplatin and the possible stage specificity require further investigation and may provide important information about mutation induction at ESTR loci by chemicals, and the possible link of ESTR mutation rates with alterations in the levels of meiotic recombination.

3.2 Experimental design

Exposure to ionising radiation is known to induce ESTR mutation. Previous studies have shown that acute exposure to 1 Gy of X-rays results in a four-fold increase in mutation rates at the two single locus ESTR loci *Ms6-hm* and *Hm-2* (Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b); therefore, the male mice from this study were acutely exposed to 1 Gy of X-rays. The effect of radiation exposure on crossover frequencies is unknown; however, a previous study has shown a 1.8 fold elevation in crossover frequency in the offspring of male mice conceived 4 weeks after exposure to 10 mg/kg of cisplatin (Hanneman *et al.*, 1997a), though nothing is known about the capability of cisplatin to induce ESTR mutations. Cisplatin induces DNA damage in a manner similar to that of ionising radiation, i.e. they both lead to DNA double-strand breaks (DSBs), although via different processes. Ionising radiation is known to produce double-strand breaks during the traversal of the nucleus by ionising particles (see Chapter 1, Section 1.5.4 for details) and cisplatin by the excision of inter-strand DNA adducts (see Section 3.1.2 for details). It was proposed therefore that cisplatin would be a good candidate drug to investigate the hypothesis that alterations in meiotic recombination may lead to changes in the mutation rates at ESTR loci after the exposure to mutagenic agents.

The mice used in the radiation study were F_1 heterozygous (C57BL/6 x CBA/H) hybrid males and CBA/H females. The cisplatin study used F_1 heterozygous (C57BL/6 x CBA/Ca) hybrid males and CBA/Ca females. CBA/H mice were used in previous studies of ESTR mutation induction after irradiation and therefore have well characterised ESTR mutation rates (Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Dubrova *et al.*, 2000b). The C57BL/6 and CBA strains were chosen as they contain differently-sized alleles at the microsatellite and ESTR loci studied, which enables the assignment of parental origin of alleles to the due to size differences. The backcross of F_1 heterozygous (C57BL/6 x CBA) hybrid males with CBA females permits the detection of

single and multiple crossover events between the microsatellite markers studied. It has been shown that at least one crossover event is required in the formation of viable gametes, a process known as interference (Sym and Roeder, 1994), therefore any increases in recombination rates would be observed as increases in double/multiple crossover events per chromosome.

Another important consideration in the design of the breeding scheme is the further investigation of the stage-specific responses for both ionising radiation and cisplatin. Conflicting data have been presented for the stage-specific mutation induction at ESTR loci (see Chapter 1, Section 1.5.3 for details), and cisplatin has been shown to influence recombination frequency only during meiotic division (Hanneman *et al.*, 1997a). To facilitate the analysis of the stage-specificity of these mechanisms a breeding scheme was set up which would allow the analysis of the relevant stages of spermatogenesis (See Chapter 1, Section 1.5.3 for details). Male mice were mated prior to exposure, to produce the control populations, and then again at 3, 4, 5 and 6 weeks after acute exposure to either 1 Gy X-rays or to 10 mg/kg of cisplatin. Offspring conceived at these time points relate to germ cells that were at specific stages of spermatogenesis at the time of exposure (see Chapter 1, Table 1.5). Previous work has shown that the offspring conceived at the 3 week time point show no increases in ESTR mutation rate but those conceived at 6 weeks post-exposure show significant increases (Dubrova *et al.*, 1998a). This raises the possibility that ESTR mutation induction can only take place in cells that are diploid at the time of exposure and suggests that the process of recombination may be important in mutation induction at these loci. This study re-analyses these time points, 3 and 6 weeks post-exposure, and also the intervening weeks, 4 and 5 weeks post-exposure. The cell types assessed at these time points represent primary spermatocytes and type B spermatogonia respectively. As the crossover events occurring during meiosis in the pachytene/diplotene phases and the time taken for these cells to reach the ejaculate is approximately 22-30 days

any increases in crossover rate should be observable in the offspring conceived 4 and 5 weeks after exposure.

ESTR analysis was performed using a standard pedigree approach, where the parents and offspring are typed together on a single gel. Mutations were scored as bands possessing a different size to the parental alleles (see Chapter 2, Section 2.10.5 for more detail). Mutation detection and statistical analysis were performed for the two single-locus probes Ms6-hm and Hm-2, which provide the basis of the data presented here (see Chapter 2, Section 2.11 for details). Multi-locus fingerprinting was also performed with the MMS10 probe. Establishing the parental origin of *Ms6-hm* and *Hm-2* was possible due to the extensive multiallelism and heterozygosity observed at these loci within the CBA inbred mice and the large differences in allele size range seen between the CBA and C57BL/6 mice strains. In contrast the DNA fingerprints produced with the MMS10 multi-locus probe show relatively homogeneous patterns shared by the parents and the offspring, which greatly confounds the assignment of the parental origin of a mutant band. The MMS10 probe therefore is not included in a number of the analyses, but provides good support for the data obtained from the other loci.

The microsatellite markers chosen for this study were known to show size polymorphism between the alleles of the C57BL/6 and C3H strains, (information obtained from the Mouse Genome Database (MDG) at http//:www.jax.org Blake *et al.*, 1998; Blake *et al.*, 1999), and were then tested for size polymorphisms between C57BL/6 and CBA mice for our in-house mouse stocks[1]. Crossover frequencies were measured using PCR analysis of a panel of 25 mouse microsatellite makers covering six chromosomes (see Figure 3.3); this provides coverage of 421.7cM, approximately 28% of the mouse genome.

[1] C3H mice were developed by Strong (1920) from a cross of a Bagg albino female with a DBA male followed by selection for a high incidence of mammary tumours. The CBA strain was derived from the same cross and was selected for a low mammary tumour incidence. From this information it can be inferred that the strains are very closely related and likely to show the same sequence polymorphisms (Festing, M., 1996).

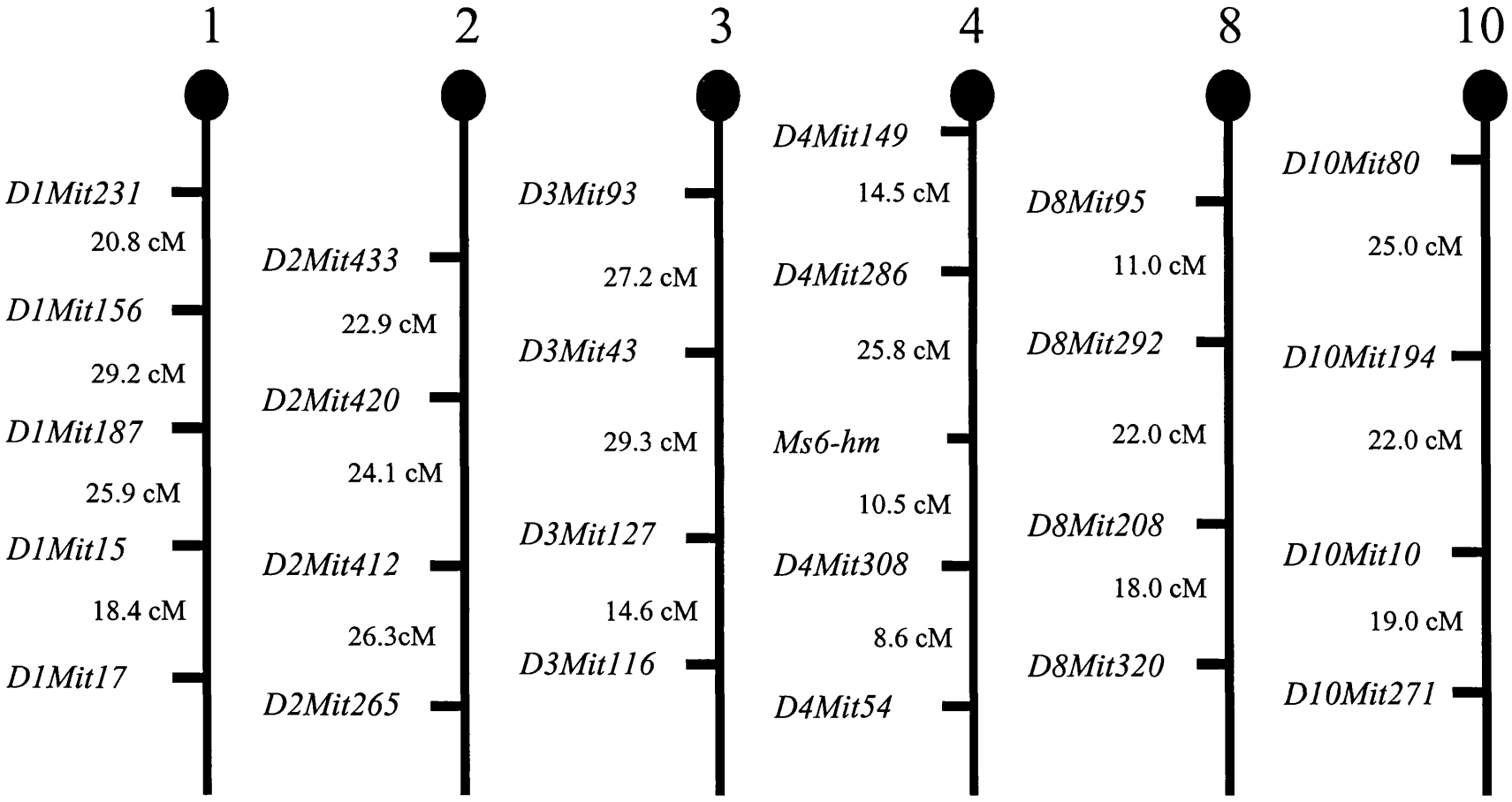


Figure 3.3 - Chromosomal positions of microsatellite loci used for crossover analysis (not to scale).

The chromosomal position of the ESTR *Ms6-hm* is also included. Sizes given represent the genetic inter-marker distances obtained from the Mouse Genome Database using 'Build a Linkage Map' at the Mouse Genome Informatics website (http://www.informatics.jax.org/).

3.3 Summary of mutation induction at ESTR loci

Table 3.1 provides a summary of the mutation data; ESTR mutation rates are shown for the control group (pre-exposure) and for the male mice mated 3, 4, 5 and 6 weeks after exposure either to 1 Gy of X-rays or to 10 mg/kg of cisplatin. Details are provided for the total number of mutants scored for each locus and also the number of independent mutations. The scoring of independent mutants takes into account apparently identical mutations that are shared by more than one offspring in a litter. These 'mutation clusters' can be seen to represent a single mutational event and can be a scored as such. Subsequent data presented here uses the total number of mutant alleles observed, as no significant difference was seen when comparing mutation rates either for the total number of mutants or the number of independent mutations. This approach is also supported by the conventional approach for the calculation of mutation rates, where the total numbers of mutations observed are used. Mutation clustering at the mouse ESTRs *Ms6-hm* and *Hm-2* has previously been reported (Kelly *et al.*, 1989; Gibbs *et al.*, 1993a). The results of these studies show that at least 12% of all observed mutations could have arisen as a result of germline mosaicism. It has also been shown that the frequency of germline mosaicism at *Ms6-hm* and *Hm-2* did not differ significantly between non-exposed and irradiated males (Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000b) suggesting that mutation induction by radiation does not alter the level of mosaicism in germ cell populations.

Exposure, stage	No. of litters	No. of offspring	Number of mutations scored by different probes[a]							
			Total mutants	MMS10	Ms6-hm		Hm-2		MS6-hm + Hm-2	
					Paternal	Maternal	Paternal	Maternal	Paternal	Maternal
X-rays										
Control	19	119	56 (46)	22 (21)	11 (7)	11 (10)	6 (3)	6 (5)	17 (10)	17 (15)
3 weeks	8	57	30 (29)	10 (10)	8 (7)	6 (6)	3 (3)	3 (3)	11 (10)	9 (9)
4 weeks	9	55	58 (54)	28 (27)	16 (14)	4 (3)	7 (7)	3 (3)	23 (21)	7 (6)
5 weeks	5	39	38 (33)	18 (17)	11 (8)	5 (4)	4 (4)	0	15 (12)	5 (4)
6 weeks	7	51	74 (70)	35 (34)	17 (16)	6 (5)	9 (8)	7 (7)	26 (24)	13 (12)
Cisplatin										
Control	14	71	44 (37)	19 (19)	8 (7)	4 (4)	6 (6)	7 (5)	14 (13)	11 (9)
3 weeks	10	54	24 (18)	12 (11)	6 (6)	1 (1)	1(1)	4 (3)	7 (7)	5 (4)
4 weeks	8	37	20 (18)	9 (9)	2 (2)	5 (4)	3 (3)	1 (1)	5 (5)	6 (5)
5 weeks	9	51	27 (22)	13 (13)	5 (4)	6 (3)	1 (1)	2 (2)	6 (5)	8 (5)
6 weeks	11	62	41 (36)	17 (17)	9 (8)	7 (5)	5 (5)	3 (3)	14 (13)	10 (8)

Table 3.1 - Summary of ESTR mutation data for mice exposed to X-rays or cisplatin.

The number of independent mutations are given in parenthesis (x).

3.4 ESTR mutation and crossover analysis in male mice exposed to ionising radiation

ESTR mutation rates in the control and exposed males are given in Table 3.2 and Figure 3.4a and b. The frequency of paternal ESTR mutations in the offspring of male mice conceived 3 weeks after exposure is similar to that observed in the control group. In contrast, statistically significant increases in paternal mutation rates can be observed at *Ms6-hm* and *Hm-2*, for the offspring conceived 4, 5 and 6 weeks post-exposure. The rates increased by 2.9, 2.7 and 3.6 fold respectively, when compared to the control population. No significant changes were observed in maternal mutation rate at any of the time points studied; this implies that the increased rates observed do indeed represent mutation induction within the germline of the exposed males.

The results obtained with the MMS10 multilocus probe (Table 3.2), where parent of origin cannot be assigned, provide additional evidence for increased germline mutation induction at ESTR loci in male mice after exposure to ionising radiation. Increases in mutation rates of 2.7, 2.5 and 3.7 fold at *MMS10* were observed in offspring conceived at 4, 5 and 6 weeks post-exposure, respectively, which are very similar to the results obtained for paternal exposure for the single locus probes, Ms6-hm and Hm-2.

	Total				MMS10 alone				Paternal[a]			Maternal[a]		
Stage	No. bands	Rate	Ratio[b]	Prob.[c]	No. bands	Rate	Ratio[b]	Prob.[c]	Rate	Ratio[b]	Prob.[c]	Rate	Ratio[b]	Prob.[c]
X-rays														
Control	3751	0.0149	-	-	3275	0.0067	-	-	0.0714	-	-	0.0714	-	-
3 weeks	1800	0.0167	1.12	0.7018	1572	0.0064	0.95	1	0.0965	1.35	0.5384	0.0789	1.10	0.8546
4 weeks	1764	0.0329	2.20	**3.65×10^{-5}**	1544	0.0181	2.70	**0.0007**	0.2091	2.94	**0.0006**	0.0636	0.89	0.9883
5 weeks	1238	0.0307	2.06	**0.0011**	1082	0.0166	2.48	**0.0083**	0.1923	2.69	**0.0066**	0.0641	0.90	1
6 weeks	1629	0.0454	3.04	**3.21×10^{-10}**	1425	0.0246	3.66	**2.06×10^{-6}**	0.2549	3.57	**1.71×10^{-5}**	0.1274	1.78	0.1498
Cisplatin														
Control	2197	0.0200	-	-	1913	0.0099	-		0.0986	-	-	0.0775	-	-
3 weeks	1682	0.0143	0.71	0.2169	1466	0.0084	0.84	0.7823	0.0648	0.66	0.4728	0.0463	0.60	0.4656
4 weeks	1165	0.0172	0.86	0.6650	1017	0.0088	0.89	0.9486	0.0676	0.68	0.6224	0.0811	1.05	1
5 weeks	1592	0.0170	0.85	0.5744	1388	0.0094	0.94	1	0.0588	0.60	0.3806	0.0784	1.01	1
6 weeks	1965	0.0209	1.04	0.9320	1717	0.0099	1	1	0.1129	1.14	0.8553	0.0806	1.04	1

Table 3.2 – ESTR mutation rates in mice before and after exposure to X-rays or cisplatin.

a) Number of paternal and maternal mutations scored by two single-locus probes Ms6-hm and Hm-2.

b) Ratio of mutation rates between exposed and control groups.

c) Probability of differences from the control group (Fisher's exact test, two-tailed; statistically significant values are in bold).

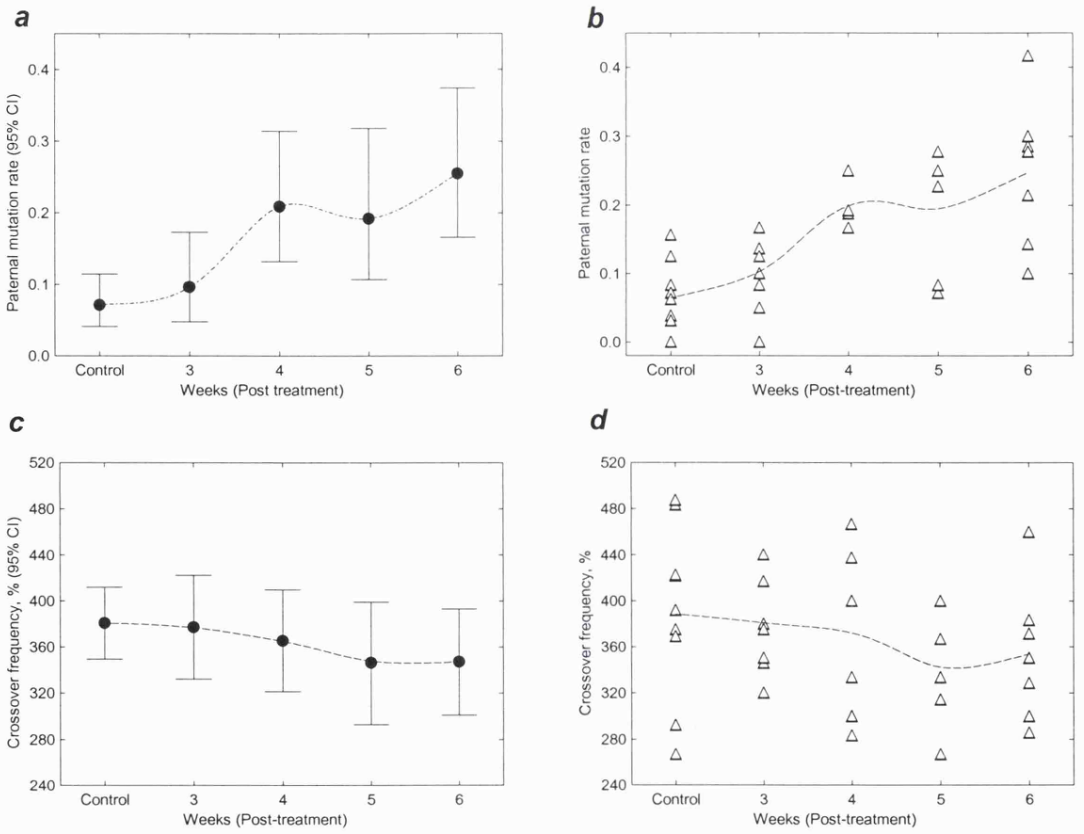


Figure 3.4 – ESTR mutation rates and crossover frequencies in male mice acutely exposed to 1 Gy of X-rays

Paternal ESTR mutation rates a) aggregated data and b) mutation rate in individual males.

Crossover frequencies c) aggregated data and d) mutation rate in individual males.

The 95% confidence intervals for mutation rate and crossover frequency are given in a) and c).

Analysis of crossover frequencies was also performed for the same irradiated males enabling a direct comparison with the mutation rates at ESTR loci. The data are summarised in Table 3.2 and graphically in Figure 3.4c and d. The frequency of meiotic recombination remains unchanged for the duration of the experiment; the same levels were observed in the control population and also in the offspring derived from males 3, 4, 5 and 6 weeks after exposure to 1 Gy of X-rays. There is no evidence for increases in crossover frequencies across any of the six chromosomes studied (Table 3.3), or with any of the males studied (Figure 3.4d). The data shown in Figure 3.4c and d represent the total number of crossover events across all the chromosomes studied; additional analysis of the frequency of crossovers was performed for each chromosome individually (Table 3.3), which did not reveal any elevated frequencies for the chromosomes included in our study (Figure 3.5). The number of double crossover events per chromosome were also assessed (Table 3.3) and showed that exposure to ionising radiation had no effect on frequency of these events. Correlation analysis was unable to reveal any associations between paternal ESTR mutation rate and crossover frequency in the control or exposed males (Figure 3.6).

It is possible to calculate the statistical power of the crossover analysis, using the data from Table 3.2, by taking the maximum standard error (26.5) from within one experimental group (data for 5 weeks post exposure) and the grand total for the control group. From these data it is possible to estimate that the data set would enable the detection of a genome-wide increase in crossover frequency of $\geq$ 15%, with 95% confidence. These calculations equally apply to the data set for male mice exposed to cisplatin.

Table 3.3 – Crossover frequencies in irradiated and control males.

		Frequency of crossovers (%)[a]								
Locus1	Locus2	Control	3 weeks	Prob.[b]	4 weeks	Prob.[b]	5 weeks	Prob.[b]	6 weeks	Prob.[b]
Chromosome 1										
D1Mit231	*D1Mit156*	20.2 (24)	26.3 (15)	0.4650	20.7 (12)	1	23.1 (9)	0.8553	33.3 (17)	0.1041
D1Mit156	*D1Mit187*	20.2 (24)	28.1 (16)	0.3375	12.1 (7)	0.2606	12.8 (5)	0.4358	13.7 (7)	0.4020
D1Mit187	*D1Mit15*	17.6 (21)	19.3 (11)	0.9420	22.4 (13)	0.5742	20.5 (8)	0.8513	15.7 (8)	0.9451
D1Mit15	*D1Mit17*	14.3 (17)	12.3 (7)	0.8144	13.8 (8)	1	20.5 (8)	0.4919	15.7 (8)	0.9819
Total± s.e.[c]		72.3±7.0	86.0±10.8	*t*=1.06 n.s.	69.0±9.9	*t*=0.35 n.s.	76.9±12.6	*t*=0.26 n.s.	78.4±10.9	*t*=0.47 n.s.
Chromosome 2										
D2Mit433	*D2Mit420*	21.8 (26)	22.8 (13)	1	25.9 (15)	0.6792	20.5 (8)	1	29.4 (15)	0.3876
D2Mit420	*D2Mit412*	24.4 (29)	24.6 (14)	1	19.0 (11)	0.5438	15.4 (6)	0.3431	11.8 (6)	0.9036
D2Mit412	*D2Mit265*	12.6 (15)	19.3 (11)	0.3439	22.4 (13)	0.1486	12.8 (5)	1	7.8 (4)	0.5364
Total± s.e.[c]		58.8±6.2	66.7±9.5	*t*=0.70 n.s.	67.2±9.5	*t*=0.74 n.s.	48.7±10.2	*t*=0.85 n.s.	49.0±8.7	*t*=0.92 n.s.
Chromosome 3										
D3Mit93	*D3Mit43*	37.8 (45)	28.1 (16)	0.2698	20.7 (12)	0.3144	28.5 (11)	0.3716	23.5 (12)	0.0995
D3Mit43	*D3Mit127*	26.1 (31)	24.6 (14)	0.9869	29.3 (17)	0.7747	30.8 (12)	0.7036	23.5 (12)	0.8879
D3Mit127	*D3Mit116*	12.6 (15)	10.5 (6)	0.8990	6.9 (4)	0.3751	12.8 (5)	1	9.8 (5)	0.8157
Total± s.e.[c]		76.5±6.7	63.6±9.2	*t*=1.11 n.s.	56.9±8.7	*t*=1.78 n.s.	71.8±11.6	*t*=0.35 n.s.	56.9±9.4	*t*=1.46 n.s.
Chromosome 4										
D4Mit149	*D4Mit286*	17.6 (21)	15.8 (9)	0.9398	25.9 (15)	0.2828	17.9 (7)	1	7.8 (4)	0.1476
D4MIT286	*D4Mit308*	31.1 (37)	35.1 (20)	0.7161	29.3 (17)	0.9524	25.6 (10)	0.6656	35.3 (18)	0.7154
D4Mit308	*D4Mit54*	8.4 (10)	10.5 (6)	0.8381	6.9 (4)	0.9825	5.1 (2)	0.7863	5.9 (3)	0.8297
Total± s.e.[c]		57.1±6.1	61.4±8.3	*t*=0.42 n.s.	62.1±8.9	*t*=0.46 n.s.	48.7±10.0	*t*=0.72 n.s.	49.0±8.4	*t*=0.78 n.s.

Chromosome 8										
D8Mit95	*D8Mit292*	8.4 (10)	8.8 (5)	1	6.9 (4)	0.9825	2.6 (1)	0.3872	7.8 (4)	1
D8Mit292	*D8Mit208*	11.8 (14)	14.0 (8)	0.8389	22.4 (13)	0.1084	15.4 (6)	0.7312	15.7 (8)	0.6400
D8Mit208	*D8Mit320*	25.2 (30)	14.0 (8)	0.1308	17.2 (10)	0.3182	20.5 (8)	0.7172	17.6 (9)	0.3833
Total± s.e.[c]		45.4±5.6	36.8±7.5	*t*=0.92 n.s.	46.6±8.1	*t*=0.12 n.s.	38.5±9.0	*t*=0.65 n.s.	41.2±8.3	*t*=0.42 n.s.
Chromosome 10										
D10Mit80	*D10Mit194*	24.4 (29)	14.0 (8)	0.1639	8.6 (5)	**0.0170**	20.5 (8)	0.7977	19.6 (10)	0.6416
D10Mit194	*D10Mit10*	16.8 (20)	29.8 (17)	0.0778	31.0 (18)	0.0521	12.8 (5)	0.7558	27.5 (14)	0.1710
D10Mit10	*D10Mit271*	29.4 (35)	19.3 (11)	0.2104	24.1 (14)	0.5821	28.2 (11)	1	25.5 (13)	0.7458
Total± s.e.[c]		70.6±6.7	63.2±9.2	*t*=0.65 n.s.	63.8±9.1	*t*=0.60 n.s.	61.5±11.1	*t*=0.70 n.s.	72.5±10.4	*t*=0.15 n.s.
Grand total± s.e.[c]		380.7±15.7	377.2±22.6	*t*=0.13 n.s.	365.5±22.1	*t*=0.56 n.s.	346.2±26.5	*t*=1.12 n.s.	347.1±23.0	*t*=1.21 n.s.
Frequency of double crossovers/chromosome[d]		0.0714 (51)	0.0672 (23)	*P*=0.9128[e]	0.0690 (24)	*P*=0.9921[e]	0.0641 (15)	*P*=0.8307[e]	0.0686 (21)	*P*=0.9909[e]

Table 3.3 continued - Crossover frequencies in irradiated and control males.

[a] The number of recombinants are given in brackets.

[b] Probability of difference from the control group (Fisher's exact test, two-tailed; statistically significant values are in bold).

[c] t-test for difference from control group (n.s., non-significant).

[d] The total number of chromosomes with double crossovers are given in brackets.

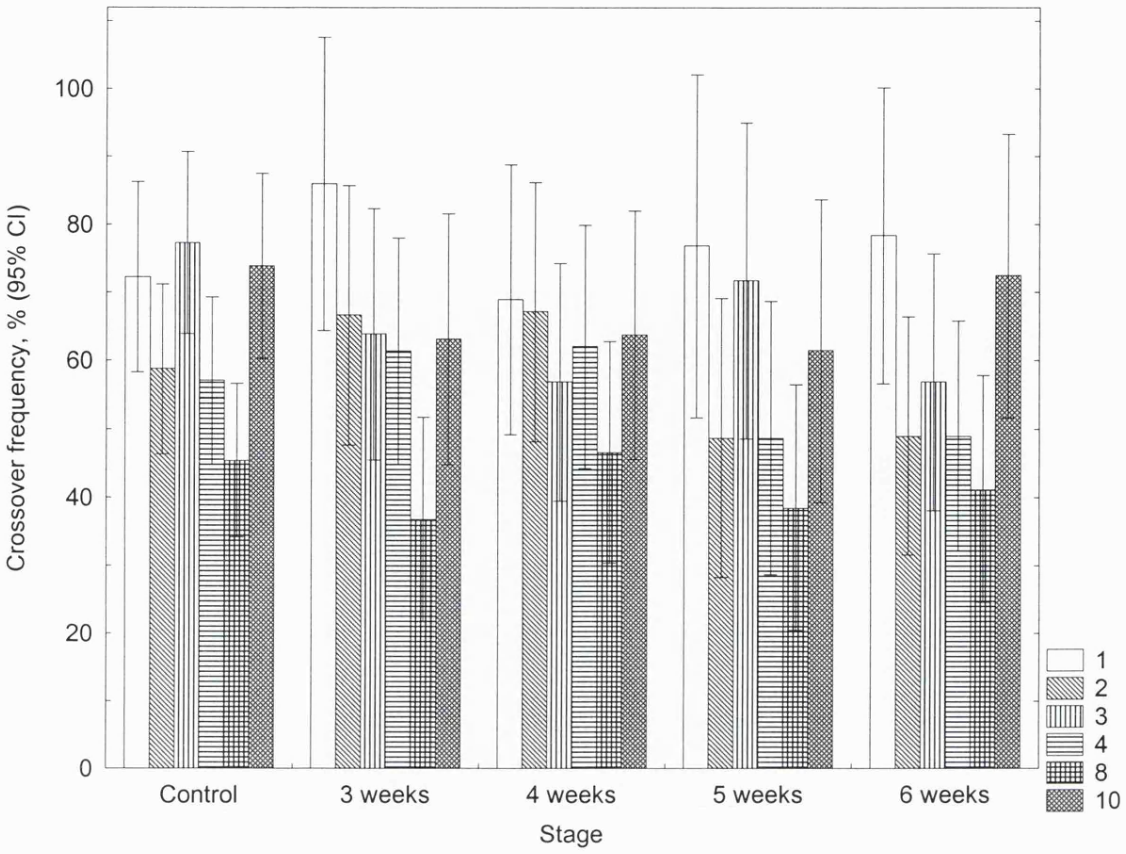


Figure 3.5 – Crossover frequency per chromosome after exposure to 1 Gy of X-rays.

Chromosomes 1, 2, 3, 4, 8 and 10 were analysed.

The 95% confidence intervals for crossover frequency are given.

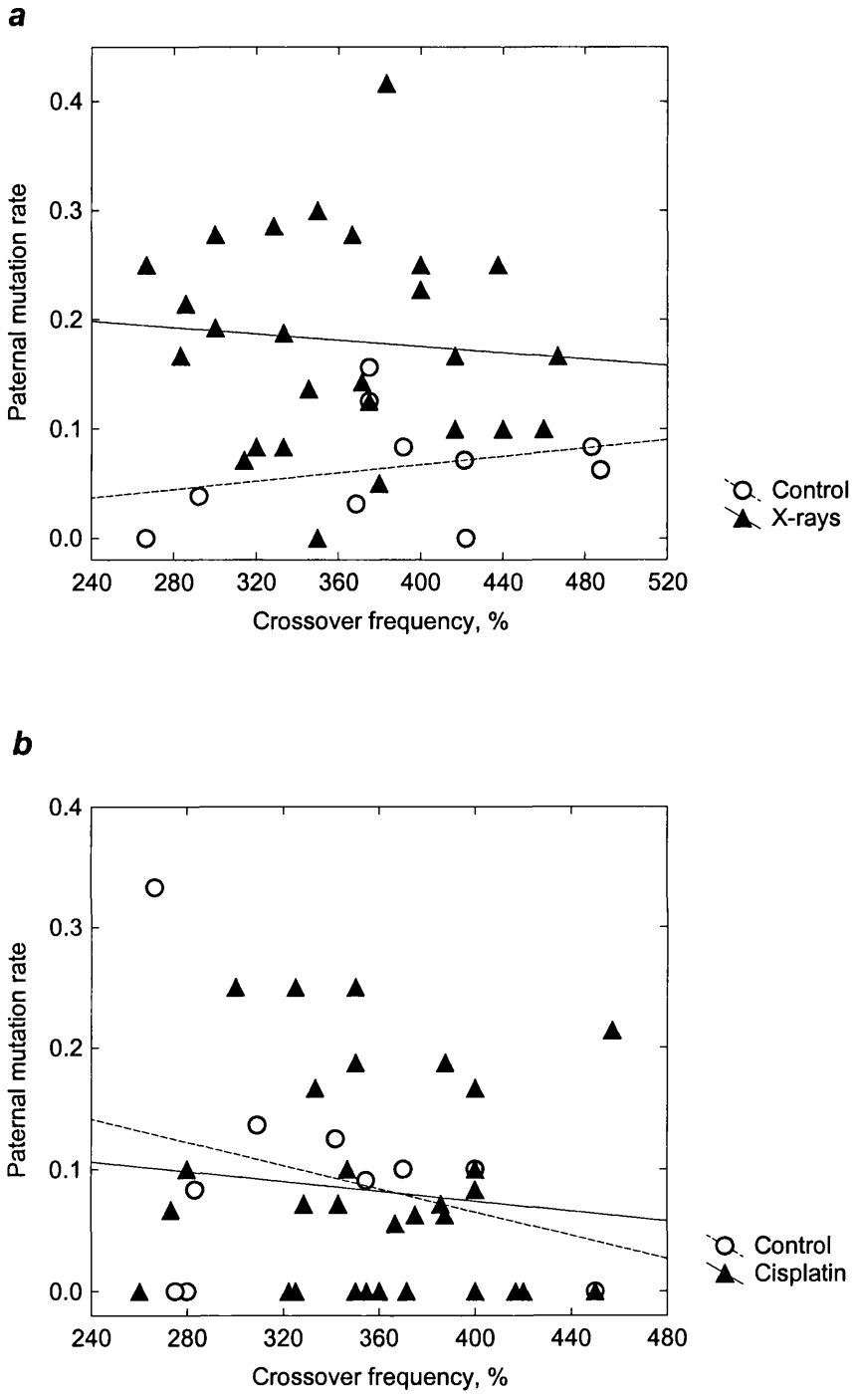


Figure 3.6 – Correlation between crossover frequencies and paternal ESTR mutation rates.

a) Correlation analysis for male mice before and after exposure to 1 Gy of X-rays (Kendal's $\tau = 0.0920$, $P = 0.7113$ and $\tau = -0.0734$, $P = 0.5911$ respectively).b) Correlation analysis for male mice before and after exposure to 10 mg/kg of cisplatin (Kendal's $\tau = -0.0698$, $P = 0.7786$ and $\tau = -0.0734$, $P = 0.4279$ respectively).

3.5 ESTR mutation and crossover analysis in male mice exposed to cisplatin

The mutation rates of ESTR loci after exposure to 10 mg/kg of the anti-cancer drug cisplatin are given in Table 3.1, and are also represented in Figure 3.7 a and b. The results presented here clearly show that exposure to cisplatin does not affect ESTR mutation rates for any of the stages of spermatogenesis studied. The lack of mutation induction holds true regardless of whether the analysis was performed on the combined data representing all males (Figure 3.7 a) or by comparing the germline mutation rates of individual male mice (Figure 3.7 b). These results greatly contrast with those seen after exposure to ionising radiation where statistically significant increases in mutation rate are seen in the time points of 4, 5 and 6 weeks after irradiation when compared to the spontaneous rate, as presented in Section 3.4 (Table 3.1 and Figure 3.4 a and b).

Treatment of male mice with cisplatin also failed to produce any alteration in crossover frequency (Table 3.4) at any of the time points studied (Figure 3.7 c), or for any of the males (Figure 3.7 d), which is similar to the situation seen with irradiated males. The analysis crossover frequencies in the six chromosomes of treated males produced random patterns similar to those seen in the germline of the control group (Figure 3.8). Again no additional analyses could produce any significant patterns showing that treatment had an effect on crossover rate; there were no alterations in the frequency of double crossovers (Table 3.4) or any significant correlation observed between ESTR mutation rates and crossover frequency (Figure 3.6).

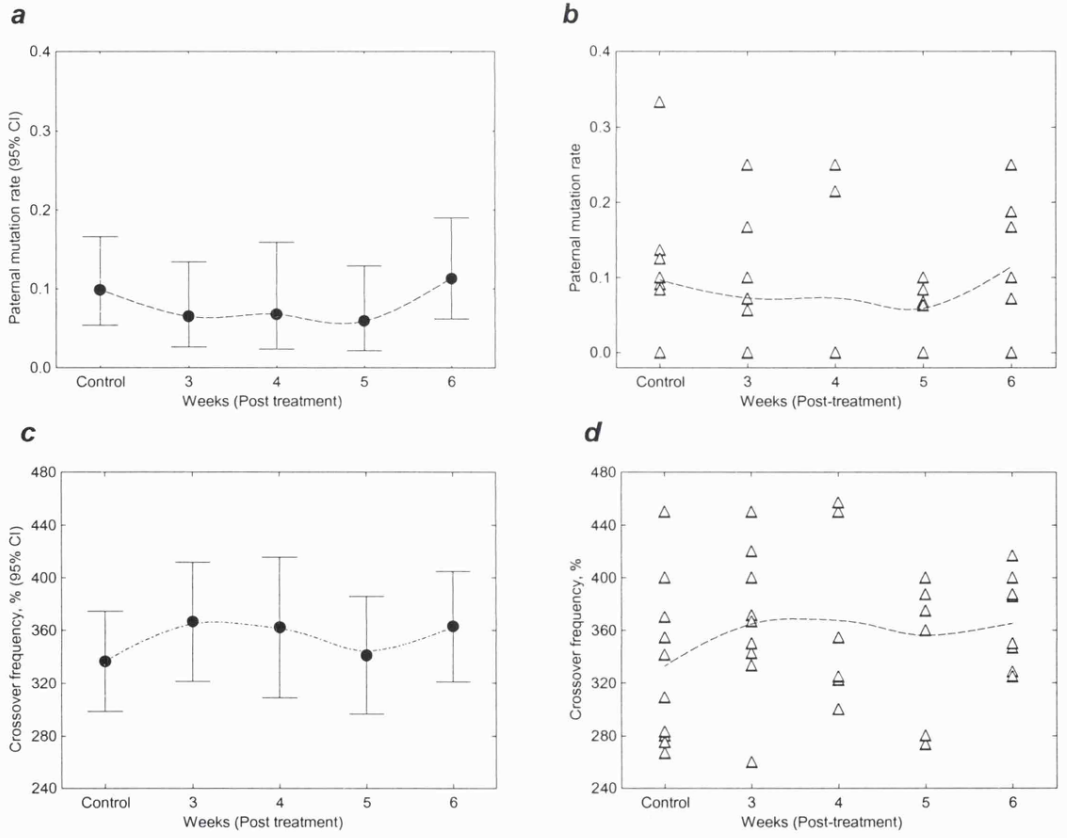


Figure 3.7 – ESTR mutation rates and crossover frequencies in male mice acutely exposed to 10 mg/kg of cisplatin.

Paternal ESTR mutation rates a) aggregated data and b) mutation rate in individual males.

Crossover frequencies c) aggregated data and d) mutation rate in individual males.

The 95% confidence intervals for mutation rate and crossover frequency are given in a) and c).

Table 3.4 – Crossover frequencies in cisplatin treated and control males.

		Frequency of crossovers (%)[a]								
Locus1	Locus2	Control	3 weeks	Prob.[b]	4 weeks	Prob.[b]	5 weeks	Prob.[b]	6 weeks	Prob.[b]
Chromosome 1										
D1Mit231	*D1Mit156*	29.6 (21)	31.5 (17)	0.9709	40.5 (15)	0.3509	15.7 (8)	0.1151	14.5 (9)	0.0601
D1Mit156	*D1Mit187*	12.7 (9)	13.0 (7)	1	8.1 (3)	0.7127	15.7 (8)	0.8274	16.1 (10)	0.7475
D1Mit187	*D1Mit15*	15.5 (11)	18.5 (10)	0.8311	18.9 (7)	0.8418	15.7 (8)	1	25.8 (16)	0.2082
D1Mit15	*D1Mit17*	15.5 (11)	18.5 (10)	0.8311	18.9 (7)	0.8418	15.7 (8)	1	22.6 (14)	0.4113
Total± s.e.[c]		73.2±9.1	81.5±10.8	*t*=0.59 n.s.	86.5±13.0	*t*=0.84 n.s.	62.8±10.2	*t*=0.76 n.s.	79.0±10.0	*t*=0.60 n.s.
Chromosome 2										
D2Mit433	*D2Mit420*	23.9 (17)	27.8 (15)	0.7767	18.9 (17)	0.7350	35.3 (18)	0.2448	19.4 (12)	0.6702
D2Mit420	*D2Mit412*	19.7 (14)	24.1 (13)	0.7105	13.5 (15)	0.6014	11.8 (6)	0.3574	25.8 (16)	0.5281
D2Mit412	*D2Mit265*	15.5 (11)	18.5 (10)	0.8311	21.6 (8)	0.5894	19.6 (10)	0.7206	17.7 (11)	0.9065
Total± s.e.[c]		59.2±8.2	70.4±10.0	*t*=1.49 n.s.	54.1±10.9	*t*=0.38 n.s.	66.7±9.8	*t*=1.08 n.s.	62.9±8.9	*t*=1.07 n.s.
Chromosome 3										
D3Mit93	*D3Mit43*	26.8 (19)	18.5 (10)	0.3867	21.6 (18)	0.7343	21.6 (11)	0.6612	19.4 (12)	0.4233
D3Mit43	*D3Mit127*	19.7 (17)	27.8 (15)	0.3983	27.0 (10)	0.5279	19.6 (10)	1	24.2 (15)	0.6784
D3Mit127	*D3Mit116*	8.5 (6)	18.5 (10)	0.1630	13.5 (5)	0.6101	5.9 (3)	0.8683	19.4 (12)	0.1138
Total± s.e.[c]		54.9±7.7	64.8±9.6	*t*=0.80 n.s.	62.2±11.4	*t*=0.53 n.s.	47.1±8.7	*t*=0.67 n.s.	62.9±8.9	*t*=0.86 n.s.
Chromosome 4										
D4Mit149	*D4Mit286*	14.1 (10)	14.8 (8)	1	8.1 (3)	0.5657	15.7 (8)	1	17.1 (11)	0.7330
D4MIT286	*D4Mit308*	26.8 (19)	29.6 (16)	0.8755	37.8 (14)	0.3378	25.5 (13)	1	30.6 (19)	0.7614
D4Mit308	*D4Mit54*	7.0 (5)	7.4 (4)	1	8.1 (3)	1	7.8 (4)	1	4.8 (3)	0.8742
Total± s.e.[c]		47.9±7.3	51.8±8.6	*t*=0.34 n.s.	54.1±10.2	*t*=0.49 n.s.	49.0±8.8	*t*=0.1 n.s.	53.2±8.1	*t*=0.62 n.s.

Chromosome 8										
D8Mit95	*D8Mit292*	4.2 (13)	1.9 (1)	0.8374	10.8 (4)	0.3610	0 (0)	0.3872	4.8 (3)	1
D8Mit292	*D8Mit208*	9.9 (7)	1.67 (9)	0.3899	13.5 (5)	0.7834	21.6 (11)	0.1252	14.5 (9)	0.5769
D8Mit208	*D8Mit320*	22.5 (16)	18.5 (10)	0.7492	16.2 (6)	0.6108	27.5 (14)	0.6794	16.1 (10)	0.4791
Total± s.e.[c]		36.6±6.5	37.0±7.6	t=0.04 n.s.	40.5±9.7	t=0.33 n.s.	49.0±8.5	t=1.16 n.s.	35.5±7.0	t=0.15 n.s.
Chromosome 10										
D10Mit80	*D10Mit194*	18.3 (13)	18.5 (10)	1	18.9 (7)	1	11.8 (6)	0.4689	21.0 (13)	0.8658
D10Mit194	*D10Mit10*	25.4 (18)	18.5 (10)	0.8974	8.1 (3)	0.0509	21.6 (11)	0.7931	12.9 (8)	0.1104
D10Mit10	*D10Mit271*	21.1 (15)	24.1 (13)	0.8571	37.8 (14)	0.1057	33.3 (17)	0.1933	35.5 (22)	0.0911
Total± s.e.[c]		64.8±8.4	61.1±9.5	t=0.37 n.s.	64.9±11.2	t=0.01 n.s.	66.7±9.8	t=0.19 n.s.	69.4±9.0	t=0.49 n.s.
Grand total± s.e.[c]		336.6±19.4	366.7±23.0	t=1.29 n.s.	362.2±27.2	t=0.93 n.s.	341.2±22.8	t=0.20 n.s.	362.9±21.4	t=1.20 n.s.
Frequency of double crossovers/chromosome[d]		0.0610 (26)	0.0802 (26)	P=0.3778[e]	0.0811 (18)	P=0.4216[e]	0.0392 (12)	P=0.2514[e]	0.0519 (22)	P=1[e]

Table 3.4 continued - Crossover frequencies in cisplatin treated and control males.

[a] The number of recombinants are given in brackets.

[b] Probability of difference from the control group (Fisher's exact test, two-tailed; statistically significant values are in bold).

[c] t-test for difference from control group (n.s., non-significant).

[d] The total number of chromosomes with double crossovers are given in brackets.

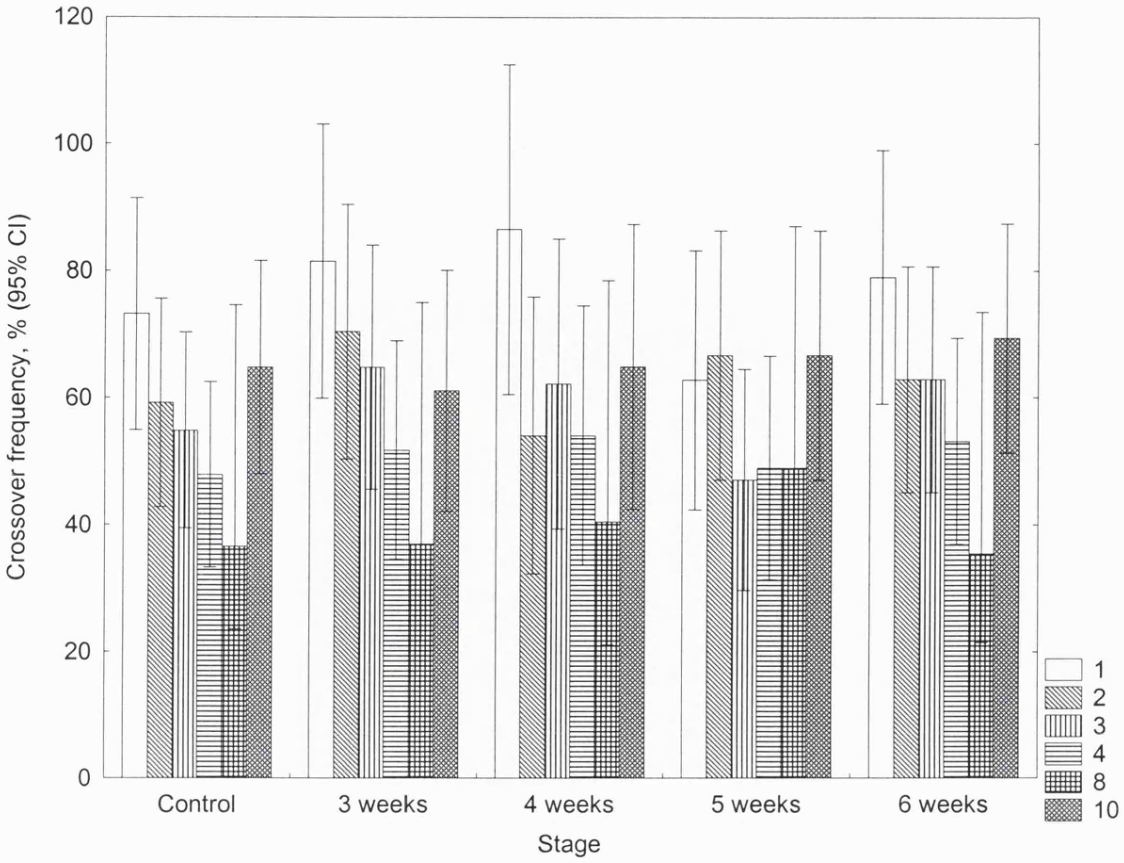


Figure 3.8 – Crossover frequency per chromosome after exposure 10 mg/kg of cisplatin.

Chromosomes 1, 2, 3, 4, 8 and 10 were analysed.

The 95% confidence intervals for crossover frequency are given.

3.6 Discussion

The aim of this study was to ask whether induced germline mutations observed at ESTR loci in mice could be attributed to a genome-wide increase in meiotic recombination. It was decided to approach this problem using the simultaneous analysis of ESTR mutation rate and crossover frequency in the germline of the same directly exposed males. Male mice were exposed to 1 Gy of X-rays, a dose that has been shown to dramatically increase germline ESTR mutation rate (Dubrova *et al.*, 1998a), but the effects on crossover frequency is unknown. Another group of male mice were exposed to the anti-cancer drug cisplatin at a dose of 10 mg/kg; this dose is known to increase crossing-over (Hanneman *et al.*, 1997a), but the effect on ESTR mutation rate is unknown. This experimental design allows the detection of any correlation between the two systems, as both rates should alter proportionally to each other in the germline of exposed mice.

Analysis of irradiated males clearly showed evidence of mutation induction at ESTR loci but no effects were seen on meiotic crossing-over, or any correlation between the two mechanisms in either control or irradiated males. This evidence strongly suggests that ESTR mutation induction cannot be a by-product of a genome-wide alteration in meiotic crossing over. It is known that exposure to ionising radiation leads to DNA damage including an increase in the number of double-strand breaks (DSBs) which act as an initiator for recombinational repair by either non-homologous end-joining (NHEJ) or by homologous recombination (Weaver, 1996). The outcome of such DNA repair however may not always result in the crossing-over of homologous chromosomes; the spermatogenic cells containing such DSBs may be arrested via cell cycle checkpoints, until repair can be achieved or lost completely via the apoptosis of damaged cells. It was initially expected that exposure of cells undergoing meiotic recombination (those cells resulting in mature sperm at approximately 4 weeks after exposure) would show the greatest sensitivity to an increase in DSBs, which would lead to greatly increased crossover

rates; however this was not the case as no evidence of increased crossover frequencies was observed in the offspring conceived 4 weeks after paternal exposure to ionising radiation. Although timed matings were performed to enrich for cells that were at a particular stage of spermatogenesis at the time of exposure, the heterogeneity of germ cells at any given point of spermatogenesis may mask any stage-specific effects and therefore explain the lack of induced crossovers. It remains possible that strict targeting of mouse germ cells at specific stages of spermatogenesis may lead to increases in meiotic crossover frequencies; however, this may be confounded further by the possibility that the timing of mutation induction may be very short and therefore more difficult to analyse.

Previous work has shown that ionising radiation induces ESTR mutation in a stage-specific manner; mutation induction was observed in pre-meiotic spermatogonia (offspring conceived 6 weeks after exposure) and also in stem cells (both at 10 and 20 weeks after exposure), but no induction was observed in the offspring conceived from cells that were post-meiotic at the time of exposure (3 weeks) (Dubrova *et al.*, 1998a). The data presented here covered two additional stages of spermatogenesis (4 and 5 weeks after exposure) and have shown significant increases in paternal mutation rate in cells that were exposed to ionising radiation during the early/mid pachytene stages of meiosis and also in type B spermatogonia; the mutation rates observed are comparable to those observed in A_s spermatogonia, cells that become mature 6 weeks post exposure (Table 3.1, probabilities of differences between these stages, $0.4 < P < 0.95$). The data also confirm the lack of mutation induction in post-meiotic cells (3 weeks post-exposure). These data strongly suggest that all stages of spermatogenesis prior to metaphase I are susceptible to germline mutation induction at ESTR loci by ionising radiation.

An increasing amount of evidence suggests that the mutation process(s) acting at mouse ESTR loci can only lead to mutation in cells that are diploid at the time of exposure. The evidence includes the stage specificity of the response to mutation induction at ESTR

loci by ionising radiation, i.e. mutation induction only takes place in cells that are pre-meiotic or during the pachytene stage of meiosis at the time of exposure; cells that are post-meiotic, and hence haploid show no alterations in mutation rate after exposure. Evidence of transgenerational instability at ESTR loci, where instability can be observed in the germline of at least 2 generations after initial paternal exposure (Dubrova *et al.*, 2000a; Barber *et al.*, 2002), further suggests that a diploid genome is required for the manifestation of induced mutations at ESTR loci. Elevated mutation rates are observed in the germline of the F_1 offspring conceived from post-meiotic spermatogonia (three weeks post-exposure); this greatly contrasts with the post-meiotic haploid spermatids, from which the F_1 offspring were derived, where no mutation induction is observed in the germline of F_0 directly exposed males (see Chapter 5, Figure 5.4). This discrepancy may be explained by the fact that the germ cells in the F_1 offspring have been diploid and have passed through numerous mitotic cell divisions prior to meiosis and becoming haploid gametes. These F_1 germ cells can then lead to statistically significant increases in ESTR mutation rate in their offspring (F_2 individuals).

The results of this study imply that genome-wide increases in recombination cannot account for the major mutation mechanism at ESTR loci which itself suggests that ESTRs do not mutate in a similar manner to human GC-rich minisatellite loci; however it remains possible that the mutation mechanism at ESTRs may be similar to those observed at other repetitive regions. It is possible that the mechanisms acting at ESTRs may be similar to those observed at microsatellite loci, as the internal structure of both repeat types are similar; details of the similarity between possible mutation mechanisms at ESTR and microsatellite loci are given in Chapter 1, Section 1.5.5.2, and the potential mutation mechanisms at microsatellite loci are covered in detail in Chapter 1, Section 1.6.2.

Surprisingly, paternal preconceptional exposure to cisplatin had no effect on the rates of meiotic crossing-over (Table 3.3) for any of the six chromosomes (Figure 3.8 a), any of

the males (Figure 3.7 d) or any time point studied 3, 4, 5 or 6 weeks (Figure 3.7 c). These findings contrast with recent evidence suggesting that exposure to the same dose of cisplatin at the primary spermatocytes could result in elevated crossover rates (Hanneman *et al.*, 1997a). The discrepancy between the data sets cannot be attributed to the statistical power of the data presented here, i.e. the 1.8-fold increase in crossover rates at 4 weeks reported by Hanneman *et al.* (1997a) should have resulted in a total crossover frequency of 606% (1.8 x 337) in the present study but the observed frequency at the 4 week time point was 362 ± 27% (data taken from Table 3.3). The difference between these values corresponds to 8.9 standard error units and the probability for obtaining this difference by chance is well below 10^{-8}. The corresponding estimates for chromosome 10 (with similar inter-marker coverage between the two experiments) yielded a probability of 2×10^{-5}, for the data presented here being unable to detect an increase in recombination rate similar to that observed in Hanneman *et al.* (1997a). The discrepancy between the studies may be due to differences in the inbred mice strains used between the two experiments, (C57BL/6J x DBA/2J) for Hanneman *et al.* (1997a) compared with (C57BL/6J x CBA/Ca) for this investigation. As mentioned in Section 3.2, CBA and DBA mice are closely related and therefore should share a common genetic background (Festing, 1996). It remains possible that F_1 hybrid mice derived from these two different strains may differ greatly in their response to cisplatin; if true, the remarkable strain differences may shed further light on the mechanisms involved in the recognition and repair of cisplatin adducts and would warrant further study.

Cisplatin is known to be a powerful cross-linking agent with the capacity to form a number of DNA adducts (Kartalou and Essigmann, 2001b, see Section 3.1.2.3 for further details). The most important type of adduct, with respect to this study, is the inter-strand adduct formed between guanine residues in the sequence d(GpC); although this type of adduct may only represent approximately 2% of the total number of adducts formed, it has

been shown to be associated with the clastogenic effects of cisplatin (Kartalou and Essigmann, 2001b). Such inter-strand adducts form cross-links between the two DNA strands of the double helix, and this causes severe bending of the backbone, leading to helix distortion and hence problems with DNA stability, replication and repair. The most likely route of repair of this type of adduct involves the formation of DNA double–strand breaks (DSBs) (De Silva *et al.*, 2000) followed by the induction of homologous recombination, via the formation of Rad51 and Rad54 nuclear foci which are thought to form the repair centres for DNA damage (Haaf *et al.*, 1995; Tan *et al.*, 1999; Tashiro *et al.*, 2000). This evidence would suggest that exposure to cisplatin should increase recombination events within mammalian cells, especially if treatment were targeted to recombinationally active cells. The exposure of somatic tissues to cisplatin shows the induction of mutations at protein-coding genes, as well as increases in recombination events, such as sister-chromatid exchange and chromosome loss (Sanderson *et al.*, 1996). Chromosome aberrations have also been observed in mouse spermatocytes (Adler and el Tarras, 1990) which have a doubling dose of 0.5 mg/kg (Adler and El-Tarras, 1989); these types of aberrations are also observed in mouse spermatogonial stem cells (Meistrich *et al.*, 1982). In contrast, the results of a number of studies provide no evidence for mutation induction in the germline of male mice exposed to cisplatin (Katoh *et al.*, 1990; Russell, 1990; Witt and Bishop, 1996). The data from these studies show that treatment with relatively high doses (10mg/kg) of cisplatin does not increase the frequency of specific-locus mutations or dominant lethals in the offspring derived from any stage of spermatogenesis from exposed males. The analysis of ESTR mutation rates in the germline of male mice also exposed to 10 mg/kg of cisplatin showed no effect on any of the stages of spermatogenesis studied. The reasons for the resistance of the mouse germline to the mutagenic effects of cisplatin remain unclear. Testicular tumours have been shown to be intrinsically sensitive to the cell-killing effects of cisplatin, whereas tumours in a number

of somatic tissues, including bladder and colon cancers, have been shown to be highly resistant to such effects (Sark *et al.*, 1995; Masters *et al.*, 1996). Differences have been noted in the cellular environment of germ cells when compared to somatic tissues and which may effect the cells' ability to cope with the DNA damage induced by cisplatin (Kartalou and Essigmann, 2001a). These differences include the levels of activated intra-cellular proteins (such as gluthionine and metallothioneins), the expression of regulatory proteins (oncogenes and tumour suppressor genes), the DNA repair status of cells (with respect to nucleotide-excision repair, recombination proficiency and mismatch repair). Testicular tumours overall seem to express lower levels of intra-cellular and regulatory proteins than other tissues (Kartalou and Essigmann, 2001a), and have also been shown to have lower levels of proteins involved in nucleotide excision repair (Koberle *et al.*, 1999). Conversely, testicular tumours have high expression levels of mismatch repair proteins when compared to somatic tissues (Mello *et al.*, 1996). These differences in the cellular environment of the testis may go some way to explain the differences observed between the somatic and germline effects of cisplatin. Another possible explanation for the resistance of the mouse germline to the mutagenic effects of cisplatin may be the existence of a very efficient DNA repair system acting in pre-meiotic cells which is capable of the high-fidelity removal of cisplatin adducts. The high mobility group proteins (HMG) have a potential role in mediating the anchoring of cisplatin-damaged DNA to the nuclear matrix where DNA repair is thought to take place; they may therefore increase the efficiency of cellular repair pathways (Boulikas, 1996). Apoptosis may also play an important role in protecting the germline against cisplatin-induced damage, as exposure of male mice to doses of cisplatin greater than 7.5 mg/kg has been shown to greatly reduce testicular weight in a dose dependent fashion, suggesting increased cellular cytotoxicity and apoptosis (Adler and el Tarras, 1990). Evidence from testicular tumours also suggests that the testicular cells readily undergo *p53*-mediated apoptosis, and show high testicular levels

of the active *p53* protein (Zamble *et al.*, 1998). Cisplatin has also been shown to activate apoptosis via the JNK pathway in ovarian cancers (Fajac *et al.*, 1996). Increased levels of apoptosis could lead to a selective loss of sperm carrying cisplatin-damaged DNA, which could result in a decrease of mutations observed within the offspring of treated males. The results obtained here show no mutation induction in sperm cells that were either pre- or post-meiotic at the time of exposure suggesting that the predominant method of the removal of cisplatin adducts is by the apoptotic pathway and other direct cell-killing effects.

The possibility still remains that recombination processes may affect instability at ESTR loci, but in a more localised context via recombination hotspots rather than at the genome-wide level, as studied here. A number of publications have shown that localised recombination hotspots appear to drive recombination in the mouse genome (Edelmann *et al.*, 1989; Shiroishi *et al.*, 1991; Bryda *et al.*, 1992), in humans (Jeffreys *et al.*, 1998a; Jeffreys *et al.*, 1999; Badge *et al.*, 2000; Jeffreys *et al.*, 2001) and in yeast (Fan *et al.*, 1997; Fox and Smith, 1998). Interestingly, a number of hotspots have also been found in association with repetitive DNA sequences, such as the human minisatellite MS32 (Jeffreys *et al.*, 1998b), MT elements and LTR elements in mice (Shiroishi *et al.*, 1991), and in GC-rich chromosomal domains in yeast (Gerton *et al.*, 2000). These data show that recombination hotspots can be found in regions of the genome containing repetitive DNA sequences, and may be associated with the high mutation rates associated with such repetitive sequences. The experiment performed here was designed to assess the genome-wide levels of recombination, and it is possible that localised hotspots of recombination may not have been detected. Chromosome 4 was analysed in the recombination assay covering the region surrounding the mouse ESTR *Ms6-hm* (40 cM away from the centromeres, see Figure 3.3) however, no changes in crossover frequency were detected in

this region. A recombination hotspot could be located near *Ms6-hm*, but the detection of any hotspot activity was not possible due to the large inter-marker distances in this region.

In conclusion the evidence presented here shows that acute exposure to X-rays results in significant increases in mutation induction at ESTR loci for all stages of spermatogenesis prior to metaphase I, whereas the treatment of male mice with the anticancer drug cisplatin has no effect on ESTR mutation rate. These data reflect previous analyses showing the highly mutagenic capability of X-rays in inducing germline mutations in male mice and the lack of germline mutations in cisplatin-treated mice (Searle, 1974; Katoh *et al.*, 1990; Russell, 1990; Witt and Bishop, 1996). These findings suggest that mutation induction at ESTR loci and protein-coding genes may follow similar patterns, further supporting the use of ESTR loci as a means of germline mutation detection in mice. Treatment with either mutagenic agent (ionising radiation or cisplatin) was unable to increase the rate of crossing-over in the germline of male mice directly exposed during the meiotic division. These data provide evidence that the mechanisms leading to mutation induction at ESTR loci and meiotic recombination are separate, and that the increases in mutations observed in ESTR after exposure to ionising radiation cannot be attributed to alterations in genome-wide levels of meiotic recombination.

Chapter 4

The analysis of germline mutation rates at expanded simple tandem repeat loci in scid miceIntroduction

4.1.1 General introduction

The results of previous studies suggest that mutation induction by ionising radiation at ESTR loci does not result from direct DNA damage (Sadamoto *et al.*, 1994; Fan *et al.*, 1995; Niwa *et al.*, 1996; Dubrova *et al.*, 1998; Dubrova *et al.*, 2000). The possibility that a genome-wide increase in meiotic recombination could account for radiation-induced ESTR mutation in the mouse germline has been also been excluded (Barber *et al.*, 2000, and Chapter 3); therefore, the mechanism(s) of mutation induction at ESTR loci by ionising radiation remains uncharacterised. It is possible that the ESTR mutation rates could be altered after exposure to ionising radiation as a by-product of DNA repair; this hypothesis may be investigated by the analysis of mice with known mutations or gene knockouts in key components of the DNA repair pathways. The following sections describe the various pathways of double-strand break repair, highlighting their role in the removal of radiation-induced DNA damage.

The presence of DNA DSBs has been shown to be highly deleterious to the cell; for example, a single un-repaired DSB in a dispensable single-copy plasmid results in cell death in yeast (Bennett *et al.*, 1993). Un-repaired DNA double-strand breaks are also highly recombinogenic and potentially mutagenic during the processes of DNA synthesis and cell division (Kanaar *et al.*, 1998; Haber, 2000; Pastink *et al.*, 2001). Checkpoint activity would normally detect DNA damage and lead to cell cycle arrest, until repair could be completed, or to apoptosis. Therefore efficient repair systems have been developed to prevent DNA damage being accumulated in cells during multiple cell divisions, which could potentially lead to genomic instability and carcinogenesis (Pastink *et al.*, 2001).

4.1.2 DNA double-strand break repair

DNA double-strand breaks can arise in a number of different ways; these may be spontaneous chromosomal breaks during replication, or by the action of specific endonucleases involved in processes such as legitimate recombination during meiosis and during V(D)J recombination in the production of antibodies. Additionally, exposure to mutagenic agents, such as ionising radiation has also been shown to lead to DNA double-strand breaks (Frankenberg-Schwager, 1990).

Two major pathways are recruited in mammalian cells for the repair of DNA DSBs, homologous recombination (HR) and non-homologous end joining (NHEJ). The processes of DNA double-strand break repair have been shown to be important for the maintenance of genome stability within a cell; however, the processes themselves are not without error. Errors in DNA repair can in turn lead to elevated mutation rate and could also result in persistent genomic instability (Pastink *et al.*, 2001).

Homologous recombination occurs via strand invasion and break-induced replication; these processes use the transfer of genetic information from an un-cleaved donor molecule to a cleaved recipient molecule (Szostak *et al.*, 1983) (see Figure 4.1). In mammals, a large number of proteins are directly involved in homologous recombination, including the RAD52 group, ATM, NBS and several RAD51-like proteins (reviewed by Pastink *et al.*, 2001). The targeted disruption of a number of the genes involved in HR, including *RAD50*, *MRE11* and *RAD51*, has shown that these genes are essential for embryogenesis, and indicates that hi-fidelity DNA DSB repair is required in early development (Pastink *et al.*, 2001).

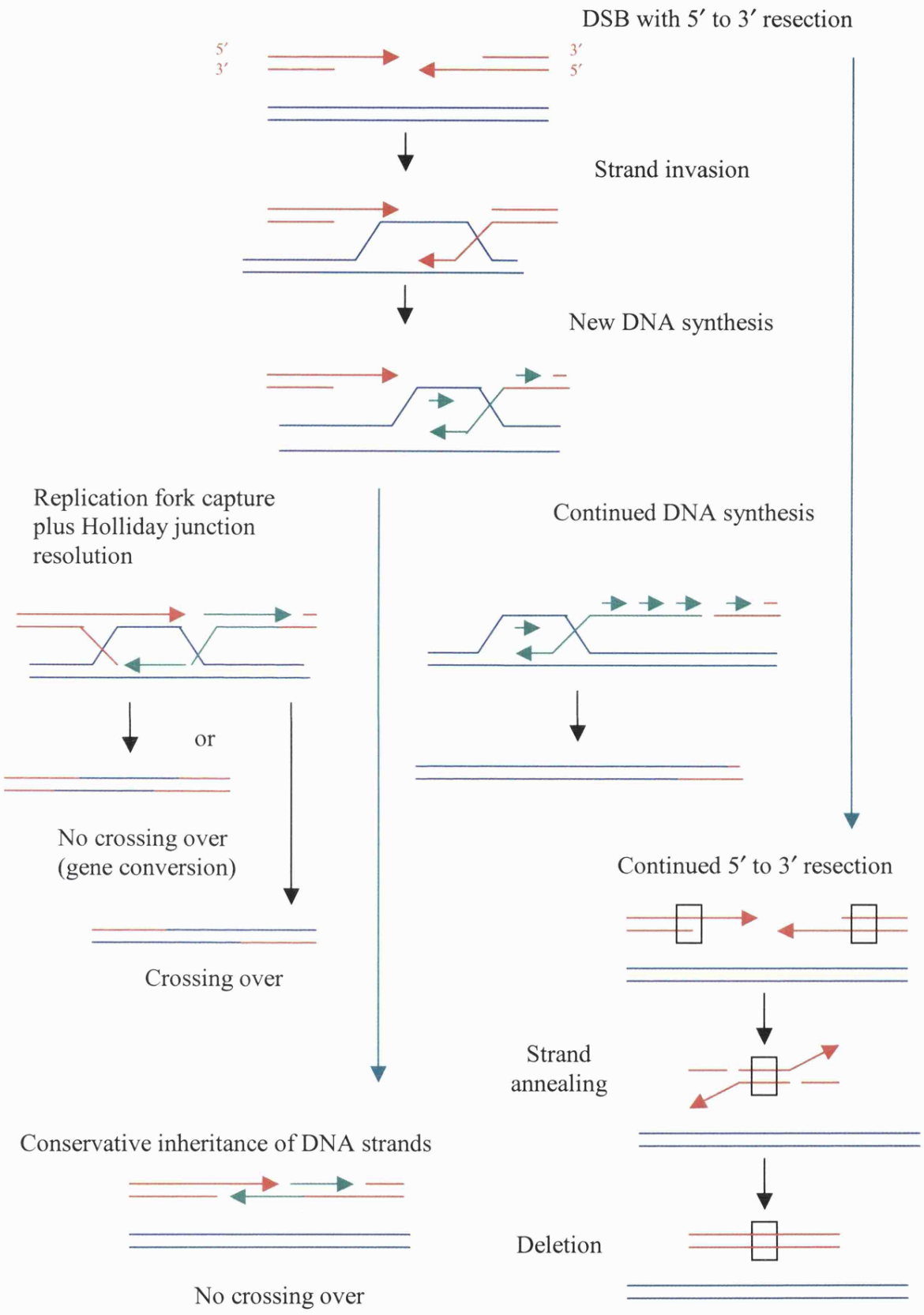


Figure 4.1 - The repair of DNA double-strand breaks via homologous recombination. Adapted from Haber (2000).

The process of single-strand annealing (SSA) represents another form of DSB repair relying on the use of homologous regions. SSA utilises small stretches of homology in the region surrounding the double-strand break for alignment of two broken DNA strands; the use of this pathway is usually restricted to cases where the homologous regions are arranged as direct repeats (Fishman-Lobell *et al.*, 1992) (see Figure 4.1). Genes associated with SSA include *RAD1*, *RAD10*, *RAD52*, *RAD59*, *MSH2* and *MSH3* (Pastink *et al.*, 2001).

Non-homologous end joining provides a mechanism of DSB repair where broken DNA ends are joined that have little or no homology. The mechanism of non-homologous end joining has been studied most extensively in rodent cell lines which are X-ray sensitive, such as those carrying the *scid* mutation (described in detail in Section 4.1.3). Such studies have shown that NHEJ has an important role in the repair of X-ray induced DSBs and is also vital for the repair of DSBs generated via V(D)J recombination for the production of immunoglobulins (Peterson *et al.*, 1995). The process of NHEJ involves a number of proteins (Figure 4.2), including ligase IV and its associated protein XRCC4, and the three components of the DNA-dependent protein kinase (DNA–PK) complex, which are Ku70 (encoded by the *XRCC6* gene), Ku80 (encoded by the *XRCC5* gene) and the catalytic subunit DNA-PKcs (encoded by the *Prkdc* gene also known as *XRCC7*) (reviewed by Pastink *et al.*, 2001).

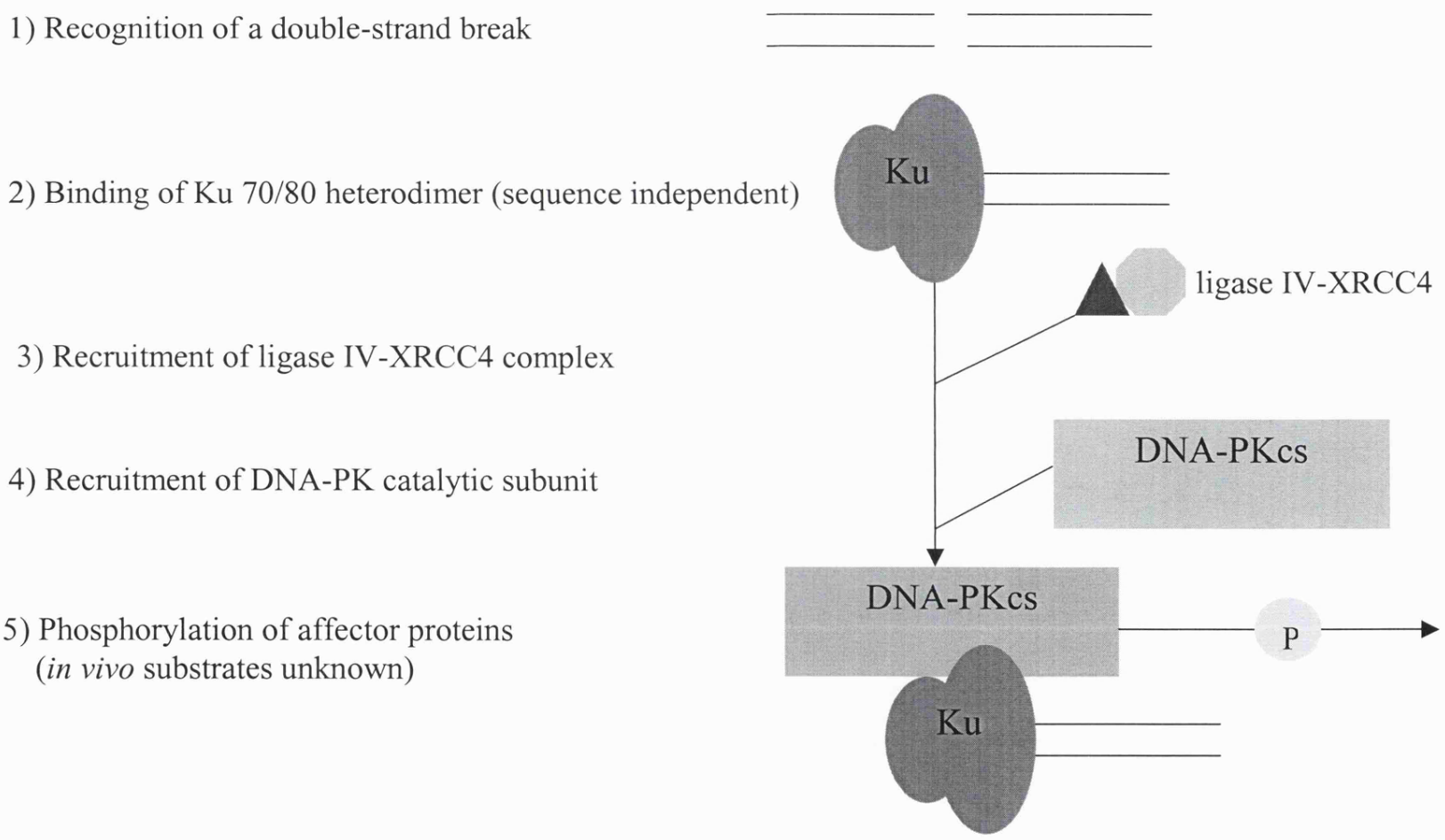


Figure 4.2 – DNA double-strand break repair via non-homologous end joining.

Adapted from Muller *et al.* (1999) with additional data from Pastink *et al.* (2001).

It is known that two of the three major repair mechanisms have low levels of fidelity; single-strand annealing can result in deletions and non-homologous end-joining is often associated with the loss or gain of a number of nucleotides (Pastink *et al.*, 2001). In contrast, homologous recombination normally leads to the correct repair of the DNA double-strand breaks, although it may result in homozygosity of the repaired region. The loss of heterozygosity (LOH) in certain regions of the genome, often where tumour suppressor genes are located, has been found to be associated with carcinogenesis (Jones and Baylin, 2002). The problems associated with the different repair pathways may explain the use of the three different mechanisms to repair DSBs in mammalian cells. It has been shown that the preference for one mechanism over the others may depend on a number of factors, including the stage of development of the organism or the phase of the cell cycle (reviewed by Haber, 2000 and Pastink *et al.*, 2001). It has also been suggested that the preference for the NHEJ pathway may exist for genomes containing large numbers of repetitive regions. It is thought that the preference of NHEJ may be especially important where the regions of homology between potential donor and recipient strands are small, and that this process may help to prevent large-scale rearrangements due to recombination between dispersed elements such as Alu elements (Haber, 2000).

Given the importance of HR and NHEJ in the maintenance of genomic stability, it may be possible that the activation of these pathways by DNA damage could indirectly affect ESTR mutation rate. The analysis of gene mutations or knockouts of the key components of these pathways may provide information about the mechanisms of radiation-induced ESTR instability in the mouse germline. The *scid* mutation in mice which severely affects the activity of the DNA-dependent protein kinase catalytic subunit, encoded by the by the *Prkdc* gene, has been chosen for the current study aiming to test the hypothesis that ESTR instability may be a by-product of repair of radiation-induced DSBs.

4.1.3 Characteristics of the scid mutation in mice

4.1.3.1 The scid phenotype

The severe combined immunodeficiency disease has been characterised for a number of years in humans and is due to a deficiency in the maturation of both T and B-lymphocytes. Human studies have demonstrated that the condition is congenital and shows an autosomal recessive mode of inheritance; a similar severe combined immunodeficiency (scid) phenotype was also found in mice (Bosma *et al.*, 1983). Further analysis of scid mice has shown that the lymphoid organs of scid mice are less that one tenth the size of those of the isogenic C.B17 strain and the lack of mature T cells is manifested by an inability of scid mice to reject unmatched skin grafts (Bosma *et al.*, 1983). Additional transplantation studies have shown that, although scid mice lack mature immunoglobulin stem cells, they are capable of supporting the development of stem cells transplanted from BALB/c mice (Bosma *et al.*, 1983). Bosma *et al.* (1983) also demonstrated some evidence that scid mice are capable of producing mature T and B cells but at a very low rate. A high proportion of the resulting mature T cells, but not the B cells, are highly susceptible to neoplastic transformations leading to the detection of T-cell lymphomas in approximately 10% of scid homozygotes of 5-9 months of age. The characteristics of the scid mutation have been further analysed due to the importance of these mice as a model system for lymphocyte differentiation and regulation, and for the analysis of the association between immunodeficiency and lymphomagenesis.

4.1.3.2 Characterisation of the mutation

Bosma *et al.* (1988) showed that, although the majority of scid mice lack functional lymphocytes, approximately 2-23% develop a limited number of B and T cells between 3 and 9 months of age. The study further demonstrated that mice with a 'leaky' scid phenotype had a higher chance of developing thymic lymphomas, approximately 40% of scid mice showing a small number of mature T cells developed lymphomas. The analysis of the processes leading to the production of mature T and B cells in mice showed that the re-arrangement of antigen receptor genes is defective in mice with severe combined immune deficiency (Schuler *et al.*, 1986). Mice carrying the *scid* mutation are unable to complete V(D)J recombination (the process leading to the assembly of antigen receptor genes in developing B and T lymphocytes) and are hence immunodeficient (Schuler *et al.*, 1986).

The *scid* gene was later mapped to the mouse chromosome 16, 9.2 cM from the centromere (Bosma *et al.*, 1989). A subsequent study shows that the *scid* gene maps to the syntenic region of human chromosome 8 which contains the gene coding for the DNA dependent protein kinase catalytic subunit (DNA-PKcs) also known as *XRCC7*. Complementation of scid fibroblasts with the region of human chromosome 8 containing the DNA-PKcs gene restored the normal phenotype (Miller *et al.*, 1995). Blunt *et al.* (1995) also demonstrated that the *scid* phenotype is attributed to the severely reduced activity of DNA-PKcs. Complementation with DNA-PKcs from yeast artificial chromosomes was able to restore normal V(D)J recombination and abolish the increased radiosensitivity normally observed in *scid* homozygous mice (see Section 4.1.1.3 for details of scid radiosensitivity). The complete inactivation of the *Prkdc* gene, encoding the DNA-PKcs protein, using an insertion in the 5′ region of the gene, the *slip* mutation, recapitulates the *scid* phenotype and demonstrates that Prkdc and scid are allelic (Jhappan *et al.*, 1997).

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is a 460 kDa protein, and is thought to be a member of the phosphatidylinositol-3 kinase (PI-3 kinase)

family due to similarity in the 110-kDa carboxyl terminus of the protein (Hartley *et al.*, 1995). DNA-PKcs possesses properties most similar to the PI kinase family members that are involved in cell cycle control, DNA repair, and DNA damage responses; these include the product of the ataxia telangiectasia gene (*ATM*), mutations in which lead to genomic instability and predisposition to cancer (Hartley *et al.*, 1995). The carboxyl-terminal of DNA-PKcs is highly conserved between humans and rodents, and therefore may be of important functional significance (Blunt *et al.*, 1996). DNA-PKcs has been shown to phosphorylate a number of substrates *in vitro* including Sp-1, c-jun, c-fos, Oct-1, p53, RNA Pol (II), the Ku components and itself (Boubnov and Weaver, 1995; Danska *et al.*, 1996); however the *in vivo* substrates have not yet been identified. Additionally, it has been shown that the DNA-PKcs can only phosphorylate proteins in the presence of DNA ends (Danska *et al.*, 1996). The results of these studies clearly suggested the scid phenotype can be attributed to a mutation affecting the activity of DNA-PKcs and provided the first experimental evidence for the involvement of this protein in V(D)J recombination and the repair of DNA double-strand breaks via the non-homologous end joining pathway (Blunt *et al.*, 1995; Miller *et al.*, 1995 Peterson *et al.*, 1995).

A nonsense mutation was identified in the gene coding for the DNA-PKcs component of the DNA-dependent protein kinase complex (Blunt *et al.*, 1996). The mutation is a single nucleotide polymorphism (SNP), a T→A transversion at amino acid residue 4045, which results in the creation of an ochre stop codon (UAA) and the loss of the terminal 83 amino acids of the DNA-PKcs protein (Blunt *et al.*, 1996). It was also noted that the SNP creates an *Alu*I restriction site, which has been used as a diagnostic test for the presence of the *scid* mutation in genomic DNA. The 'leaky' nature of the *scid* mutation may also be explained by the suppression of the ochre stop codon created by the T→A transversion at amino acid residue 4045 (Danska *et al.*, 1996). The terminal deletion of DNA-PKcs has no direct effect on the kinase domain, but it has been proposed that the deletion may affect protein stability

and sub-cellular location possibly leading to impaired protein function (Danska *et al.*, 1996). Following the discovery of the *scid* mutation, the gene coding for DNA-PKcs was renamed *Prkdc*; however a number of other synonyms are commonly used in the literature including, DNA-PKcs, DNAPDcs, slip, scid, DNA-PK, DNAPK, DNPK1, HYRC1, XRCC7 or severe combined immunodeficiency (from the Mouse Genome Database, www.jax.org/, Blake *et al.*, 1998; Blake *et al.*, 1999).

4.1.3.3 The sensitivity of scid mice to ionising radiation

One of the major characteristics of mice homozygous for the *scid* mutation is an increase in radiosensitivity. The effect of the *scid* mutation on radiosensitivity was first demonstrated by Fulop and Phillips (1990). This study demonstrated that myeloid cells and fibroblasts from scid mice show a marked increase in sensitivity to ionising radiation, indicating that the scid mutation leads to an inability to repair DNA damage induced by ionising radiation. Many studies have further analysed the effect of the *scid* mutation on the radiosensitivity of mouse cells in both *in vitro* and *in vivo* experiments.

***In vitro* analysis of *scid* radiosensitivity**

Further *in vitro* evidence of the increased sensitivity of *scid* cells in culture was obtained by Biedermann *et al.* (1991). In this study, scid, C.B17 and BALB/c fibroblasts were γ-irradiated and the effect of ionising radiation was determined using a number of different endpoints. The LD_{90} test demonstrated that scid cells were two to three-fold more sensitive than either C.B17 or BALB/c, the doses required to produce only a 10% survival of cells after exposure being 2.5, 5 and 7.1 Gy respectively. The levels of DNA repair were also assessed and it was shown that scid cells were only able to repair approximately 37% of cellular double-strand breaks after 24 hrs; conversely the levels of repair in the BALB/c lines were approximately 90-95% after only 6 hrs. These data suggest that scid cells have a

reduced ability to repair radiation-induced DNA double-strand breaks, which may account for the increased radiosensitivity of these cells.

Scid cells were shown to be sensitive to the effects of agents that lead to an increase in DNA double-strand break formation, such as bleomycin, but no effect was observed after treatment with mitomycin C or UV irradiation, both of which do not result in direct DSB formation (Biedermann *et al.*, 1991). The data from this study clearly demonstrate that *in vitro* scid cells show sensitivity to the DNA-damaging effects of ionising radiation and strongly suggest that this is due to a reduced ability in the repair of DNA double-strand breaks.

Scid cells also show differences in sensitivity to ionising radiation over certain stages of their cell cycle. It has been demonstrated that contrary to normal cells, scid cells show an increased sensitivity to both chromosome and chromatid-type aberrations if irradiated during the G_1 stage of the cell cycle. This G_1 sensitivity is similar to that for other radiosensitivity mutants, such as ataxia telangiectasia and Nijmegen Breakage Syndrome (van Buul *et al.*, 1998). It has also been demonstrated that the levels of DNA-PK activity during the cell cycle alter, and that in scid mice this loss of the activity in G_1/early S phase of the cell cycle correlates with an increased sensitivity to the effects of ionising radiation (Lee *et al.*, 1997). Additionally, the lack of DNA-PK activity in the G_2 phase of the cell cycle appears to have an effect on the cell cycle checkpoint control, with an increased number of scid cells showing cell cycle arrest at the G_2 checkpoint (Lee *et al.*, 1997).

In vivo analysis of *scid* radiosensitivity

The analysis of the *in vivo* levels of radiosensitivity in scid mice was performed by Biedermann *et al.* (1991). The results of this study show a two-fold increase in mortality and a three-fold increase in skin sensitivity in scid mice compared to those in congenic BALB/c mice. Bone marrow cells of scid mice are also two to three times more sensitive to cell killing than those in BALB/c or C.B17 (Biedermann *et al.*, 1991). It was also noted that scid mice showed a whole body sensitivity to the effects of ionising radiation, where as in other immunoglobulin mutants, such as wasted (*wst*), the increased sensitivity is restricted to the bone marrow. These data suggest that the *scid* mutation may be responsible for an increase in radiosensitivity *in vivo* as well as in cell culture.

The induction of chromosomal aberrations in scid mice after exposure to ionising radiation was also studied. The induction of structural chromosomal aberrations was shown to be greatly enhanced in bone marrow cells of scid mice compared to controls (Disney *et al.*, 1992; Grigorova *et al.*, 1995), and high levels of cell killing were observed in the bone marrow (van Buul *et al.*, 1995; van Buul *et al.*, 1998). Surprisingly, the recovery of stable chromosomal translocations from scid bone marrow cells was extremely low (Grigorova *et al.*, 1995), whereas normal levels of radiation-induced micronuclei in bone marrow polychromatic erythrocytes were recorded (van Buul *et al.*, 1995; van Buul *et al.*, 1998; Kobayashi *et al.*, 1997). It should be stressed that the majority of the studies assessing the effect of the *scid* mutation on radiosensitivity in mice have concentrated on the analysis of somatic cells, and the issue of radiosensitivity of the germ cells in scid mice has not yet been investigated in detail. The results of several studies have demonstrated the reduction of both testis weight and the survival of spermatogonial stem cells in scid mice, after irradiation, when compared with C.B17 mice (van Buul *et al.*, 1995; van Buul *et al.*, 1998, van Buul and van Duyn-Goedhart, 1996). Furthermore the enhanced induction of chromosomal aberrations observed for scid somatic G_2 phase cells was also present at meiotic Diakinese-

Metaphase I spermatocytes (van Buul *et al.*, 1998; van Buul *et al.*, 1999). These data suggest that the *scid* mutation also confers an increased sensitivity to the effects of irradiation on the mouse germline. However, as found for bone marrow, the recovery of radiation-induced chromosomal translocations was extremely low from scid stem cell spermatogonia (van Buul *et al.*, 1995, van Buul.and van Duyn-Goedhart, 1996; van Buul *et al.*, 1998).

Additional analysis of the effects of ionising radiation on the scid phenotype demonstrated that the exposure of scid cells in long-term bone marrow culture to ionising radiation (4 Gy) can lead to the reconstitution of normal lymphocytes by inducing colony-forming B cell development and normal cytotoxic T cell activity after transplantation into an ablated mouse host (Fulop and Phillips, 1986). Neonatal exposure to ionising radiation, and a number of other DNA-damaging agents, has been shown to reverse at least part of the scid phenotype; the adult mice show normal T cell development, but no B cell maturation (Danska *et al.*, 1994). This study also demonstrated that all scid mice treated with DNA-damaging agents developed thymic lymphoma; this suggests a correlation between V(D)J recombination, DNA double-strand break repair, and lymphomagenesis.

4.1.3.4 The effect of the *scid* mutation on genome stability

The results of two recent studies show that the scid mutation affects spontaneous somatic and germline mutation rates at the mouse ESTR locus *Ms6-hm* (also known as *Pc-1*, see Imai *et al.*, 1997; Yamauchi *et al.*, 2002). It has been suggested that there may be a link between the inactivation of the family of protein serine/threonine phosphatases (of which the DNA-PK complex is a member) and tumourigenesis, and that this could be associated with a decrease in the cells' ability to repair DNA double-strand breaks, leading to genomic instability (Imai *et al.*, 1997). The analysis of the spontaneous mutation rates of *Ms6-hm* in a scid fibroblast cell line showed an increase in ESTR instability compared to a normal cell line. Additionally when the scid cell line was complemented with the part of human chromosome 8, known to contain the *Prkdc* gene, much lower spontaneous ESTR

mutation rates were observed than in the scid cell line which suggests a possible role for DNA-PK in the maintenance of genomic stability (Imai *et al.*, 1997). The effect of the *scid* mutation on spontaneous mutation at *Ms6-hm* in the mouse germline has also been investigated (Yamauchi *et al.*, 2002). The results of this study demonstrate that the germline mutation rate at *Ms6-hm* is also highly elevated *in vivo* when compared to the isogenic C.B17 mouse strain, further supporting the hypotheses that the function of DNA-PK is important for the maintenance of genome stability.

The possibility that DNA-PK may be important in the mechanism of instability at ESTR loci in the soma and in the germline of mice (Imai *et al.*, 1997; Yamauchi *et al.*, 2002) and the fact that the *scid* mutation confers increased radiosensitivity suggests that the investigation of the effect of ionising radiation on the germline of male scid mice may be useful in elucidating the mechanism of radiation-induced germline instability at ESTR loci.

4.2 Experimental design

Male C.B17 and scid mice, which were exposed to testicular irradiation of 1 Gy of X-rays, or control (untreated males) were mated to untreated C57BL/6 females. Testicular irradiation was performed to minimise the adverse effects of the whole-body irradiation on the survival of the highly radiosensitive scid mice. The C57BL/6 females were chosen because the size range of alleles at both ESTR loci in this strain clearly differs from that in the scid/C.B17 mice. This substantially facilitated mutation scoring and allowed unambiguous establishment of the parental origin of mutant bands. All parents and offspring were profiled using two mouse-specific hypervariable single-locus ESTR probes, Ms6-hm and Hm-2. Additionally an RFLP analysis, using the *Alu*I restriction site created by the *scid* single nucleotide polymorphism, was used to confirm *scid* status of all individuals.

4.3 Summary of mutation induction at ESTR loci

Table 4.1 provides a summary of the ESTR mutation data. The ESTR mutation rates for the loci *Ms6-hm* and *Hm-2* are shown for the C.B17 and scid control males (non-exposed), and for the male mice of both strains after exposure to testicular irradiation of 1 Gy of X-rays.

4.4 A comparison of spontaneous ESTR mutation rates between C.B17 and scid males

The data presented in Table 4.2 and Figure 4.3 clearly show differences in the spontaneous ESTR mutation rate in the germline of C.B17 and scid male mice. The spontaneous ESTR mutation rate for scid males is statistically significantly elevated; it is almost double that of the C.B17 isogenic strain of mice. A comparison of the spontaneous ESTR mutation rates for the C.B17 and scid strains with the rates obtained for other strains shows that scid mice have the highest spontaneous mutation rate observed to date (Figure 4.4). As expected, the spontaneous mutation rates for the C.B17 and BALB/c mice are very similar. C.B17 mice were derived from a backcross of (BALB/c x C57BL/Ka) x BLAB/c, and have been shown to have characteristics similar to those of BALB/c mice (Festing, 1996).

Strain	Dose	No. of males	No. of offspring	ESTR loci*					
				Ms6-hm		*Hm-2*		*Ms6-hm + Hm-2*	
				Paternal	**Maternal**	**Paternal**	**Maternal**	**Paternal**	**Maternal**
C.B17	Control (0 Gy)	7	94	14 (14)	9 (4)	10 (10)	9 (7)	24 (24)	18 (11)
scid	Control (0 Gy)	14	190	42 (38)	11 (6)	48 (38)	3 (3)	90 (76)	14 (9)
C.B17	Exposed (1 Gy)	7	102	29 (29)	4 (4)	21 (17)	6 (4)	50 (46)	10 (8)
scid	Exposed (1 Gy)	11	114	28 (20)	4 (4)	23 (23)	4 (4)	51 (43)	8 (8)

Table 4.1 – Summary of mutation data for C.B17 and scid male mice.

* Number of individual mutation are given in parenthesis (x).

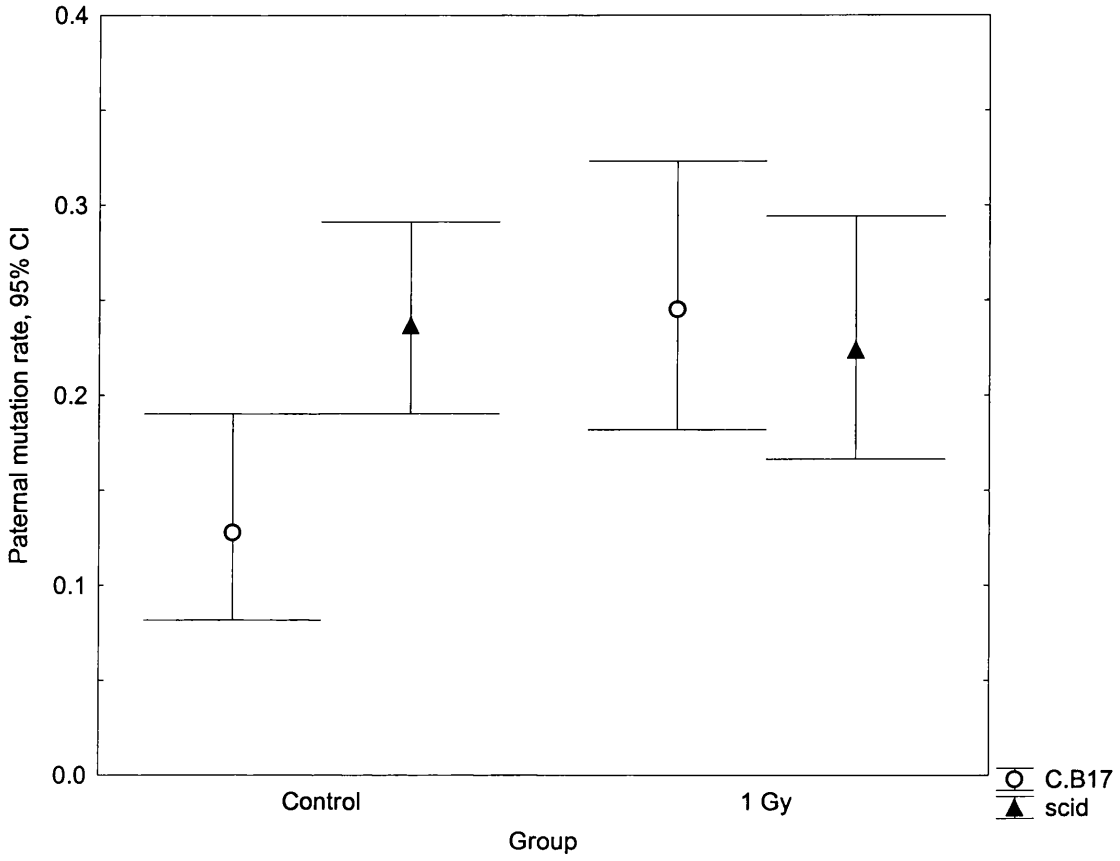


Figure 4.3 – Spontaneous and induced ESTR mutation rates in the germline of C.B17 and scid mice.

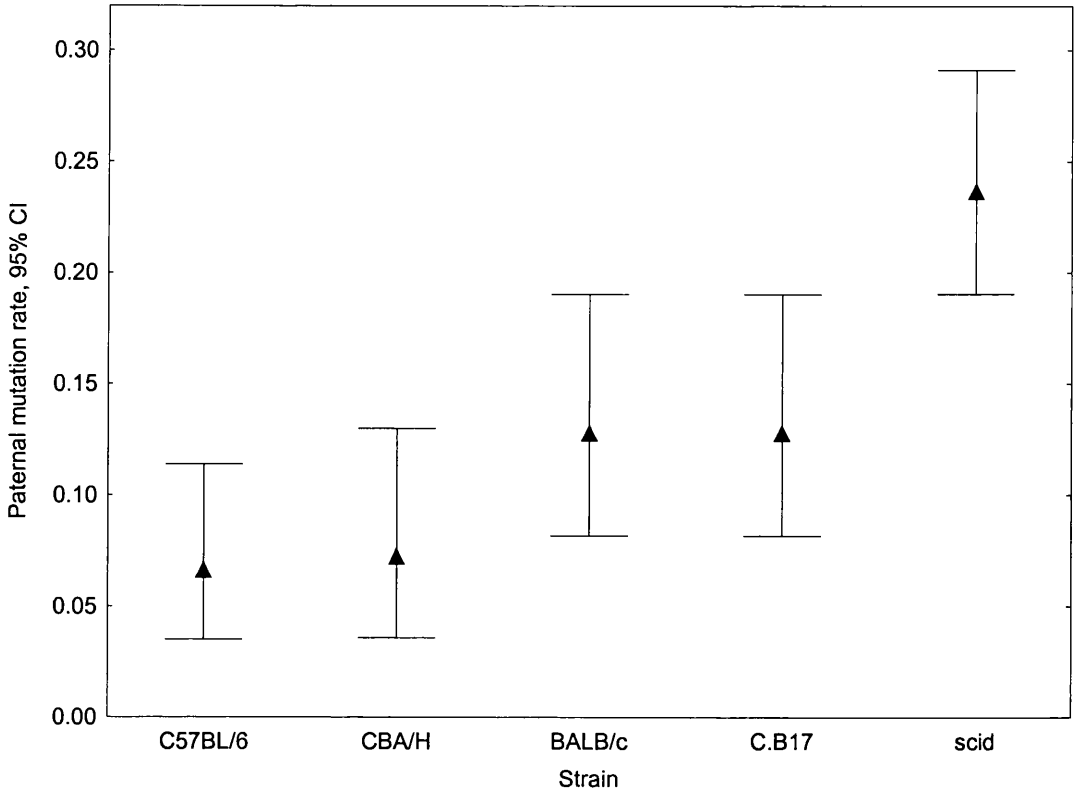


Figure 4.4 – Comparison of the spontaneous ESTR mutation rate in the male germline of five different inbred strains of mice.

(Data for C57BL/6, CBA/H and BALB/c are given in Chapter 5, Table 5.1).

4.5 ESTR mutation induction in C.B17 and scid mice

ESTR mutation rates in the control and exposed C.B17 and scid mice are shown in Table 4.2 and Figure 4.3. The data for C.B17 mice clearly show evidence for mutation induction at ESTR loci after exposure to 1 Gy of X-rays. The mutation rate in the germline of exposed C.B17 males is significantly increased (almost 2 fold) when compared to the unexposed males. In contrast, scid males show no evidence of mutation induction after irradiation; the mutation rates in the non-exposed and exposed males are similar.

A correlation analysis between the effect of radiation exposure and ESTR mutation rate was also performed (see Figure 4.5). C.B17 mice show a positive correlation, i.e. the mutation rate in the germline for most male mice increases after irradiation. The scid mice however show no correlation between radiation exposure and ESTR mutation rate; the mutation rates observed in all males do not alter significantly between the non-exposed and exposed groups.

Strain	Dose	Mutation rate	Ratio	Probability[¶]
C.B17	Control (0 Gy)	0.1277	-	-
scid	Control (0 Gy)	0.2368	1.86[†]	0.0025
C.B17	Exposed (1 Gy)	0.2451	1.92[†]	0.0042
scid	Exposed (1 Gy)	0.2237	1.75[†]	0.0151
			0.94[‡]	0.7880[‡‡]

Table 4.2 - Germline mutation rates in the C.B17 and scid males.

[†] Ratio compared to mutation rate in non-exposed C.B17 males.

[¶] Probability of difference from the non-exposed C.B17 males (Fisher's exact test, two-tailed).

[‡] Ratio of exposed scid compared to mutation rate in non-exposed scid males.

[‡‡] Probability of difference from the non-exposed scid males (Fisher's exact test, two-tailed).

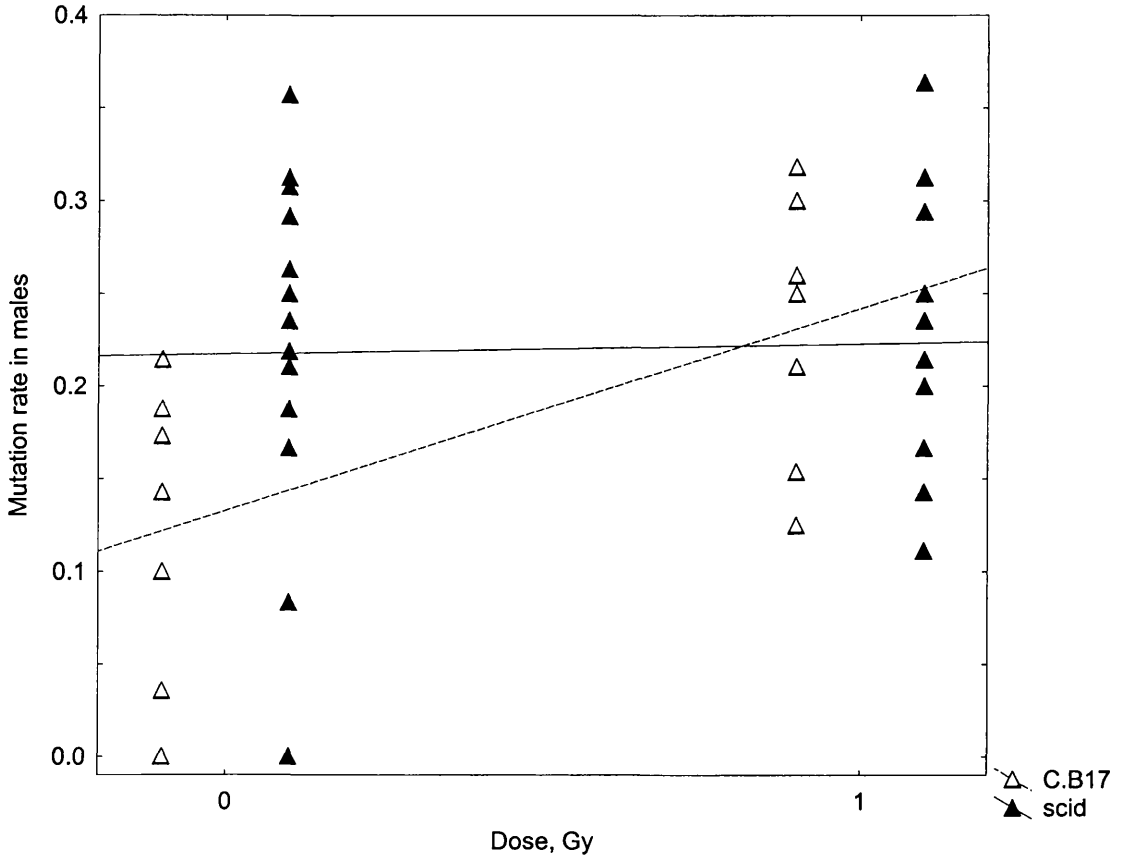


Figure 4.5 – Scatterplot of ESTR mutation rate in C.B17 and scid male mice before and after exposure to X-rays.

Each point represents the mutation rate in the germline of an individual male.

The figure shows a lack of mutation induction after exposure to 1 Gy of X-rays for scid males.

4.6 Genotyping for the *scid* mutation

Due to the unexpected result which showed a lack of mutation induction at ESTR loci in scid mice after exposure to ionising radiation, an RFLP analysis was performed to assess the *scid* status of all individuals.

The analysis of the parental strains demonstrated that that all of the proposed scid males were homozygous positive for the *scid* mutation, and that all proposed C.B17 males and C57BL/6 mothers were homozygous negative for the *scid* mutation. The offspring were also analysed – those derived from a cross between C.B17 males and C57BL/6 mothers were homozygous negative for the *scid* mutation and those derived from crosses between *scid* homozygous positive males and C57BL/6 mothers were heterozygous for the *scid* mutation.

4.7 Discussion

The aim of this study was to investigate the effect of mutations in genes involved in the repair of DNA damage on the levels of mutation induction at ESTR loci. It has been proposed that this work might help to elucidate the mechanism of mutation induction at ESTR loci. The mutation chosen for this study was the *scid* mutation. The *scid* mutation is a naturally occurring single nucleotide polymorphism leading to the truncation of the catalytic subunit of the DNA-dependent protein kinase complex (Blunt *et al.*, 1996). It has been shown that the *scid* mutation, which is found in the *Prkdc* gene, reduces the ability of cells to repair DNA double-strand breaks (DSBs) which leads to an immunodeficient phenotype due to an inability to produce mature T and B cells via V(D)J recombination; it also leads to an increase in radiosensitivity due to a decrease in DNA repair by non-homologous end-joining (Schuler *et al.*, 1986; Fulop and Phillips, 1990; Biedermann *et al.*, 1991).

The data presented here show that the C.B17 mouse strain, the isogenic strain for the *scid* mutation, has relatively high spontaneous ESTR mutation rates and shows statistically significant increases in ESTR mutation rate after exposure to ionising radiation. In contrast, scid mice show a highly elevated spontaneous ESTR mutation rate, but no evidence of mutation induction after exposure to ionising radiation. These data suggest that non-homologous end joining may be involved in the maintenance of ESTR stability in the mouse germline.

The fact that scid mice show a high spontaneous mutation rate at ESTR loci may be explained by the inefficient repair of endogenous DNA double-strand breaks. It has also been demonstrated *in vitro* that minimal levels of DNA-PK activity can substantially restore the *scid* defect associated with V(D)J recombination but that normal cellular levels are required to correct the radiosensitivity phenotype, possibly due to the much large number of DNA double-strands within the cell (Kienker *et al.*, 2000).

The high spontaneous level of ESTR mutations in scid mice, approximately 24% for the combined data for *Ms6-hm* and *Hm-2* (see Table 4.2 and Figure 4.3), does not represent a saturation of the mutation detection system. Previous studies have shown much higher mutation rates after exposure; for example, ESTR mutation rates in the germline of CBA/H male mice exposed to 0.5 Gy of fission neutrons and BALB/c males exposed to 1 Gy of X-rays showed ESTR mutation rates of 32% and 28%, respectively (Dubrova *et al.*, 2000a; Barber *et al.*, 2002). These data clearly demonstrate that the current detection system for ESTR mutations in the germline of male mice is not saturated at the 24 % spontaneous ESTR mutation rate observed for the scid mice; however, the saturation point of the system is yet to be determined.

The *scid* mutation has previously been reported to result in a high spontaneous mutation rate at the *Ms6-hm* locus, in both cell culture and in the mouse germline, and therefore it was proposed that DNA-PKcs may be important in the maintenance of genome stability (Imai *et al.*, 1997; Yamauchi *et al.*, 2002). It may be proposed that the lack of mutation induction at ESTR loci after irradiation of mice carrying the scid mutation is caused by an inability of the cells to repair the large number of DNA DSBs caused by the exposure. It has been shown *in vitro* that normal levels of DNA-PK activity are required to abolish the increased radiosenstivity of scid cells, which may be linked with the cells inability to repair DNA double-strand breaks (Kienker *et al.*, 2000).

The lack of mutation induction at ESTR loci in the germline of scid males after irradiation is paralleled by the lack of induced chromosomal translocations in spermatogonial stem cells. Low levels of chromosomal aberrations are observed in the cells of scid mice even after relatively low dose exposures (0.25 to 0.5 Gy of acute X-rays), where the amount of cell killing is minimal (van Buul *et al.*, 1995). The authors suggest that in scid mice both chromosome and chromatid-type aberrations are induced after irradiation during in the G1 phase of the cell cycle; this differs from the situation

observed in wild type (non-scid) mice where only chromosomal aberrations are induced. Chromatid-type aberrations have been shown to be more damaging to cells than the chromosome-type aberrations; it is therefore possible that cells containing chromatid-type aberrations may be preferentially lost (van Buul *et al.*, 1998). The loss of cells containing chromatid-type aberrations may be able to account for the low yields of chromosomal translocations observed in scid cells after irradiation; this may also be able to explain the apparent lack of ESTR mutation induction after exposure to X-rays.

Previous studies have demonstrated that the irradiation of scid males leads to a reduction in testis weight and a decreased number of spermatogonial stem cells; this suggests that the *scid* mutation may confer an increased sensitivity to the cell-killing effects of irradiation on the mouse germline (van Buul *et al.*, 1995; van Buul *et al.*, 1998). Data from this study also show a significant reduction in litter size in the exposed scid males, providing further indirect evidence for the loss of spermatogonial stem cells in these mice (see Table 4.3). Radiation-induced cell killing has also been demonstrated *in vivo* for somatic tissues, including bone marrow and intestinal crypt cells (Biedermann *et al.*, 1991). The effect of the *scid* mutation on the activation of p53-induced apoptosis has also been studied and has shown that the levels of apoptosis in scid mice are at least as efficient as in wild-type C.B17 mice (Candeias *et al.*, 1997). Recently, it has been demonstrated that DNA-PKcs plays an important role during early meiotic prophase; this role may be linked to a meiotic checkpoint and leads to an increase in the spontaneous levels of apoptosis in the germline of scid males (Hamer *et al.*, 2002).

Mouse strain	Litter size pre-irradiation	Litter size post-irradiation
C.B17	8.8 ± 0.5	7.2± 0.5
scid	7.7 ± 0.3	**5.8 ± 0.5***

Table 4.3 – Litter sizes of C.B17 and scid male mice before and after acute exposure to 1 Gy of X-rays.

* Statistically significantly different from scid males pre-irradiation, t-test $P=<0.05$.

The study of the *slip* mutation, a DNA-PKcs null mutant, has also demonstrated increased apoptosis in mice with this mutation (Jhappan *et al.*, 2000). The *slip* mutation was created in the FVB/N mouse strain using an inactivating mutation in the 5′ region of the *Prkdc* gene (Jhappan *et al.*, 1997), and studies have shown that *slip* mice are also more sensitive to radiation-induced apoptosis than either *scid* mice or the isogenic FVB/N strain (Jhappan *et al.*, 2000). The results of this study show that the *slip* mice have higher than normal rates of apoptosis in untreated thymus cells, and that exposure to ionising radiation (4 or 8 Gy of acute γ-irradiation) leads to a dramatic increase in the number of apoptotic cells in the thymus (Jhappan *et al.*, 2000). The authors have also found that an accumulation of p53 can be observed in irradiated slip cells which is similar to that previously described for scid cells in culture (Burma *et al.*, 1999). It has therefore been proposed that the accumulation of p53 in the presence of un-repaired DNA double-strand breaks in a cell may lead to the activation of ATM and apoptosis (Jhappan *et al.*, 2000). It is also possible that DNA-PK may possess a role in the negative regulation of apoptosis in the thymus by preventing p53 accumulation, thus explaining the increased levels of apoptosis observed in cells containing the *scid* and *slip* mutations (Jhappan *et al.*, 2000).

It has been suggested that *Prkdc* may be a potential candidate for a radiation-induced apoptosis susceptibility gene *Rapop* due to the co-localisation of the *Prkdc* gene with the 0.45 cM critical interval of the apoptosis susceptibility locus on chromosome 16 (Mori *et al.*, 2001). Mouse strains containing the *scid* mutation or allelic variants of the *Prkdc* gene, which result in reduced DNA-PK activity, (such as those described by Yu *et al.*, 2001), show increased levels of apoptosis when compared to strains carrying the normal *Prkdc* gene and have been linked with increased cancer susceptibility (Mori *et al.*, 2001).

It remains possible that cell cycle checkpoints may be important in the removal of damaged scid cells from the germ cell population, which would result in the observed lack of mutation induction at ESTR loci in germline of irradiated scid mice. Work by Lee *et al.*

(1997), analysing scid cells in culture, has demonstrated that DNA-PK may also function at the G_2 checkpoint, and this may be essential for cell survival following DNA damage. The data from this study suggest that the G_2 cell cycle arrest in scid cells that occurs after irradiation (3.75 or 6 Gy of X-rays) was normal, but that the cells were subsequently impaired or defective in their ability to resume cell cycling, which finally results in cellular necrosis. Additional data analysing a mutant hamster cell line sxi-3, that is defective in Ku80, also show G_2 cell cycle arrest after X-ray irradiation and are unable to re-enter the cell cycle (Lee *et al.*, 1997). These data suggest that DNA-PK activity may be required for the correct cell cycle progression after exposure to irradiation, and may therefore provide a possible explanation for the lack of mutation induction at ESTR loci in scid mice where DNA-PK activity is greatly reduced. The loss of DNA-PK activity may prevent cell cycle progression after irradiation *in vitro* via a secondary role for the protein complex itself or as a by-product of un-repaired DNA double-strand breaks (Huang *et al.*, 1996; Lee *et al.*, 1997); however, no data are currently available to support this hypothesis within *in vivo* systems. Therefore it remains possible that the lack of mutation induction observed at ESTR loci in the germline of scid mice after exposure to X-ray irradiation may be due to the loss of damaged cells by either apoptosis or cell cycle checkpoint arrest, or as a combination of the two processes. Further *in vitro* studies are required to clarify the mechanism of radiation-induced cell killing in scid mice.

The data presented here show that the non-homologous end-joining pathway is directly involved in the maintenance of ESTR stability in the mouse germline. This work has, however, failed to ascertain whether the activation of NHEJ could explain the non-targeted increases observed in ESTR mutation rates in the germline of irradiated mice. As mentioned above, the lack of mutation induction in the exposed scid males could be attributed to the loss of cells from their germline. Future studies would require the analysis of mice defective in other essential components of DNA double-strand break repair,

including subunits of the DNA-PK complex (Ku 70 and Ku 80) and other components of recombination repair.

Chapter 5

A transgenerational analysis of the transmission of radiation-induced germline instability

5.1 Introduction

5.1.1 General introduction

Currently little is known about the mechanism(s) of radiation-induced mutations at ESTR loci in the germline of male mice (see Section 1.5.5 for details). The magnitude of increases in mutation rate at ESTR loci in the germline of exposed male mice provides evidence for indirect targeting of ESTR loci by ionising radiation. There is a large discrepancy between the levels of DNA damage induced per nucleus, per Gy of ionising radiation demonstrated by Frankenberg-Schwager (1990) and the actual elevation in mutation rates observed; the difference between the actual and expected yield of mutation per Gy has been calculated to be about 10,000 times (Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a). This evidence suggests that mutations do not arise as a result of direct damage to the ESTR loci themselves, but instead may result as a by-product of alterations elsewhere in the genome (Sadamoto *et al.*, 1994; Fan *et al.*, 1995; Niwa *et al.*, 1996; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a). The analysis of mammalian cells in culture after exposure to ionising radiation has also been demonstrated to cause increases in mutation rates which are greater then can be explained by direct DNA damage (Morgan *et al.*, 1996). The increased mutation rate can be observed not only in directly exposed somatic cells, but also leads to elevated mutation rates for many cell divisions after initial radiation exposure (reviewed by Morgan *et al.* (1996), see Section 5.1.2 for the characteristics of genomic instability). It is possible that radiation exposure of the parental germline can also result in genomic instability and that the delayed effects of instability may be manifested in their offspring.

A number of studies have suggested that germline exposure to ionising radiation may lead to an effect that is manifested in subsequent generations – a transgenerational effect. The first evidence for such an effect was demonstrated by Luning *et al.* (1976); they showed that levels of dominant lethal mutations were elevated in F_1 offspring of males injected with plutonium salts, indicating that germline mutation induction occurred in the directly exposed males. However, increased levels of dominant lethal mutations were also observed in the non-exposed F_2 offspring irradiated males, showing that increases in germline mutation rate were also present in the F_1 germline. Additional evidence from Vorobtsova *et al.* (1993) showed that the incidence of skin cancer was increased in the F_1 and F_2 offspring of X-ray irradiated males after secondary challenge with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a chemical known to induce skin papillomas in mice. These studies suggest that alterations caused to the germline by exposure to ionising radiation may be passed on to subsequent generations.

Dubrova *et al.* (2000b) analysed the possibility that paternal exposure to high-LET fission neutrons could lead to a transgenerational increase in mutation rates at ESTR loci in the mouse germline. An elevation of ESTR mutation rate was observed in the germline of both male and female F_1 offspring of directly exposed male mice. These data provide the first direct experimental evidence for the transmission of genomic instability through the germline to subsequent generations. Such a transmission of instability may have important implications for the long-term effects of pre-conceptional paternal exposure to ionising radiation within human populations, in the form of the delayed effects associated with genomic instability (see Section 5.1.2 for details), which may in turn lead to an increased health risk.

The results of the study by Dubrova *et al.* (2000b) also provide important clues relating to the possible mechanisms of transgenerational instability. The F_1 offspring show increases in ESTR mutation in alleles inherited equally from the unexposed F_0 mothers and

the irradiated F_0 fathers (excluding the possibility that ESTR mutation rates in the F_1 result from the inheritance of a 'damaged' allele from the irradiated F_0 male). The data also suggest that a signal is produced which results in a genome-wide, rather than localised effect on the F_1 germline. All of the F_1 offspring analysed showed similar levels of germline instability at the ESTR loci *Ms6-hm* and *Hm-2*; this non-Mendelian pattern of the inheritance of genomic instability provides evidence to exclude the possibility that the F_1 offspring of irradiated males may inherit mutations in genes involved in DNA repair. The likelihood of all ten offspring, derived from different sperm cells, carrying the same gene mutation is very low, which suggests that the radiation-induced signal leading to genomic instability may be inherited in an epigenetic fashion (Dubrova *et al.*, 2000b).

The results from Dubrova *et al.* (2000b) raise a number of important issues concerning the mechanisms of transgenerational instability at ESTR loci. Firstly, it is important to assess whether transgenerational effects can be observed in other inbred strains of mice in order to exclude the possibility that such effects are only observed in the germline of CBA/H mice. Secondly, given the results of recent publications showing the differential efficiency of high- and low-LET irradiations in the induction of genomic instability *in vitro* (reviewed by Limoli *et al.*, 2000), it remains to be seen whether low-LET exposure can also affect ESTR mutation rate in the offspring of irradiated males. Finally, it would be interesting to assess the possibility that transgenerational germline instability may also be observed in the F_2 germline, the grand-offspring of the directly exposed F_0 males. The details of the work addressing these issues are presented in Sections 5.2 onwards in this Chapter.

5.1.2 Genomic instability

Radiation-induced genomic instability is characterised by an increased mutation rate in the progeny of irradiated cells. A number of effects are manifested in the progeny of initially irradiated cells for many generations after the initial exposure to ionising radiation; these delayed effects include reproductive cell death, reduced plating efficiency, giant cell formation, cell fusions, decreased cellular adhesion, delayed mutation, clonal heterogeneity, malignant transformation, and delayed chromosomal instability (reviewed by Morgan *et al.*, 1996).

The analysis of genomic instability has predominantly been performed in tissue culture systems following exposure to high-LET radiation, such as α-particle irradiation (Morgan *et al.*, 1996). The results of initial *in vitro* low-LET studies were inconclusive, with a number of studies demonstrating an absence or reduced levels of genomic instability after low-LET exposure (reviewed by Limoli *et al.*, 2000). Later studies however, suggest that low-LET radiation can lead to genomic instability, but that the longevity of radiation-induced genomic instability from low-LET sources may be reduced in comparison to that induced by high-LET radiation, *i.e.* the effects of low-LET exposure diminish after fewer population doublings, and that lethal effects are only observed after a certain threshold level (Mothersill *et al.*, 2000). It has also been suggested that the relationship between the different manifestations of radiation-induced genomic instability is not a simple one; there is no direct correlation between the different end-points of genomic instability after exposure to different sources of ionising radiation (Mothersill *et al.*, 2000).

The biological significance of radiation-induced instability may be reflected in an increased risk of cancer after radiation exposure. It may be possible to link the induction of genomic instability, by ionising radiation, to an increase in the likelihood of the accumulation of multiple genetic events within a cell, which may in turn lead to the induction of oncogenesis (Little, 2000). This assumption is backed in part by the findings

of epidemiological studies which suggest that some types of radiation-induced cancers may follow a relative risk model, such that exposure to radiation enhances the rate at which cancers develop rather than inducing a specific cohort of new tumours (Little, 2000). Therefore the investigation of a possible *in vivo* effect on the germline of mice after exposure to ionising radiation, which can be transmitted to subsequent generations, may be significant in the understanding the long term risks of radiation exposure. The transmission of genomic instability may also provide valuable information about the mutation mechanisms occurring at ESTR loci in the germline of mice exposed to ionising radiation.

5.2 Experimental design

To assess the transmission of germline instability in the subsequent generations of male mice exposed to different sources of ionising radiation, a number of interlinked experiments were designed. The experimental procedure was based on the measurement of the frequencies of ESTR mutation at the loci *Ms6-hm* and *Hm-2* in the F_1, F_2 and F_3 offspring of initially irradiated males; these measurements yield germline mutation rates for the F_0, F_1 and F_2 generations, respectively (see Figure 5.1). To ensure the random assignment of F_1 and F_2 parents all genotyping was performed after the end of the three-generational breeding scheme.

Using this mating scheme, three experiments were designed to address a number of issues concerning the transgenerational passage of germline instability. The first experiment assesses the effect of different sources of ionising radiation on the germline mutation rate at ESTR loci, by measuring the mutation rates in the germline of offspring of CBA/H male mice exposed to either 0.4 Gy of high-LET fission neutrons or 2 Gy of low-LET X-rays. The second experiment was designed to evaluate the transgenerational effects of exposure of pre- and post-meiotic spermatogenic cells. Mutation rates were established for the germline of the first-generation of CBA/H offspring and their descendants conceived at either 3 or 6 weeks after initial paternal exposure to neutrons or X-rays. The litters conceived 3 weeks after irradiation were derived from cells that were at the post-meiotic spermatids stage of spermatogenesis at the time of exposure, whereas those conceived 6 weeks after exposure were derived from irradiated pre-meiotic A_s spermatogonia. The final experiment was designed to compare transgenerational effects between three inbred mouse strains. The mutation rates of the F_1 and F_2 germline were compared between CBA/H, C57BL/6 and BALB/c mice; these particular strains have previously been shown to differ significantly in both their radio-sensitivity (Roderick,

1963) and the radiation-induced instability in their somatic cells (Ponnaiya *et al.*, 1997; Watson *et al.*, 1997; Mothersill *et al.*, 1999)

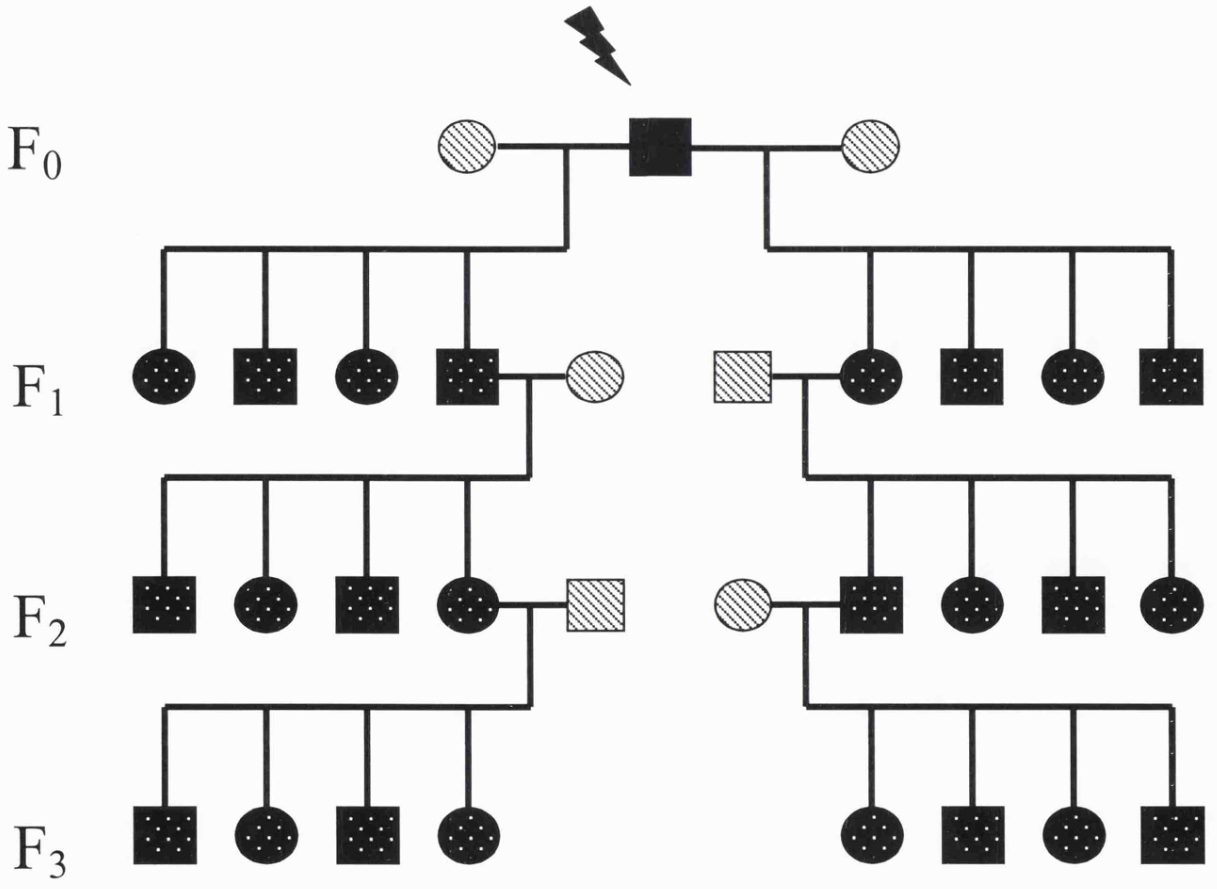


Figure 5.1 – Mating scheme for the transgeneration study.

irradiation, ■ initially irradiated male, ⊠ partners without any history of irradiation

■ represent offspring descended from the initially irradiated male

5.3 Mutation induction in the exposed F_0 males

The exposure of pre-meiotic spermatogonia to either high-LET fission neutrons or low-LET X-rays resulted in an elevation of ESTR mutation rate in the germline of all exposed CBA/H males (Table 5.1). In contrast irradiated post-meiotic spermatids showed mutation rates similar to those of the control (non-irradiated) group. Significantly elevated mutation rates were also found in pre-meiotically exposed C57BL/6 and BALB/c males. The maternal mutation rate in the F_0 non-irradiated females was not affected by either pre or post-meiotic exposure of the male germline, and remained similar to the control rates (Figure 5.2 a and b). These data agree with a number of previously published results on the stage-specificity of mutation induction at mouse ESTR loci (Dubrova *et al.*, 1998a; Barber *et al.*, 2000).

5.4 Assessment of germline instability in the male and female germlines

Due to the presence of multi-allelism and heterozygosity at the ESTR loci *Ms6-hm* and *Hm-2* in all inbred strains included in this study, it was possible to establish the parental origin of most of the mutant bands in the F_1 offspring. The ratio of paternal to maternal mutation rates did not differ for the control parents of any strain studied (Figure 5.2). As discussed in the previous section, the mutation rate was elevated in the germline of the directly exposed males but no effect was observed for the non-exposed F_0 females. However, the mutation rates from either male or female offspring, of both the first (F_1) and second generations (F_2) descended from irradiated F_0 males, showed similarly elevated mutation rates in their germline (Figure 5.2). Additionally the elevation in mutation rate observed in the germline of F_1 and F_2 offspring was as high as those in the germline of directly exposed F_0 males; this demonstrates that the mutation rate in the germline of F_1 and F_2 offspring of irradiated males is significantly elevated. As no difference in the

transmission of instability through the male or female germlines was observed it was possible to combine the data for the different sexes to produce single estimates for the control groups, and the F_1 and F_2 offspring of irradiated males (Table 5.1).

Strain, group*	Number of: offspring	mutations†	Mutation Rate	Ratio to control	Prob.‡
CBA/H					
Control (8♂, 8♀)	76	22 (20)	0.072	-	-
Neutrons, 0.4 Gy					
- F_0, 3 weeks (5♂)	18	1 (1)	0.028	0.4	0.55
- F_0, 6 weeks (5♂)	43	18 (16)	0.209	2.9	0.001
- F_1 (7♂, 9♀)	83	42 (30)	0.253	3.5	2.1×10^{-7}
- F_2 (9♂, 7♀)	84	33 (25)	0.196	2.7	0.0002
X-rays, 2 Gy					
- F_0, 3 weeks (7♂)	18	4 (2)	0.111	1.5	0.58
- F_0, 6 weeks (7♂)	47	18 (16)	0.192	2.6	0.003
- F_1 (13♂, 8♀)	106	47 (39)	0.222	3.1	2.1×10^{-6}
- F_2 (13♂, 11♀)	157	75 (57)	0.239	3.3	1.2×10^{-8}
C57BL/6					
Control (4♂, 4♀)	98	25 (21)	0.064	-	-
Neutrons, 0.4 Gy					
- F_0, 6 weeks (3♂)	45	24 (22)	0.267	4.2	5.5×10^{-7}
- F_1 (3♂, 5♀)	58	18 (18)	0.155	2.4	0.006
- F_2 (5♂, 5♀)	63	19 (19)	0.151	2.4	0.006
BALB/c					
Control (11♂, 12♀)	94	45 (43)	0.120	-	-
X-rays, 1 Gy					
- F_0, 6 weeks (5♂)	32	18 (16)	0.281	2.4	0.003
- F_1 (5♂, 10♀)	68	42 (36)	0.309	2.6	2.7×10^{-6}
- F_2 (8♂, 6♀)	86	43 (35)	0.250	2.1	0.0003

Table 5.1 - Mutation rates in the germline of controls and offspring of irradiated males

* The number of male and female parents is given in brackets.

† Number of singleton mutations is given in parentheses.

‡ Probability of difference from the control group (Fisher's exact test, two-tailed).

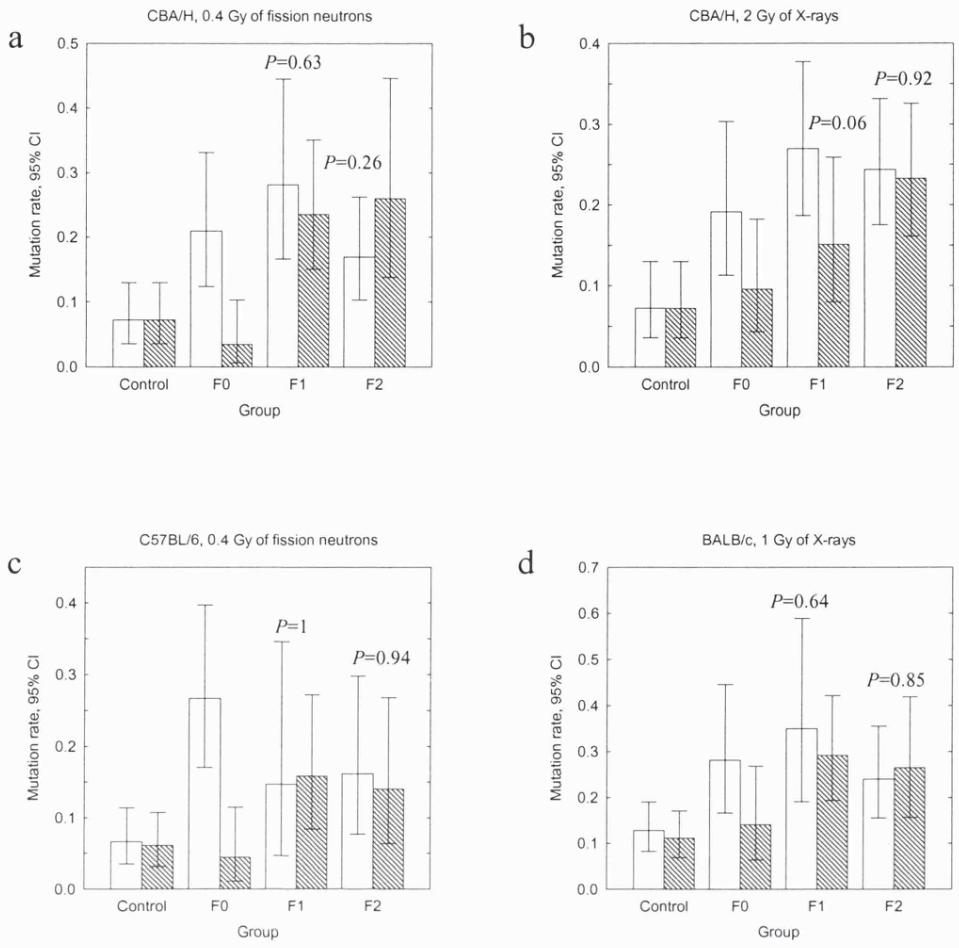


Figure 5.2 – Paternal and maternal ESTR mutation rates in the germline of control individuals and the offspring descended from irradiated males.

ESTR mutation rates in the male germline (open boxes) and female germline (hatched boxes). The data are given for controls, F_0-exposed males, non-exposed F0 females, F_1 and F_2 offspring of irradiated males. The F_0 paternal germline mutation rate is shown for pre-meiotic exposure (6 weeks after irradiation) only. The 95% confidence intervals (CI) for mutation rates are estimated from the Poisson distribution and the probabilities of differences between male and female mutation rates in the F_1 and F_2 parents (Fisher's exact test, two-sided) within each generation are shown.

5.5 Effects of high- and low-LET exposure

All the offspring from CBA/H male mice exposed to either 0.4 Gy of high-LET fission neutrons or 2 Gy of low-LET X-rays showed statistically significant increases in germline mutation rates at ESTR loci (Table 5.1). The magnitudes of the transgenerational increase for the two doses of high- and low-LET irradiations were similar. These data suggest that both high- and low-LET exposure to ionising radiation are capable of inducing germline instability in the F_1 and F_2 offspring of directly irradiated males (Figure 5.3); therefore data for both types of exposure were combined for further analyses.

5.6 Stage specificity for transgenerational instability

The germline ESTR mutation rates in the CBA/H F_1 offspring derived from post-meiotic spermatids (3 weeks) or pre-meiotic spermatogonia (6 weeks) show similar increases (Figure 5.4). Similarly elevated ESTR mutation rates are also observed in the germline of the grand-offspring (F_2) of directly exposed males. These data contrast with the levels of mutation induction observed in the germline of directly exposed males (F_0), where no elevation of ESTR mutation rate was observed in post-meiotic spermatids (Table 5.1).

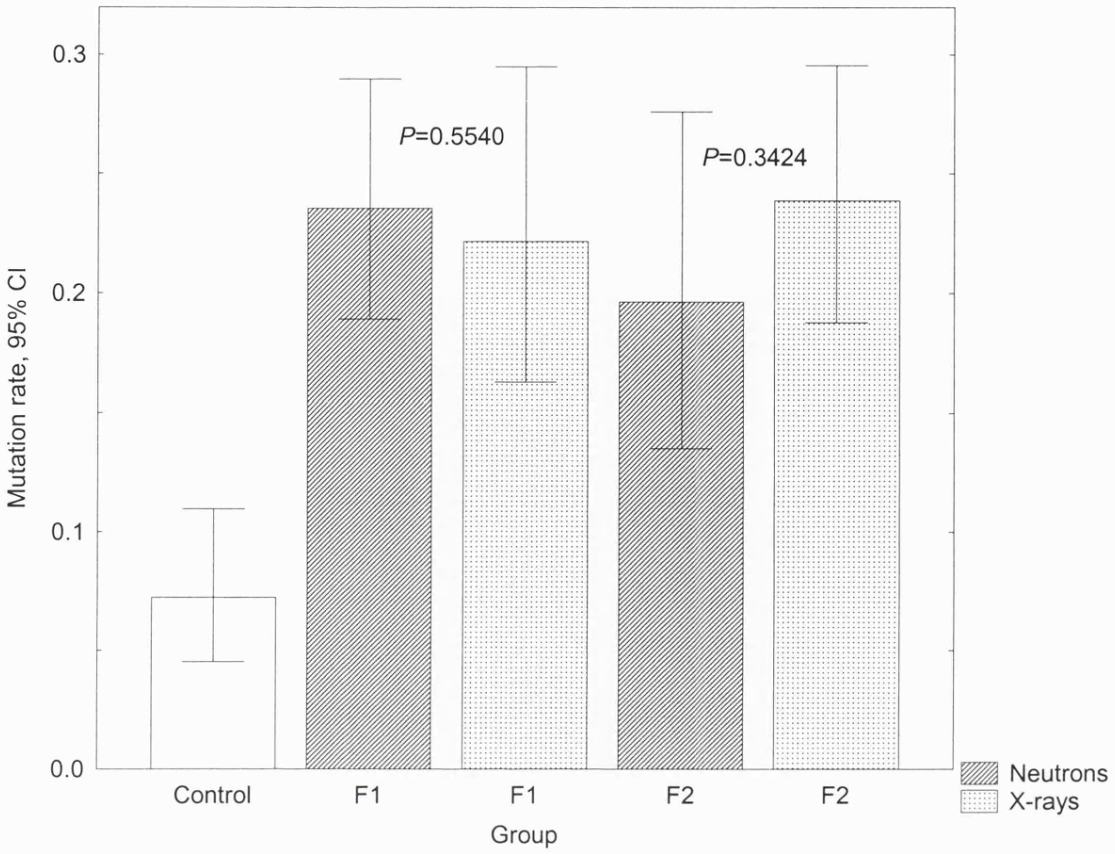


Figure 5.3 - Mutation rates in the germ line of F_1 and F_2 offspring CBA/H male mice exposed to either 0.4 Gy of fission neutrons or 2 Gy of X-rays.
Probability of difference between the different types of exposure are given.

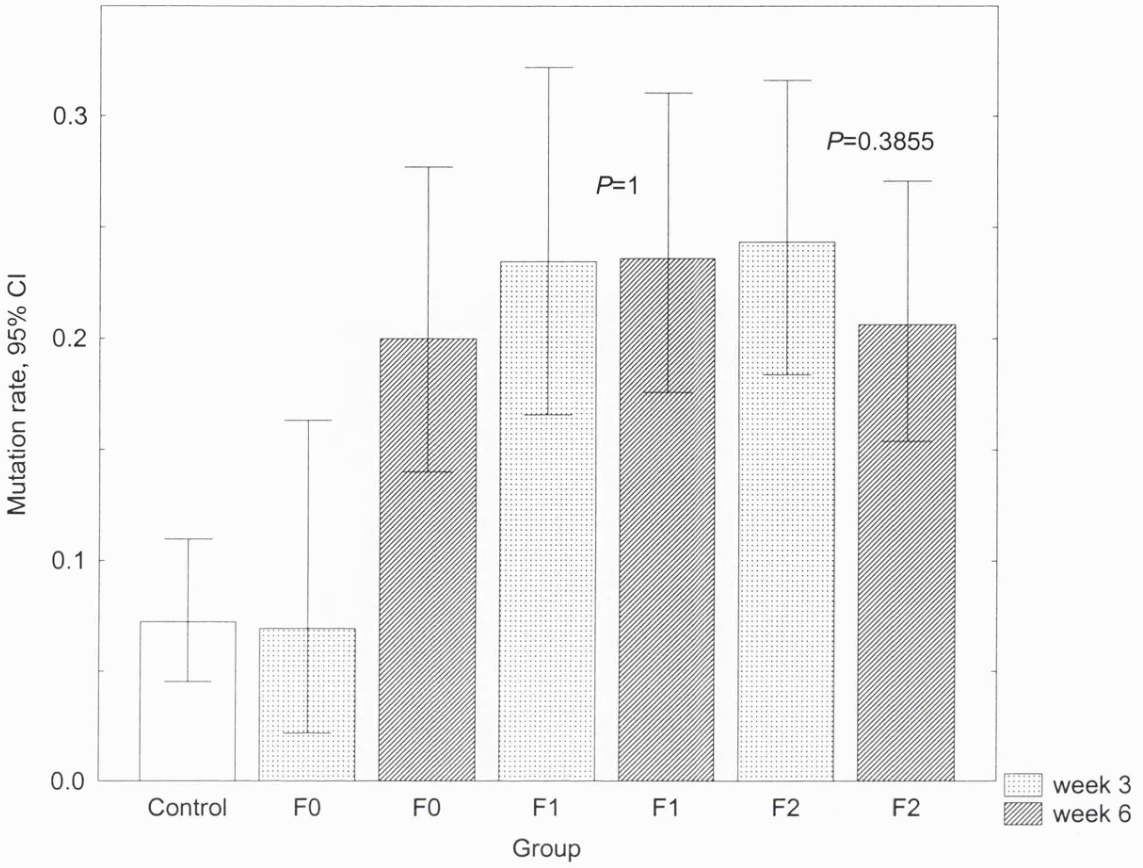


Figure 5.4 – Stage specificity for transgenerational instability.

Mutation rates in the germline of controls, directly exposed males (3 and 6 weeks after exposure) and their F_1/F_2 offspring conceived 3 or 6 weeks after exposure are shown.

The probabilities of difference between mutation rates within each generation are shown.

5.7 Strain specificity for transgenerational instability

The combined data for the CBA/H mice germline mutation rates were compared to those for the C57BL/6 and BALB/c strains (Table 5.1, Figure 5.5). Increases in germline mutation rate were observed in the F_1 and F_2 offspring of all inbred mice; however, the magnitude of both spontaneous mutation and transgenerational instability clearly varied between strains. The highest and lowest mutation rates were detected in BALB/c and C57BL/6 mice strains respectively, with CBA/H mice showing intermediate mutation rates.

Several amino-acid substitutions in genes involved in either cell cycle checkpoint control, *Cdkn2*, or DNA repair, *Prkdc,* have recently been identified in the BALB/c inbred strain (Zhang *et al.*, 1998; Yu *et al.*, 2001). The *Cdkn2a* encodes the cell cycle control protein p16 (Quelle *et al.*, 1995) and the *Prkdc* gene encodes the catalytic subunit of the DNA-dependent protein kinase complex (Fujimori *et al.*, 1997). The amino-acid substitutions in these two genes result in reduced activity of the both proteins in BALB/c mice (Zhang *et al.*, 1998; Yu *et al.*, 2001). Using direct sequencing BALB/c, CBA/H and C57BL/6 mouse strains were typed for the allelic variants of the *Cdkn2a* and *Prkdc* genes (Figure 5.6). The Harwell BALB/c colony contains a C→ A transversion at base 134 (His→ Pro) and a G→ A transition at base 232 (Val→ Ile) within the *Cdkn2a* gene. Also two changes were confirmed in the *Prkdc* gene, a C→ T transition at base 6418 (Arg→ Cys) and A→ G transition at base 11530 (Met→ Val). These polymorphisms were not found in the CBA/H and C57BL/6 mice which is in agreement with previous reports (Zhang *et al.*, 1998; Yu *et al.*, 2001).

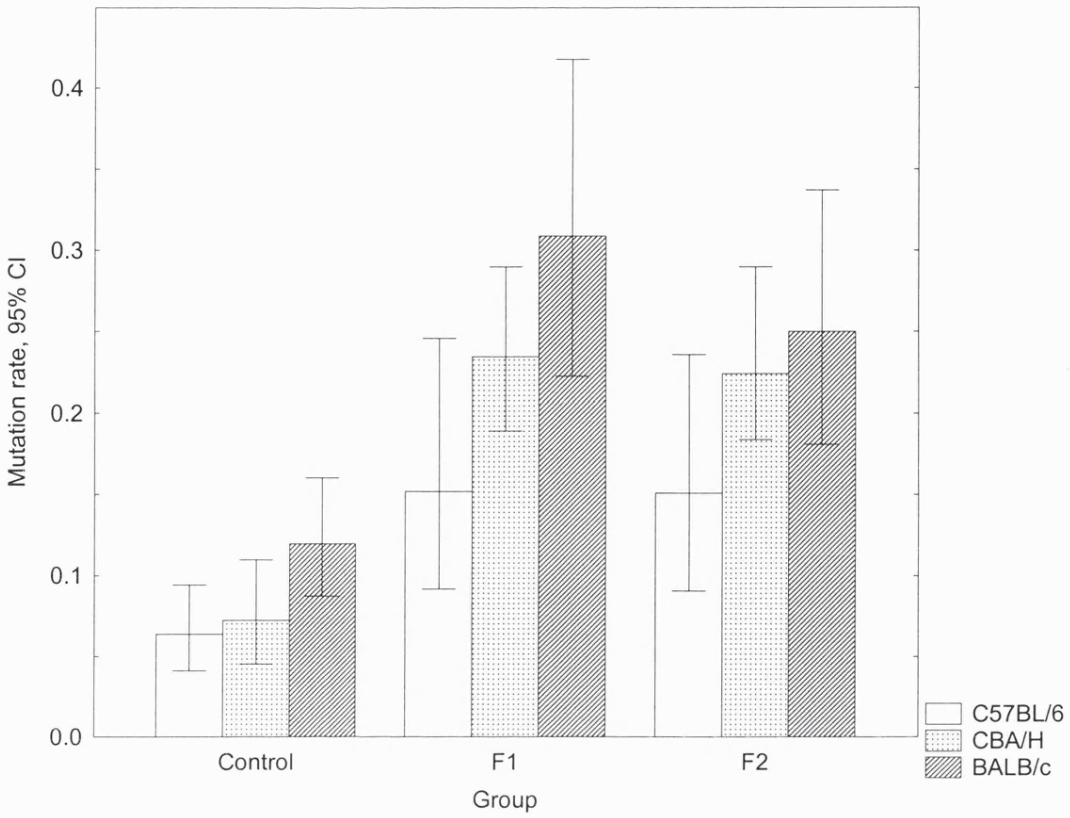


Figure 5.5 - Comparison of ESTR mutation rates in the germline of controls and the F_1/F_2 offspring of irradiated males in three different inbred strains of mice.

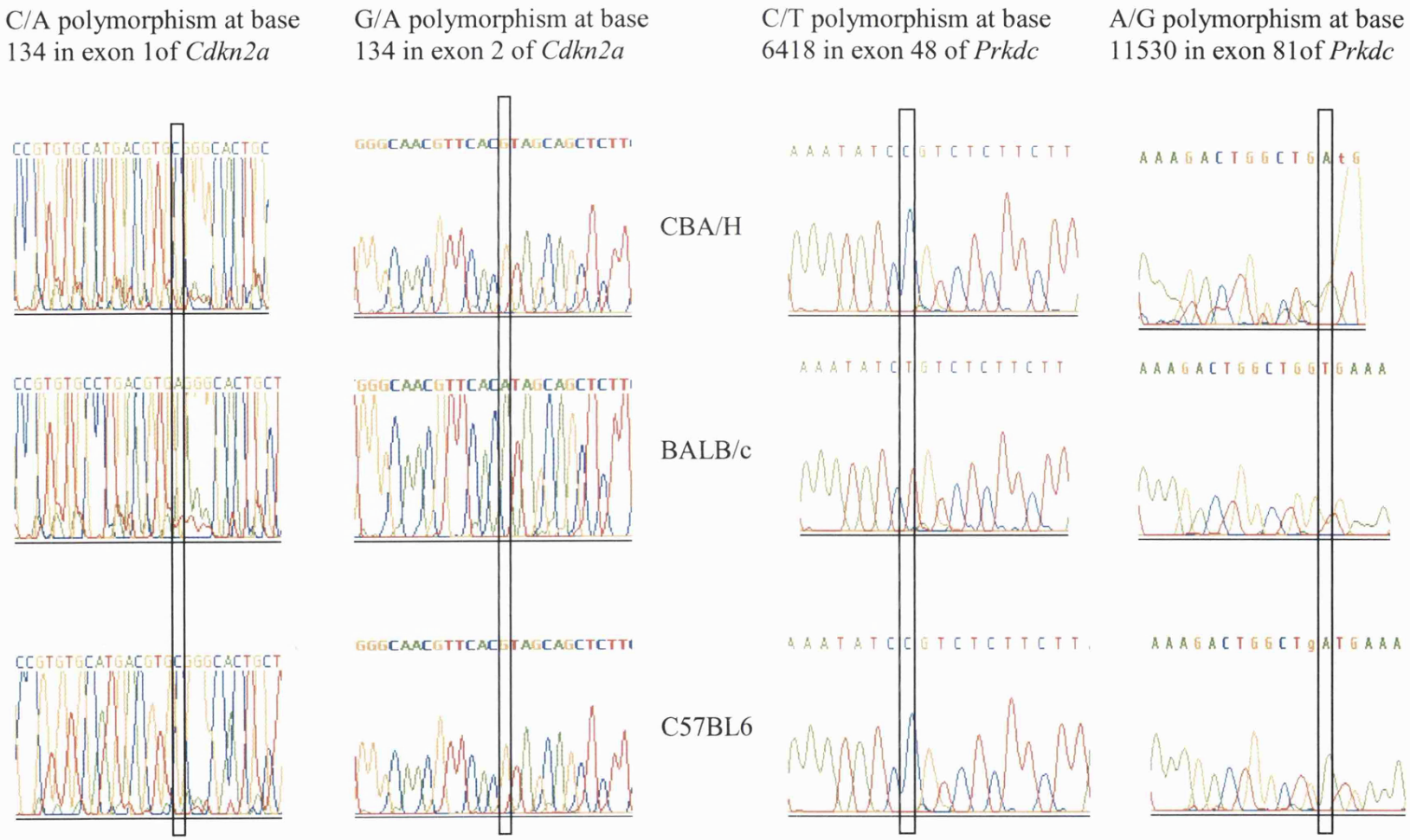


Figure 5.6 – DNA sequences showing the functional polymorphisms in the *Cdkn2a* and *Prkdc* genes, in three different mouse strains.

5.8 Transgenerational instability in the F_1 and F_2 parents

The frequency of ESTR mutation was established in the F_2 and F_3 litters; this provided estimates of mutation rates for the germline of F_1 and F_2 individuals for the three mouse strains studied (Figure 5.5). Most of the F_1 and F_2 parents showed elevated mutation rates (Figure 5.7), resulting in a relatively homogeneous distribution of rates within each strain (chi-square test for homogeneity of the Poisson distribution, $P>0.95$, data not shown). The statistical analysis of the homogeneity of mutation rate for all the offspring of irradiated parents, using a two-way ANOVA (with arc-transformation), showed that mutation rates differed significantly between strains ($F=6.97$, df=2, 118, $P=0.001$) but remained similar between generations within a strain ($F=0.61$, df=1, 118, $P=0.4350$; interaction $F=0.01$, df=2, 118, $P=0.99$). These data show that, within each strain, there is no evidence of a decrease in ESTR germline mutation rate between the first and second generations.

5.9 Parental origin of alleles and mutations in the F_1 generation

The F_1 offspring contained alleles derived from both the irradiated F_0 father and the non-irradiated F_0 mother. In most cases it was possible to establish whether an ESTR mutation occurring in the F_1 generation and detected in the F_2 offspring, was derived from the allele inherited from the irradiated F_0 father or non-irradiated F_0 mother (Figure 5.8a). Among 149 *de novo* mutants transmitted from the F_1 parents to their offspring, 63 were clearly traced to the allele derived from the irradiated F_0 father and 53 were derived from the non-irradiated F_0 mother. The origin of remaining 35 mutants could not unambiguously be established (Figure 5.8b). It is therefore possible to conclude that radiation-induced instability affects the ESTR alleles derived from both irradiated and non-irradiated F_0 parents.

The F_1 parents can be divided into two groups: those with and without ESTR mutation derived from irradiated fathers. The mutation rates in the germline of the F_1 parents from the two groups were compared using a one-way ANOVA and showed that at both *Ms6-hm* and *Hm-2* the rates of those individuals containing mutations transmitted from the irradiated fathers were similar to those without paternal mutations (*Ms6-hm*: F=0.85, df=1, 57, P=0.36; *Hm-2*: F=0.11, df=1, 57, P=0.74). These data indicate that the presence of mutations transmitted from the irradiated fathers does not affect ESTR stability in the germline of the F_1 offspring.

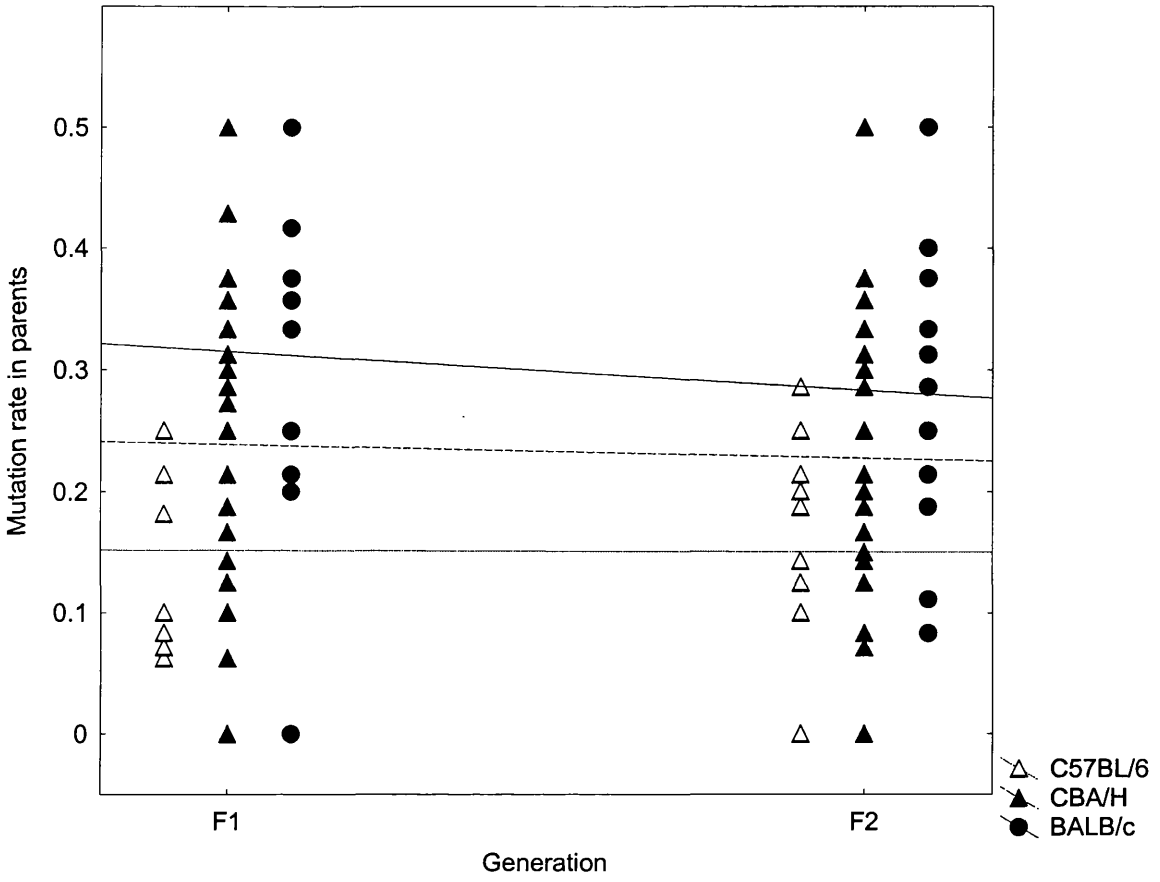


Figure 5.7 - Individual mutation rates in the germline of F_1 and F_2 parents of exposed males.

Each point represents the germline mutation rate of a single individual.

The mutation rates from both male and female offspring are represented.

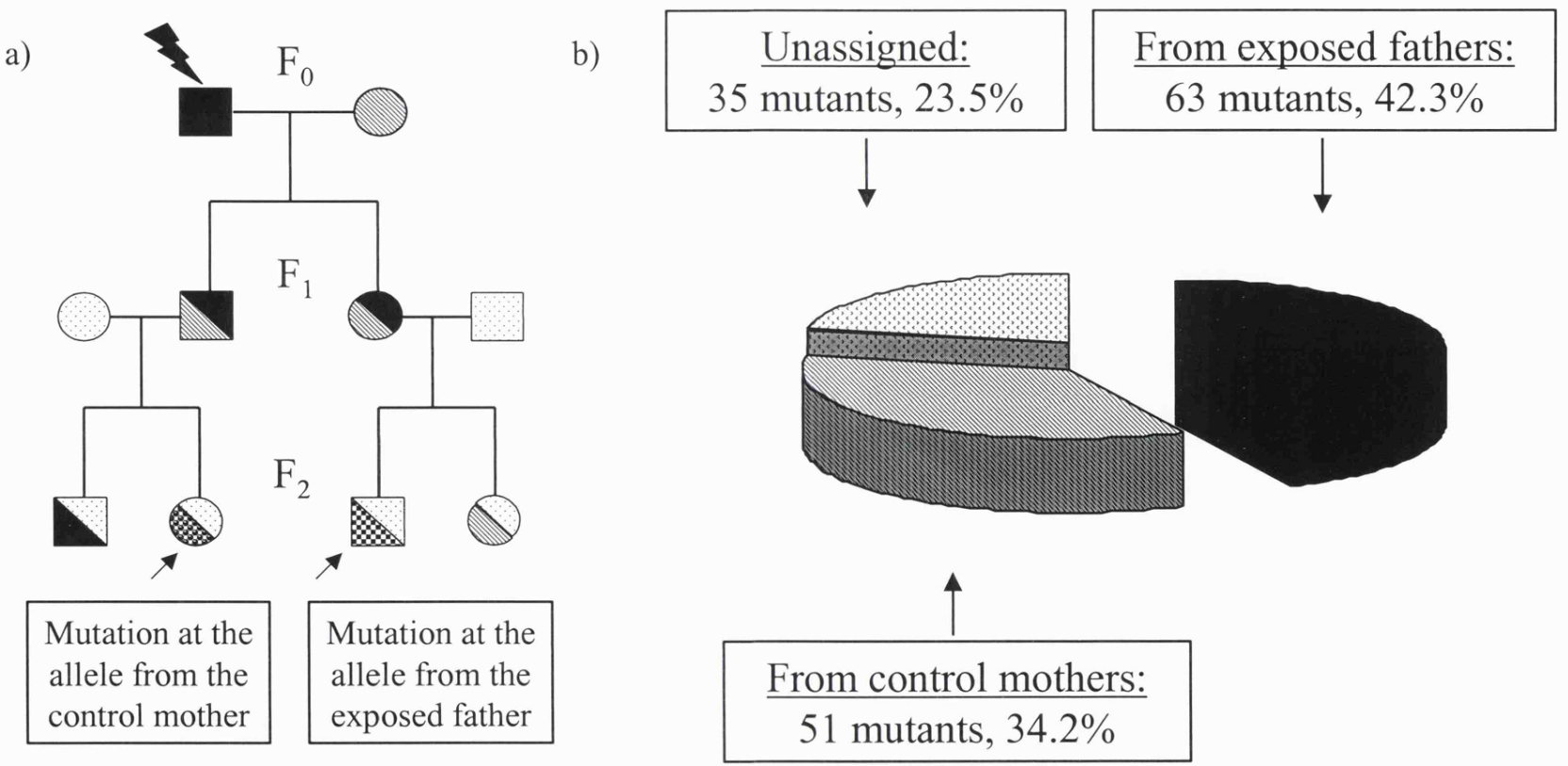


Figure 5.8 - Parental origin of ESTR mutations in the first- and second-generation offspring of exposed males

a) Pedigree showing the F_0 parental origin of mutant alleles observed in the F_2 offspring

b) Distribution of parental origin of mutant alleles in the F_2 offspring.

5.10 Discussion

The analysis of ESTR mutation in the germline of first- and second-generation offspring of irradiated males have revealed a number of important issues relevant for the understanding of mechanisms of transgenerational instability. They include: (i) similarly elevated ESTR mutation rates in the germline of F_1 and F_2 offspring of irradiated males; (ii) the same pattern of transmission of transgenerational instability through male and female germline; (iii) the similar efficiency of both high-LET (0.4 Gy of fission neutrons) or low-LET (2 Gy of X-rays) in the induction of transgenerational instability; (iv) the similar efficiency of pre- and post-meiotic exposures in the induction of transgenerational instability; and (v) the inter-strain differences in the magnitude of transgenerational instability.

A number of studies have previously examined the long-term effects of parental exposure on the health and viability of subsequent generations. These studies have been able to provide some preliminary evidence for increased mutation rates in the germline of the F_1 offspring of irradiated males using a number of different end points, such as dominant lethality, or the assessment of congenital malformations in the F_2 offspring of directly exposed F_0 parents (Luning *et al.*, 1976; Hales *et al.*, 1992; Pils *et al.*, 1999). The response of the F_1 and F_2 offspring of irradiated fathers to secondary challenges by mutagenic or carcinogenic compounds has also been studied. The F_1 offspring of irradiated fathers when challenged with MNU show an increased incidence of leukaemia compared to those without paternal radiation history (Hoyes *et al.*, 2001). The incidence of skin tumours was increased in F_1 and F_2 offspring of irradiated F_0 males after secondary challenge with TPA, a promoter of skin tumours in mice (Vorobtsova *et al.*, 1993). One study has analysed the effect of paternal irradiation on the signalling activities of a number of kinase enzymes in F_3. The hepatic activities of tyrosine kinase, protein kinase C and MAP were elevated in the F_3 offspring with radiation history (Baulch *et al.*, 2001). The

data from these different studies provide indirect evidence that parental exposure to ionising radiation may have long-term effects on mortality, malformations and cancer incidence in subsequent generations. The data presented here and the previously published study of transgenerational mutation induction at ESTR loci in mice (Dubrova *et al.*, 2000b) provide the first direct evidence for an increase in mutation rates in the F_1 and F_2 germline of offspring derived from irradiated F_0 males.

The analysis of high- and low-LET sources of ionising radiation within the same strain of mice (CBA/H), over two generations, has demonstrated that both sources of ionising radiation are capable of inducing a similar transgenerational response within the mouse germline. The data suggest that radiation-induced genomic instability can be initiated by exposure of the male germline to both fission neutrons and X-rays leading to increases in mutation rate for a number of generations after the initial exposure in an *in vivo* model; these increases in mutation rate are reminiscent of the *in vitro* situation within mammalian tissue culture systems (reviewed by Morgan *et al.*, 1996 and Limoli *et al.*, 2000).

The fact that irradiation at different stages of spermatogenesis can lead to differences in mutation induction at ESTR loci may provide important information about the mechanism(s) of mutation at these loci. The data from the current study (Figure 5.4) show that post-meiotic exposure to ionising radiation does not affect ESTR germline mutation rate in the directly exposed males F_0, and supports the data obtained in previous studies (Dubrova *et al.*, 1998a; Barber *et al.*, 2000). The lack of mutation induction at this stage has been explained by assuming that spontaneous and radiation-induced mutations at ESTR loci can occur only in diploid cells, and therefore mutations cannot occur in haploid post-meiotic cells. In contrast, the data from the germline of F_1 offspring of exposed males conceived from either post-meiotic (3 weeks) or pre-meiotic (6 weeks) stages of spermatogenesis show similar elevations in mutation rate, which persist in the germline of

the second generation (Figure 5.4). These data therefore indicate that both pre- and post-meiotic paternal exposure to ionising radiation can affect ESTR mutation rates in the germline of the F_1 and F_2 offspring of irradiated males. It should be stressed that the issue of stage-specificity of radiation-induced transgenerational effects in mice has not yet been properly addressed as a number of publications report conflicting data on the efficiency of post-meiotic paternal exposure (Vorobtsova *et al.*, 1993; Burruel *et al.*, 1997; Luke *et al.*, 1997; Wiley *et al.*, 1997; Vorobtsova, 2000; Hoyes *et al.*, 2001). The apparent discrepancy between these findings remains unexplained and future work should directly address in greater detail the issue of the stage-specificity of transgenerational effects.

The data presented here show elevated germline mutation rates in the F_1 and F_2 offspring of irradiated males belonging to three inbred strains, CBA/H, C57BL/6 and BALB/c; these data clearly demonstrate that transgenerational instability is not restricted to one particular inbred strain of mice, although significant inter-strain differences in spontaneous mutation rates and radiation-induced transgenerational instability were observed (Figure 5.5). Previous studies of the same inbred strains of mice have also revealed profound differences in their response to ionising radiation, i.e. BALB/c and CBA mice are significantly more radiosensitive and show higher levels of radiation-induced genomic instability in somatic cells than C57BL/6 mice (Roderick, 1963; Ponnaiya *et al.*, 1997; Watson *et al.*, 1997; Mothersill *et al.*, 1999; Yu *et al.*, 2001). It is possible that the high level of radiation-induced genomic instability in BALB/c mice could be attributed to the strain-specific single nucleotide polymorphisms in the *Prkdc* and *Cdkn2a* genes leading to decreased activity of the DNA-dependent protein kinase catalytic-subunit protein and the p16 protein respectively (Zhang *et al.*, 1998; Yu *et al.*, 2001). The activity of the DNA-dependent protein kinase complex, including the catalytic-subunit (*Prkdc*), is required for the repair of DNA double-strand breaks via the non-homologous end-joining pathway. The p16 protein is important in cell cycle checkpoint control via interactions with the

retinobalstoma protein. Reduced activity of these proteins may lead to higher levels of mis-repair or incorrect checkpoint control in cells that have been damaged by exposure to ionising radiation. The analysis of the known polymorphisms at the *Cdkn2a* and *Prkdc* genes in the Harwell colony of BALB/c mice showed them to be the same as those published previously (Zhang *et al.*, 1998; Yu *et al.*, 2001) and may partially explain the increased levels of spontaneous and radiation-induced transgenerational instability in the BALB/c mice. Additional, as yet unidentified polymorphisms may be present in DNA repair or checkpoint related genes in the other mouse strains similarly affecting their response to ionising radiation.

The results of this study also provide important clues relating to the possible mechanisms of transgenerational instability. If the transgenerational signal inducing instability in the F_1 germline were caused by the direct action of radiation, in the form of DNA damage within the ESTR loci themselves, then mutations in the F_1 germline should occur predominantly in the damaged allele transmitted to them from the irradiated F_0 fathers. As equally elevated mutation rates at alleles derived from the irradiated F_0 fathers and the unexposed F_0 mothers were detected in the germline of F_1 offspring, this explanation can be excluded and a global elevation of mutation rate can be inferred (Figure 5.8). The persistence of elevated mutation rates in the germline of two consecutive generations excludes the possibility that transgenerational effects are due to radiation-induced mutations at any specific set of genes in the exposed F_0 males. For example, mutations in some DNA-repair genes may be transmitted from irradiated males to their offspring which could then potentially affect genome stability in the first generation; however, the further mating of F_1 offspring to control animals should result in Mendelian segregation of wild type and mutant alleles, causing an overall reduction of mutation rate in the second generation. In contrast, ESTR mutation rates in the germline of both generations are similar and significantly exceed those for the control parents (Figure 5.7),

clearly implicating an epigenetic mechanism for the process of transgenerational instability.

It is possible that alterations in DNA methylation patterns in the genome may account for the epigenetic changes responsible for radiation-induced transgenerational instability. It is known that cellular DNA methylation patterns are established in a complex manner involving at least three independent methyltransferases DNMT1, DNMT3A and DNMT3B (Robertson and Wolffe, 2000). The DNMT3 family of methyltransferases have been shown to be important in *de novo* methylation during embryogenesis (Okano *et al.*, 1998) and DNMT1 has been implicated in maintaining methylation patterns in the genome (Robertson and Wolffe, 2000). Furthermore, it has been proposed that DNA methylation may be involved in the maintenance of genomic integrity (Robertson and Wolffe, 2000). For example, ES cells containing a homozygous knockout of the *Dnmt1* gene have only 30% of the normal methylation levels and show a ten-fold increase in the rate of gene rearrangements (Robertson and Wolffe, 2000). Human tumour samples have been shown to exhibit global hypomethylation of the genome in conjunction with the hypermethylation of CpG islands. Such alterations in methylation patterns have been shown to be associated with increased levels of genomic instability which manifest as chromosomal instability, the activation of endogenous parasitic sequences, the loss of imprinting, illegitimate expression patterns of genes, aneuploidy, mutations, and the transcriptional silencing of tumour suppressor genes (Esteller and Herman, 2002). Methylation has also been implicated in the maintenance of genome stability via interactions with repetitive DNA sequences, possibly by 'masking' repeat regions or by inhibiting homologous recombination (reviewed by Robertson and Wolffe, 2000). It has been demonstrated in the fungus *Ascobolus immerses* that methylation of a known recombination hotspot could reduce the frequency of crossing-over in that region several hundred-fold (Maloisel and Rossignol, 1998). An effect of methylation has also been observed on V(D)J

recombination in mammalian cells, which shows a greater than one hundred fold reduction in crossing-over when the recombination substrate is methylated (Hsieh and Lieber, 1992). The processes of homologous recombination and V(D)J recombination (via the non-homologous end joining pathway) are the two major mechanisms involved in the repair of DNA double stand breaks; these are the most biologically relevant DNA lesions caused by exposure to ionising radiation and it may therefore be possible that alterations in methylation patterns may affect DNA repair leading to a destabilisation of the genome. The mechanism by which methylation can effect homologous recombination remains unknown; however, potential mechanisms have been postulated to involve the masking of recombination initiation sites and/or interference with the assembly of the recombination machinery (Robertson and Wolffe, 2000).

Alterations in methylation patterns may affect genome stability and subsequently result in oncogenesis due to transcriptional repression via the hypermethylation of promoter regions. The hypermethylation of promoter regions is one of the best-characterised epigenetic events associated with many types of human neoplasm, and has been shown to be associated with the inappropriate transcriptional silencing of genes. It is thought that promoter hypermethylation may be at least as common in silencing tumour suppressor genes as the classical disruption of these genes by mutations (reviewed by Jones and Baylin, 2002). The silencing of a gene by promoter hypermethylation increases the chance of the inactivation of both copies of a gene, which according to Knudson's two-hit model is essential for the loss of tumour-suppression (Jones and Baylin, 2002). The study of hereditary non-polyposis colon cancer (HNPCC) was the first to show the importance of epigenetic silencing in cancer and that such changes may actually predispose to increased mutations in the genome during tumour progression. The hypermethylation of the promoter region of the *MLH1* gene has been observed in tumours of patients with HNPCC that also show microsatellite instability (MSI) (Kane *et al.*, 1997; Herman *et al.*,

1998). Changes in the 5′ methylation of the *MLH1* gene have also been characterised in apparently normal colon epithelium of patients with colon cancer associated with MSI; this suggests that alterations in methylation may be associated with the development of MSI and not the consequence of it (Nakagawa *et al.*, 2001). The data from studies of HNPCC and endometrial cancer (Esteller *et al.*, 1999) have shown an association of the hypermethylation of the promoter of *MLH1* with tumourigenesis. Additionally the hypermethylation of other genes involved in a number of different cellular pathways, such as DNA repair (*BRCA1*, *MGMT*, and others), cell cycle checkpoint (*INK4a, ARF, INK4b* and others) and apoptosis (*DAPK, APAF-1* and others) (as reviewed by Esteller and Herman, 2002). The data from such studies provide evidence that alterations in methylation pattern may lead to genome-wide disruptions resulting in genomic instability and an increased risk of carcinogenesis. It is therefore possible that alterations in methylation patterns may affect the mechanisms leading to mutations at ESTR loci in the mouse germline; however, no direct evidence exists linking exposure to ionising radiation with changes in methylation patterns in the genome. Additionally the evidence provided from the transgenerational studies of genomic instability in the mouse germline strongly suggests an epigenetic mechanism for the transmission of radiation-induced germline instability from directly exposed parent to subsequent generations (Dubrova *et al.*, 2000b).

If the transmission of radiation-induced germline instability is due to alterations in methylation patterns, how are these alterations passed on into subsequent generations? It has been generally assumed that the imprint of the parental germlines were removed during waves of demethylation during the blastocyst stage in early embryogenesis, and that only the methylation marks of imprinted genes remained (Tilghman, 1999; Oswald *et al.*, 2000). It has also been suggested that the paternal genome is especially susceptible to active demethylation during embryogenesis, whereas maternally derived chromosomes seem to be targets for *de novo* methylation followed by passive demethylation later in

development. These data would seem to argue against the passage of alterations in methylation through the male germline to subsequent generations; however, a number of studies have shown that some genes resist the general demethylation of embryogenesis and can carry methylation signals to the subsequent generation. The correct imprinting of a number of genes has been shown to be essential, and alterations in the pattern of methylation of these genes can lead to disease, such as Angleman and Prader-Willi syndromes. The disease phenotypes of these syndromes are observed when there is a lack of active maternal or paternal genes, respectively, which can be caused by deletions, duplications or incorrect imprinting of the 15q11q13 region on human chromosomes. Using mice that are homozygous knockouts for the methyltransferase *Dnmt1* it has been possible to demonstrate that the *Igf2r* gene becomes methylated during oogenesis (Stoger *et al.*, 1993), the *H19* gene during spermatogenesis (Tremblay *et al.*, 1995), and that both of the imprints remain in the F_1 offspring. It has been proposed that some kind of 'imprinting box' provides these genes with resistance to demethylation during blastocyst stage and also resistance to remethylation later in development, therefore allowing the maintenance of imprinting integrity within the somatic tissue of the offspring (Bartolomei and Tilghman, 1997). Such a mechanism could facilitate the passage of alterations in methylation from F_0 irradiated males to their F_1 offspring. It has also been proposed that the imprint may then be removed during next round of gametogenesis to allow the establishment of the correct maternal or paternal imprint for passage to the subsequent generation (Ruvinsky, 1999). This evidence is unable to explain the radiation-induced germline instability observed in the F_1 and F_2 generations of mice derived from directly exposed F_0 males; however, a number of studies have shown that epigenetic alterations can be passed through the F_1 germline into the subsequent F_2 generation (reviewed by Ruvinsky, 1999). These studies have mainly concentrated on the analysis of imprinting of transgenes; Allen *et al.* (1990) assessed the methylation patterns of the transgene TKZ751

and were able to demonstrate that the expression of the transgene was suppressed in BALB/c mice following maternal transfer. The effect of methylation on the transgene was cumulative over successive generations, leading to irreversible methylation in BALB/c mice after three consecutive passages through the female germline. Another transgene, DM2 has recently been analysed and incomplete imprinting was also observed; the transgene was either completely silenced, or the expression levels reduced after passage through the female germline (Kearns *et al.*, 2000). Additionally work has been performed on endogenous loci analysing the epigenetic inheritance of the agouti locus (Morgan *et al.*, 1999); this study was able to demonstrate that a maternal epigenetic effect on the agouti locus resulted from incomplete germline erasure of methylation. The agouti phenotype, caused by allele A which contains a retrotransposon, remains silenced when inherited through the maternal germline. The authors suggest that this may be due to alterations in the epigenetic modification of the retrotransposed element, possibly via CpG methylation. Although these examples only cite evidence for the incomplete erasure of methylation status in the female germline it remains possible that a similar mechanism may also be observed in the male germline.

The analysis of methylation levels during sperm development has shown that they begin to decrease in meiotic cells, and continue decreasing through to the elongated spermatid stage of spermatogenesis (del Mazo *et al.*, 1994). Methyltransferase *Dnmt1*, the enzyme normally active to maintain methylation in somatic cells and most stages of spermatogenesis (pre-meiotic, meiotic and post-meiotic), has been shown to be absent from this stage of meiosis; where an alternative methyltransferase enzyme, which has not been characterised, was found to be active only during this stage of meiosis (Jue *et al.*, 1995). The exact stage of spermatogenesis where the methylation patterns leading to genomic imprinting of specific sets of genes occurs, remains unknown, although it has been confirmed that imprinting is complete by the round spermatid stage (Shamanski *et al.*,

1999). Treatment of rat spermatogenic cells with 5-azacytidine, a drug that blocks the action of methyltransferase enzymes, resulted in abnormal embryo development when the germ cells were exposed throughout spermatogenesis (mitotic, meiotic and post-meiotic), but showed no effect when administered only post-meiotically (Doerksen *et al.*, 2000). Rats that were treated for 6 weeks with 5-azacytidine (4.0 mg/kg) showed an increase in levels of the preimplantation loss of embryos, but no effect on testicular morphology or methylation levels of sperm. Conversely the same dose administered over an 11 week period (encompassing the mitotic, meiotic and post-meiotic stages of spermatogenesis) demonstrated preimplantation loss, severe abnormalities of the seminiferous tubules, the degradation and loss of germ cells, and a 22-29% decrease in the methylation of mature sperm (Doerksen *et al.*, 2000). This study also examined the DNA methylation levels of isolated germ cells, demonstrating that spermatogonia were more susceptible to the hypomethylating effects of 5-azacytidine than were spermatocytes. The data from these studies suggest that the action of methyltransferase enzyme(s) can alter methylation patterns during the pre-meiotic or meiotic stages of spermatogenesis and that these alterations have an effect on the subsequent generation; this is manifested as an increase in the preimplatation loss of embryos from males with a reduced ability for the correct methylation of germ cells due to treatment with 5-azacytidine (Doerksen *et al.*, 2000). It is possible that the action of ionising radiation during the pre-meiotic or the meiotic stages of spermatogenesis may also affect the cells' ability to correctly methylate developing germ cells; this may explain the increased levels of mutation at ESTR loci observed in the germline of male mice exposed at these stages of spermatogenesis (Dubrova *et al.*, 1998a; Barber *et al.*, 2000), and the transgenerational passage of germline instability shown in this study and the previous study by Dubrova *et al.* (2000b).

Finally, the results of the transgenerational analysis of germline instability in the mouse genome may have far-reaching implications for the evaluation of genetic risks of

ionising radiation for human populations. If germline instability is able to persist in populations for several generations after the initial exposure to ionising radiation, this may lead to a significant increase in mutation load. As ESTRs are non-coding loci, with no apparent function, the germline mutations studied here can be considered selectively neutral and it may be possible to argue that instability at these loci should not affect the fitness of a population. However, recent studies provide strong evidence for health-related transgenerational effects, which affect predisposition to cancer, mortality, somatic mutation, fertility, and behaviour (Table 5.2). Altogether, these data raise the important issue of the delayed transgenerational effects of ionising radiation for humans, and may provide, for example, a plausible explanation for the apparent leukaemia cluster near the Sellafield nuclear plant (Gardner *et al.*, 1990).

Strain	Paternal exposure	Generation	Endpoint	Effect*	Reference
Mice					
- ICR	X-rays (216 rad)	F_1	Lung tumours after treatment with urethane	Elevated	Nomura (1983)
- SHR	X-rays (4.2 Gy)	F_1, F_2	Skin tumours after treatment with TPA	Elevated	Vorobtsova *et al.* (1993)
- CBA/H	Injection with ^{239}Pu	F_1	Lympho-hemopoeitic malignancies after treatment with MNU	Elevated	Lord *et al.* (1998)
- DBA2	Injection with ^{55}Fe	F_1	Lympho-hemopoeitic malignancies after treatment with MNU	Elevated	Hoyes *et al.* (2001)
- CBA	Injection with ^{239}Pu	F_1	Pre-/post-implantation loss among offspring	Elevated	Luning *et al.* (1972)
- HLG/Zte	X-rays (1 Gy)	F_1	Pre-/post-implantation loss and malformations among offspring	Elevated	Pils *et al.* (1999)
- CBA/H	Neutrons (0.4 Gy)	F_1	Germline mutation rate at two ESTR loci	Elevated	Dunrova *et al.* (2000)
- C57BL/6	X-rays (1-4 Gy)	F_1	Somatic mutation rate at *lacI* gene	Elevated	Luke *et al.* (1997)
- C57BL/6J	X-rays (1 Gy)	F_1	Somatic reversions at p^{un} locus	Elevated	Carls & Schiestl (1999)
- CD1	γ-rays (1 Gy)	F_1	Proliferation of early embryonic cells in offspring	Decreased	Wiley *et al.* (1997)
- CD1	γ-rays (1 Gy)	F_1	Fertilization rate for spermatozoa	Decreased	Wiley *et al.* (1997)
- CD1	γ-rays (1 Gy)	F_3	Levels of p53 and $p21^{waf1}$	Elevated	Baulch *et al.* (2001)
Rats					
- Sprague	Cyclophosphamide	F_1	Pre-/post-implantation loss and malformations among offspring	Elevated	Hales *et al.* (1992)
- Swiss	X-rays (4.5 Gy)	F_1	Chromosome aberrations after treatment with X-rays or cyclophosphomide	Elevated	Vorobtsova (2000)
- Wistar	Cyclophosphamide	F_1, F_2	Learning capacity	Decreased	Auroux *et al.* (1988)

Table 5.2 – Transgenerational changes in the offspring of male mice and rats exposed to ionising radiation or chemical mutagens.

* Compared with the offspring of non-exposed parents.

Chapter 6

Thesis summary

The aim of this thesis was to investigate the mechanisms of radiation-induced germline mutation at mouse ESTR loci. The first part of my work evaluates the possibility that mutation induction at mouse ESTR loci may result from a genome-wide increase in meiotic recombination. This study was initiated because meiotic recombination is known to be one of the major mechanisms responsible for spontaneous mutation at human minisatellite loci (Jeffreys *et al.*, 1999). Human hypervariable minisatellite loci share a number of characteristics with mouse ESTR loci (see Section 1.3, for details), and it therefore appeared possible that the mutation mechanisms at both types of tandemly repeated loci may be similar. Male mice were exposed to ionising radiation (1 Gy of acute X-rays) or the anticancer drug cisplatin (10 mg/kg). Radiation exposure has previously been shown to result in increased ESTR mutation rates in the germline of directly exposed males (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a). It has also been demonstrated that treatment with cisplatin can increase the rate of meiotic recombination in the mouse germline (Hanneman *et al.*, 1997a). The data presented in my thesis (Chapter 3) showed no evidence for mutation induction or an elevation in the meiotic recombination rate in male mice exposed to cisplatin (Figure 3.7). The study of males exposed to ionising radiation showed no correlation between the rates of meiotic crossing over and ESTR mutation rate (Figure 3.6). It was therefore concluded that the mutation induction at ESTR loci cannot be attributed to a genome-wide increase in meiotic recombination rates. This study also examined the stage-specific response of ESTR mutation induction by ionising radiation during a number of key stages of spermatogenesis. The data demonstrate that irradiation of all stages of spermatogenesis prior to the metaphase I stage of meiosis results in mutation induction at ESTR loci, whereas exposure during post-meiotic stages has no effect on ESTR mutation rates. These

results provide further information on the stage-specificity of mutation induction at mouse ESTR loci and suggest that this induction can only occur in diploid male germ cells. The work presented here detail in Chapter 3 and has been published as Barber *et al.* (2000).

The second part of my work analyses the possibility that mutation induction at ESTR loci may result as a by-product of DNA repair. The effect of the *scid* mutation was assessed; this mutation is a single-nucleotide polymorphism in the *Prkdc* gene, which encodes the catalytic subunit of the DNA-dependent protein kinase complex (DNA-PKcs). The mutation results in a truncation of the DNA-PKcs protein with the loss of the terminal 83 amino acids. The *scid* mutation causes an immunodeficient phenotype in mice, resulting from the inability to perform V(D)J recombination which prevents the maturation of T and B cells (Schuler *et al.*, 1986). The *scid* mutation is also associated with an increased sensitivity to ionising radiation, due to a greatly reduced activity of the DNA-PK complex which is involved in the repair of DNA double-strand breaks (DSBs) via non-homologous end-joining (NHEJ) (Blunt *et al.*, 1995). The analysis of the effect of the *scid* mutation involved the assessment of the spontaneous and radiation-induced ESTR mutation rates in the germline of male scid mice and the isogenic C.B17 strain. The data obtained from this study showed that scid mice had the highest spontaneous ESTR mutation rate of any strain analysed to date, and that the isogenic C.B17 strain had a much lower spontaneous mutation rate, which is similar to that observed for the closely related BALB/c strain (see Figure 4.4). C.B17 mice showed a significant increase in ESTR mutation rate after exposure to 1 Gy of acute testicular irradiation. In contrast, no radiation-induced increase was observed in the germline of the scid males (Figure 4.3). The results of this study show therefore that non-homologous end-joining is important in the maintenance of genomic stability at ESTR loci in the germline of non-exposed male mice, but fail to ascertain whether the activation of the NHEJ pathway could provide a plausible explanation for the non-targeted increases in ESTR mutation rates in the germline of irradiated mice. Further

work is clearly required to clarify the role of NHEJ in the process of mutation induction at ESTR loci, including the analysis of other components of this pathway. The work presented in Chapter 4 is in preparation for publication.

The final part of work presented in this thesis examines the effect of ionising radiation on the long-term stability of the mouse genome. The analysis of ESTR mutation in the germline of first- and second-generation offspring of irradiated males has revealed that: (i) exposure to 2 Gy X-rays and 0.4 Gy of fission neutrons results in similar increases in both generations of CBA/H mice (Figure 5.3); (ii) the magnitude of transgenerational increases for both post-meiotic (3 weeks) and pre-meiotic (6 weeks) exposures were similar, and the mutation rates for the second-generation offspring of pre- and post-meiotically exposed grandfathers were also similar (Figure 5.4); (iii) germline mutation rates were significantly elevated in both generations in all inbred strains studied (Figure 5.5); (iv) the extent of transgenerational increase clearly varied with the different strains but was observed in all of the three inbred mouse strains studied (Figure 5.5); (v) no differences could be observed in the transmission of instability through the male or female germline (Figure 5.2). The results obtained from this study provide important clues relating to the possible mechanisms of transgenerational instability. If the transgenerational signal that induces instability in the F_1 germline was due to direct radiation-induced DNA damage at the ESTR loci themselves, then mutations in the F_1 germline should occur predominantly in the damaged allele transmitted to them from the irradiated F_0 fathers. As equally elevated mutation rates were found at alleles derived from both the irradiated F_0 fathers and the unexposed F_0 mothers were detected in the germline of F_1 offspring, then the explanation above can be excluded and a global elevation of mutation rate is inferred. The persistence of elevated mutation rates in the germline of two consecutive generations rules out the possibility that transgenerational effects are due to radiation-induced mutations at any specific set of genes in the exposed F_0 males. For example, mutations in

DNA-repair genes transmitted from irradiated males to their offspring could potentially affect genome stability in the first generation; however, the further mating of F_1 offspring to control animals should result in Mendelian segregation of wild type and mutant alleles, causing an overall reduction of mutation rate in the second generation. In contrast, the mutation rates in the germline of both generations are similar and significantly exceed those for the control parents, clearly implicating an epigenetic mechanism for the transgenerational instability. This work has been published as Barber *et al.* (2002).

The work presented in this thesis represents an initial survey of a number of potential mechanisms for radiation-induction mutation at mouse ESTR loci and provides a basis for the exploration of new and exciting directions in the analysis of ESTR mutation induction.

Chapter 7

7.1 Future Work

The work presented in this thesis has provided the initial information for a currently ongoing project to characterise the mutation mechanism(s) at ESTR loci. The work needed to continue this study would include the further analysis of the transgenerational effects of exposure to ionising radiation, and an investigation of the possibility that exposure to chemical mutagens may also result in transgenerational effects at ESTR loci. Additional work should also address the issue of the possible epigenetic effects of ionising radiation and further characterise the influence of a number of DNA repair pathways on the stability of ESTR loci.

7.1.1 Transgenerational effects in the offspring of irradiated males

As the increases in transgenerational ESTR mutation rates were similar for both the first- and second-generation offspring of irradiated males (Barber *et al.*, 2002), it will be possible to continue the transgenerational analysis using the first-generation offspring of irradiated males only. This would allow a reduction in the number of individuals required for analysis.

It would be important to assess ESTR mutation rates in somatic and germline tissues of offspring of irradiated males in order to establish whether transgenerational changes at ESTR loci can be correlated with somatic mutation events at protein coding genes; this could be achieved by simultaneously assessing mutation rates in the germline, soma and at chosen protein coding genes.

The use of two mouse strains for all additional experiments would show that any information obtained did not represent a strain-specific phenomenon. The mouse strains CBA/H and BALB/c show significant differences in germline ESTR mutation rate as

assessed by Southern blotting (Barber *et al.*, 2002), and possess easily amplifiable alleles at *Ms6-hm* locus (Yauk *et al.*, 2002).

7.1.1.1 SM-PCR analysis of the first generation offspring of irradiated males

SM-PCR analysis of the *Ms6-Hm* ESTR locus for the CBA/H and BALB/c mouse strains may help to further characterise the mechanisms of transgenerational genomic instability by providing robust estimates for the mutation rate within individual males. The SM-PCR analysis of the mutation rates at *Ms6-hm* in the germline of directly exposed male mice, and control (non-exposed) individuals, would be able to provide useful comparisons of ESTR mutation spectra, between the exposed and non-exposed individuals, in the germline rates of the F_0 and F_1 generations, and between the germline and somatic tissues.

7.1.1.2 Mutation rates at protein coding genes in the first-generation offspring of exposed males

The simultaneous analysis of mutation rates at protein coding genes and ESTR loci may be able to establish whether a correlation exists between these two systems, and thereby provide additional support for the analysis of ESTR mutation rate as a model system to assess DNA damage after exposure to ionising radiation.

One possible method for detecting somatic mutations at protein coding genes involves the measurement of the frequency of thioguanine-resistant (TG^r) mutations at the hypoxanthine guanine phosphoribosyl transferase (*hprt*) locus in primary lymphocyte cultures either derived from directly exposed F_0 males, their offspring or control individuals. The frequency of (TG^r) mutant cells can be assessed by [^{3}H] thymidine labelling of the culture cells, grown in a selective media containing thioguanine, followed by scintillation counting. The frequency of partial and complete deletion could be assessed by multiplex PCR and the mutation spectra compared between the groups.

7.1.2 Transgenerational effects in the offspring of male exposed to chemical mutagens

The analysis of the transgenerational effects of chemical mutagens may help to elucidate additional information about the pathways associated with mutation induction at ESTR loci. The alkylating agent ethylnitrosourea (ENU) could be a good candidate to analyse, as it is known to induce ESTR mutations in the germline of directly exposed males (unpublished data). The analysis of F_0 males treated with ENU, their offspring (F_1), grandoffspring (F_2), and control individuals, should be performed using SM-PCR approaches similar to those outlined for the radiation study. The data obtained from this type of study should be able to establish whether exposure to mutagenic chemicals as well as ionising radiation can also lead to transgenerational genomic instability.

7.1.3 Assessing the epigenetic effects of ionising radiation

The work presented in Chapter 5 provides compelling evidence for the involvement of epigenetic factors in the transgenerational inheritance of radiation-induced genomic instability. It has been suggested that the transgenerational increases may be attributed to an alteration in the expression profiles of genes associated with DNA repair and cell cycle checkpoints in the offspring of irradiated males (Barber *et al.*, 2002). It should be possible to further investigation the likelihood of epigenetic modification of gene expression using microarray technology.

7.1.3.1 Gene expression profiling in the offspring of irradiated males

The profiling of total RNA from germline (spermatogonia) and somatic tissues (brain and spleen) from directly irradiated males, their first-generation offspring and control individuals would provide information on any potential changes in gene expression in the germline and soma after radiation exposure. Any such alterations could be verified by Northern blotting or possibly by semi-quantitative RT-PCR. If any alterations in gene

expression are identified, they may be further characterised to assess the type of epigenetic alteration.

The use of a whole genome subtraction hybridisation approach, followed by cloning and sequencing may also identify regions that are altered between control and exposed individuals. Similar approaches can be employed for the identification of the nature of these alterations as those used for the microarray study.

7.1.3.2 Establishment of the epigenetic basis of alterations in gene profiles

If alterations in gene expression are discovered then techniques such as bisulphite modification and/or methylation-specific PCR analysis (Herman *et al.*, 1996; Yuanxiang *et al.*, 1997) would be able to identify whether alterations in methylation can account for changes in the gene expression profile of directly exposed F_0 male and their F_1 offspring.

7.1.3.3 The effect of mutations in DNA repair genes on mutation induction at ESTR loci

The continuation of the study assessing the effects of mutations in DNA repair genes, which was initiated by the analysis of the effect of the *scid* mutation on mutation induction at ESTR loci, may be able to further clarify which repair pathways are important in the maintenance of stability at ESTR loci. The genes chosen for analysis need to play key roles in the process of DNA repair; however, the number of potential genes available for analysis are limited by the need for viable and fertile mouse models. Mouse knock-outs defective at *p53*, *MLH2* and *ATM* genes would appear to be good candidates for this analysis. *p53* (or *trp53*) has been termed 'the guardian of the genome' and is known to be associated with a number of cellular functions, including the control of apoptosis after DNA damage (Morris, 2002). MSH-2 plays a pivotal role in mismatch repair, and the lack of MSH-2 activity is associated with genomic instability, and carcinogenesis (Wei *et al.*,

2002). The ATM protein is involved in the repair of DNA double-strand breaks by the homologous recombination pathway (Pastink *et al.*, 2001). Mutations in the *ATM* gene lead to ataxia telangiectasia, a condition associated with genomic instability, increased cancer risk, and increased sensitivity to the DNA damaging effects of ionising radiation (Gatti *et al.*, 2001). The effect of these genes on ESTR mutation induction by ionising radiation can be assessed by the comparison of the ESTR mutation rates in mutant mouse strains and the strains isogenic for each mutation or knockout; these would be determined before and after exposure to ionising radiation, using SM-PCR analysis of the germline and somatic mutation rates in directly exposed individuals. Pedigree-based Southern blotting would also provide a direct comparison for the data previously obtained for germline mutation induction by ionising radiation.

7.2 Feasibility of proposed future work

The work outlined in the previous section has received grant support from the Wellcome Trust and will be pursued by myself and others within Dr Dubrova's research group over the next three years.

The work analysing the effect of DNA repair gene knockouts on mutation induction by ionising radiation is currently being undertaken by Karen Burr, a PhD student with Dr Yuri Dubrova.

Appendix 1 PCR primers

Microsatellite primers for crossover analysis

Primer	Forward primer sequence	Reverse primer sequence	MGI Accession ID
Chromosome 1			
D1Mit231	ACCCACAATTGCCTGTGG	TCTTTGCAAGCCACCAAATG	MGI:706737
D1Mit156	TCTGCTGCCACTTCTGAGAA	TGTGTGTCTATGGACATGGATG	MGI:700451
D1Mit187	ACTGCAAAAGAATATGAAGAATCTTT	TATATGTACATTCGTGTGAACACACA	MGI:705531
D1Mit15	TCCACAGAACTGTCCCTCAA	ATACACTCACACCACCCCGT	MGI:703258
D1Mit17	GTGTCTGCCTTTGCACCTTT	CTGCTGTCTTTCCATCCACA	MGI:703264
Chromosome 2			
D2Mit433	CTGTCTATCCTCATATTAGGAAATGG	AACTTTTAAAGACCATTTTATAGCCTT	MGI:701478
D2Mit420	ACATATCTGTATGTGAATGTTTGCG	GTTCCTTCTTCACAGGGAAGC	MGI:703281
D2Mit412	ACAGGGCTGCAGAGACCTAA	GACTATCAAAAAGATGGTATTGATGG	MGI:705052
D2Mit265	AATAATAATCAAGGTTGTCATTGAACC	TAGTCAAAATTCTTTTGTGTGTTGC	MGI:707486
Chromosome 3			
D3Mit93	TCAATCAGTTTCATGTGCTGTG	TTTTTGCCTTCAAAGGATTTAT	MGI:705079
D3Mit43	TGACCTCCAGAGAGTCTTCCA	CTGTGCATGAGACCACTACCA	MGI:703724
D3Mit127	CCTTCTGACAAGCAGGATTTG	TTTCTAGCATCTCCAAGCAGG	MGI:702440
D3Mit116	TCACTGCCCATCTTTGTAACC	CCCAGAGACCCGGAATAGAA	MGI:700686
Chromosome 4			
D4Mit149	TGAATTCAGAAGGATGTGTGTATG	ATGTGAGAATCAACACCTGAGG	MGI:702001
D4Mit286	ATGGGGTCTAGGAAAACATGG	AAATTATGAGTATTTCACCTGAGTGTG	MGI:706166
D4Mit308	TATGGATCCACTCTCCAGAAA	CAAAGTCTCCTCCAAGGCTG	MGI:707340
D4Mit54	CTGCCATCCTGTAGTTTCACTG	ACCCCCACATATGTCTCCCT	MGI:700659

Chromosome 8			
D8Mit95	AGCACATTCCTTACTACCCACC	CCAAAAATTGAGTTGTTTAAGGC	MGI:700671
D8Mit292	AGTCAAGGCATTTAAAATTAACTG	CTGGGTTTGCTAGTGAAAGATG	MGI:102181
D8Mit208	CTACTATTCACCCGCTCACTCC	CTAGCCCTGTTTATCTTGGTGG	MGI:705319
D8Mit320	TGACCCCTGCCTCAACAT	GCTGTGTGTGTGCTTGTTCA	MGI:702619
Chromosome 10			
D10Mit80	CAAAAAAAACCCTGATTCTACCA	GTGTGCATATGGCAGTAACTTTG	MGI:700662
D10Mit194	GATTGTTTGTAAAGACATGATCACG	AGATGTGGAATAGGAAGTATGATCG	MGI:705101
D10Mit10 (pA & pB)	CCAGTCTCAAAACAACAACAAAC	TTGCACCTAGATTGCCTGA	MGI:701596
D10Mit271	ACAACCAAAGGTCTTTGTAGAAGA	AATATATAGGCACACCTTAATAGCCA	MGI:706179

All sequences were obtained from the Mit Database at http://www.jax.org

Primers for probe production

Sequence similarities
HM1 G|GGCA|
HM2 |GGCA|
MMS10 G|GGCA|GA (80% of repeats)
G|GGCA|GAGGA (20% of repeats)

Loci	Primer 1		Primer 2	
Ms6-hm	**HMA**	GGGCAGGGCAGGGCAGGGCA	**HMB**	CCGTCCCGTCCCGTCCCGTC
Hm-2	**HM2FOR**	GGCAGGCAGGCAGGCAGGCA	**HM2REV**	GTCCGTCCGTCCGTCCGTC
MMS10	**MMS10F**	GGCAGAGGCAGAGGCAGA	**MMS10R**	TCTGCCTCTGCCTCTGCC

All primer sequences were designed by members of the laboratory group.

RFLP and sequencing primers

Loci	Primer 1		Primer 2	
scid (Y4046X)	**scidF**	TGGTATCCACAACATAAAATACGC	**scidR**	AGTTATAACAGCTGGGTTGGC
P16 exon 1	**P161f**	ACTGAATCTCCGCGAGGAAAGCGAACT	**P161r**	CTGAATCGGGGTACGACCGAAAG
P16 exon2	**P162f**	GTGATGATGATGGGCAACGTTCA	**P162r**	TGAGCTGAAGCTATGCCCGTC
Prkdc (M3844V)	**3844F***	TGTCACAAGAGGAGAAAGTGGC	**3844R**	TGTACATTAGCACATAGGATCC
	PK3.1F^{+}	GCCATGCAGTATACCTCCTT		
Prkdc (R2140C)	**2140F**	GCCATGATCCTTAGCAAGTG	**2140R**	GCCTAAGGTAAGGTGCT

*Used for sequencing only, a new primer $^{+}$ was designed from newly obtained sequences for RFLP analysis. For all other loci the same primers were used for sequencing and the subsequent RFLP analysis scid primer sequences were taken from Kosugi *et al.* (1999), p16 primer sequences were taken from Zhang *et al.* (1998), and the *Prkdc* primer sequences were taken from Yu *et al.* (2001).

Appendix 2 Radiation units

Name	Symbol	Status	Definition	Calculation
Curie	Ci	Old unit	Unit of radioactivity	3.7×10^{10} (nuclear) disintegrations per second (dps).
Becquerel	Bq	SI unit		Equivalent to one disintegration per second.
Roentgen	R	Old unit	Unit of radiation exposure	The amount of x- or γ-rays required to produce one electrostatic unit of electric charges in 1 cm^3 of dry air under standard conditions
Roentgen absorbed dose	Rad	Old unit	Unit of absorbed energy dose	Equivalent to an energy absorption of 10^{-2} J/kg.
Gray	Gy	SI unit		Equal to 100 Rad.
Roentgen equivalent man	Rem	Old unit	A measure of the dose equivalent	The effective dose is obtained by summing the equivalent doses in all tissues and organs of the body weighted by their sensitivity to radiation.
Sievert	Sv	SI unit		Equal to 100 Rem.

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