Analysis of Germline Mutation Rates in DNA-repair Deficient Mice

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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September 2005

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Abstract

Expanded simple tandem repeat (ESTR) loci provide a useful and sensitive tool for the analysis of germline mutation induction in male mice; however the mechanisms that act at ESTR loci causing instability remain unknown. Understanding of mutational processes at ESTR loci may provide important information about the damaging effects of exposure to mutagens at the genome level. To investigate the possibility that mutations at ESTR loci may be a by-product of DNA damage responses mice with mutations at genes involved in DNA repair/apoptosis have been analysed. Through this analysis it was hoped to determine whether DNA repair/apoptosis were involved in the mechanism of mutation at mouse ESTR loci.

Four gene mutations were studied; p53, PARP-1, XPC and polκ. The analysis of p53 deficient mice demonstrated that p53 status, therefore p53-dependent apoptosis in mice has no effect on the spontaneous or radiation induced ESTR mutation rates, indicating p53 has no influence on ESTR stability and therefore has no role in the mechanisms of mutation acting at these loci. Three DNA repair deficient mice strain have been analysed (PARP-1, XPC and polκ) and it was seen that in all three strains the spontaneous ESTR mutation rates were elevated when compared to wild-type counterparts. This has led to the proposal that delay or lack of repair could lead to polymerase slippage due to enhanced replication fork pausing. From this study it has been suggested that the mechanism of mutation at ESTR loci is polymerase slippage due to replication fork pausing, similar to the mechanism proposed for microsatellites. The work presented shows for the first time that DNA repair deficiencies can cause germline instability in mice and suggested a model for the mechanism of mutation at ESTR loci.

Work from this thesis has been published separately as:

Barber, R. C., Miccoli, L., van Buul, P. P. W., Burr, K. L-A., van Duyn-Goedhart, A., Angulo, J. F., and Dubrova, Y. E. (2004). Germline mutation rates at tandem repeat loci in DNA-repair deficient mice. *Mutat Res* **554**, 287-295.

Burr, K. L-A., Smith, A. G., and Dubrova, Y. E. (2005). p53 deficiency does not affect mutation rates in the mouse germline. *Oncogene* **24**, 4315-4318.

Acknowledgements

Firstly, I would like to thank both my supervisors, Dr. Andy Smith and Prof. Yuri Dubrova for the opportunity to undertake this project and their help and support during my time in Leicester. Also I would like to thank everyone in G18 and G19 for being a great bunch of people to work and socialise with and their help towards this thesis.

I would like to thank all the people we collaborated with during the work presented in this thesis; Dr. G. de Murcia and Dr. L. Miccoli for the mouse breeding and irradiations performed at Ecole Soperieure de Biotechnologie de Strasbourg, Illkrich, France; Dr. E. Freidberg, Department of Pathology, UT Southern Medical Centre, Dallas, Texas, for providing the polκ mice. I would also like to thank the staff from the Biomedical Science, for their help with the p53 study.

I would like to thank the MRC and the Wellcome Trust for their financial support.

Thanks to my friends and family for their constant support during my PhD and keeping me sane. A big thank you has to go to my Fiancé, Alistair for just being there when I needed him to be. Finally I would like to thank my Grandpa, for his final words of encouragement.

I would like to dedicate this thesis to Alexander Ballantyne, my wonderful, much missed Grandpa.

Abbreviations

AAF- Acetylaminofluorene

Aprt – Adenine phosphoribosyltransferase

ATM – Ataxia telangiectasia mutated

BBN – N-butyl-N-(4-hydroxlbutyl)nitrosamine

bp – Base pairs

DMH – 1,2-Dimethylhydrazine

dNTP – Deoxynucleoside triphosphates

DNA – Deoxyribonucleic Acid

DNA-PK – DNA-protein kinase

DNA-PK$_{cs}$ – DNA-protein kinase catalytic subunit

DSB – DNA double strand break

ENU – Ethylnitrosourea

ESTR – Expanded simple tandem repeat

F$_{(0,1)}$ – Filial generation, F_0 parental, F_1 first generation

FISH – Fluorescence *in situ* hybridisation

GGR – Global genomic repair

Gy – Gray

HNPCC - Hereditary non-polyposis colorectal cancer

HDB – Homeodomain protein binding

Hprt - Hypoxanthine guanine phosphoribosyl transferase

HR – Homologous recombination

IGH – Immunoglobin heavy chain

kb – Kilo-base pairs

kDa – Kilo-Daltons

LET – Linear energy transfer

LTR – Long terminal repeat

Mb – Mega-base pairs

MEF – Mouse embryonic fibroblast

MMR – Mismatch repair

MNU – Methylnitrosourea

MMS - Methylmethanesulfonate

MVR – minisatellite variant repeats

NER – Nucleotide excision repair

NHEJ – Non-homologous end joining

NLS – Nuclear localisation signal

PAD – Polymerase associated domain

PARP-1 – Poly (ADP-ribose) polymerase-1

PCR – Polymerase chain reaction

Polκ- Polymerase kappa

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

RPA – Replication protein A

SCE – Sister chromatid exchange

scid – severe combined immunodeficient

SLT – Specific locus test

SM-PCR – Single molecule polymerase chain reaction

SP-PCR – Small pool polymerase chain reaction

SSA – Single-strand annealing

SSB – DNA single-strand break

STR – Simple tandem repeats

TCR – Transcription coupled repair

TLS – Translesion synthesis

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

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

UV - Ultraviolet

VNTR – Variable number tandem repeat

XP- Xeroderma pigmentosum

XPC – Xeroderma pigmentosum complementation group C

1 INTRODUCTION

We live in an environment where we are constantly exposed to ionising radiation, both from natural sources from the ground, food and cosmic rays as well as from man made sources from nuclear reactors, sterilisation of food, therapeutic and diagnostic medical procedures (UNSCEAR, 2000).

It is well known that radiation is harmful to humans and other living organisms. There are two types of mutations that can occur due to radiation exposure, somatic and germline mutations. Somatic mutation can lead to the development of cancer or other problems in the exposed individual, whereas germline mutations could lead to miscarriages, birth defects, physical and mental development defects, and a broad range of diseases in the offspring of exposed parents. These outcomes indicate that there is a need to understand the genetic risk of exposure to radiation within the population. Estimating the genetic risk of radiation in humans has been a difficult task. Epidemiological studies have been carried out to analyse the effects of ionising radiation on the human population by examining the atomic bomb survivors and individuals exposed to radiation by accident (Hiroshima, Nagasaki and the Chernobyl disaster) or exposed to medical sources (radiotherapy). These results give limited information on the genetic risk of low doses of radiation as low dose risks have been extrapolated from high dose mouse data. Limitations such as control of variables within subject and control groups in the human studies has led to the increasing use of model systems to analyse genetic risk of exposure to ionising radiation.

Model systems use genetically homogeneous populations in identical environmental conditions exposed to controlled doses of radiation. The most commonly used model system to analyse the effects of low dose radiation is the laboratory mouse. By using the mouse model a large quantity of data has been gathered on the genetic risks of radiation exposure that has been extrapolated to human situations. The data obtained for low dose exposure has relied on the extrapolation of data from higher dose exposure, which may not be entirely accurate. Recently a more sensitive germline mutation detection system has been developed utilising mouse expanded simple tandem repeat (ESTR) sequences. ESTRs have given an insight into the effects of low dose exposure to ionising radiation in mice without the need to extrapolate the data from higher doses. It has been verified that ESTRs provide a good germline mutation monitoring system in mice, although

their use is limited as the mechanism of mutation at these loci is unclear. It was the main aim of this study to provide more information on the mechanism of mutation at ESTR loci.

1.1 Analysis of germline mutation induction

One of the most important types of environmental mutagens for the human population is ionising radiation. It is therefore important to understand how ionising radiation as a mutagenic agent affects biological organisms.

1.1.1 The sources of human exposure to ionising radiation

Ionising radiation has been shown not only to be able to destroy cancers but also to cause them. In fact the genetic effect of ionising radiation on living organisms has become an increasingly important issue over the past few decades due to the increased use of ionising radiation. Ionising radiation is used in nuclear facilities to produce electricity leading to an increased in the number of people being occupationally exposed. There is also concern of an increase in environmental exposure of those people that live near by such nuclear facilities. Accessibility to nuclear weapons raises concern over the increase in environmental exposure to ionising radiation after the dropping of the atomic bombs on Hiroshima and Nagasaki and the awareness of nuclear bomb testing sites. Accidental exposure has also been highlighted as a threat, as seen after the Chernobyl disaster. Another concern is that the use of ionising radiation as a treatment for cancer has risen considerably over the years.
Taking into account the increased use ionising radiation and the threat and availability of nuclear weapons highlights an increased risk of exposure and therefore a need to understand the effect of ionising radiation on living organisms.

To evaluate the effect of exposure to ionising radiation on the human population epidemiological studies have been carried out on individuals exposed in the work place, accidentally, environmentally and through medical exposure. Through these studies many health implications of exposure to ionising radiation have been revealed in individuals exposed directly and indirectly to ionising radiation. Most epidemiological data that have been obtained to date examined individuals that were directly exposed to

ionising radiation, and analysing their somatic tissue alterations by observing cancer frequencies (both solid tumours and leukaemia) in exposed individuals (Cardis et al., 1995; Kelleler, 2000 & reviewed in Wakeford, 2004). Fewer studies have focussed on what the effect of germline exposure to ionising radiation has on the offspring of directly exposed individuals. To study the effects on the germline data has been gathered on the rate of congenital malformation (Otake et al., 1990; Doyle et al, 2000), and on evaluating the cancer risk in the offspring of directly exposed parents (Gradner et al., 1990). Such epidemiological studies have shown that parental exposure to ionising radiation can increase the health risks in their offspring, but were unable to show that parental exposure is related to an increased risk of congenital malformations or cancer in the next generation.

Findings from such epidemiological studies have led to changes in work practice and legislation. An advisory committee named the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) was established in 1955 and appointed to address concerns regarding the effects of radiation (natural and man-made) on human health and the environment. The committee issues annual reports that governments and organisations rely on for radiation protection and safety standards and for the regulation of radiation sources. The annual report outlines important issues concerning long-term health implications of radiation exposure, mutation induction monitoring and the calculation of risk estimates. UNSCEAR reports have assessed issues such as the sources and effects of ionising radiation, the genetic and somatic effects of ionising radiation and hereditary effects of exposure to ionising radiation (UNSCEAR,2000). Based on these reports Health and Safety Regulations relating to working with ionising radiation have been changed, for example, the Management of Health and safety at Work Regulations 1999 . The Ionising Radiation Regulations 1999 (IRR99) provide information on effective dose limits, areas of key risk and information on approved dosimetry (The Ionising Radiation Regulations, 1999).

Epidemiological studies supply a great source of data and information on the effects of ionising radiation on the human populations, but there are a few problems and limitations associated with this type of survey. Firstly, the assessment of doses and dose rates that individuals have been exposed to is not always possible. This is not a problem for exposure at work as records are kept, but environmental and accidental exposure cannot be measured accurately. When trying to estimate environmental exposure,

individuals may be exposed internally through food and water as well as externally and therefore possibly producing different biological effects. Secondly, during epidemiological studies well matched controls are required to ensure observed variations between the groups are due to exposure to the mutagen and not to any other factor. Various factors have to be considered including lifestyle, occupation, smoking habits, parental age and ethic background. Thirdly, the estimation of the risk of low dose irradiation remains unclear due to the requirement of large sample sizes. To date risk of low doses exposure to ionising radiation is based on extrapolation from data from intermediate to high doses. Since most humans are only exposed to low doses of radiation these are the values of most interest. Finally, epidemiological studies are not always reproducible. For instance, contradicting results have been obtained when different cohorts within the same exposed population have been analysed for congenital malformations of the children of parents exposed after the Chernobyl accident (Little, 1993).

Epidemiological studies, although they provide valuable information, cannot be relied on alone to give accurate genetic risks of exposure to ionising radiation. It is evident that there is need to analyse the effect of ionising radiation using a system that is powerful, more reliable and reproducible to backup and clarify the data obtained from epidemiological studies alone. This has been done through the use of various model systems to analyse the effects of ionising radiation.

1.1.2 Model systems and ionising radiation

To analyse the effects of ionising radiation, various *in vivo* model systems have been used including *Drosphila*, yeast, plants and laboratory rodents. The use of model systems eliminates many of the complications encountered in epidemiological studies by reducing the number of variables within the cohort. When using model systems it is possible to control the dose and the dose rate of mutagenic agent, the genetic background (by using genetically homogeneous inbred strains) and the environmental conditions. The most commonly used model system to analyse the effects of ionising radiation is the laboratory mouse. Mice provide a useful experimental system as they have a short life span, reproduce rapidly and their genome can be easily manipulated.

Ionising radiation was first reported to have mutagenic properties in *Drosphila* in 1927 by Muller. X-rays have been shown to be mutagenic in mice by numerous studies

over the years that have provided data that indicate ionising radiation can induce germline mutations (reviewed in Searle, 1974). These observations demonstrated that radiation induced damage can be transmitted to the next generation via the germline, highlighting the need to investigate the transmission of induced damage through the germline and the implications of environmental exposure. A number of germline mutation detection systems have been developed over the years to do just this.

1.1.3 Assays used in model systems to detect germline mutations

Traditionally germline mutation detection is carried out using pedigree analysis i.e. analysing the offspring of the parents in question. Some germline detection systems however, analyse the germ cells directly eliminating the need for breeding. Some of the most widely used germline mutation monitoring systems will be outlined in this section, highlighting their drawbacks and requirements for improvement to allow the development of a new monitoring system.

1.1.3.1 Dominant visible mutations

Donald Charles first documented through phenotypic changes that ionising radiation could cause dominant mutations in the offspring of exposed parents (Charles, 1950). Dominant mutations were monitored by comparing the phenotype of the F1 offspring to their exposed parents or to F1 offspring of untreated parents. The main difficulty with this method is distinguishing between variants (deviations from normal phenotype) and mutants. Another drawback to this method is that the number of dominant mutations that could give phenotypic changes are likely to be low compared to the total number of dominant mutations caused by exposure to ionising radiation. Other assays have been designed to monitor other dominant mutations including lethality, skeletal malformations and cataract formation (Searle, 1974, Ehling, 1991).

1.1.3.2 Specific locus test (SLT)

The specific locus test was developed to improve the sensitivity and scope of the previously designed phenotype monitoring tests as it not only detects dominant

mutations but also recessive mutations. The specific locus tested was introduced by William Russell and colleagues (Russell, 1951), and is also known as the Russell-7-locus test. The specific locus test has been the most widely used germline mutation induction monitoring system to date and has provided a large proportion of the data available to date on mouse radiation genetics. The test is designed to detect mutations at specific loci in mice that were chosen as they cause clearly visible phenotypical changes in the offspring of parents being tested.

There are two specific locus stocks available, one of which is more commonly used the T stock (test stock) created by W.L. Russell in 1948, which is homozygous recessive at seven loci (Table 1.1). The second specific locus stock was developed and used at MRC Harwell (Lyon and Morris, 1966).

The test involves the mating of homozygous mice, where mice homozygous for the dominant wild-type alleles are crossed with mice homozygous for the recessive alleles (Figure 1.1). By analysing the phenotypes of the offspring from these crosses the specific locus test allows the estimation of the germline mutation rates in the wild-type parents. All the offspring produced show either dominant wild-type phenotype or mutant (deviations from wild-type) phenotype arising from mutation in the wild-type parent. The wild-type parents can be untreated or irradiated, and thus the spontaneous and the radiation-induced germline mutation rates can be evaluated.

The specific locus test provides a non-subjective and reproducible method for monitoring germline mutation induction due to clear cut phenotypic changes in the offspring that can be attributed to mutations occurring at a specific loci in the germline of the control or irradiated wild-type parent. The specific locus test does have its drawbacks; due to the low spontaneous mutation rates large numbers of animals need to be profiled to detect an increase in mutation rate, high doses of ionising radiation have to be used and there is a lack of direct application to the human population.

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 locus test loci

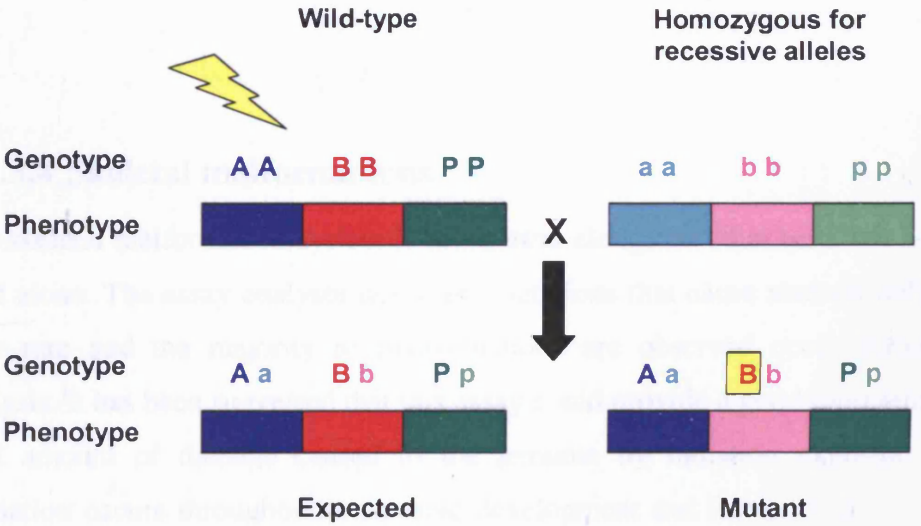


Figure 1.1: Schematic of the specific locus test

1.1.3.3 Dominant lethality

The dominant lethal test analyses the number of *in utero* deaths of the offspring of exposed parents. The increase in lethality is due to an increase in chromosomal aberrations that do not affect the fertilisation ability of the germ cell but the viability of the embryo resulting in death before or after implantation (Lyon, 1964; Phillips and Searle, 1964). The dominant lethality test is based on the mating of exposed males with

untreated virgin females. Pregnant females are sacrificed, approximately 12-17 days after conception and the contents of their uteri are analysed. The number of living and dead implants are used to estimate the germline mutation rate. Several studies have shown an increase in the number of *in utero* deaths with increasing paternal exposure to ionising radiation (Searle and Beechey, 1981; Kirk and Lyon, 1984).

The dominant lethality test does not require as many offspring as the specific locus test to obtain statistically significant data, but a large sample size is still required (several hundreds). This assay does provide information on germline mutation but does not supply any data on the induction of non-lethal mutations that would not prevent implants from developing fully. In addition, *in utero* death could be caused by other factors such as maternal health and environment that undermine the robustness of the test.

1.1.3.4 Skeletal malformations

The skeletal malformation system is often used alongside other tests but has also been used alone. The assay analyses dominant mutations that cause skeletal defects, but are very rare and the majority of malformations are observed occur through internal analysis. It has been suggested that this assay could provide a good representation of the total amount of damage caused to the genome by radiation exposure as skeletal formation occurs throughout embryonic development and is modified by a number of genes (Ehling, 1991). It has been shown that skeletal malformation does increase in the offspring of irradiated parents (Selby, 1979; Ehling, 1991). The skeletal malformation assay requires a large number of animals due to low spontaneous rate of skeletal malformations (Searle, 1974). The identification of internal variants/malformations requires skilled techniques and is difficult to perform.

1.1.3.5 Dominant cataracts

Detection of dominant cataracts is a mutation system that is often used as part of a multiple endpoint approach. The dominant cataract system is based on the observation of cataract formation (opacity of the lens) in offspring of treated parents and occurs in offspring after parental exposure to ionising radiation (Ehling et al., 1982; Graw et al., 1986; Ehling, 1991). The main advantage of the dominant cataract system is that

morphologically similar cataracts in human and other mammals seem to have the same mode of inheritance, this suggests that results from mice may be comparable to that which may be observed in humans (Ehling, 1991). The disadvantage of dominant cataracts as a germline mutation monitoring system is that it is difficult to perform due to the requirement of specialised techniques and the mutation rates are very low.

1.1.3.6 Recessive mutations

Monitoring of recessive mutation in the grand-offspring of treated parents has been used to monitor germline mutations in mice. This has been done in two ways; firstly by monitoring the recessive lethal mutations of the grand-offspring (as described in section 1.1.3.3) or secondly by observing recessive visible mutations in the grand-offspring (Charles, 1950; Lyon et al., 1964; Luning, 1971; Luning and Eiche, 1975). Few studies have concentrated on this method of germline mutation monitoring as the phenotypic changes are less clearly visible than those observed in dominant mutation assays and the requirement of further breeding to analyse the grand-offspring.
Recessive visible mutations have however been observed in the offspring of treated parent using the specific locus test (Graw et al., 1986).

1.1.3.7 Enzyme activity

The analysis of enzyme activity assesses the overall biochemical 'fitness' of the offspring produced after paternal exposure to ionising radiation (Pretsch et al., 2000). A number of enzyme systems have been employed, but the most commonly used enzyme system to date is the monitoring of alterations to the erythrocyte enzyme function. This system involves 10 enzymes encoded by 14 loci (Charles and Pretsch, 1986). Using this system an increase in the alterations of enzyme activity has been observed in the offspring of irradiated mice (Pretsch, 1994; Pretsch, 2000). This method has a number of advantages over other systems used to monitor the induction of germline mutations, including; the enzyme activity assay has a similar sensitivity as the mouse specific locus test, the assay is not dependent on the use of precisely matched strains or species allowing comparative studies of different species and strains (Pretsch, 2000). The limitation of this type of assay is that the loci involved have low spontaneous mutation

rates, therefore require a large sample size. Another downfall is the high cost of this type of assay.

1.1.3.8 Semi-sterility

The semi-sterility assay measures the effect of paternal exposure to ionising radiation on the fertility of male mice in the subsequent generations. Male mice are irradiated and then mated with untreated females of a different strain and the male offspring's fertility is then assessed by mating them with out-bred female mice. The males showing semi-sterility are then bred with females from a recessive stock to demonstrate the possibility of heritably of semi-sterility (Lyon, 1964).

The semi-sterility assay has been used in the past to show that paternal exposure to acute ionising radiation can cause an increase in male semi-sterility of the offspring of exposed fathers (Lyon et al., 1964). During this study Lyon was also able to show that the semi-sterile male mice all carried heterozygous reciprocal translocations. When Phillips and Searle (1964) analysed the effect of chronic paternal exposure on semi-sterility of the male offspring they found that there was no significant increase. Evidence has been produced through the analysis of semi-sterility in mice showing that DNA damage (reciprocal translocations) can be passed through the germline onto subsequent generations. This assay however requires a complex mating scheme and a fairly large number of animals to be screened. Also it has been shown not to be sensitive enough to detect germline mutations after chronic exposure to ionising radiation which is the most common type of human exposure.

1.1.3.9 Chromosomal aberrations

Structural alterations of the chromosomes account for a large proportion of the DNA damage caused by ionising radiation (van Buul, 1983). The analysis of such aberrations in the germ cells after exposure to ionising radiation has been undertaken to provide information on the effect of ionising radiation on the germline. The scoring of germline aberrations is often done on self-renewing stem cell spermatogonia. The most relevant type of aberrations that are monitored are reciprocal translocations that are known to be efficiently transmitted through the germline. Chromosomal aberrations are scored visually using conventional cytogenetic techniques or FISH during metaphase.

The analysis of chromosomal aberrations has been done on the offspring conceived from exposed spermatogonia. The analysis provided evidence that the rate of chromosomal aberrations increases with increased dose of ionising radiation (Leonard and Deknudt, 1967). Analysis of the directly exposed germ cells has also been investigated (Griffin and Tease, 1988; Tease and Fisher, 1996).

It has been suggested that data from chromosomal aberrations in mice can be extrapolated to humans if several correction factors are taken into account (van Buul, 1983). van Buul suggested that due to the complexity of the formation of chromosomal aberrations data should be obtained from the germline of species more closely related to humans (higher primates) to get quantitative estimations of human genetic risks.

1.1.3.10 Micronucleus assay

Micronuclei are formed during mitosis via various mechanisms including acrocentric fragments, multi-centric chromosomes, damaged kinetochores and spindle fibre defects. Micronuclei can be detected in the cytoplasm as small nucleus-like particles near the nucleus. The micronucleus assay was developed to provide a fast simple assay to monitor DNA damage. The majority of radiation-induced damage analysis using the micronucleus assay has concentrated on the measurement of DNA damage in bone marrow cells. There have however been studies on the effects of ionising radiation in the germline using this assay (Pampfer et al., 1989). By analysing the 2-cell stage embryos conceived from irradiated spermatozoa, Pampfer *et al.* found that the number of micronuclei increased linearly with dose. The micronucleus assay can not provide any information on the consequence of damage seen on the viability of the cells or the type of mutations within the cells nor is this assay able to provide any information on the mutation rate.

1.1.3.11 Comet assay

The comet assay uses gel electrophoresis of a single cell to detect the amount of DNA damage using microscopic measurements of the degraded DNA. During gel electrophoresis, cells take the shape of a comet where the nucleus appears as the head and tail, which is visualised by fluorescence. The length and the width measurement of the tail indicate the amount of DNA damage (increase proportionally with the amount of

damage). Studies have shown that the amount of DNA damage increases with increased dose of radiation (Schindewolf et al., 2000; Haines et al., 2001). The Comet assay is able to provide information on the quantity of *in vivo* damage caused to DNA but it is unable to detect the type of mutations or the viability of the cells analysed and it does not measure the mutation rate.

1.1.3.12 Transgenic mouse constructs (lacI, lacZ)

Transgenic mice have been engineered to detect mutations *in vivo* in any tissue. There are two commercially available transgenic mouse constructs, named Muta^{TM}Mouse and Big Blue mice. They were generated by using a λ shuttle vector containing the *lacI* gene (Big Blue mice) or the *lacZ* gene (Muta^{TM}Mouse). Mutation analysis is performed by recovering the vector from the treated animals' genomic DNA; the extracts are then plated on bacterial agar containing β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). *LacZ* or *LacI* mutations appear as blue plaques on a clear background of non-mutated plaques. The number of blue plaques in the control individuals is compared to the number from the exposed individual. Exposure to ionising radiation has been shown to increase the frequency of mutants observed in the germline using the *lacZ* reporter system (Katoh et al., 1994). Using the *lacI* reporter system a slight increase in germline mutation rate was observed after exposure to ionising radiation (Hoyes et al., 1998). The main advantage of this system is that by using transgenic mouse constructs the analysis of spontaneous and induced mutations rates in the germline can be compared to those in other tissues. However, Hoyes *et al.* (1998) suggest that the *lacI* system may not be suitable to detect mutation induction due to deletions that would be larger than the *lacI* transgene.

1.1.3.13 Summary of germline mutation detection systems

As illustrated there are many approaches for monitoring germline mutation induction in mice. The phenotype based assays have provided data that proves that exposure of male mice to various sources of ionising radiation results in elevated germline mutation rates detectable as elevated frequency of germline mutations transmitted to the offspring (Searle, 1974). These phenotype based assays have been shown to be valuable *in vivo* methods to assess radiation induced germline mutations in mice. Although they have

provided such a powerful tool these techniques require relatively high-dose exposure to radiation to induce mutations due to the low resolution of these techniques. Also using these phenotype based assays no information on the total amount of DNA damage produced by irradiation can be gathered. Since extensively damaged germ cells may not be viable or lost via apoptosis or cell cycle arrest these types of mutations would not be apparent in the phenotype based assays. New techniques have overcome this by analysing germ cells from directly exposed individuals. A few of these techniques allow the analysis of the damage caused directly to the DNA. Since there are so many different mutation systems around to detect germline mutation induction in mice their sensitivity and ability to monitor radiation induced mutations need to be evaluated. Comparison data produced using six of these are shown in Table 1.2.

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 the most commonly used germline mutation detection systems for the analysis of radiation induced mutations. (Taken from Dubrova *et al.*, 1998a). † Minimal sample size for detection of 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.

An efficient method of comparing the sensitivity of these systems is to compare the doubling dose. The doubling dose is the dose of ionising radiation required to produce a two-fold increase over the spontaneous mutation rate. The doubling dose estimates of

some of the tests are presented in table 1.2. The estimates of the doubling dose range from 0.17 Gy, for the dominant visible test to 0.56 Gy, for the dominant cataracts test. These estimates do not differ greatly from the average value which is 0.35Gy. However, the most reliable assay that has been used to date is the specific locus test and the doubling dose estimate of this system is very similar to that of the average value (0.34 Gy).

Although these systems discussed here have produced a great deal of valuable information on mutagenicity of ionising radiation in the germline of mice, they still have a number of marked limitations. One of the problems is the need to profile a large number of animals to produce significant results. Another is that animals have to be exposed to relatively high doses of ionising radiation (3-12 Gy) to induce mutations that are detected at a phenotypic level due to the low spontaneous mutation rates of the protein coding genes involved. These high levels of exposure are well in excess of those to which the human population are exposed. To date the data obtained from mouse models after high dose exposure has been extrapolated to provide information on mutation rates at lower doses that would be more relevant to human exposure. Since analysis of radiation induced mutation in human presents a variety of difficulties the use of the mouse system to assay genetic risk of exposure to ionising radiation is of great interest.

Unfortunately, the extrapolation from high doses could prove to be problematic. In addition, there are a number of other problems concerned in the extrapolation of data from the mouse systems for the evaluation of human risks after exposure to ionising radiation (Bridges, 2001). The specific locus test is the most reliable source of data to date on radiation induced mutation in the germline of mice, but due to the specific strains and breeding schemes used it is uncertain how far this system can be extrapolated to humans. A number of the other phenotype based techniques are applicable to humans, although it would be difficult to assess the mutation components of such changes within the human population (Sankarananrayanan, 1998).

After evaluation of the available germline mutation monitoring systems in mice it was proposed that a new test system was required. The new system would have to take into account the drawbacks of the previously used methods and try to overcome some of their limitations. The previously used assays have utilised the analysis of a small number of loci and assumed that these loci are representative of the whole genome. A

new system would need to take into account that the extrapolation of the data obtained for the loci studied to the genome as a whole would be relatively accurate. A new system would have to be able to analyse mutagenic agents that are known to affect humans, such as ionising radiation and chemicals, and would need to be quick, easy and reproducible. The test system would be required to have high spontaneous mutation rates at the chosen loci to allow the reduction in the numbers of offspring required to achieve significant results. Also the mutation rates at the test loci would have to increase after exposure to known mutagens.

Taking all the requirements into account the analysis of mutation rates at a DNA level seems the most convenient. Protein coding genes would require large numbers of animals therefore not providing an alternative to the specific locus test. The use of repetitive DNA on the other hand seems to comply with the highlighted requirements; repetitive loci are found throughout the mammalian genome, they have high spontaneous mutation rates and can be found in the genome of most organisms. The analysis of repeat loci mutations would be easily monitored as mutations at these loci involve the gain or loss of repeat (length polymorphisms) and would therefore be easily detected using Southern blotting analysis of genomic DNA or by PCR based technology making the analysis reproducible. The following section gives a summary of the characteristics of repetitive DNA found within the mammalian genome and some of their known uses to date.

1.2 Repeat DNA

A large proportion of many genomes contains repetitive DNA sequences; therefore the analysis of mutation induction at repetitive loci may be a more representative potential monitoring system for the damage caused to the genome as a whole. There are different types of repetitive DNA and these have been divided into classes. The classes of repetitive DNA that would prove most useful as a tool for monitoring mutation induction are the tandemly repeated sequences. Tandem repeat sequences are divided into three major groups; satellite, minisatellite and microsatellite DNA. The distinction between the groups is based on the size of repeat unit and the repeat array. There are two other classes of tandem repeat sequences. One that is found in the mouse genome that does not fall neatly into any of the satellite groups as they have characteristics of both mini- and microsatellite DNA. These loci have been termed Expanded Simple

Tandem Repeat loci (ESTRs). The second class of tandem repeat DNA found consists of telomere repeats which are found at the end of chromosomes. Telomere repeats differ greatly between mouse and humans (Wright and Shay, 2000) and are therefore unlikely candidates for use as a monitoring system to examine mutation induction. The other four classes will be reviewed in this section.

1.2.1 Satellite DNA

Satellite DNA sequences are highly repetitive with lengths of one to several thousand base pairs and account for several percent of the eukaryotic genome. Satellite array lengths can be up to 5Mb and are composed of single repeat units. Satellite DNA is usually located at very specific sites near the centromeres and telomeres of chromosomes. Due to their large size satellite DNA is rarely used for genotyping, but they have proved useful as anchors for genome mapping.

1.2.2 Minisatellites

Minisatellites, also known as variable number of tandem repeats (VNTRs), are found within the genome of most eukaryotes. It has been estimated that there are over a thousand minisatellites in both the human and mouse genomes (Jeffreys, 1987a; Bois et al., 1998a). Minisatellites are generally GC-rich with array sizes from 0.5 to 30 kb and are composed of tandemly repeated units of between 8 and 100bp (Armour et al., 1990; Wong et al., 1987). Minisatellites are highly polymorphic due to variations in the number of copies of repeats within the arrays and variant repeat units found within the arrays (Bois and Jeffreys, 1999; Jeffreys, 1985b). Minisatellites are classified as hyper-mutable or hypervariable if the mutation rate observed is over 0.5%, although there is a large number of stable minisatellites with mutation rates well below 0.5%. Germline mutation rates at minisatellite loci are locus specific and have been extensively studied at some human minisatellites including MS205, MS32, B6.7 and CEB1, which have germline mutation rates of 0.4%, 0.8%, 5% and 13% per sperm, respectively (Jeffreys et al., 1997). A subset of human minisatellites contain a 'core' repeat sequence of 10-15bp that is similar to the generalised recombination signal (chi) of *Esherichia coli* (GCTGTGG), which might contribute to the generation of hypervariablity of minisatellites (Jeffreys, 1985b). Alternative 'cores' have been identified that are

associated with other sets of minisatellites such as AT-rich minisatellites (Jarman et al., 1986; Nakamura et al., 1987).

Minisatellites have been detected in the genome of most higher eukaryotes including humans, mice, rats, cats, dogs, birds and pigs (Jeffreys et al., 1985b; Burke and Bruford, 1987; Jeffreys and Morton, 1987; Schonian, 1993; Vergnaud et al., 1993; Archibald et al., 1995; Andersen and Nilson-Tillgren, 1997). Minisatellites are mainly found in the non-coding regions of the genome. The chromosomal distribution of the repeat arrays varies between different species. In humans minisatellites are clustered in the sub-telomeric regions (~90%) (Amarger et al., 1998), while in the pig, rat and mouse genomes minisatellites show less telomeric clustering (66%, 30% and 15%, respectively) (Amarger et al., 1998; Jeffreys, 1987).

Distinct mutational processes operate in somatic and germline cells generating minisatellite variability. Somatic mutations are rare, simple intra-allelic events caused by replication slippage or unequal crossing-over between sister chromatids (May et al., 1996; Jeffreys and Neumann, 1997). Germline minisatellite mutations on the other hand arise through complex inter-allelic conversion-like events that probably take place during meiosis (Jeffreys et al., 1994; May et al., 1996; Bois and Jeffreys, 1999).

The function of the majority of minisatellites is unknown, although some minisatellites have been found in the coding regions of the human genome. These minisatellites vary in size altering the length of proteins and altering the protein function. Such alterations have been associated with disease. One example of this is the variation in size of the *apo(a)* gene producing a protein of various molecular weight ranging from 400 to 800 kDa (Koschinsky, 1990). Other examples are the variation in number of repeat units in minisatellites in the epithelial mucin gene and the D4 dopamine receptor (DRD4) causing variations of protein size (Lancaster et al., 1990; Lichter et al., 1993; Asghari et al., 1994).

The majority of minisatellites however are found within the non coding region of the genome and although their function remains unclear, a number of functions have been suggested. It has been proposed that minisatellite instability in non coding regions close to gene rich areas may disturb the expression of neighbouring genes (Bois and Jeffreys, 1999). VNTRs located 1kb downstream of the proto-oncogene H-ras (HRAS1) influence the risks of heritable forms of cancers (Phelan et al., 1996). Furthermore, the onset of progressive myoclonus epilepsy (EMP1) has been shown to be influenced by

the expansion of a minisatellite located upstream of the *cystatin B* gene, possibly in the promoter (Lalioti et al., 1998; Lalioti et al., 1999). Another minisatellite that may affect transcription is one that is found within the human immunoglobulin heavy chain (IGH) locus. The IGH locus contains a sequence that is similar to that of the binding site in the adenovirus major late promoter (MLP); minisatellite variation at this site may be associated with the suppression of transcriptional activity (Trepicchio and Krontiris, 1993). A minisatellite found within the intronic region of a gene has been shown to interfere with exon splicing. This may be due to the similarity of the splice donor repeat with the consensus sequence of the minisatellite, as seen in the human interferon-inducible gene 6-16 (Turri et al., 1995). Other proposed functions have included a role in imprinting (Chaillet et al., 1995) and a role in chromosomal pairing (Ashley, 1994; Sybenga et al., 1999).

Although the function of minisatellites still remains unclear due to their large number, their persistence within the genome and the fact that they are found in most eukaryotes suggests that they are not just 'junk' and that they do have a function or some evolutionary value.

Traditionally, minisatellite germline mutation detection in humans was achieved by pedigree based analysis. This approach has been used to detect the rate at which the formation of new length polymorphisms occur within the germline (Jeffreys et al., 1988a). Minisatellites are easily analysed using Southern blot hybridisation of genomic DNA using either single locus or multilocus probes. Single locus probes detect unique minisatellite sequences whereas multilocus probes consist of tandem repeats of the core sequence allowing simultaneous detection of several minisatellites (Jeffreys et al., 1985a; Wong et al., 1986). Multilocus probes produce a complex pattern of bands described as a 'DNA fingerprint' as the chance of two individuals giving the same pattern is very small. Multilocus probes were the type of probe initially used for analyses; however single locus probes were later isolated and used to generate 'DNA profiles'. Single locus probes produce much simpler patterns consisting of two allelic bands per individual. The pattern per probe is not individual specific unlike the multilocus probe patterns but high levels of individual specificity can be obtained if used in conjunction with up to five different single locus probes.

Single locus probes eventually became the probe of choice for many reasons; they require smaller quantities of DNA, and they generate simpler and more easily

interpreted results. When combinations of several different single locus probes are used they are highly discriminating.

The discovery of hypervariable minisatellites and multilocus and single locus probes has led to the development of 'DNA fingerprinting' and DNA profiling for the identification of individuals (Jeffreys et al., 1985b; Jeffreys et al., 1985c; Wong et al., 1987). This approach has been used in individual identification for paternity testing (Jeffreys et al., 1992; Smith et al., 1990) and for forensic analyses (Gill et al., 1985). The single locus approach has also been used for population studies (Balazs et al., 1989) and human evolutionary histories (Jobling et al., 1998). This approach was found to have limitations due to the inefficiency of mutation detection and the inability to provide any information on the processes acting on minisatellite loci (Jeffreys et al., 1997).

PCR-based techniques have been developed to analyse the mechanism of mutation and allow the detection of rare mutations. Two techniques are used to detect mutations in sperm directly. Small-pool PCR (SP-PCR) involves the amplification of 10-200 molecules (based on Poisson analysis of single molecule dilutions) (Jeffreys et al., 1994). This approach is able to give reliable estimates of mutation rates above 10^{-3} per progenitor molecule, although below this PCR artefacts become problematic (Jeffreys et al., 1994; May et al., 1996). This difficulty has been overcome by incorporating another technique, size enrichment of the genomic DNA before amplification. The approach is very sensitive, detecting mutation rates as low as 10^{-7}, but is only semi-quantitative (Jeffreys et al., 1990; Jeffreys and Neumann, 1997). Southern blot hybridisation only allows the detection of length polymorphism. Minisatellite variant repeat typing by PCR (MVR-PCR) was developed to analyse the pattern of variant interspersed repeat units along a minisatellite. MVR-PCR has provided information on the processes taking place within minisatellite creating new mutant alleles (Jeffreys et al., 1991a; Jeffreys et al., 1994; May et al., 1996). The use of SP-PCR followed by MVR-PCR allows the isolation of unlimited *de novo* minisatellite mutants from sperm, which can then be further characterised (Armour et al., 1999; Buard et al., 2000b; May et al., 1996; May et al., 2000; Monckton et al., 1995; Tamaki et al., 1999).

Minisatellite analysis has also been used to detect mutation induction after exposure to mutagenic agents, such as the analysis of human germline mutation rates after exposure to ionising radiation (Dubrova et al., 1996; Dubrova et al., 1997; Dubrova et al., 2002).

Minisatellites have proven to be a powerful tool in the analysis of germline mutation rates in humans. On the other hand 'true' mouse minisatellite have been shown to be less successful due to their low mutation rate ($<10^{-3}$ per gamete) and most mutations are thought to be simple, intra-allelic events (Bois et al., 2002). Since mouse minisatellites show low mutation rates and mutate differently to humans the data are not comparable so analysis of minisatellites in model systems is not considered a useful tool for monitoring germline mutation induction in mice.

1.2.3 Microsatellites

Microsatellites, or simple tandem repeats (STRs) are ubiquitous in both prokaryotic and eukaryotic genomes (Hancock, 1996; Field and Wills, 1996). Microsatellites are composed of repeat units of 1-6bp tandemly repeated to form short arrays (10bp- 1kb), containing mainly identical repeat units (Toth et al., 2000). Microsatellites are highly polymorphic. Large variations in microsatellites have been observed between and within species (Hearne et al., 1992). Spontaneous mutation rates at microsatellite loci range from 10^{-5} to 10^{-3} per locus per generation (Dietrich et al., 1992; Weber and Wong, 1993; Heyer et al., 1997). The microsatellite mutation rate is thought to be correlated to the array size (Wierdl et al., 1997; Pupko and Graur, 1999). Around 90% of the mutations at microsatellite DNA are thought to be due to a gain or loss of a single repeat unit (Brinkman et al., 1998).

Microsatellites can be found in both the coding and the non-coding regions of the genome, although they are predominantly observed in the latter (Toth et al., 2000).
The abundance of microsatellite DNA is species specific, where rodent genomes contain more repeat loci of many different types and yeast and fungi have less than other organisms (Toth et al., 2000). The repeat type varies between species; in humans the most abundant repeat types are A, AC, AAAN, AAN and AG (decreasing order of abundance), and in rats the most abundant repeats are AC, AG, A, AAAN and AAGG (Beckman and Weber, 1992). Toth et al (2000) examined the abundance of different types of microsatellite in different genomes. They found that in primates the most abundant type of microsatellite was mononucleotide repeats, accounting for 42% of repeat types in the genome (poly (A/T) tracts). They also observed that the rodent genome contained the highest proportion of di-nucleotide repeats, accounting for 39%

of all repeat types. Rodents were also shown to have the largest number of microsatellite sequences compared to the other organisms. Differences in maximum length of microsatellite arrays have been observed. Prokaryotes tend to have shorter repeat arrays than those observed in eukaryotes (Katti et al., 2001). This distribution of variation in length of array is also observed within eukaryotes, 43% of rat microsatellites have array sizes of 40bp or greater, whereas only 12% of human microsatellite are that size (Beckman and Weber, 1992).

Although there is a lack of direct experimental data, it is generally accepted that replication slippage plays a major role in mutation at microsatellite loci (Levinson and Gutman, 1987). Evidence that supports replication slippage as the mechanism of mutation at microsatellite loci comes from a study on the effect of sex and age on the spontaneous mutation rate. Brinkman et al (1998) found that female germline mutation rates were lower than in males and that germline mutation rates were increased in older men. These differences can be explained by the number of mitotic divisions occurring before entering meiosis. Oogonia only undergo about 22 divisions before they enter meiosis, however the sperm cells of men have undergone more. Likewise the older a man is the more divisions cells have undergone before enter meiosis. Further evidence that replication slippage may be responsible for microsatellite mutation comes from the similarities observed in rates and patterns between autosomal and Y-linked microsatellites (Heyers et al., 1997). This shows that microsatellite mutations are not due to intra-allelic exchanges or any recombination events as these are absent across most of the Y-chromosome.

Another hypothesis that has been proposed to explain microsatellite mutation involves the ability of repeat units to form secondary structures such as, hairpin structures and possibly triplexes and tetraplexes (Mitas, 1997). It is possible that these secondary structures may promote recombination or interfere with enzymes involved in DNA replication creating instability (Mitas, 1997). A nuclease that is known to be involved in Okazaki fragment processing, flap-endonuclease-1 (FEN-1), has been suggested to play a role in large repeat expansions (Gordenin et al., 1997). FEN-1 has been shown to be responsible for removal of the 5` flap of an Okazaki fragment that can be generated during synthesis of the lagging strand (Lieber, 1997). Deletion of the yeast homologue of FEN-1, RAD27 causes an increase in the rate of tri-nucleotide expansion (Freudenreich et al., 1998). Gordenin et al (1997) suggested that some sequences form

FEN-1 resistant secondary structure and would result in inefficient excision of aberrant flaps on the lagging strand, therefore resulting in the generation of large expansions.
Mutation detection at microsatellite loci is most commonly done using pedigree analysis where mutants show length polymorphisms compared to the progenitor.
The analysis involves the use of simple PCR approaches followed by separation of product by gel electrophoresis. Polyacryamide gel electrophoresis is usually used alongside the use of radioactively or fluorescently labelled primers. This allows multiplex PCRs to be performed on up to 20 alleles at one time (Wang et al., 1996) and a resolution of down to 1bp.
Analysis of microsatellites is achieved using PCR-based techniques of both *in vitro* and *in vivo* samples. Small-pool PCR has been used to assess germline mutation rates in the human genome (Bacon et al., 2001) and to characterise the mutation spectra for repeat expansion diseases (Monckton et al., 1995).

The function of microsatellites is to date unknown. Many studies have shown that a number of human genetic diseases are caused by expansion of microsatellites, mainly tri-nucleotide repeats. To date fourteen tri-nucleotide repeat disorders have been identified (Cummings and Zoghbi, 2000). Tri-nucleotide disorders are usually neurodegenerative and can be divided into two subclasses based on their location within the genome. The first subclass of disorders occurs due to tri-nucleotides that occur in the non-coding sequences and account for six diseases, including Fragile X syndrome (FRAXA). This disease is a consequence of the expansion of beyond 230 repeats of a CGG motif in the 5` untranslated region of the *FMR1* gene. The expansion leads to the loss of the normal gene function due to hypermethylation (Cummings and Zoghbi, 2000).
The second subclass contains those diseases that are caused by exonic (CAG) n repeats that are known to code for polyglutamine tracts. Expansions at the CAG repeat cause diseases such as Huntington's disease (Pearson, 2003), Spinal and Bulbar muscular atrophy (La Spada et al., 1991), Machado-Joseph disease (Kawaguchi et al., 1994) and spinocerebellar ataxia types 1,2,6 and 7 (Orr et al., 1993; Imbert et al., 1996; Zhunchenko et al., 1997; Vuillaume et al., 1998).
The expansion of other classes of microsatellite repeats can also result in human disease. These include a large intronic expansion repeat within the spinocerebellar ataxia type 10 gene (*SCA 10*) (Matsuura et al., 2000) and an expansion that causes

Myotonic Dystrophy Type 2 (DM2) (Liquori et al., 2001). Spinocerebellar ataxia type 10 is caused by an expansion of a penta-nucleotide repeat (ATTCT) in intron 9 of the *SCA10* gene. Myotonic Dystrophy Type 2 is caused by an expansion in a tetra-nucleotide repeat (CCTG) located in intron 1 of the zinc finger protein 9 (ZNF9) gene.

Due to their high mutation rates and the simplicity of detection microsatellites have been utilised as DNA markers for a number of applications. Microsatellites have been used for a number of forensic applications, including the identification of individuals (Hagelberg et al., 1991; Jeffreys et al., 1992), and paternity testing (Papiha and Sertedaki, 1995; Ingvarsson et al., 2000). To make the analysis of microsatellite variation easier multiplex short tandem repeat (STR) systems have been developed for forensic analysis (Urquhart et al., 1994; Budowle et al., 1997). Microsatellites have also been used to map genes involved in diseases (Bergthorsson et al., 1995), study the evolution of populations (Ellegren, 2000; Tautz, 1989) and to study to association of microsatellite instability with human genetic diseases (Stallings, 1994).

Due to the relatively low mutation rate of microsatellites in mouse germline these loci would be of no use for mutation monitoring as large sample sizes would be required at low doses.

1.2.4 Expanded simple tandem repeat loci (ESTRs)

Expanded simple tandem repeat, or ESTR loci were originally identified by DNA fingerprinting and were classified as hypervariable mouse minisatellites (Jeffreys et al., 1987). Further analysis called for the reclassification of these repeats due to a number of fundamental differences between these newly identified repeats and 'true' human minisatellites. These mouse repeats were then renamed ESTRs (Bois and Jeffreys, 1999). The first difference is that ESTRs consist of homogeneous relatively short repeats of 4 to 9 bp (Jeffreys et al., 1987; Kelly et al., 1989; Kelly et al., 1991; Gibbs et al., 1993) which are more similar to microsatellite loci (1-6 bp) than minisatellite loci that show variation and have longer repeat units (10-100bp). Another fundamental difference between the two types of repeat loci is the lengths of array ESTRs tend to be 1 to 22 kb closer to the length of minisatellites (0.5-30kb) than microsatellites. A final difference observed between true human minisatellites and ESTRs is that human

minisatellite mutate solely in the germline, whereas ESTRs mutate in both the germline and somatic cell (Kelly et al., 1989; Gibbs et al., 1993; Yauk et al., 2002).

To date ESTRs have only been found in the mouse genome, but are thought to be present in other genomes. The mouse ESTRs that have been characterised include three single locus repeats, *Ms6-hm* (also known as *Pc-1*), *Hm-2* and *Pc-2*, also a repeat family, *MMS10*. A summary of what is known about each ESTR locus is given in the table below (Table 1.3).
The large family of mouse ESTRs, *MMS10* and the *Pc-2* locus have both expanded within the B1 family of rodent specific short interspersed elements (SINE) (Bois et al., 1998b; Suzuki et al., 1993). The other two loci, *Ms6-hm* and *Hm-2* have expanded from the mammalian retrotransposon-like (MaLR) long terminal repeat (LTR) sequence of the mouse transcript (MT) family (Kelly et al., 1989; Gibbs et al., 1993; Kelly et al., 1994).

ESTRs are detected by simple Southern hybridisation analysis of mouse pedigrees to analyse both somatic and germline mutation rates. This type of analysis has been used successfully to monitor spontaneous and induced mutation rates (Dubrova et al., 1993; Sadamoto et al., 1994; Fan et al., 1995; Niwa et al., 1996; Bois et al., 1998b; Dubrova et al., 1998b). More recently a more sensitive system has been developed that uses a single molecule PCR based approach (Yauk et al., 2002). The use of single molecule PCR has enabled further analysis of the spectrum of spontaneous and radiation mutations at the *Ms6-hm* loci (Yauk et al., 2002). The analysis of the other ESTR locus has not been possible due to the problems with the resolution of small mutations at the larger loci.

To conclude human minisatellite loci and ESTR loci in mice may provide useful mutation induction monitoring systems due to their high spontaneous mutation rates, reproducibility and ease of methods used to detect mutations.

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 1.3: Summary of characteristics of known mouse expanded simple tandem repeat (ESTR) loci.
Data from [ℓ] Suzuki *et al.* (1993), * Bois *et al.* (1998a), [‡] Bois *et al.* (2001) and [†] Unpublished data. ND, not determined.

1.3 Repeat DNA as a tool for monitoring spontaneous and radiation induced germline mutation

Originally, germline mutation induction has been analysed using systems that monitor phenotypic changes. These systems have many drawbacks including the large sample numbers required. These drawbacks highlighted the need for the development of a new more sensitive germline mutation monitoring system. This led to the proposal that repetitive DNA sequences might provide a good system to monitor mutation. To be used as a germline mutation monitoring system repetitive DNA sequences would need to show an increase in mutation rates after exposure to mutagens, such as ionising radiation. Mutation induction at repetitive regions has been studied in various organisms, the results of which are described in this section.

1.3.1 Mouse

Human studies present a number of problems including difficultly obtaining suitable subjects and controls and the difficulty in controlling factors such as ethnicity, age and occupation. To overcome these problems the initial studies performed on germline mutation induction after exposure to ionising radiation at repetitive regions were done in mice. The laboratory mouse is the most commonly used model system. The use of the mouse allows complete control of population, environment and dose through the use of genetically homogeneous populations in identical environmental conditions exposed to controlled doses of radiation.

The first study to show that germline mutation rates at repeat regions increases after exposure to ionising radiation was done by Dubrova et al (1993). This study analysed approximately 200 offspring of male mice that had been acutely exposed to either 0.5 or 1 Gy of γ-radiation. The offspring were analysed using two human multilocus minisatellite probes (33.6 and 33.15) and two mouse specific single locus ESTR probes (Ms6-hm and Hm-2). Using these probes, a two fold increase in mutation rate at these loci after exposure to ionising radiation was found. Many other studies have also shown that after exposure to ionising radiation the mutation rate at ESTR loci increases (Sadamoto et al, 1994; Fan et al, 1995; Dubrova et al, 1998a; Dubrova and Plumb, 2002) and will be presented in section 1.4.

1.3.2 Human

The initial studies analysing the effect of ionising radiation on minisatellites in the human population were performed on the survivors of the atomic bombs at Hiroshima and Nagasaki (Kodaira et al., 1995; Satoh et al., 1996; Kodaira et al., 2004). It was found that there was no significant effect on the germline mutation rate of the minisatellite loci analysed.

In contrast Dubrova et al (1996) performed a study on the germline mutation rates of individuals from the Mogilev district of Belarus exposed to ionising radiation after the Chernobyl accident. Using the human mutlilocus probe 33.15 and four hypervariable single locus probe individuals showed an increase in germline mutation rate of 2 fold after exposure. A second study analysing the germline mutation rates in individuals from Belarus was performed using three independent sets of minisatellites (including five additional minisatellite probes not used in the initial study) to confirm the results

observed in the previous study (Dubrova et al., 1997). The additional data verified a two fold increase in germline mutation rate in exposed families from this contaminated region. These findings were criticised due to the use of controls from the United Kingdom and the lack of control of confounders. The study was expanded to include contaminated areas in Ukraine and the groups were matched with appropriate controls (Dubrova et al., 2002a). To overcome the problem of the control group used in the first study the control group consisted of offspring from the same area conceived before the Chernobyl accident. A 1.6 fold increase in paternal germline mutation rate was observed. All three of these studies provide evidence that the germline mutation rates at minisatellites is elevated in the Chernobyl area since the accident.

Human populations exposed to ionising radiation around the Semipalatinsk nuclear testing site in Kazakhstan, were analysed using eight hypervariable single locus probes (Dubrova et al., 2002b). From this study it was seen that F_0 individuals exposed to slightly higher doses had 1.8 fold increase in germline mutation rate, whereas the F_1 individuals showed a less marked 1.5 fold increase in mutation.

Other studies including the atomic bomb survivor studies have shown no evidence of an increase in germline mutation rate at minisatellite loci after exposure to ionising radiation (Kodaira et al., 1995; Satoh and Kodaira, 1996). These include the studies of the families of the clean up workers after the Chernobyl accident (Livshits et al., 2001; Kiuru et al., 2003) and the sperm analysis of radiotherapy patients (May et al., 2000).

The discrepancies between the atomic bomb studies and the Chernobyl studies could be the result of a number of reasons. The atomic bomb survivors were exposed externally to a single acute high dose of radiation, whereas the families near Chernobyl were exposed chronically to external and internal doses of radiation. The majority of children analysed in the atomic bomb studies were born more than ten years after paternal exposure allowing time for radiation induced DNA alterations to be repaired. The children studied from the Chernobyl area were conceived shortly after the accident in areas by parents that have constantly been irradiated. Furthermore, the atomic bomb survivor data are derived from families in which mainly a single parent was exposed, whereas the Chernobyl data were obtained from families where both parents were chronically irradiated.

Two studies performed on the minisatellite mutation rates in the children of the Chernobyl clean up workers (liquidators) that were exposed to radiation found that there was no significant increase in mutation rate compared to a control population (Livshits

et al., 2001; Kiuru et al., 2003). The differences seen in these studies compared to the studies involving individuals living in the contaminated areas may be due to the heterogeneity of the doses received by the group of clean up workers. In contrast to the individuals living in the contaminated areas, the majority of the participants involved in the decontamination work were exposed externally to relatively uniform doses of γ-irradiation of less than 0.25 Gy (Pitkevitch et al., 1997), with a relatively minor contribution from the intake of radionuclides. The exposure of the clean up workers is less than the calculated doubling dose in mice (0.33 Gy). The Chernobyl clean up workers were exposed to repeated small daily (fractionated) doses of ionising radiation of the period of the clean up. Previous studies in male mice have shown that the yield of germline mutations after an acute external fractionated exposure was less than the same dose given in a single acute exposure (Lyon et al., 1972b). Since the clean up workers were exposed to relatively low doses and the effects of dose fractionation, the expected increase in this group may be too small to detect.

One further group of individuals that have been observed to study the effects of radiation on minisatellites in humans are radiotherapy patients. This study has used a small pool-PCR technique to analyse two hypervariable minisatellite loci (B6.7 and CEB1) in the sperm of three seminoma patients before and after treatment (May et al., 2000). During this study there was no evidence of an increase in mutation induction after radiotherapy. The lack of mutation induction in these patients compared to the individuals exposed after the Chernobyl accident may be due to the duration and type of exposure. The radiotherapy patients were acutely exposed once externally, whereas the individuals in contaminated areas around Chernobyl were exposed to chronic internal and external sources.

Although the human analysis of the effects of ionising radiation on minisatellite loci has produced various results they have shown that the type of exposure and the dose are important factors in the induction of mutation at repeat loci. These studies have also shown that human minisatellites can be used to detect germline mutation induction after exposure to ionising radiation.

1.3.3 Other model systems

Germline mutation rates in barn swallows (*Hirundo rustica*) breeding in the contaminated area close to Chernobyl were analysed using two hypervariable

microsatellite loci (*HrU6* and *HrU9*) (Ellegren et al., 1997). Only the *HrU6* locus showed an increase in germline mutation rate in the exposed population compared to a population from an uncontaminated area in Ukraine. Both loci showed an increase in mutation rate compared to a similar population of barn swallows from Italy; this suggests that microsatellites could be used to monitor germline radiation induced mutations. The microsatellites used for this study are not typical microsatellites due to their large size (tetra-nucleotide repeat) and they may be more similar to mouse ESTRs than other microsatellite loci.

Kovalchuk et al (2000) used thirteen microsatellite loci to estimate the mutation rate of wheat plants grown in heavily contaminated plots near the Chernobyl power plant. These plants showed a significant increase in the number of heterozygous mutations compared to control plants grown in a clean area with similar agrochemical properties.

These studies are consistent with human data by Dubrova et al and show that radiation exposure increases the mutation rate at repeat DNA loci. This suggests that the use of repetitive DNA may provide a powerful tool to monitor radiation induced mutations.

1.4 Analysis of germline mutation induction at ESTR loci in the mouse model

There have been numerous studies analysing the effects of environmental mutagens (mainly ionising radiation) on ESTR mutation rates in the mouse germline. Some of these studies have produced conflicting results, but it has been established through these studies that ESTR loci provide a useful tool for monitoring induced germline mutation in mice. By studying ESTR mutation induction several discoveries have been made which aid our understanding of mutation induction at these loci.

1.4.1 Stage specificity

Once it was established that ionising radiation could induce mutations at ESTR loci (Dubrova et al., 1993), studies were designed to analyse mutation induction at ESTRs in more detail. The stage of spermatogenesis at which ESTRs are most sensitive was analysed by several groups (Sadamoto et al., 1994; Fan et al., 1995; Dubrova et al., 1998a). Spermatogenesis is the process of production of mature sperm cells from the primordial cells. The details of the different stages of spermatogenesis can be seen in

Table 1.4. Several studies have demonstrated that different stages of spermatogenesis show differing sensitivity to the DNA damaging effects and cell killing caused by ionising radiation (reviewed by Searle, 1974). To understand the results from the studies on the possibility of a stage specific response to ionising radiation knowledge of the processes of spermatogenesis and meiosis is required (Table 1.4). Furthermore, to analyse the differing levels of sensitivity of maturing sperm cell the knowledge of the dynamics of mouse spermatogenesis has been important (Table 1.4).

Stage of development	**Days taken to reach ejaculate**	**Sensitivity to irradiation**	**Mating scheme**
Primordial germ cells	Over 42	Intermediate	> 6 weeks
As stem cells	Over 42	Low	
Type A spermatogonia	Over 42	High	
Intermediate spermatogonia	35-37	V. High	5 weeks
Type B spermatogonia	34-36	V. High	
Primary spermatocytes	23-33	Low/Intermediate	
Secondary spermatocytes	21-22	Low/Intermediate	3 weeks
Spermatids	7-21	Low	
Spermatozoa	0-7	V. Low	1 week

Table 1.4: Stages of spermatogenesis (adapted from Searle, 1974)

Traditional methods of mutation monitoring have been used to analyse the effects of ionising radiation on different stages of spermatogenesis in mice (Searle, 1974). Using these methods it has been shown that post-meiotic spermatids are more radiosensitive than other stages of sperm development; this is possibly due to suppression of DNA repair at the post-meiotic stages of spermatogenesis (Alder, 1996; Russell et al., 1958).

The analysis of the stage specificity of mutation induction at mouse ESTR loci has generated conflicting results. The analysis of the *Ms6-hm* ESTR locus found that exposure to ionising radiation affects both pre-meiotic and post-meiotic stages of spermatogenesis, although the post-meiotic spermatids showed the highest sensitivity (Sadamoto et al., 1994; Fan et al., 1995; Niwa et al., 1996). It has been suggested that the high sensitivity in spermatids is due to an indirect mechanism of mutation at the ESTR loci where the damage caused by ionising radiation in the germline causes ESTR mutations after fertilisation (Fan et al., 1995). Data obtained from other studies including additional ESTR loci detected no mutation induction in the post-meiotic stages of spermatogenesis, but showed a significant increase in ESTR mutation rate in the pre-meiotic stages of spermatogenesis (spermatogonia and stem cells) (Dubrova et al., 1998a). Further analysis by Barber et al (2000) showed that there was an increase in radiation induced ESTR mutations after irradiation at all stages of spermatogenesis prior to metaphase I (3 weeks). The discrepancies between the findings could possibly be explained by the scoring of mosaics as mutants. It was reported in one of the initial studies that a high level of mosaicisms was observed (Sadamoto et al., 1994). It is known that a high degree of somatic mosaicism takes place early in embryogenesis for *Ms6-hm* (Kelly et al., 1989; Kelly et al., 1991), therefore mosaic bands in the offspring of irradiated males probably arise through somatic mutational events and do not represent germline mutations. Another discrepancy seen between the data obtained to date is that some studies have reported an elevation in maternal germline mutation rate after paternal exposure (Sadamoto et al., 1994; Fan et al., 1995), whereas an effect on the maternal mutation rate has never been observed in any other studies (Dubrova et al., 1993; Dubrova et al., 1998a; Dubrova et al 2000a; Dubrova et al., 2000b; Dubrova and Plumb, 2002). The difference in observed maternal mutation rates may also be explained by elevated somatic mutation during embryogenesis leading to mosaicism.

A further study to verify which stages of spermatogenesis ESTR loci are most radiosensitive was performed by Yauk *et al* (2002) using a novel single-molecule PCR approach to analyse ESTR mutation rates of sperm DNA samples from both control and irradiated male mice. Data from this study show an elevated mutation rate in the sperm of male mice exposed during the pre-meiotic stages of spermatogenesis. This study provides evidence that ESTR mutation induction occurs in pre-meiotic diploid stem cells and supports the findings of Dubrova et al (1993, 1998a, 2000a, 2000b, 2002). In addition, this study does not support the proposed mechanism of post-meiotic mutation

at ESTR loci that mutations arise through mis-repair of DNA lesion in sperm during fertilisation or embryogenesis as ESTR mutations are detectable in sperm.

The explanation for the elevated sensitivity to ionising radiation observed in post-meiotic cells at protein coding genes detected by traditional methods is through lack of repair (Russell et al., 1958; Alder, 1996). The DNA repair ability of a cell during spermatogenesis reduces with time after the meiotic division. The suppression of DNA repair function during spermatogenesis may also be used to explain ESTR mutation induction at various stages of spermatogenesis. ESTR mutations probably happen in the diploid cell stages of spermatogenesis (spermatogonia and stem cells) that have fully proficient DNA repair and DNA replication. It is possible that ESTR mutations are a result of repaired DNA damage or the DNA repair process, therefore requiring a functional repair system to produce mutations at ESTR loci.

From this data it is accepted that during the pre-meiotic stages of spermatogenesis ESTR loci are most sensitive to ionising radiation. The information gained through these studies has led to various hypotheses in the hope to improve the understanding of mutation induction at ESTR loci (see section 1.5).

1.4.2 ESTR mutation induction in pre-meiotic cells

Once it was established that ionising radiation induced ESTR mutations during the pre-meiotic stages of spermatogenesis further investigations of the effects of dose and type of radiation were performed.

1.4.2.1 Different source of radiation

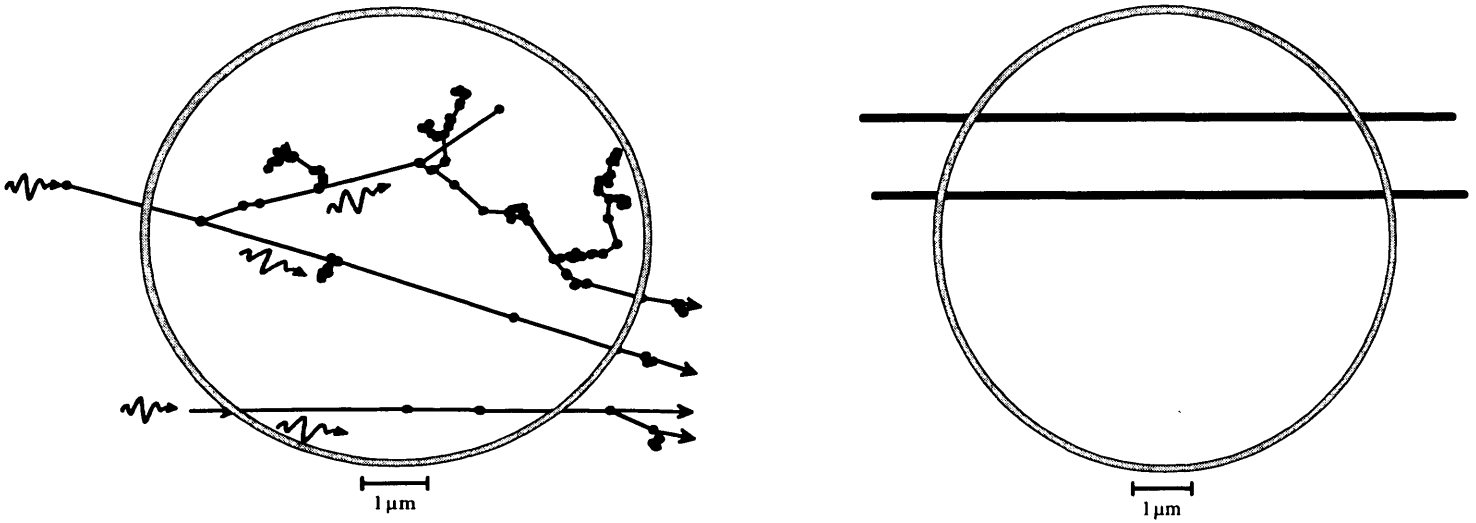
Ionising radiation can be divided into two types; electromagnetic or particulate radiation. Both types of radiation result in ionisation and the release of energetic electrons. The most common types of electromagnetic radiation encountered are X- and γ-rays. Both X- and γ-rays act as a stream of particles that have no charge or mass, known as photons (clusters of energy). When X- and γ-rays are absorbed into a medium (living material) the energy of the photons are deposited in the cells causing excitation and ionisation. The second type of radiation is particulate radiation. Particulate radiation sources include radiation of particles other than photons, including electrons, α-particles, neutrons and heavy ions. Neutron and α-particles are of greatest importance to

radiobiological research. α-particle emission occurs during the decay of naturally occurring radionuclides, such as uranium, thorium and radium contributing to the natural background radiation to which the general public are exposed. Radon gas is a derivative of these radionuclides and can seep through the ground and build-up in houses. From a study carried out in the US it has been estimated that approximately half of the lung cancer cases in the US may be attributed to the inhalation of radon (BEIR VI, 1999). Neutrons are of interest as they are produced as a by product of the fission of heavy radionuclides in nuclear reactors. Neutrons are also an important component of cosmic radiation, contributing to high altitude exposure during air travel. Both types of radiation are absorbed into the cells of living materials causing excitation of cell or production of free radicals.

Ionisation caused by radiation can damage DNA either directly or indirectly through the production of chemically reactive free radicals that may lead to DNA damage. By both direct and indirect processes ionising radiation can cause a broad spectrum of DNA lesions including base damage, cross-linking, and single and double strand breaks (Frankenberg-Schwager, 1990).

Radiation can be further classified by its ability to deposit energy in a medium through which it traverses. This is termed the linear energy transfer (LET) of the radiation. There are two types; high-LET and low-LET. Low-LET radiation (i.e. X-rays and γ-rays) deposits less energy per unit path length than high-LET radiation (i.e. α-particles & neutrons) to the medium through which it is passing. Low-LET radiation is said to be 'sparsely ionising' with the damage being distributed throughout the cell, while high-LET radiation (neutrons and α-particles) is 'densely ionised' and the damage produced being more concentrated (Figure 1.2). Exposure to high-LET radiation leads to mainly direct damage, but indirect effects of radiation are also observed and the energy deposition (damage) is more confined than that observed for the same exposure of low-LET. When deposition occurs after exposure to low-LET radiation it takes the form of single ionisations, excitations or small clusters of small amounts (~2 keV), but when energy is deposited after exposure to high-LET radiation it is much greater with larger energies (~10 keV) (Goodhead, 1988). This means that high-LET radiation has a greater biological effect than low-LET radiation. There are a number of factors that are important in estimating the relative biological effectiveness (RBE) of exposure to various sources of ionising radiation, including the radiation quality (LET), type of exposure (internal or external), dose and dose rate.

Studies have been performed on the effects of the different types of radiation (LET) and different types of exposure (chronic and acute) on germline mutation induction. Several experiments using traditional mutation monitoring systems have been used to monitor the effects of the dose rate of high doses (3-6 Gy) (Lyon et al., 1972a; Russell and Kelly, 1982a; Russell and Kelly, 1982b). The data from these studies of spermatogonia irradiation indicate that acute doses of 3 Gy delivered at or above 0.24 Gy min^{-1} gives rise to a 3 fold higher mutation rate than the same dose delivered at or below 0.008 Gy min^{-1}. When the intermediate dose-rate ranges were analysed it was suggested that there is a sharp transition point from low to high mutation rates. From the data it was concluded that acute low-LET is more efficient in causing germline mutation induction than chronic exposure. It has been proposed that the differences may be explained by acute exposure to high dose irradiation leading to saturation of the DNA repair system capacity, therefore leaving a number of damaged sites being un-repaired before fixation leading to an increase in mutation rate in the offspring (Russell et al., 1958). After chronic exposure the repair systems are less saturated and are able to repair a higher proportion of the induced damage before fixation, therefore individuals have a lower mutation rate. The analysis of post-meiotic germ cells further supports this hypothesis, as in the post-meiotic cell DNA repair efficiency is suppressed and the frequencies of mutation after exposure to acute or chronic low-LET irradiation are similar (Russell et al., 1958). The effects of high-LET irradiation have also been studied using traditional methods and found that high-LET exposure is more efficient at inducing mutations in the germline than low-LET sources (Russell, 1965; Batchelor et al., 1966; Batchelor et al., 1967; Niwa et al., 1996). Extrapolation of high-LET exposure data are not all that relevant to the human population, except the atomic bomb survivors of Hiroshima and Nagasaki, as acute exposure to high-LET radiation is uncommon within the environment. The main concern to the human population is low dose chronic low-LET irradiation as this is the most common type of exposure whether occupational, therapeutic or environmental. Since the human situation is the main concern all the data to date on low dose exposure has been extrapolated from data obtained at high doses. It is important to be able to verify experimentally the extrapolated data for low dose irradiation risks. This has been done using ESTR loci to monitor radiation induced germline mutation rates after exposure to various sources of ionising radiation (Dubrova et al., 1993; Dubrova et al., 1998a; Dubrova et al., 2000a; Dubrova et al., 2000b). The results from these pre-meiotic exposure studies are presented below.



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 1.2: Energy deposition for high- and low-LET irradiation (Adapted from Goodhead, 1988)

1.4.2.2 Acute exposure to low-LET radiation

Pre-meiotic acute exposure to low-LET X-rays delivered at 0.5 Gy min^{-1} to doses of 0.5-1 Gy caused a significant increase in paternal ESTR mutation rates (Dubrova et al., 1998a). The data from this study indicated that there is a linear dose response for the dose range used (see Figure 1.3), as previously observed in earlier studies (Dubrova et al., 1993; Dubrova et al., 1998a). From the regression slope and the paternal mutation rate of the control group the doubling dose was estimated to be 0.33 Gy. This estimate is very similar to the estimate produced from the specific locus test (0.35 Gy). All previous studies exposed males to doses of 3-6 Gy of acute low-LET radiation, whereas the studies by Dubrova *et al* (1993, 1998a) are the first to present data on low dose acute exposure to low-LET radiation. The use of ESTR loci as a germline mutation monitoring system allows a more robust estimation of the doubling dose without extrapolating from data obtained from high doses of radiation.

1.4.2.3 Chronic exposure to low-LET radiation

Pre-meiotic chronic exposure to low-LET radiation was studied by exposing male mice to 0.5-1 Gy γ-rays delivered at a dose-rate of 1.66×10^{-4} Gy min^{-1} (Dubrova et al., 2000a). It was found that pre-meiotic chronic exposure caused a significant increase in paternal mutation rate and that there was a linear dose response (Figure 1.3). When the results of chronic exposure to γ-rays were compared to the results from the acute X-ray exposure experiment it was found that there was no significant difference in the efficiency of the two types of irradiation on the mutation induction at the spermatogonial stages of spermatogenesis. In previous studies using high doses of radiation it was predicted from extrapolation of the data that acute exposure to low-LET caused a higher mutation rate than chronic exposure. This suggests that the extrapolation of data from the traditional methods may not hold true when estimating the genetic risks of lower doses. The extrapolated data suggests that chronic exposure produces only a third of the mutations as the same total dose that acute radiation would cause, whereas data obtained from the analysis of lower doses suggests that there is no difference in the level of mutation induction after exposure to chronic or acute low-LET radiation. This may be due to the fact that after low dose acute exposure the DNA repair systems may not be saturated. This could mean that the

response after acute exposure could approach that of the chronic exposure response as the dose decreases.

1.4.2.4 Exposure to high-LET radiation

Chronic paternal exposure to high-LET fission neutrons gave rise to a very high increase in ESTR mutation rate (Dubrova et al., 2000a). After chronic exposure to a range of doses (0.125-0.5 Gy) delivered at 0.003 Gy min^{-1} a linear dose response was observed. It was found that exposure to high-LET fission neutrons was more efficient at inducing germline mutations than acute or chronic low-LET exposure (Figure 1.3). Previous studies have also shown that high-LET radiation is more efficient than low-LET radiation in the induction of germline mutations (Russell, 1965; Batchelor et al., 1966; Batchelor et al., 1967).

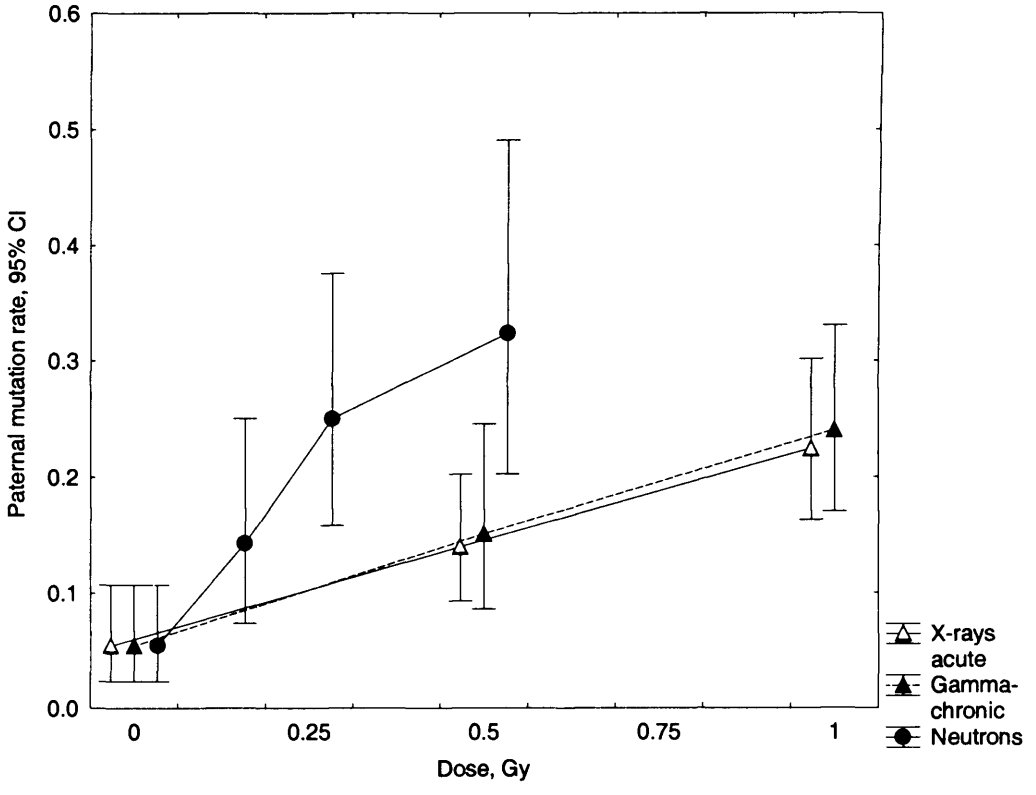


Figure 1.3: Dose response curve for paternal ESTR mutation induction in mouse spermatogonia after exposure to different sources of ionising radiation. (Adapted from Dubrova and Plumb, 2002)

1.4.3 Exposure to chemicals

Chemical mutagens could potentially present a significant genetic hazard to the human population. Chemical mutagens affect the general population through exposure to chemicals from air pollutants and in diets, as well as industrial workers and cancer patients (treatments). Since chemical mutagens affect the human population the genetic risk of exposure to certain chemicals has been analysed using ESTR loci. There have been very few studies carried out to establish whether exposure to individual chemicals can induce ESTR germline mutations in mice. The first study to examine the effect on ESTR germline mutation induction of the exposure to a chemical mixture of polychlorinated biphenyls and diesel exhaust (Hedenskog et al., 1997). This study showed that there was an increase in ESTR mutation rate detected after exposure to the chemical mix. Barber et al (2000) studied the effect of the anticancer drug cisplatin on ESTR mutation rates and found that there was no evidence that cisplatin induced ESTR mutations at any stage of spermatogenesis. A study of laboratory mice was designed to allow exposure to air pollutants released by a steel mill (Somers et al., 2002). It was found that pre-meiotic exposure to air pollutants from the steel mill caused a significant increase in ESTR mutation rates. The analysis of three chemical mutagens with different DNA reactivity was designed to determine stage specificity and dose response of germline ESTR mutation induction in male mice exposed to chemical mutagens (Vilarino-Guell et al., 2003). Mutation rates at ESTR loci were monitored after males were exposed to two monofunctional alkylating agents, ethylnitrosourea (ENU) and isopropyl methanesulfate (iPMS) and a topoisomerase II inhibitor, etoposide. Both ENU and iPMS showed pre-meiotic mutation induction and a linear dose response reaching a plateau at higher concentrations. Etoposide however did not show mutation induction at pre-meiotic stages but did show a significant increase in ESTR mutation induction after exposure to the drug during meiotic stages of spermatogenesis (weeks 4 and 5). The analysis of the effects of chemical mutagens on germline ESTR mutation rates in male mice has shown that different chemicals induce different levels of ESTR mutation induction. The studies also showed that ESTR loci provide a sensitive system for monitoring chemical-induced germline mutations and that each chemical mutagen needs to be considered separately.

1.4.4 Transgenerational effect

Barber et al (2002) showed for the first time that ESTR radiation induced instability can persist for at least two generations. The study involved exposing male mice to high-LET and low-LET radiation and observing the offspring and the grand-offspring of the directly exposed males. They found that after exposure to both sources of ionising radiation that ESTR mutation rates in the germline of first- and second-generation non-exposed offspring were elevated (Dubrova et al., 2000b; Barber et al., 2002). From the data it was concluded that there is an existence of transgenerational radiation induced genomic instability in the germline of offspring of irradiated parents. The persistence of the elevated germline mutation rates in two consecutive generations rules out the possibility that this effect may be attributed to radiation induced mutations at any specific genes in the exposed males (Barber et al., 2002). The authors suggest that the transgenerational genomic instability at ESTR loci may be due to epigenetic mechanisms.

1.4.5 Limitations of ESTR system

ESTRs have proven to be a highly sensitive system for monitoring germline mutation induction by ionising radiation and chemical mutagens. The use of ESTRs as a mutation monitoring systems allows the analysis of lower doses and smaller numbers of offspring than previously used techniques. The use of the ESTR system has allowed the analysis of low doses of exposure and eliminated the need to extrapolate data obtained from higher doses, allowing more accurate estimates of genetic risks of exposure.

Although the ESTR system has proven to be a sensitive and robust system for monitoring mutation induction, there is a down-side to the system. To date the mechanism of spontaneous and induced mutation at ESTR loci remains unknown. The lack of information on the mechanism of mutation limits the use of ESTRs for the analysis of mutation induction and prevents the extrapolation of the ESTR mutation induction data to a level of DNA damage to the genome as a whole. Better understanding of mutational processes at ESTR loci would provide better comprehension of the action of mutagenic agents on the genome and possibly enable the prediction of which mutagens may lead to mutation induction at ESTR loci.

1.5 Mechanisms of mutation induction at tandem repeat DNA loci

Mutation mechanisms at minisatellite loci have been analysed by utilising the complexity of their internal structure, containing variant repeats within a single array. The simplicity of ESTR internal structure containing single repeat units means that such methods can not be used and would be uninformative. The little that is known about the mechanism of mutation at ESTR loci has been gained through the analysis of mutation rates at these loci and the spectrum of mutation.

1.5.1 Non-targeted effects

Previous studies have shown that the observed radiation induced increase in ESTR mutation rates in the germline of irradiated male mice cannot be attributed to the direct effects of radiation induced damage (Sadamoto et al., 1994; Fan et al., 1995; Dubrova et al., 1998a; Barber et al., 2002). It has been suggested that ESTR mutation induction may be attributed to some as yet unknown non-targeted event elsewhere in the genome, which could lead to an increase in genomic instability and indirectly causing mutations at ESTR loci.

It has been calculated that the levels of ESTR mutation induction observed after exposure to ionising radiation are too high to be accounted for by random direct targeted events at these small loci (Dubrova et al., 1998). This is verified by considering the amount of damage that is induced per haploid cell per 1 Gy irradiation. The estimated levels of DNA damage induced by 1 Gy of ionising radiation is approximately 70 double strand breaks, 1000 single strand breaks and 2000 damaged bases (Frankenberg-Schwager, 1990). Assuming that ESTR loci are random targets for radiation induced damage, according to the data after 1 Gy pre-meiotic irradiation 45,000 extra points of damage per haploid genome would be required to produce a 4-fold increase in paternal mutation rate at the *Ms6-hm* and *Hm-2* loci, which together represent a 1.6×10^4 bp target in the mouse genome (3×10^9 bp) (Dubrova et al., 1998). This unrealistically high number of extra damage points per genome required to produce the mutation rates observed provides the main source of evidence for the mechanism of radiation induced mutation at ESTR loci being initiated by a non-targeted event.

Further evidence for a non-targeted mechanism of mutation induction at ESTR loci comes from the increase in paternal mutation rates observed after exposure to fission neutrons (Dubrova et al., 2000a). It is estimated that the expected number of traversals per cell for a dose of 0.5 Gy would be less than 6. Considering the low number of traversals per cell and the small size of the target (1.6×10^4 bp), it is improbable that a six-fold increase in paternal mutation rate can be attributed to a direct effect at ESTR loci.

All the evidence to date points toward the possibility that the radiation induced increase in ESTR mutation rates results from radiation induced damage elsewhere in the genome/cell or the cells response to the induced damage.

1.5.2 Spectra

Until recently the spectrum of mouse germline ESTR mutations was poorly characterised. The observation of the spectra of ESTR mutation in the control and irradiated males are important for the understanding of the mechanisms of spontaneous and radiation induced ESTR mutation.

Initial studies of the mutation spectra of *Ms6-hm* showed that the size changes were similar in the germline and somatic tissues and the size changes were small, less than 200 repeats (Kelly et al., 1989). A study by Yauk et al (2002) also found that the majority of mutations at *Ms6-hm* resulted from small changes (3-5 repeats). Analysis showed that the mutation spectra for *Ms6-hm* were similar for spontaneous and radiation induced mutations in both somatic tissue and the male germline. This study also highlighted the fact there appeared to be a significant bias towards gains of repeats, 69% of somatic mutants and 59% of germline mutants showing gains of repeats. Further analysis of the spectra of radiation induced ESTR mutations in five different mouse strains has been carried out and reviewed by Dubrova (2005). Dubrova found that the majority of the mutation at the ESTR loci involved small gains or losses of repeats. When the spectra of ESTR mutation for spontaneous and radiation induced mutations were analysed it was observed that the spontaneous and radiation induced spectra of ESTR mutation were indistinguishable, in all five strains. It was also shown that the distribution of ESTR mutations did not differ between strains or ESTR loci. Through the analysis of the distribution of ESTR mutations it was found that there was a bias towards gains of repeats (60%) as previously reported.

This bias was observed in all strains and at each locus (*Ms6-hm* and *Hm-2*). A more detailed analysis of the distribution of size changes observed that larger alleles involved the gain and loss of more repeats than smaller alleles. It was also shown that the incidence of gains and losses differed between small and large alleles, in that the bias towards gains was only detected in the smaller alleles. However, there is a lack of data on very small changes (1-2 repeats), which are likely to be very frequent, meaning that it is possible that the mutation rates may be greatly underestimated and the bias towards array expansion may not be accurate (Yauk et al., 2002).
The fact that the mutation spectra are similar in control and irradiated male germline supports the evidence that the mechanism of mutation induction at ESTR loci is a non-targeted process. If mutation induction was due to a direct targeted mechanism of mutation it might be expected that differences in control and induced mutation spectra would be seen, as observed for the direct targeting of protein coding genes (Nelson et al., 1994). This indicates that both spontaneous and radiation induced ESTR mutations can be attributed to the same mechanism of mutation and that radiation does not cause mutation induction at these loci directly.

As the mechanism of ESTR mutation is still unknown, it may be useful to consider the mutation processes at other repeat loci.

1.5.3 Mutation induction at human minisatellites

Mutation processes at repeat loci have been most well characterised for minisatellite loci, with a clear difference between somatic and germline mutational processes. Mutation processes at human minisatellite loci have been analysed using two experimental approaches; SP-PCR and SM-PCR.
MVR-PCR analysis to observe repeat unit variation within an array has shown that the mutation process in somatic tissue is completely different to the processes involved in germline mutation (Jeffreys and Neumann, 1997). The study of somatic minisatellite mutation showed that the type of mutations that were most commonly recovered were simple, perfect intra-allelic duplications or deletions of blocks of repeats and the mutation events occur randomly along the allele. Further analysis of somatic minisatellite mutations revealed a slight bias towards losses of repeat units. The evidence suggests that the somatic mutational process is a simple mode of

mutation induction, possibly via a recombination-based process (Jeffreys and Neumann, 1997).

It has been proposed that somatic minisatellite mutations could arise due to replication slippage or by mitotic recombination. Evidence has shown that it is more likely that the mutational process involves a recombination-based process rather than replication slippage. Firstly, the analysis of the mutation rates in mismatch repair deficient cells show no increase in minisatellite mutation rate, although there is an increase in microsatellite mutation rate in these cells and microsatellites are thought to mutate via replication slippage (Aaltonen et al., 1993; Parsons et al., 1993; Thibodeau et al., 1993). Secondly, somatic minisatellite mutations are larger than mutations observed in microsatellites therefore excluding the mutation process involving Okazaki fragments and their slippage. Lastly, minisatellite mutations are usually found in heterogeneous arrays suggesting mitotic recombination or unequal sister chromatid exchange rather than replication slippage which is thought to occur in homogeneous arrays (Jeffreys and Neumann, 1997; Jeffreys et al., 1999).

The mechanism of somatic minisatellite mutation is most likely to be due to unequal sister chromatid exchange and intra-allelic (mitotic) recombination, although replication slippage cannot be entirely excluded.

The situation in the germline is more complex due to the different mutation patterns observed at different loci (Jeffreys et al., 1999). Through extensive MVR analysis different features of minisatellite mutations in the human germline have been identified, although all mutations involve gene conversion-like repeat transfers between alleles. The types of rearrangements observed in sperm mutants were more complex than those observed in somatic tissues. The observed rearrangements included target site duplications or deletions in the recipient allele, scrambling of donor repeat segments and multiple, imperfect reduplications of donor and/or recipient alleles at the mutation junction (Jeffreys et al., 1994). It was found that these rearrangements predominantly occur at one end of the array, indicating mutation polarity (Jeffreys et al., 1994; Jeffreys et al., 1998b). The analysis of the mutation rates and spectra showed that there is a high degree of variation of mutation rates between loci and the spectrum is not dependent on locus or allele size (Jeffreys et al., 1997). It was seen that there was a bias towards the gain or loss of a small number of repeat units with a slight bias towards gains and only very large alleles being prone to large deletions (Jeffreys et al., 1997). When the mutation rates in sperm were

compared to the mutation rates in the somatic tissues it was seen that sperm mutation rates were 2-3 fold higher (Jeffreys and Neumann, 1997). This indicates that minisatellite instability is restricted to the germline.

Germline mutation at minisatellite loci are complex and involve intra-allelic and inter-allelic events. Both intra- and inter-allelic events have been observed in all five minisatellites analysed to date (MS31, MS32, MS205, CEB1 and B6.7), although mutation type varies between loci and therefore mutation processes may vary between 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 mutation process at minisatellite loci involves the copying and transfer of blocks of repeats from one allele to another. Evidence has shown that this process is not conservative, in that the donor allele is not altered by the mutation process indicating that the transfer block must be copied (Jeffreys et al., 1994). Since the minisatellite mutational process involves either gene conversion or crossing over recombination events and that complex rearrangements are restricted to the germline it is possible to suggest that the process of mutation may occur during meiotic recombination (Jeffreys et al., 1997). Further evidence indicating the mutation process is meiosis specific is that there is no evidence of germline mosaicisms and most conversion sperm mutants in a single male are different (Jeffreys et al., 1999).

There have been various models of recombinational instability developed, but not one single model has been developed to account for the wide variety of rearrangements observed at human minisatellite loci (Jeffreys et al., 1999). The current model (see Figure 1.4) suggests that mutations may be induced by the introduction of a double strand break (DSB) or by staggered nicks in the repeat array. Single-stranded DNA from the resulting gap invades the other minisatellite allele (inter-allelic exchange) or a sister chromatid (intra-allelic exchange). This is followed by DNA synthesis, DNA strand extrusion and then gap bridging in the recipient allele. This process results in a mutation of the recipient allele without alterations to the donor allele. This model would account for the large duplications and deletions, and multiple rounds could lead to complex patchwork insertions seen at some loci, but it is not able to explain more complex event, such as the imperfect reduplications seen in some loci.

It has also been revealed that there may be another process affecting the instability of minisatellites that show mutation polarity. Characterisation of a recombinational

hotspot in the 3′ flanking region of minisatellite MS32 that extends into the beginning of the repeat array (Jeffreys et al., 1998a; Jeffreys et al., 1998b). Recombinational hotspots may drive high levels of minisatellite instability and explain the mutation polarity observed at some minisatellite loci (Jeffreys et al., 1998a; Jeffreys et al., 1999).

Recombination processes are known to be important in the induction of mutations at minisatellite loci, although the mechanisms are still not clearly characterised. The current model (Figure 1.4) of meiotic recombination could explain the majority of the mutation types observed at minisatellite loci.

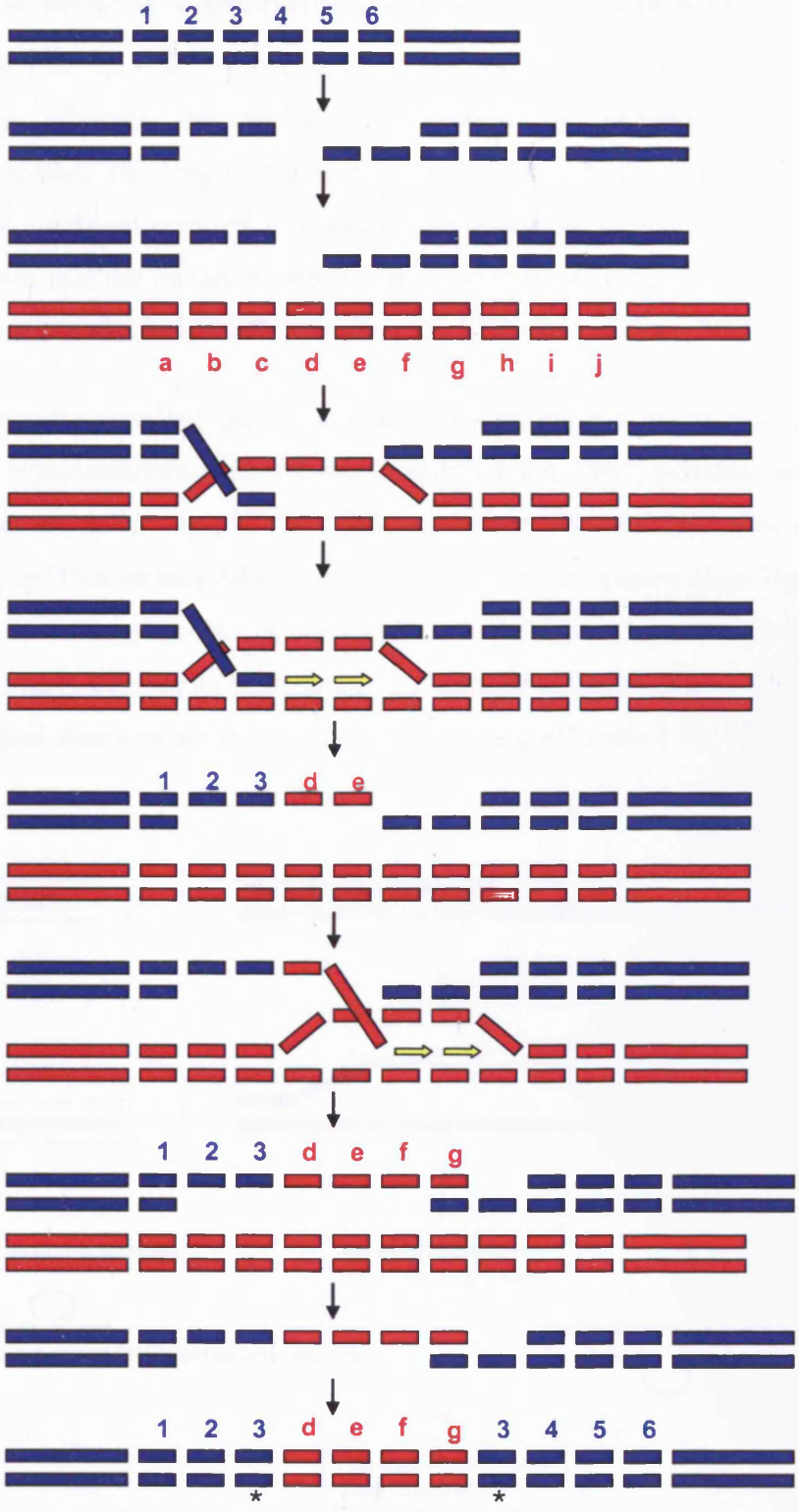


Figure 1.4: Current model of meiotic recombination.
Blue lines represent recipient allele containing a double strand break, red lines represent donor allele, yellow arrows represent DNA synthesis and the asterisk represents duplicated segments in the mutant allele. Adapted from Jeffreys *et al* (1999).

1.5.4 Mutation mechanisms at microsatellite loci

Microsatellite mutation processes are not as well characterised as minisatellite processes. Although the mechanism of mutation is unknown several models have been proposed. The length of uninterrupted repeat units has been shown to be one of the most important features in mutation rate regulation at microsatellite loci. Analysis has shown that the mutation rates decrease with the number of repeats with an array (Levinson and Gutman, 1987; Wierdl et al., 1997; Bacon et al., 2000).

It is generally accepted that replication slippage plays a major role in the mutation process at microsatellite loci (Levinson and Gutman, 1987; Schlotter and Tautz, 1992; Strand et al., 1993). Replication slippage occurs due to a transient dissociation of replicating DNA strands followed by misaligned reassociation (See Figure 1.5). When the two strands realign out of register a loop structure may be formed on either, the nascent strand causing an increase in array length or the template strand leading to the synthesised strand being shorter than the progenitor (Figure 1.5).

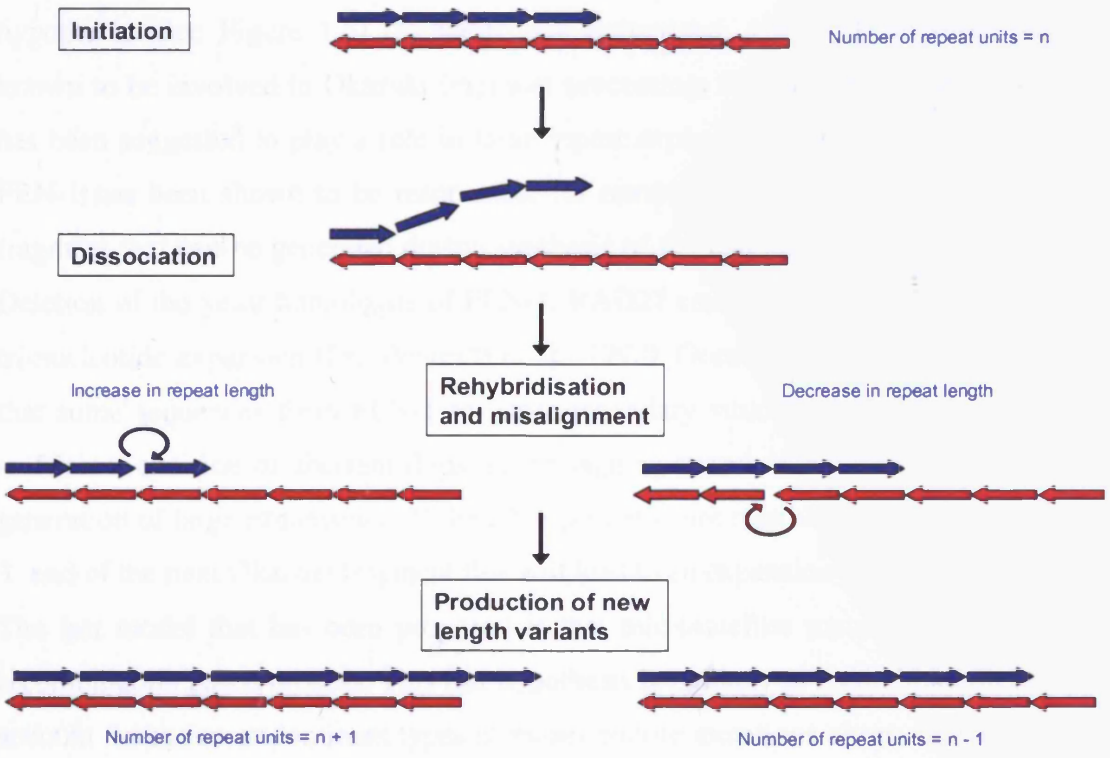


Figure 1.5: Schematic of replication slippage (Adapted from Ellegren, 2004)

Evidence that supports replication slippage as the mechanism of mutation at microsatellite loci comes from a study on the effect of sex and age on the spontaneous mutation rate. Brinkman et al (1998) found that female germline mutation rates were lower than in males and that germline mutation rates were higher in older men. These differences can be explained by the number of mitotic division before entering meiosis. Further evidence that replication slippage may be responsible for microsatellite mutation comes from the similarities observed in rates and patterns between autosomal and Y-linked microsatellites (Heyers et al., 1997). This shows that microsatellite mutations are not due to intra-allelic exchanges or any recombination events which are absent across most of the Y-chromosome.

Another hypothesis that has been proposed to explain microsatellite mutation involves the ability of repeat units to form secondary structures such as, hairpin structures and possibly triplexes and tetraplexes (Mitas, 1997). It is possible that these secondary structures may promote recombination or interfere with enzymes involved in DNA replication causing polymerase stalling followed by polymerase slippage creating instability (Mitas, 1997, McMurray, 1999).

A third hypothesis to explain large repeat expansions is the Okazaki fragment hypothesis (See Figure 1.6) (Richards and Sutherland, 1994). A nuclease that is known to be involved in Okazaki fragment processing, flap-endonuclease-1 (FEN-1) has been suggested to play a role in large repeat expansions (Gordenin et al., 1997). FEN-1 has been shown to be responsible for removal of the 5` flap of an Okazaki fragment that can be generated during synthesis of the lagging strand (Lieber, 1997). Deletion of the yeast homologue of FEN-1, RAD27 causes an increase in the rate of tri-nucleotide expansion (Freudenreich et al., 1998). Gordenin et al (1997) suggested that some sequences form FEN-1 resistant secondary structure and would result in inefficient excision of aberrant flaps on the lagging strand, therefore resulting in the generation of large expansions. (If the RNA primer is not removed but is ligated to the 3` end of the next Okazaki fragment this will lead to an expansion in the allele).

The last model that has been proposed is that microsatellite mutations occur via a recombination based process. This last hypothesis is unlikely as recombination cannot account for the most dominant types of microsatellite mutations observed and studies of yeast have shown that mutations in genes involved in recombination have no effect on microsatellite instability (Henderson and Petes, 1992).

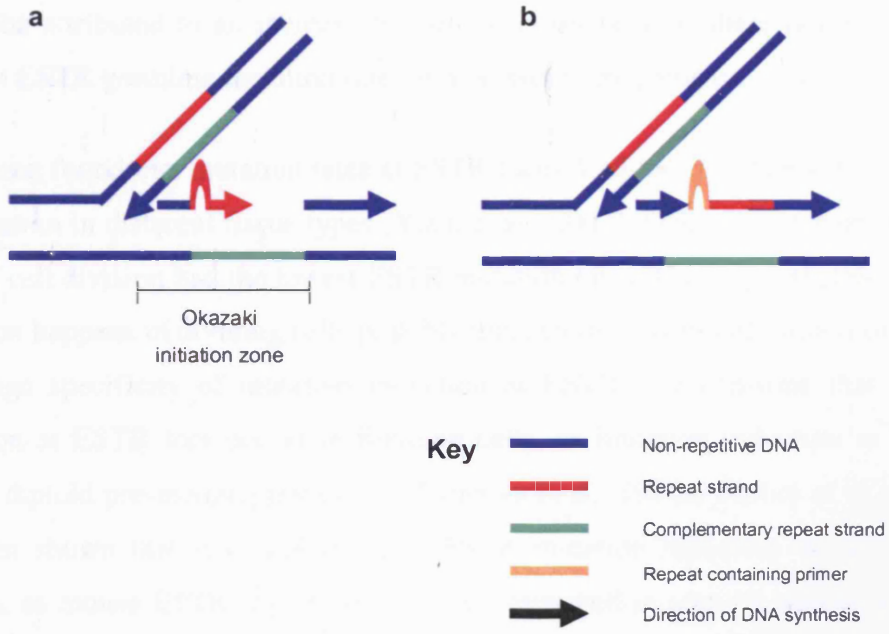


Figure 1.6: Schematic of Okazaki fragment hypothesis (Adapted from Mirkin and Smirnova, 2002)
a) Hairpin formation.The formation of a hairpin in the lagging strand, followed by misalignment of the lagging strand and the template causes expansion.
b) RNA displacement. Expansion due to the RNA primer is not removed but is ligated to the 3′ end of the next Okazaki fragment.

1.5.5 Proposed mutation mechanism

It has been generally accepted that the most potentially mutagenic type of DNA damage induced by irradiation is DNA double strand breaks (Frankenberg-Schwager, 1990). Radiation induced DNA double strand breaks (DSBs) could lead to an increase in crossovers at the crossover proficient stages of meiosis. To test the hypothesis that ESTR loci mutate in a similar fashion to minisatellite loci a study was designed to determine whether mouse germline ESTR mutation induction could be attributed to a genome wide increase of meiotic recombination events (Barber et al., 2000). The study compared the crossover frequencies with the germline ESTR mutation rates of male mice acutely exposed to 1 Gy of X-rays or 10 mg/kg of cisplatin, an anticancer drug known to increase meiotic crossover rates in mice (Hanneman et al., 1993). The analysis of crossover frequencies showed that there was no increase in crossovers

after exposure to X-rays or cisplatin, but there was an increase in ESTR mutation rate after exposure to X-rays only. This indicated that mutation induction at ESTR loci cannot be attributed to an increase in meiotic crossovers, as there is no correlation between ESTR germline mutation rates and crossover frequencies.

It has been found that mutation rates at ESTR locus *Ms6-hm* correlate with the rate of proliferation in different tissue types (Yauk et al., 2002). DNA from tissues with low rates of cell division had the lowest ESTR mutation rates. This suggests that mutation induction happens in dividing cells possibly through the process of mitosis or meiosis. The stage specificity of mutation induction at ESTR loci confirms that mutation induction at ESTR loci occurs in dividing cells, as mutation induction is observed only in diploid pre-meiotic germ cells (Dubrova et al., 1998a; Barber et al., 2000). It has been shown that it is unlikely that ESTR mutation induction occurs solely at meiosis, as mouse ESTR instability has been observed in somatic tissues as well as germ cells (Kelly et al., 1989; Gibbs et al., 1993; Yauk et al., 2002). From the data it can be said that ESTR mutation induction occurs in diploid cells.

It has been proposed that mutation induction is possibly due to the accumulation of structural damage to DNA at the pre-meiotic stages of spermatogenesis leading to the activation of certain processes during meiosis that may enhance ESTR mutation induction. It has been seen that double strand breaks can stimulate not only recombination but they can also initiate repair and apoptosis. It is possible that one of these processes is involved in the non-targeted events that influence ESTR instability. It has been hypothesised that ESTR mutation induction could be a by-product of the activation of DNA repair in the response to DNA damage. The study of DNA repair deficient mice has shown that DNA repair is involved in microsatellite instability (Umar et al., 1994; Baker et al., 1995; Baker et al., 1996), and it is also possible that ESTR instability could be affected in the same way. Further evidence that suggests ESTRs may be affected in a similar manner to microsatellites is highlighted by the similarities observed between the two. They both consist of small repeat units, 1-6 bp and 4-6 bp for microsatellite and ESTRs, respectively. Therefore ESTRs may be regarded as highly expanded microsatellites. Both loci show similar spectra of mutation with a slight bias towards gains of repeat units (Ellegren, 2004; Dubrova, 2005). Another similarity is that the spontaneous mutation rates at both ESTRs and microsatellite loci increases with allele size (Bois et al., 2001; Dubrova, 2005). Two

microsatellite repeat expansions identified at *DM2* and *SCA10* show high similarities to mouse ESTR loci, *Ms6-hm* and *Hm-2* (Kelly et al., 1989; Gibbs et al., 1993; Matsuura et al., 2000; Liqouri et al., 2001). They have similar repeat unit size, *SCA10* and *Ms6-hm* have pentanucleotide repeats and *DM2* and *Hm-2* have tetranucleotide repeat units. Additionally the expansion sizes are comparible, *Ms6-hm* has repeat arrays up to 15 kb, *Hm-2* up to 22kb, *SCA10* up to 22 kb and *DM2* up to 44 kb. These similarities suggest that ESTRs may be affected by DNA repair deficiency in the same way as microsatellites, also that it is possible that the mutation mechanisms at human expanded microsatellite repeat loci and mouse ESTR loci may be similar.

The hypothesis that ESTR instability may be a by-product of the activation of DNA repair could be used to explain the lack of mutation induction observed in post-meiotic stages of spermatogenesis, through the lack of active DNA repair at these stages. This raises the proposal that an intact DNA repair system is required to induce ESTR mutations and therefore can only occur in DNA repair proficient diploid cells. The hypothesis that ESTR mutations are induced via the activation of DNA repair could be tested by using mice that are deficient in DNA repair and testing them for ESTR instability.

An additional hypothesis that has been proposed is that ESTR stability may be influenced by the high cell killing effect of radiation or induction of DSB, and this in turn could be tested by analysing ESTR mutation frequencies in mice deficient in the apoptosis gene, p53.

Both these hypotheses have been tested during this project and the DNA repair or apoptosis genes that have been analysed are described briefly in the following section.

1.6 The use of transgenic mouse models to analyse the mechanism of germline mutation at expanded simple tandem repeat loci

Mice carrying mutations in DNA repair genes have been shown to have increased sensitivity to mutagens and carcinogens (Freidberg and Meira, 2003). The majority of work with DNA repair deficient mice has concentrated on the development of cancers and somatic mutations, although to date not much is known about the effects of DNA repair deficiencies on spontaneous and induced germline mutations.

To test the hypothesis that ESTR instability could be caused by a non-targeted event such as the activation of DNA repair or the high killing effect of radiation through apoptosis, mice deficient in various DNA repair systems or apoptosis have been studied in the work for this thesis and by other investigators. The genes that have been investigated are described below.

1.6.1 DNA-PKcs (*scid*)

The only DNA repair deficient mouse strain that had been analysed for spontaneous and radiation induced ESTR germline instability previous to this study was the severe combined immunodeficient (*scid*) mouse. *scid* is a recessive, autosomal mutation and *scid* mice are severely deficient in functional lymphocytes because of the failure of V (D) J recombination (production of antibodies) (Bosma et al., 1983). The scid mutation has been mapped to the centromeric region of chromosome 16 (Bosma et al., 1989). The *scid* mouse has now been shown to carry a nonsense mutation in the C-terminal region of the catalytic subunit of DNA-protein kinase, DNA-PKcs, also known as XRCC7 (Biedermann et al., 1991; Blunt et al., 1996). The T to A transversion causes the introduction of an ochre stop codon and a loss of 83 amino acids from the C-terminal end of the protein. Some *scid* mice show a characteristic 'leaky' phenotype that could be explained by the possibility that the truncated DNA-PKcs protein may have weak but functional activity, allowing the development of some T-cells (Blunt et al., 1996).

DNA dependent protein kinase, DNA-PK is a multiprotein complex, containing the DNA-end binding heterodimeric Ku protein (Ku70 and Ku80) and a large catalytic subunit (DNA-PKcs). The DNA dependent protein kinase catalytic subunit (DNA-PKcs) is a 465 kDa protein, which is thought to be part of the phosphatidylinositol 3-kinase (PI 3-Kinase) superfamily due to the sequence similarity at the carboxyl terminus to the 110-kDa subunit of PI 3-Kinase (Hartley et al., 1995). Other PI kinases are known to be involved in cell cycle control, DNA repair and damage response, including the product of ataxia telangiectasia (ATM). It has been shown that DNA-PKcs is involved in both V(D)J recombination and the repair of DNA double-strand breaks via non-homologous end joining (NHEJ) (Blunt et al., 1995; Miller et al., 1995; Peterson et al., 1995). The *scid* mutation in DNA-PKcs has also been shown

to be responsible for an increase in radiosensitivity due to a decrease in DNA repair via non-homologous end joining (Biedermann et al., 1991).

Double strand breaks can be induced by mutagens such as ionising radiation or they can be spontaneous occurring during replication or during V(D)J recombination. Double strand breaks can be repaired via two major pathways, homologous recombination (HR) or non-homologous end joining (NHEJ). The repair of double strand breaks is vital to the maintenance of genomic stability. Non-homologous end joining involves end joining reactions between broken ends. This reaction joins end with little or no homology, leading to NHEJ being regarded as an inaccurate method of double strand break repair. The process of NHEJ can be seen in Figure 1.7.

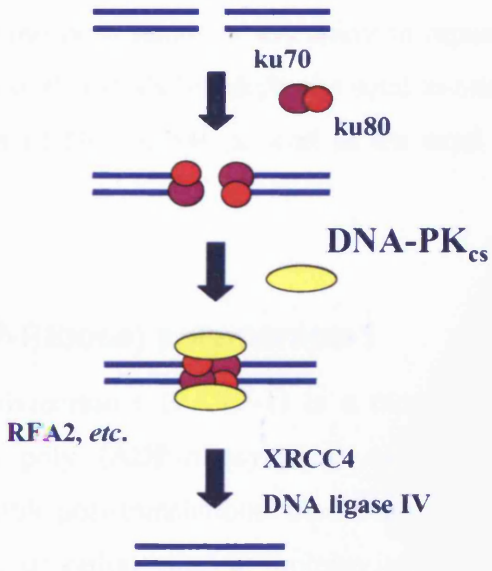


Figure 1.7: Repair of DNA double strand breaks via non-homologous end joining

It is known that *scid* mice are deficient in the recognition and repair of double strand breaks via the non-homologous end joining pathway (Biedermann et al., 1991; Blunt et al., 1996). Analysis has been carried out to determine whether ESTR instability is affected by a deficiency in NHEJ. It has been seen previously that the *scid* mutation causes an increase in spontaneous mutation rate in both cell culture (Imai et al., 1997)

and in the mouse germline (Yamauchi et al., 2002). More recently, in our laboratories, a study was designed to analyse whether the *scid* mutation has any effect on the spontaneous or radiation induced germline mutation rates at ESTR loci (Barber et al., 2004). Barber et al. found that there was a 2-fold increase after pre-meiotic exposure in the control population, likewise they observed a two-fold increase in the spontaneous mutation rate in the *scid* mice when compared to the isogenic control. Surprisingly the mutation rate after pre-meiotic exposure of the scid mice showed no detectable increase compared to the non-exposed group. The lack of increase in germline mutation rates after pre-meiotic exposure to ionising radiation may be explained by the high killing effect of radiation in *scid* mice via DNA-PK independent activation of p53 and p21 (Jimenez, et al., 1999). These results indicate that NHEJ is involved in the maintenance of ESTR stability in the mouse germline, possibly through the inability to repair or the delay in repair of DNA DSB via this pathway. The results from this study highlight the need to analyse the effects of other DNA repair systems on ESTR stability as well as the need to analyse the radiation killing effect.

1.6.2 Poly (ADP-Ribose) polymerase-1

Poly (ADP-Ribose) polymerase-1 (PARP-1) is a member of a family of nuclear enzymes that possess poly (ADP-ribosylation) catalytic capacity. Poly (ADP-ribosylation) is a reversible post-translational modification process. PARP-1 catalyses poly (ADP-ribosylation) of various nuclear proteins including itself using NAD^+ as a substrate. PARP-1 is an abundant and conserved nuclear protein that has been shown to bind rapidly and directly to both single and double strand breaks (Herceg et al., 2001).

The role of PARP-1 has been debated over the past years but it is thought to be involved in multiple cellular processes via PARP-1 dependent poly (ADP-ribosylation). Some of the processes in which PARP-1 is thought to be involved include DNA repair, cell cycle control, cell death and thus maintaining genomic stability (Herceg et al., 2001; Uchida et al., 2001; Soldani and Scovassi, 2002). PARP-1 is known to be involved in early DNA repair response, through a 100 fold increase in activity within two minutes after damage (Herceg et al., 2001).

PARP-1 has a molecular weight of 133 kDa and comprises three major functional domains (reviewed in Shall and de Murcia, 2000; Tong et al., 2001; Chalmers, 2004); (i) the N-terminal DNA-binding domain (DBD) containing two zinc fingers that act as molecular nick sensors and as an interface with protein partners, such as PARP itself, XRCC1 and histones. Within the DBD there is a nuclear localisation signal (NLS) which is interrupted by a caspase-3 cleavage site at which PARP-1 is cleaved by caspases during apoptosis to preserve the NAD^+ and ATP for use during apoptosis. (ii) PARP-1 has a central automodification domain which contains the auto-poly (ribosylation) sites that are acceptors of ADP-ribose polymers and are involved in the negative regulation of PARP-DNA interactions. This domain also contains a BRCT (BRCA-1 C-terminus) motif which is involved in protein-protein interactions that are essential for the assembly and activation of DNA repair complexes. (iii) Lastly there is the C-terminal catalytic domain which contains the 'PARP' signature sequence or the NAD^+ binding site. This site is responsible for the nick binding dependent poly (ADP-ribose) synthesis. The molecular structure of PARP-1 can be seen in Figure 1.8.

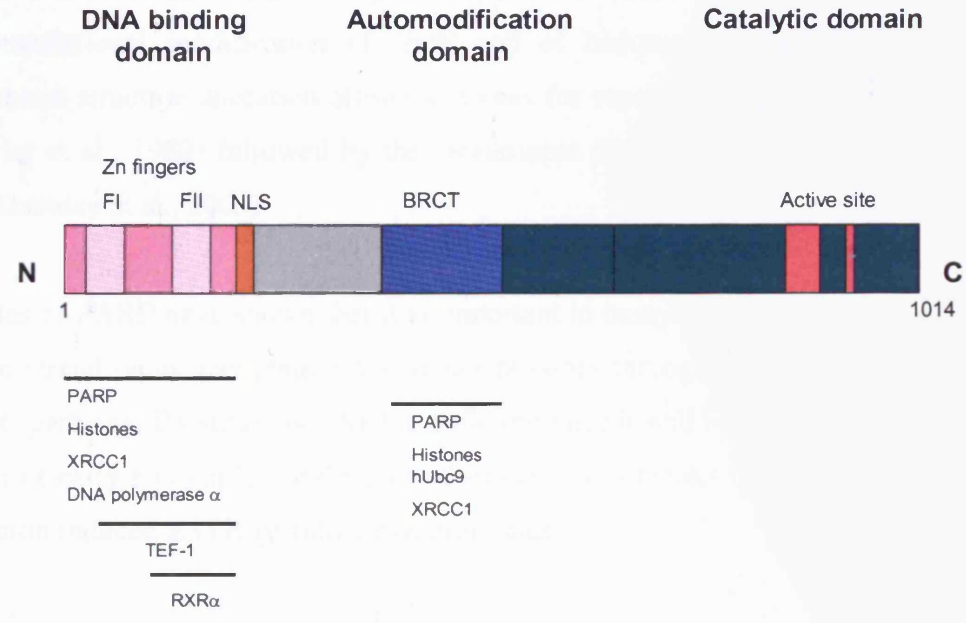


Figure 1.8: Schematic of molecular structure of PARP-1 illustrating position of functional domains. (Adapted from Shall and Murcia, 2000)

The enzymatic activity of PARP-1 has been found to increase up to 500 times in the presence of DNA strand breaks within the first two minutes of damage (Herceg et al., 2001). This indicates that PARP-1 is involved in very early DNA damage response.

After exposure to free radicals, alkylating agents or ionising radiation PARP rapidly binds to single strand breaks in DNA competing with other repair enzymes, including exonucleases and other proteins involved in the DNA repair pathway homologous recombination. On binding to DNA strand breaks PARP-1 becomes enzymatically active and using NAD^+ as a substrate to synthesise a long branched poly (ADP-ribose) polymer on itself at the automodification site. Automodification of PARP-1 results in it becoming negatively charged and subsequently lowering its affinity for DNA and dissociates allowing access to other DNA repair enzymes, such as XRCC1 which is involved in base excision repair. Poly (ADP-ribose) is short lived *in vivo* and is readily degraded by PARG (Friedberg et al., 1995). See Figure 1.9 for details of PARP-1 function during DNA repair of single strand breaks. PARP-1 has been referred to as a "nick protection" molecule that binds to SSBs preventing accidental homologous recombination events allowing other repair pathways to repair damage. PARP-1 is involved in the sensing of DNA breaks (Gradwohl et al., 1990), then the posttranslational modification of itself and of histones H1 and H2B triggering chromatin structure alteration allowing access for repair enzymes to the break point (Poirier et al., 1982) followed by the recruitment of XRCC1 via the BRCT domain (El-Khamisy et al., 2003).

Studies of PARP have shown that it is important in many cellular functions including single strand break recognition and repair possibly through the base excision repair (BER) pathway. By studying PARP-1 deficient mice it will be possible to identify the effect of early recognition and repair of single strand breaks on the spontaneous and radiation induced ESTR germline mutation rates.

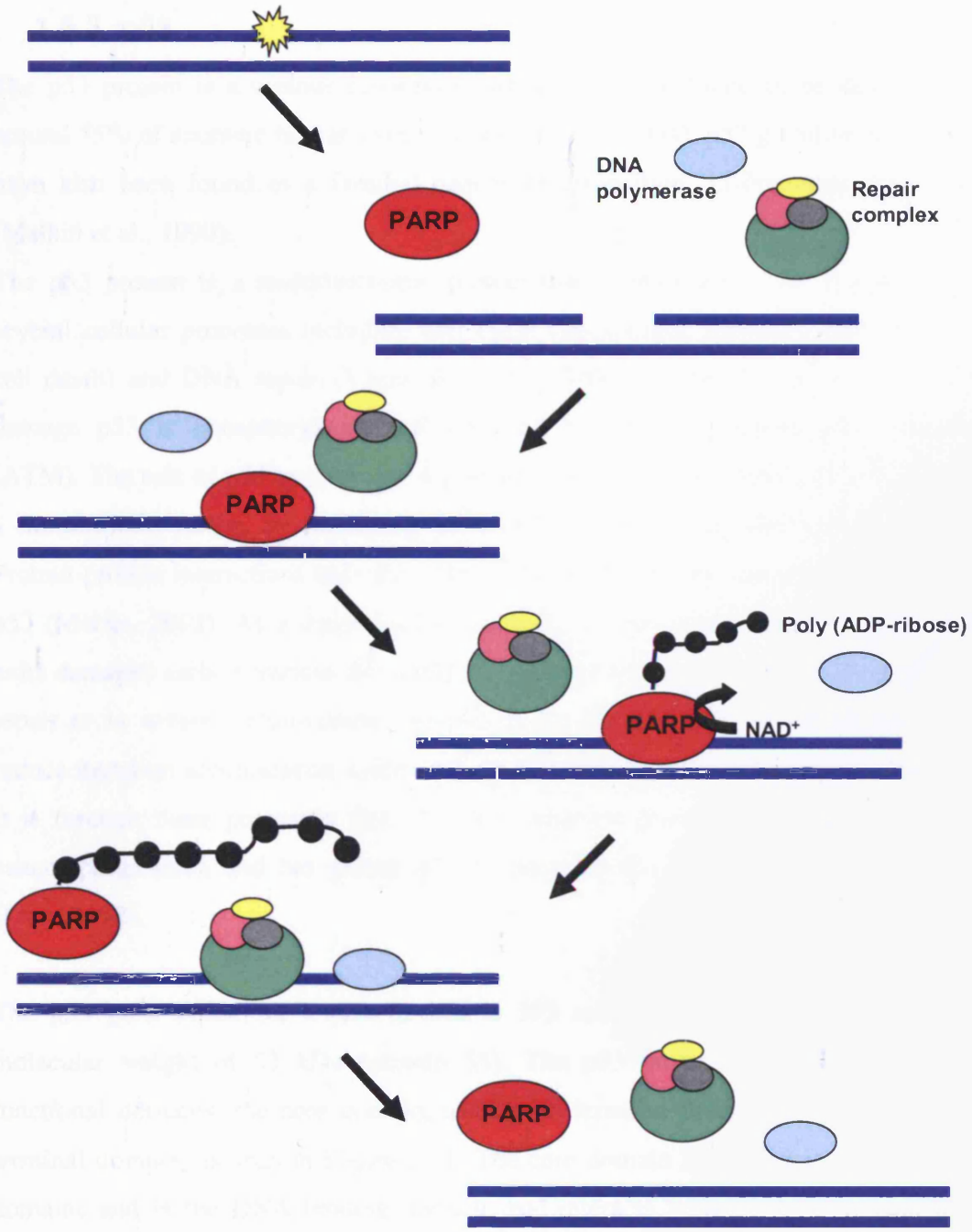


Figure 1.9: Function of PARP-1 during repair of DNA single strand breaks. (Adapted from Freidberg et al., 1995)

1.6.3 p53

The p53 protein is a tumour suppressor, which has been found to be defective in around 55% of sporadic human cancers (Levine et al., 2004). p53 germline mutations have also been found in a familial cancer predisposition, Li-Fraumeni syndrome (Malkin et al., 1990).

The p53 protein is a multifunctional protein that is involved in the regulation of several cellular processes including cell cycle checkpoints, apoptosis (programmed cell death) and DNA repair (Vogelstein et al., 2000). On the recognition of DNA damage p53 is phosphorylated and activated by Ataxia Telangiectasia Mutated (ATM). The role of p53 as a tumour suppressor can be mainly attributed to its role as a transcription factor, by regulating over one hundred genes (Zhoa et al., 2000). Protein-protein interactions may also play a role in the tumour suppression ability of p53 (Morris, 2002). As a transcription factor and as part of a protein complex, p53 halts damaged cells at various cell cycle checkpoints allowing cell cycle arrest, DNA repair or in severe circumstances, apoptosis (see Figure 1.10). All these processes reduce mutation accumulation within the genome, in turn influencing genetic stability. It is through these processes that p53 may maintain genomic stability and inhibit cancer progression and has gained p53 its name as the 'guardian of the genome' (Lane, 1992).

The p53 gene codes for a protein that is 393 amino acids in length and has a molecular weight of 53 kDa (protein 53). The p53 protein contains three main functional domains, the core domain, the amino terminal domain and the carboxyl terminal domain, as seen in Figure 1.11. The core domain is the largest of the three domains and is the DNA binding domain, and interacts with DNA in a sequence-specific manner (El-Diery et al., 1992). This central DNA binding region is where almost all of the mutations observed in p53 can be found, with the majority of which occur within three of the five highly conserved regions (III-V) (Prives and Hall, 1999). The amino terminal domain downstream of the core is responsible for the transcriptional activity of p53. This domain targets specific DNA sequences and once bound transcriptional machinery is recruited to transcribe new mRNA, therefore activating the expression of target genes (Prives and Hall, 1999). The amino terminal

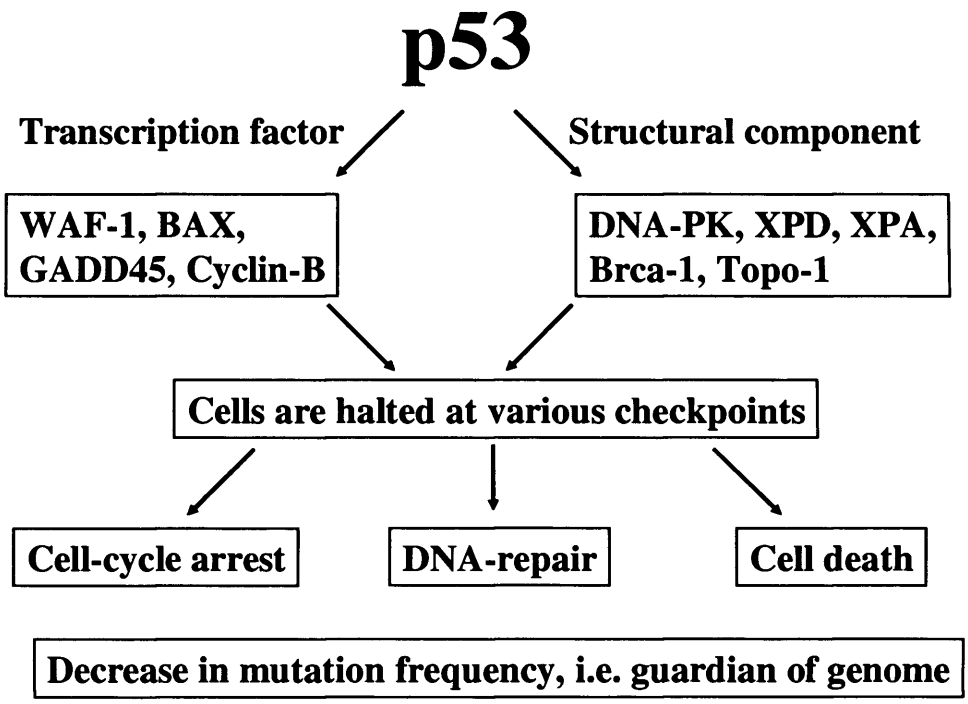


Figure 1.10: Function of p53 after DNA damage induction, adapted from Morris, 2002)

domain is also involved in the regulation of the stabilisation and activation of p53 via the interaction of proteins including mdm2. Binding of mdm2 at this region prevents p53 interacting with the transcriptional machinery and also stimulates the addition of ubiquitin groups which then encourage degradation of the p53 protein (Vogelstein et al., 2000). The amino transcriptional activation domain is separated from the core domain by a region of proline-rich sequence; through this region p53 is influenced by signalling molecules such as the c-abl oncogene (Yuan et al., 1996). The final domain of p53 is the carboxyl terminal domain containing a tetramerisation subdomain, a basic terminal subdomain and nuclear localisation signals (NLSs). The NLSs are short amino acid sequences that are responsible for the passage of p53 through the nuclear membrane (Prives and Hall, 1999). The tetramerisation domain is responsible for the oligomerisation of the p53 protein. The native p53 exists as a tetramer and oligomerisation is required for optimal p53 function (Jeffreys et al., 1995). The basic part of the C-terminal domain function is to control sequence-specific DNA binding

and is the domain responsible for DNA damage recognition (Steegenga et al., 1996). The p53 protein undergoes extensive post-translational modifications during activation. Modification sites have been identified mainly in the amino and carboxyl terminal domains, affecting transcriptional activity and DNA binding properties, respectively.

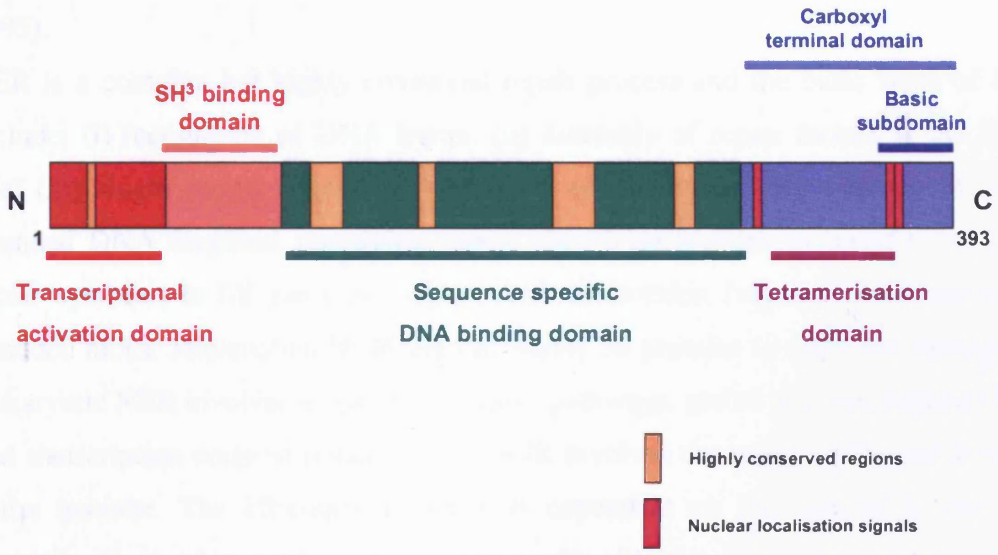


Figure 1.11: Molecular structure of p53, indicating functional domains. (Adapted from Prives and Hall, 1999)

Once DNA damage occurs p53 is modified and then regulates the synthesis of proteins that function to eliminate damage. Several genes are under transcriptional control by p53 or interact with p53, including p21 involved in the G1 checkpoint of the cell cycle, bax, bcl-2 and GADD45 all involved in apoptosis and genes such as p48, XPB and XPD which are involved in DNA repair (Reviewed by Morris, 2002).

All studies to date on p53 deficient mice have been carried out on cancer incidence and mutations within the somatic cells, and nothing is known about how germline stability is affected by the p53 status. During this project it was proposed to study the effects of p53 status on the germline stability of ESTR loci in mice.

1.6.4 Xeroderma pigmentosum group C

Xeroderma pigmentosum group C (XPC) is one of seven complementation groups known to cause the hereditary disease xeroderma pigmentosum (Freidberg et al., 1995). Xeroderma pigmentosum (XP) is a rare autosomal recessive disorder which is characterised by an increased risk of cancer in sun exposed areas of the body and in some cases neurological deficiencies (Cleaver and Kraemer, 1989). XP has been directly associated with a defect in nucleotide excision repair (NER) (Friedberg et al., 1995).

NER is a complex but highly conserved repair process and the basic steps of NER include; (i) recognition of DNA lesion; (ii) assembly of repair factors at the lesion site; (iii) single strand incision at both sides of the lesion; (iv) excision of single stranded DNA fragment containing lesion (24-32 nucleotides in length); (v) DNA repair synthesis to fill gap replacing excised nucleotides; (vi) ligation of the single stranded nicks. Mammalian NER requires nearly 30 proteins to carry out these steps. Eukaryotic NER involves at least two distinct pathways, global genomic repair (GGR) and transcription coupled repair (TCR). GGR involves the repair of lesions over the entire genome. The efficiency of GGR is dependent on the type of lesions; for example, (6-4) photoproduct (most structurally distorting) are removed from the global genome faster than cyclobutane pyrimide dimers (Mitchell and Nairn, 1989). The second pathway, TCR, removes lesions that block RNA polymerases in the transcribed strand of expressed genes (Bohr et al., 1985). This pathway repairs different lesions at a similar rate and ensures rapid recovery of transcriptional activity after damage allowing the maintenance of normal cellular functions and survival (Freidberg, 1996).

Mutations in XP genes lead to defective NER but the severity of UV-induced cytotoxicity varies within and between complementation groups reflecting the levels of residual repair (Batty et al., 2000; Hoffen et al., 2003). Cells lacking functional XPC are less sensitive to the effects of UV than the other XP complementation groups (Tyrrell and Amaudruz, 1987). The residual repair in cells lacking functional XPC is mostly restricted to transcribed regions of the genome possibly explaining the high level of UV survival (Venema et al., 1991). XPC has been shown to be the only NER protein that is not required for TCR, indicating that XPC is a GGR specific NER factor (Venema et al., 1991).

The most delicate step of NER is the recognition of the DNA lesions and therefore it is not surprising that the recognition mechanisms of the two pathways of NER are different and require different proteins. GGR lesion recognition relies on helix distortion and local DNA context for recognition and binding of the proteins rather than the lesions themselves (Sugasawa et al., 2001; Sugasawa et al., 2002). XPC has been shown to initiate GGR by sensing and binding lesions, locally distorting the DNA double helix and recruiting the other factors of the global NER system (Figure 1.12) (Sugasawa et al., 1998). It has been found that the XPC protein was bound tightly to another protein, HR23B (Matsutani et al., 1994). HR23B is human homologue of the yeast NER factor Rad23 (Matsutani et al., 1994). HR23B stimulates XPC via binding (Matasutani et al., 1997). As a complex XPC-HR23B binds to various types of damaged DNA based on the recognition of helix distorting properties of DNA lesions. The XPC-HR23B complex shows similar affinity for both single and double-stranded DNA with a strong preference for UV damaged DNA (Masutani et al 1994; Reardon et al., 1996).

A two step damage recognition process for GGR has been proposed; XPC-HR23B first recognises DNA helix distortion, following verification of damaged bases. A basal transcription factor, TFIIH (transcription factor IIH), has been suggested to play a role in the verification (Sugasawa et al., 2001).

XPC-HR23B complex specifically binds to a variety of lesions including UV-induced (6-4) photoproduct (6-4PP), cyclobutane pyrimide dimers (CPDs) and various bulky chemical adducts.

XPC is the human homolog of yeast RAD4. Structure-functional domains of XPC are to date not fully understood. The XPC gene product is 940 amino acids in length and has a molecular weight of 125kDa (Masutani et al., 1994).

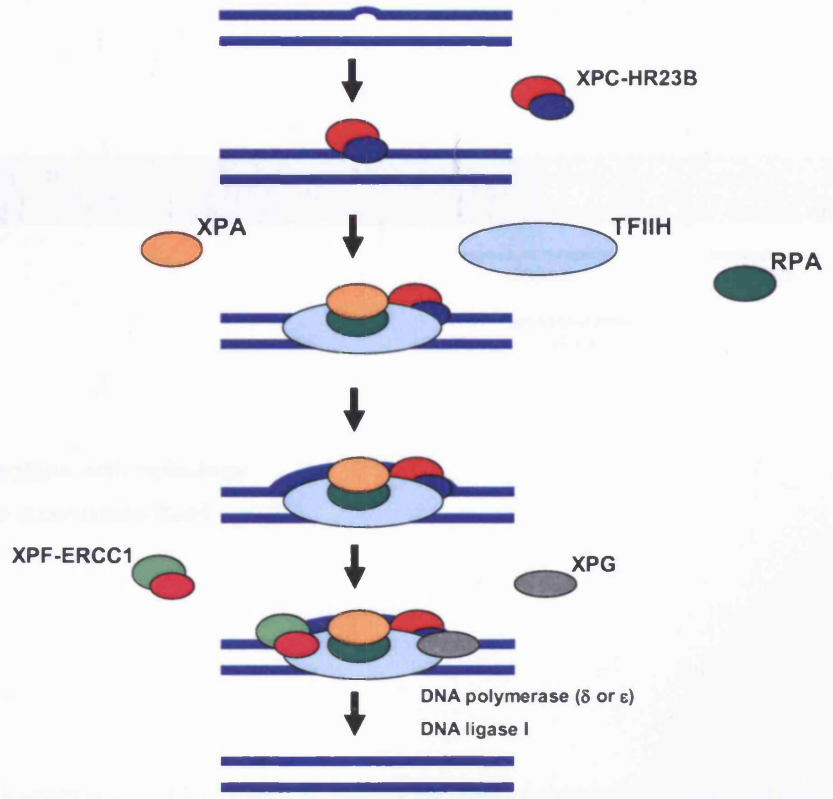


Figure 1.12: Schematic of global genomic repair. (Adapted from van Hoffen et al., 2003)

Although the structure-function relationship is poorly understood it is known that the C-terminal part of the protein is more highly conserved than the N-terminal probably due to its functional importance (Li et al., 1996). A recent study by Uchida et al. (2002) used a series of truncated proteins to investigate the domains responsible for several biochemical activities of the XPC protein. The N-terminal part of the XPC protein appears to be non-essential for repair function. Loss of 117aa of the N-terminal leads to partial reduction of the activity of NER. It has been suggested that the first 54 amino acids of the N-terminal may be involved in the specific recognition mechanisms of certain types of lesions (6-4PPs) and the amino acids between 54-117 are involved in the recognition of CPDs (Uchida et al., 2002). It was also found that the C-terminal is responsible for the NER activity as the loss of the first 118 amino acids does not affect single strand DNA, HR23B and TFIIH binding activities, as well as specific affinity for damaged DNA. What is known about the structure-functional domains of XPC is illustrated in Figure 1.13.

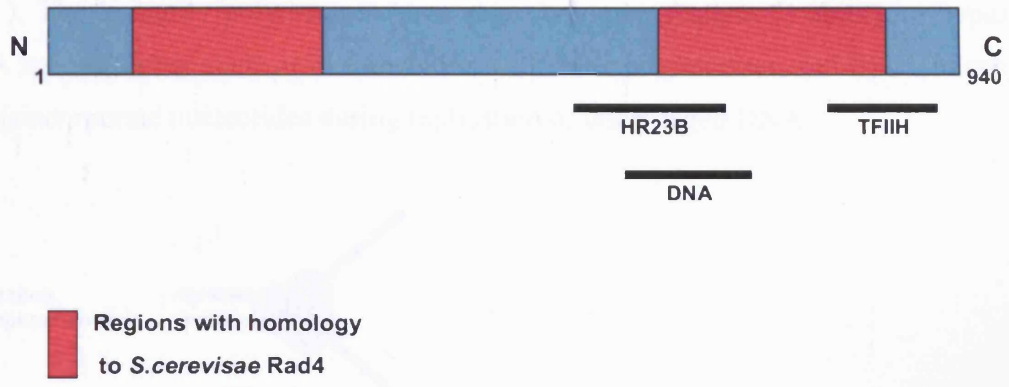


Figure 1.13: Summary of functional domains of XPC mapped to date. (adapted from Uchida et al., 2002)

1.6.5 Polκ

Most DNA lesions are repaired by one of the repair pathways found in living organisms. However, repair can be slow and lesions for some reason may escape repair pathways. These persistent lesions may cause structural distortions that may result in the stalling or complete arrest of DNA replication. This is due to the high selectivity of the replicative DNA polymerases for the correct base-pairing and structure of DNA and their inability to insert any base opposite most lesions. To prevent cell death by blockage to DNA replication the stalled polymerase is replaced by another polymerase that is capable of bypassing the lesion (Woodgate, 1999) as illustrated in Figure 1.14. The mechanism by which these polymerases are recruited to DNA lesions is yet unknown. The action of polymerases during lesion bypass can be separated into two steps I) nucleotide insertion opposite the lesion; and II) extension DNA synthesis from opposite the lesion. This process is called translesion synthesis (TLS) and the polymerases involved lack 3`-5` proof-reading exonuclease activity and have low fidelity (Ohmori et al., 2001), therefore are accompanied with an increased

frequency of mutation that depends on the polymerase and type of lesion. Recently a family of polymerases that have evolved to carryout TLS has been discovered. These polymerases have been designated the Y-family DNA polymerases (Ohmori et al., 2001). The Y-family polymerases have some common features I) ability to bypass DNA lesions, either alone or in association with other polymerases, and II) a tendency to misincorporate nucleotides during replication of undamaged DNA.

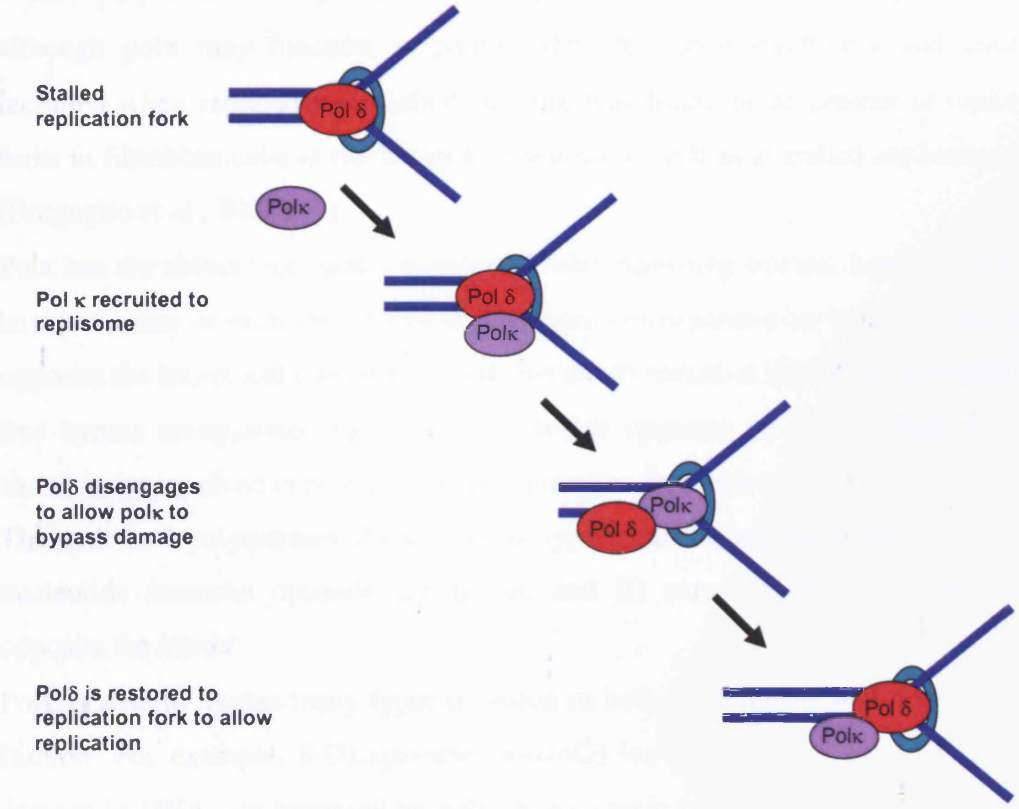


Figure 1.14: Schematic of translesion synthesis (adapted from Toueille and Hubscher, 2004)

Polymerase kappa (polκ) is a member of the Y-family polymerases. Polκ is a product of the POLκ (DINB1) gene in humans and the Polκ gene in mice (Gerlach et al., 1999). Human POLκ is a homologue of the *Esherichia coli* DinB (Pol IV) protein, a SOS protein involved in untargeted UV-induced mutagenesis (Gerlach et al., 1999). Polκ lacks intrinsic 3′-5′ exonuclease activity, therefore lacks the ability to proof-read its mistakes (Ohashi et al., 2000). Polκ has been shown to synthesise DNA with extremely low fidelity (Ohashi et al., 2000; Zhang et al., 2000). Human polκ was

shown to make one error every 200 nucleotides, with predominance towards T-G tranversions (Zhang et al., 2000). When the error rate for polκ was compared to that of another low fidelity polymerase polβ, polκ has been found to be 33 times more inaccurate than polβ (Zhang et al., 2000). Unlike other members of the Y-family polκ can processively synthesise chains of 25 nt or more (Ohashi et al., 2000). Taken together the low fidelity and moderate processivity of polκ it has been suggested that it may play a role in spontaneous mutagenesis. The role of polκ is not known, although polκ may function as part of the replication machinery and could be recruited when replication is stalled, as polκ was found to be present at replication forks in fibroblast cells in the absence of lesions as well as at stalled replication forks (Bergoglio et al., 2002).

Polκ has the ability to bypass a number of helix distorting lesions. Lesion bypass can be error-prone or error-free. Error-prone bypass incorporates an incorrect nucleotide opposite the lesion and constitutes a mechanism of mutation induction, whereas error-free bypass incorporates the correct nucleotide opposite the lesion. Polκ has been shown to be involved in both error-prone and error-free lesion bypass.

The action of polymerases during lesion bypass can be separated into two steps I) nucleotide insertion opposite the lesion; and II) extension DNA synthesis from opposite the lesion.

Polκ is able to bypass many types of lesion in both a mutagenic and non-mutagenic fashion. For example, 8-Oxoguanine (8-oxoG) lesions, a major form of oxidative damage in DNA, are bypassed by polκ in mutagenic fashion inserting mainly A; AP sites bypass by polκ is mutagenic but the efficiency is dependent on the base at the lesion site. Polκ bypasses AP-T lesions most efficiently; AAF (acetylaminofluorene) adducted guanine bulky lesions in DNA which block many polymerases are bypassed by polκ in an error-prone manner (Zhang et al., 2000). Polκ is also able to perform error-free lesion bypass opposite benzo(*a*)pyrene adducted guanine. Benzo(*a*)pyrene is a known carcinogen (Zhang et al., 2000;Ogi et al., 2002). Polκ has also been shown to be active in the extension DNA synthesis opposite these lesions (Zhang et al., 2002). Both these results suggest that polκ plays a role in suppressing mutations at DNA lesions generated by benzo(*a*)pyrene by either bypassing the lesion alone or in combination with another polymerase. Thymine glycol (Tg) form of oxidative base

damage, inhibits replication. Fischhaber et al. (2002) found that polκ was able to bypass the lesion in an error-free manner and was also able to extend the primer template beyond the lesion. These results suggest that polκ may have a role in the error-free and error-prone bypass of oxidative damage.

Human polκ has been shown to be unable to bypass T-T dimers (CPD) or (6-4) T-T photoproducts induced by UV irradiation (Zhang et al., 2000). Although polκ is unable to insert a nucleotide opposite these DNA lesions, it has been shown that polκ can extend from a G nucleotide opposite the 3'T of a T-T dimer (Washington et al., 2002). This phenomenon was also observed with m6G lesions; polκ is unable to insert a nucleotide opposite the lesion but can efficiently extend from a T or C base placed opposite the lesion (Haracska et al., 2002). Although polκ is able to bypass 8-oxo-G lesions without error and then extend from it, it was found that bypass of this lesion as well as m6G lesions were more proficient when the replicative polymerase Polδ is combined with polκ (Haracska et al., 2002). These results suggest that polκ promotes efficient lesion bypass by extending from the nucleotide incorporated opposite the lesion site by another polymerase.

Human polκ has also been shown to have a role in DNA synthesis from mismatched termini during normal DNA replication (Washington et al., 2002), therefore indicating a role of polκ in mutagenic replication in undamaged DNA. During extension of mispaired primer termini on undamaged DNA polκ generates single base substitutions by misincorporation of bases or single base deletions using template-primer misalignment (Wolfe et al., 2003). The ability of polκ to extend mispaired primer termini sets it apart from the other Y-family polymerases as they are unable to extend from mismatched primer termini (Wolfe et al., 2003).

Recently polκ has been implicated in tumourigenesis, as polκ was seen to be over expressed in 21 of 29 lung cancer patients (O-Wang et al., 2001). Data from the study of these patients indicates that polκ contributes to lung tumour development by accelerating the accumulation of mutations and therefore polκ could possibly act as an oncogene.

Human POLκ encodes for a protein with an 870 amino acid protein with a molecular weight of 100kDa (Gerlach et al., 1999).The protein is comprised of three portions, the N-terminal, the homeodomain protein binding (HDB) site and the C-terminal (see Figure 1.15). The HDB region contains all five of the motifs conserved amongst the Y-family homologues, therefore suggesting that the HDB region is essential for DNA polymerase activity (Ohashi et al 2000b). In eukaryotes it was found that the polκ carboxyl-terminal contains two copies of zinc-cluster (Gerlach et al., 1999). The carboxyl-terminal region has been shown not to be required for the catalytic activity of the polymerase, but it has been proposed that it may be involved in the increased processivity through DNA binding by the Zinc-cluster and/or interaction with other protein within the cell (Ohashi et al., 2000b).

A major determinant for base substitution and frameshift fidelity is encoded within the N-terminal two thirds of the human polκ gene (Ohashi et al., 2000). Human polκ is set apart from the other Y-family polymerases by an extension to the N-terminal of approximately 75 amino acids (Uljon et al., 2004).

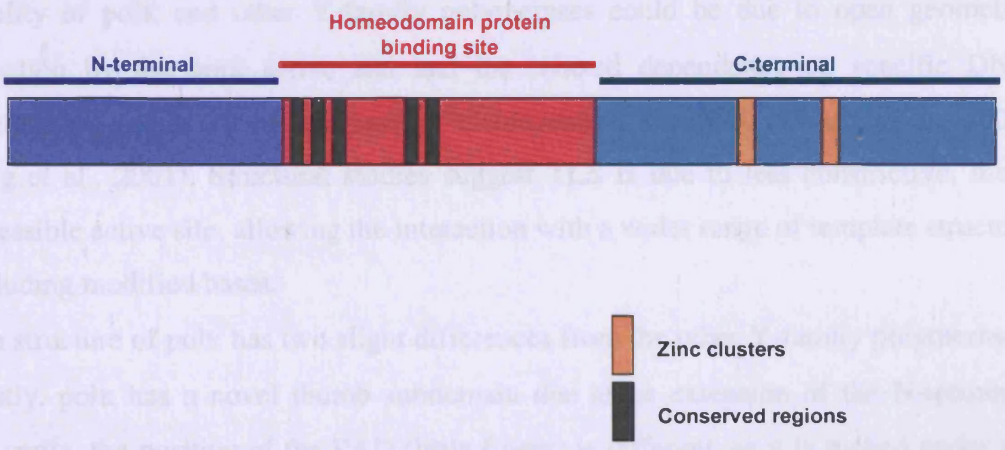


Figure 1.15: Structure of Polκ protein (adapted from Gerlach et al., 1999 and Ohashi et al., 2000b)

Y-family polymerases share common motifs in their primary structure that are different to A-, B-, C- and X-family polymerases (Ito and Braithwaite, 1991), although they have the tertiary structures conserved in most DNA polymerases, right hand architecture with fingers, palm (catalytic active) and thumb subdomains. The finger domain tightly envelops the Watson-Crick base pair between the template base and the incoming nucleotide and is responsible for the 'induced fit' mechanism for checking the fidelity of replication, when the fit is perfect the polymerase catalyses the phosphatidyl transfer reaction (Ling et al., 2001; Fleck and Schar, 2004). Unlike other polymerase families the Y-family polymerases contain an additional domain, termed PAD, polymerase associated domain which is often referred to as the 'little finger' or 'wrist' (see Figure 1.16) (Ling et al., 2001). The arrangement of the domains varies between families; the A-family polymerases the thumb domain which is followed by the finger and the palm domains, whereas in the Y-family polymerases the finger domain is first followed by the palm, thumb and the little finger (Ling et al., 2001). The little finger and the thumb subdomains grip the minor and major grooves respectively (Fleck and Schar, 2004). The little finger subdomain is the least conserved of the four subdomains and plays a role in determining the enzymatic and biological role of each of the Y-family polymerases (Bousocq et al., 2004). The low fidelity of polκ and other Y-family polymerases could be due to open geometric selection by the polκ active site and the relaxed dependency on specific DNA interactions results in the tolerance of distortions in the DNA (Ohashi et al., 2000; Ling et al., 2001). Structural studies suggest TLS is due to less constrictive, more accessible active site, allowing the interaction with a wider range of template structure including modified bases.

The structure of polκ has two slight differences from the other Y-family polymerases. Firstly, polκ has a novel thumb subdomain due to an extension of the N-terminal. Secondly, the position of the PAD (little finger) is different, as it is tucked under the palm domain instead of being anchored to the finger subdomain as in the other Y polymerases (Uljon et al., 2004).

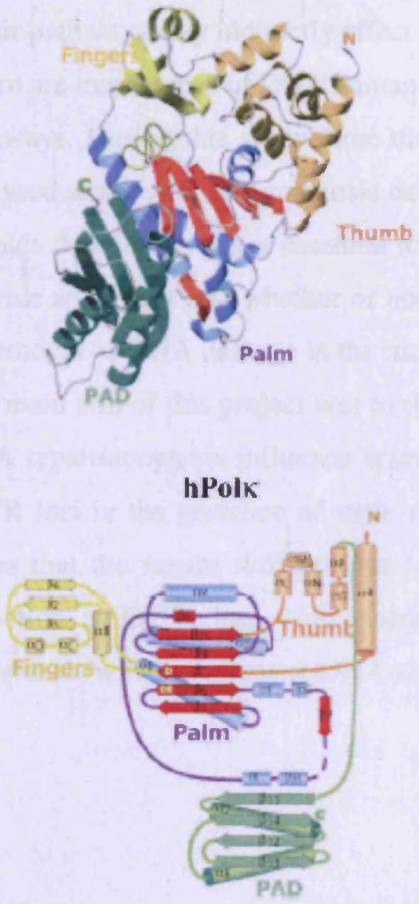


Figure 1.16: Tertiary structure of Polκ (Taken from Uijon et al., 2004)

To date nothing is known about the effect of polκ on the germline mutation rate, and to study this during this project polκ deficient mice were used.

1.7 Conclusions/aims

Several studies on radiation mutation induction at ESTR loci in the mouse germline have shown that ESTR loci provide a useful and sensitive tool for monitoring germline mutation in mice. Several of these previous studies suggest that mutation induction by ionising radiation at ESTR loci is not a result of direct damage to these loci. Although the mechanism of mutation remains unknown at these ESTR loci a number of hypotheses have been proposed for the mutation processes at repetitive DNA. The first hypothesis to be tested was that ESTR loci may mutate in the same manner as minisatellites via meiotic recombination (Barber et al., 2000). This hypothesis was rejected and the next hypothesis to be tested is that mutation at ESTR

loci may be a by product of DNA repair. It is possible that the activation of DNA repair pathways may indirectly affect ESTR mutation rates.

There are many types of DNA damage which are all dealt with by various DNA repair pathways. During this study three different repair deficient mouse strains have been analysed along with one apoptosis deficient mouse strain. It is hoped that the analysis of mice deficient in genes essential to the DNA repair or the apoptotic pathways, may provide an insight into whether or not ESTR instability may be a by product of repair or removal of DNA damage in the mouse germline.

The main aim of this project was to determine whether mutations at genes involved in DNA repair/apoptosis influence spontaneous and radiation-induced mutation rates at ESTR loci in the germline of male mice. It is hoped that through the study of these genes that the results will provide important clues on the mechanisms of mutation induction at ESTR loci, also giving an estimate of genetic risk for a cohort of individuals with mutations at DNA-repair genes.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical reagents

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

Molecular reagents were obtained from ABgene (Epsom, UK), Ambion, Inc. (Warrington, UK), Bio-rad (Hemel Hempstead, UK), Biowhittaker 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), Roche Diagnostics, Roche applied science (East Sussex, UK), Sigma-Aldrich Company (Poole, UK), Stratagene (Amsterdam, The Netherlands) and United States Biochemical Corp (USB) (Cleveland, USA).

Specialised equipment was obtained from Applied Biosystems (Warrington, UK), Bio-Rad (Hemel Hempstead, UK), Eppendorf (Hamberg, 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), Thermo Shandon (Pittsburgh, USA), Turner Designs (Sunnyvale, USA) and Ultra violet Products (UVP) Life Sciences (Cambridge, UK).

2.1.2 Enzymes

Restriction enzyme *AluI* was supplied by New England Biolabs (NEB) (Hitchin, UK), using reaction buffer supplied with the enzyme.

Proteinase K for DNA extractions was supplied by Sigma-Aldrich Company (Poole, UK).

For radioactive labelling of probes for Southern blotting Klenow, the large fragment of Polymerase I of *Escherichia coli* was cloned by USB (Cleveland, USA) and supplied by Amersham Biosciences UK Ltd (Bucks, UK).
The *Taq* DNA polymerase was supplied by ABgene (Epsom, UK).

2.1.3 Molecular weight markers

100 bp DNA ladder was supplied by Promega (Southampton, UK) and 1 Kb DNA ladder was supplied by Invitrogen UK (Paisley, UK). ΦX174 DNA digested with *Hae*III and λ DNA ladder digested with *Hind*III were supplied by ABgene (Epsom, UK).

2.1.4 Oligonucleotides

Oligonucleotides for PCR amplification of ESTR probes, genotyping and ESTR analysis were synthesised in house (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, UK).

2.1.5 Mice

PARP-1 study

PARP-1 mice were provided by Dr. G. de Murcia (Ecole Superieure de Biotechnologie de Strasbourg, Illkrich, France).
CBA/J females were purchased from Elevage Janvier, France.

p53 study

CBA/ca female mice were purchased from Charles River UK Ltd (Kent, UK).
C57BL/6 TSG-p53® N12 heterozygous mice (B6.129-Trp53^{tm1} N12) were purchased from Taconic (Germantown, NY, USA).

XPC study

XPC mice were provided by Prof. E. Freidberg (Department of Pathology, UT Southern Medical Centre, Dallas, Texas, USA).
CBA/J females were purchased from Elevage Janvier, France.

polκ study

Polκ mice (on a 129/Ola and C57BL/6 mixed background) were provided by Prof. E. Freidberg (Department of Pathology, UT Southern Medical Centre, Dallas, Texas, USA).

Balb/c females were purchased in USA.

2.1.6 Standard solutions

Southern blotting solutions (depurinating, denaturing and neutralising), 20x Sodium Chloride Sodium-Citrate (SSC) buffer and 10x Tris Borate/EDTA (TBE) electrophoresis buffer, were prepared as described by Sambrook (Sambrook and Russell, 2001).

All standard solutions were supplied by the media kitchen (Department of Genetics, University of Leicester).

2.2 Methods

2.2.1 Mice

2.2.1.1 Housing

Leicester University

Mice for the p53 study, both control and exposed, were maintained by the Biomedical Service staff at University of Leicester and fed RM1 diet (special Diet Services, Witham, UK). Animals were kept at 21°C with a 12 hour light/dark cycle.

All animal procedures were carried out under guidance issued by the MRC in 'Responsibility in the use of animals for medical research' (July 1993) and Home Office licence 80/1564.

Paris, France

Mice used in the PARP-1 and XPC studies, both control and exposed, were bred and maintained in the facilities at the Commissariat à l'Energie Atomique (CEA), Laboratoire Génétique de la Radiosensibilité, Départment de Radiobiologie et de Radiopathologie, Paris.

Animal procedures performed were carried out under the guidance issued by the French government.

Texas, USA

Mice used in the polκ study were bred and maintained in the facilities at Department of Pathology, UT Southern Medical Centre, Dallas, Texas, USA.

2.2.2 Irradiation of mice

PARP-1

PARP-1$^{+/+}$ and *PARP-1*$^{-/-}$ males at 10-12 weeks old were given whole body acute irradiation of 1Gy of γ-rays delivered at 1.97Gy min^{-1} (Cs-137 source, IBL 637 CisBio International). Irradiated males were mated with untreated CBA/J females 10 weeks after exposure, ensuring that pre-meiotic germ cells were analysed.

p53

p53$^{+/+}$, *p53*$^{+/-}$ and *p53*$^{-/-}$ males at 8 weeks old were given whole body acute irradiation of 1Gy of X-ray delivered at 0.5 Gy min^{-1} (250 KV constant potential, HLV 1.2 mm Cu) pantak industrial X-ray machine. Irradiated males were mated with untreated CBA/ca females 6 weeks after exposure.

XPC

XPC$^{+/+}$ and *XPC*$^{-/-}$ males at 10-12 weeks old were given whole body acute irradiation of 1Gy of γ-rays delivered at 1.97Gy min^{-1} (Cs-137 source, IBL 637 CisBio International). Irradiated males were mated with untreated CBA/J females 10 weeks after exposure, ensuring that pre-meiotic germ cells were analysed.

2.2.3 Preparation of mouse tissue for DNA

For genotyping, tissue was obtained under anaesthetic. Tissue was obtained by ear punching the animals, allowing the identification of each individual.

Tissue samples for the pedigree analysis 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 were the preferred tissue for extraction, but spleen was used if the initial extraction failed.

Tissue samples were taken from the mice housed in Leicester. Samples from Paris and Texas were received as DNA.

2.2.4 Genotyping of mice

Genotyping was carried out throughout the p53 study to maintain a breeding colony. Genotyping was also done to verify the genotype of the animals purchased from Taconics and of the parents at the end of the study.

2.2.4.1 Preparation of DNA for genotyping

Tissue samples were obtained from mice whilst under anaesthetic. Ear punches were used as this was the preferred method of marking mice during the study.

Proteinase K digestion

The whole ear punch was suspended with 500 µl Lysis solution A (0.1 mM NaCl, 25 mM EDTA (pH 8.0), 20 mM Tris-HCL (pH 8.0)) and 500 µl Lysis solution B (1% (w/v) SDS, 12.5 mM EDTA (pH 8.0), 10 mM tris-HCl (pH 8.0)) and 15 µl Proteinase K (25 mg/ml) in a 2 ml eppendorf. The contents were mixed by inversion and incubated overnight at 55°C. DNA was extracted by phenol/chloroform extraction and then ethanol precipitated.

Phenol/chloroform extraction

DNA was extracted from the proteinase K digested ear punches using phenol/chloroform extraction. 1 ml phenol/chloroform (Phenol:Chloroform:Isoamyl alcohol in the ratio 25:24:1, equilibrated with Tris-HCL pH 8.0) was added to each sample and the samples were mixed by repeated inversion. The samples were separated by centrifugation in a bench top microcentrifuge for 10 mins at 13,000rpm to remove any tissue debris. The upper aqueous phase was transferred to a fresh 2 ml eppendorf tube and 1 ml chloroform was added. The samples were mixed by inverting and separated by centrifugation at 13,000rpm for 10 mins in a bench top microcentrifuge to remove any traces of phenol. The upper aqueous phase was again transferred to a fresh 2 ml eppendorf tube for precipitation.

Ethanol precipitation

1 ml 100% (v/v) ethanol and 100 µl 3 M NaAc were added to the extract and the solution was inverted gently to precipitate the DNA. The eppendorf tubes were then incubated at -20°C for at least 1 hour to aid the precipitation. Following incubation at -20°C the samples were pelleted by centrifugation in a bench top microcentrifuge for 5 min at 13,000rpm. The ethanol was removed using a pipette and 500 µl 80% (v/v) ethanol was added to wash away any excess salt. The DNA was pelleted by centrifugation for a further 5 mins at 13,000rpm and the ethanol was removed using a pipette and the pellets were left to air dry. The dry DNA was resuspended in 30 µl ultrapure water. Dilutions of 1/10 and 1/100 were made ready for genotyping PCR.

2.2.4.2 Genotyping PCR

DNA was amplified using the Polymerase Chain reaction (PCR). Reactions were performed using a PTC-220 DNA Engine Peltier thermal cycler (MJ Reseach).

All genotyping PCRs were performed using 10x reaction buffer (750mM Tris-HCl (pH8.8), 200mM $(NH)_4SO_4$. 0.1% (v/v) Tween20) with $MgCl_2$, supplied with the *Taq* polymerase (ABgene).

p53

PCR reactions were set up in 10 µl reactions containing 0.2 mM dNTPs, 0.5 µM pI4F primer, 1 µM pE5R primer, 0.6 µM OPT-21 primer, 0.5 Units of Taq polymerase (ABgene), 1x PCR buffer (ABgene), 2 mM $MgCl_2$. For template DNA 1/10 and 1/100 dilutions of the DNA extracted from ear punches were used.

Amplification was performed in thin-walled 96 well-plates or thin-walled 8 strip PCR tubes (ABgene). Amplification was achieved by first denaturing at 96°C for 7 mins, then cycled at 96°C for 30 secs, 61°C for 30 secs and 70°C for 1 mins for 30 cycles finally ending with incubation at 70°C for 5 mins. The products were run on 2% agarose gels (section 2.5.1) to separate the wild-type allele (520bp) from the knockout allele (730bp).

XPC

PCR was carried out in 10 µl reaction containing 0.2 mM dNTPs, 1 µM XPCin primer, 1 µM XPCex primer, 1 µM XPCneo primer, 0.5 Units of *Taq* polymerase (ABgene), 1x buffer and 1.5 mM $MgCl_2$. Tail DNA from the southern blotting analysis was used at 1/10 and 1/100 dilutions as template DNA.

Amplification was performed in thin-walled 96 well-plates (ABgene). Amplification was performed by 95°C for 2 mins followed by a cycle of 95°C for 30 secs, 55°C for 30 secs and 72°C for 1 mins for 35 cycles finally an incubation at 72°C for 10 mins. The PCR products were run on a 2 % (w/v) agarose gel (section 2.5.1) to separate the mutant (390 bp) and wild-type (205 bp) alleles.

2.3 Methods for DNA extraction

2.3.1 Proteinase K digestion

Approximately half of the tissue sample (tail tissue) was chopped finely using a scalpel and suspended in 1ml Lysis solution A (0.1 M NaCl, 25 mM EDTA (pH 8.0), 20 mM Tris-HCl(pH 8.0)), 1ml Lysis solution B (1% (w/v) SDS, 12.5 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0)) and 30 µl Proteinase K (25 mg/ml) in a 15 ml Eppendorf Phase Lock Gel ™ Light tube*. The contents were mixed by inverting and incubated at 55°C for at least 5hrs or preferentially overnight with occasional mixing. DNA was then separated from proteins and bone debris by phenol/chloroform and chloroform extractions (section 2.3.2). DNA was then precipitated and resuspended in ultrapure water.

* The majority of the studies were carried out using the Light tubes, however due to manufacturing problems Eppendorf Phase Lock Gel ™ Heavy tubes were used in the later stages of the study.

2.3.2 Phenol/chloroform extraction

Phenol/chloroform and chloroform were used to purify genomic DNA from the proteins and bone debris following the initial proteinase K digestion (Section 2.3.1). 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 each sample and the samples were mixed by repeated inversion. The samples were separated by centrifugation using Phase Lock Gel™ tubes, which rely on physical separation of the aqueous phase from the contaminating solvents and protein phases. Occasionally additional phenol/chloroform extractions were required to obtain a clear colourless aqueous phase. A final extraction with chloroform alone was performed to remove any traces of phenol. The DNA was then ethanol precipitated (section 2.3.3).

2.3.3 Ethanol precipitation

Following the phenol/chloroform extractions in order to recover DNA, ethanol precipitation was performed. The aqueous phase was transferred into a 15 ml polypropylene tube containing two volumes 100% (v/v) ethanol and 1/10 volume 3 M NaAc (pH 5.5). The solution was gently inverted to precipitate the DNA. The DNA was removed using a pipette from the solution 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 13,000rpm. The ethanol was removed using a pipette and the pellets were air dried. The DNA was resuspended in ultrapure water to obtain a stock DNA concentration of 800-1500ng/µl.

2.4 Methods for DNA manipulation

2.4.1 Estimation of DNA concentration

DNA concentration was estimated using a spectrophotometer (Biophotometer, eppendorf), using 2 µl of genomic DNA in 98 µl ultrapure water. Using the absorbency at 260 nm the spectrophotometer calculated the concentration of DNA. If the concentration was less than 800 ng/µl the samples were vacuum dried and requantified before digestion.

2.4.2 DNA digestion

Digests were performed by first balancing the samples, so that all samples were at the same concentration. 10 µg-20 µg of each DNA sample was digested depending on the

study and the quality of the DNA. Digests were performed using 20 Units of *Alu*I (NEB) using the appropriate reaction buffer (NEBuffer 2). The samples were incubated at 37°C for at least 5 hours or preferentially overnight to ensure complete digestion.

5µl of loading dye (5x TBE, 49% (v/v) Glycerol, 0.1% (w/v) SDS, 0.1% (w/v) Bromophenol Blue) was added to the samples to stop the reaction.

2.4.3 PCR for Southern blot analysis

In the p53 study it was discovered that analysis of the *Hm-2* allele in these mice was impossible by standard *Alu*I digestion of genomic DNA followed by southern blotting analysis. This was due to the unusually small size of the allele. The small size meant that it could be amplified by PCR.

PCR was performed using Expand High Fidelity PCR system, a kit supplied by Roche Diagnostics. A PCR enhancing agent, betaine was required for the PCR to amplify the DNA successfully. Betaine is an isostabilising agent that equalises the contribution of GC- and AT-base pairing to allow easier separation of GC-rich regions of DNA during PCR.

PCR reactions were set up in 10 µl containing 1x buffer (with $MgCl_2$, 1.5 mM), 0.2 mM dNTPs, 0.4 µM primers (Hm2C & Hm2D), 1 M betaine, 1 Unit enzyme (mix of *Taq* polymerase and *Tgo* polymerase) and 500 pg of template DNA.

PCR was performed in thin-walled 96 well-plates (ABgene), after denaturing for 5 mins at 96°C, PCRs were cycled at 96°C for 30 secs, 58°C for 30 secs and 68°C for 6 mins for 30 cycles, ending with a 10 min incubation at 68°C.

PCR products were run on 1 % (w/v) agarose gels as described in section 2.5.2.2.

2.5 Agarose gel electrophoresis

2.5.1 Genotyping

7 µl of the PCR products were electrophoresed through 2% (w/v) LE (SeaKem™, BMA) agarose gel in 0.5x TBE (22.25 mM Tris-borate (pH 8.3), 0.5 mM EDTA), containing 0.5 µg/ml ethidium bromide for 1-2 hours at 200V using Bio-Rad power packs, to allow the separation of the two alleles (see section 2.2.4.2). The marker ΦX174 DNA digested with *HaeIII* was used providing the adequate size range to

identify the size of the two alleles. Electrophoresis tanks were made in-house (Bio/Medical Joint Workshop, University of Leicester).

2.5.2 Southern blotting analysis

2.5.2.1 Genomic DNA

Electrophoresis tanks were manufactured in-house (as in section 2.5.1). Gel electrophoresis was carried out using a 40cm horizontal submarine 0.8% (w/v) LE (SeaKem™, BMA) agarose gel in 1x TBE (44.5 mM Tris-borate (pH 8.3), 1 mM EDTA) buffer containing 0.5 μg/ml ethidium bromide. 10-20 μg of the *Alu*I digested DNA samples were run alongside a 1 kb DNA ladder for sizing. DNA samples were run for 36-48hours at 110-130V depending on the allele size using Bio-rad and Fisher Scientific power packs. DNA was visualised over the course of the run using a UV wand (Chromato-vue UVM-57, UVP Life Sciences). DNA samples were run until the 2-4 Kb band of the 1 Kb ladder was at the end of the gel (depending on the size of the allele to be scored).

2.5.2.2 PCR products

Electrophoresis tanks were manufactured as in section 2.5.1. Gel electrophoresis was carried out using a 40 cm horizontal submarine format. Samples were run on a 1% (w/v) LE (SeaKem™, BMA) agarose gel in 1x TBE (44.5 mM Tris-borate (pH 8.3), 1 mM EDTA) buffer containing 0.5 μg/ml ethidium bromide. 7 μl of the PCR products were run alongside 100 bp DNA ladder for allele sizing. PCR products were run at 110V for 24-28 hours using Bio-rad and Fisher Scientific power packs. PCR products were run until the 500 bp band had run off the end of the gel.

2.6 Southern blotting

2.6.1 Genomic DNA

Following electrophoresis, the bottom 30cm of agarose gel was cut and inverted before the blotting procedure. The gel was depurinated in 0.25 M HCl for 2x10 mins. Depurinating the DNA allows it to be cleaved more readily by NaOH. The gel was then alkali-denatured in 0.5 M NaOH, 1 M NaCl for 2x20 mins, which denatures and

cleaves the DNA into smaller fragments and neutralised in 0.5 M Tris-HCl pH 7.5, 3 M NaCl for 2x10 mins. DNA was transferred to a pre-soaked (2x SSC) MAGNA nylon mambrane (GRI, Osmonics Laboratory Products) by capillary transfer using 20x SSC as a transfer buffer (Southern, 1975). Blotting was done for 5 hours or overnight. Following blotting the membrane was rinsed in 2x SSC, dried at 80°C and the DNA was covalently linked to the membrane by exposure to $7x10^4$ J/cm^2 of UV light in a RPN2500 ultraviolet crosslinker (Amersham Biosciences).

2.6.2 PCR products

Following electrophoresis, the PCR gels were blotted in the same manner as the genomic gels (see section 2.6.1) except for the time in each of the blotting solutions. The gels were depurinated for 2x5 mins, denatured for 1x8 mins and 1x15mins and neutralised for 1x8 mins and 1x20 mins.

2.7 Synthesis and purification of synthetic repeat probes

2.7.1 Probe synthesis

Synthetic repeat probes for the ESTR loci *Ms6-hm* and *Hm-2* were produced by PCR amplification of synthetic primers. All probe synthesis PCR reactions were done using 11.1x PCR buffer (Jeffreys el al., 1990). This buffer was produced within the laboratory by R. Neumann using the components in Table 2.1.

Ms6-hm probe synthesis was carried out in 20 µl PCR reactions with 2 µl 11.1x PCR buffer (Table 2.1), 1 µM of each primer and 2.5 Units *Taq* polymerase.
PCR reaction for Hm-2 probe synthesis were performed in 7 µl reactions containing 0.63 µl 11.1x PCR buffer, 0.4 µM of each primer and 0.07 Units *Taq* polymerase.
The repeat specific primers act as a template, producing varying lengths of pure repeat sequence.

Component	Concentration of stock solutions	Final concentration in 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	4.4µM
dATP	100 mM	1 mM
dCTP	100 mM	1 mM
dGTP	100 mM	1 mM
dTTP	100 mM	1 mM
BSA	50 mg/ml	13µg/ml

Table 2.1: Components of 11.1x PCR buffer

2.7.2 Probe purification

Synthesised PCR products for both Ms6-hm and Hm-2 were purified using a QIAquick PCR Purification kit (Qiagen) as per the manufacturer's instructions.

2.8 DNA hybridisation

2.8.1 Random oligonucleotide labelling of DNA fragments

Pure repeat DNA for each locus was produced as in section 2.7, and 10 ng of this double stranded DNA was labelled by the random primed labelling reaction (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) which uses randomly generated hexamers and the *E. coli* DNA polymerase Klenow fragment to incorporate α-^{32}P-dCTP (1000Ci/mmol, NEN, Belgium) into newly synthesised DNA.

The labelling reactions were carried out in a reaction volume of 30 µl and incubated at 37°C for 1-5 hours or at room temperature overnight. The probe was recovered from the unincorporated deoxyribonucleotides by ethanol precipitation using 100 µg high molecular weight salmon sperm DNA (sigma-Aldrich) as a carrier. The probes were

resuspended in 400 µl of ultrapure water, and boiled for 6 minutes to denature prior to use.

During the course of the study the above technique was found to be inadequate for some of the genomic Southern blots, so a kit was introduced.

The random Primers DNA Labelling System supplied by Invitrogen works in the same way as the previous method, using randomly generated hexamers and the *E. coli* DNA polymerase Klenow fragment to incorporate α-^{32}P-dCTP (1000Ci/mmol, NEN, Belgium) into newly sysnthesised DNA.
The labelling reaction was carried as per the supplier's instructions in a 30 µl reaction and incubated for 1 hour at room temperature. The probes were recovered from the unincorporated deoxyribonucleotides by passing them through a nick column™ containing Sephadex® G-50 DNA grade using column wash (1x TE (pH8.0), 0.1% (w/v) SDS). The probes were collected from the columns (400 µl) and boiled for 6 minutes to denature prior to use.

2.8.2 Hybridisation

Membranes were pre-hybridised for at least 30 mins at 65°C in 7% (w/v) SDS, 0.5 M Na2PO4 (pH 7.2), 1 mM EDTA, modified from Church and Gilbert (1984). Approximately 15ml of hybridisation solution (7% (w/v) SDS, 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA) containing the appropriate labelled probe was used for hybridisation. Membranes were hybridised at 65°C for 5 hours or overnight in Maxi 14 or Mini 10 hybridisation ovens (ThermoHybaid).

2.8.3 Post-hybridisation washing

Following hybridisation membranes were washed once for 10 mins in phosphate solution (40 mM sodium phosphate, 0.5% (w/v) SDS) and then 2-5x in high stringency wash solution (0.1% (w/v) SSC, 0.01% (w/v) SDS) for 10 mins each, until the counts/second of the wash solution recovered was less than 5 counts/second.

2.8.4 Autoradiography

Membranes 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 2 hours to one week depending on the strength of signal.

2.8.5 Mutation scoring

Autoradiographs were scored over a well-resolved region, 2-20 Kb (region depends on study). The scoring criteria for all studies was that where a band showed a shift of greater than 1 mm from the size of the parental allele it was considered to be a mutant. Mosaic bands (individuals with greater than two alleles per loci) were not included in the final analysis.

In the results section data is presented for both the total number of mutations scored per locus and for the number of independent mutations scored.

Independent/singleton mutations are mutations that only occur once within a litter. The analysis of independent mutations rather than total mutations takes into account the number of identical mutations shared by more than one offspring in a litter, or mutation clusters. Mutation clusters can be explained by a single mutational event that occurred during gametogenesis and therefore can be scored as a single mutation. When mutation rates for independent mutations and total mutations were compared it was found that there was no significant difference between the two sets of data. Total number of mutations observed was used for statistical analysis. To prevent any bias during scoring all samples were coded and scored blind. All autoradiographs were scored and then checked by a second individual.

Examples of mutations can be observed in Figure 2.1 and Figure 2.2.

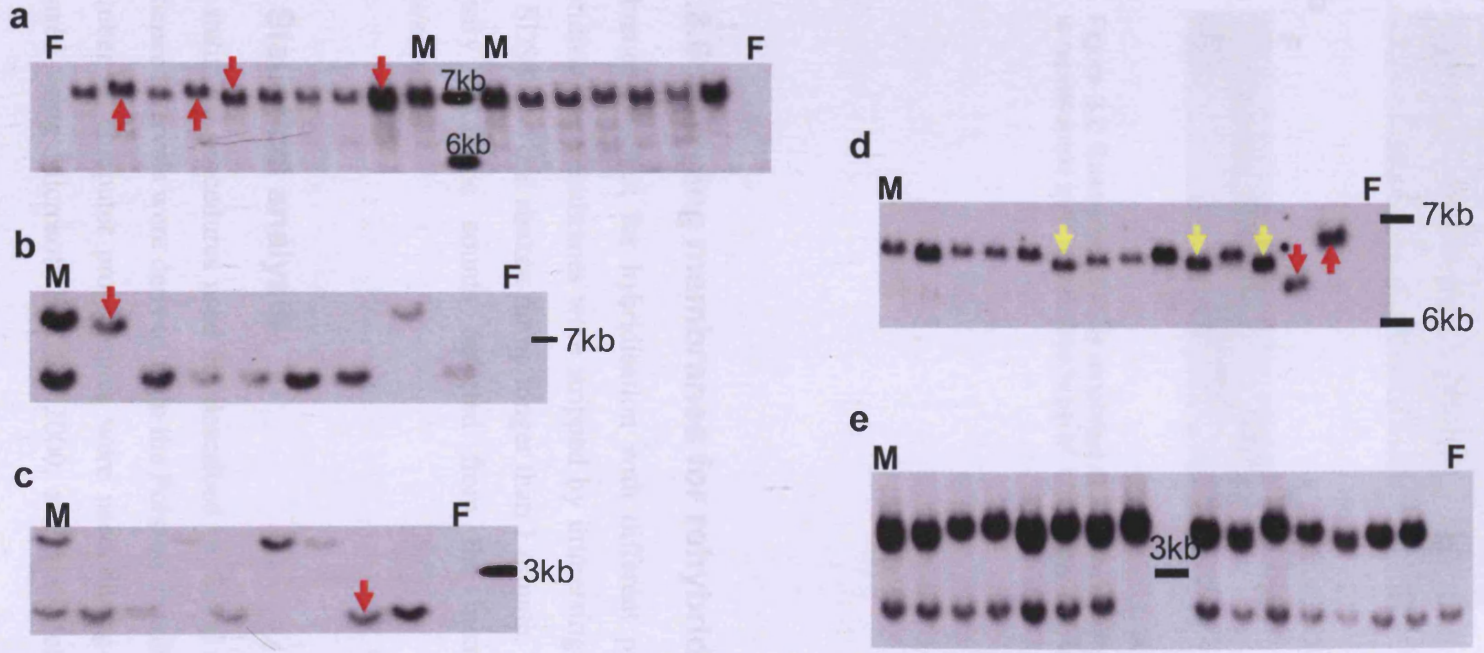


Figure 2.1: Examples of ESTR mutations of the paternal allele, detected by Southern blotting .
Father's allele is represented by F and mother's by M. Mutations are represented by red arrows and shared mutations are indcated by yellow arrows.
a, b and c examples of *Ms6-hm* alleles from DNA repair deficient strains and d and e examples of *Hm-2* alleles from DNA repair deficient strains.

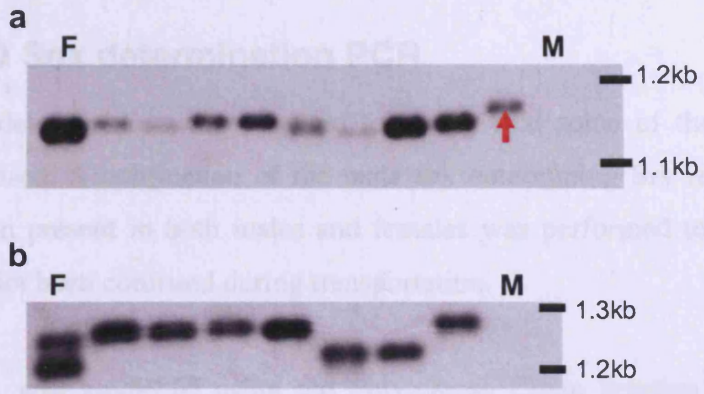


Figure 2.2: Examples of PCR amplified *Hm-2* alleles of p53 deficient mice. Father's allele is represented by F and mother's by M. Mutations are represented by red arrows.

2.8.6 Stripping membranes for rehybridisation

Membranes required for hybridisation with different probes were stripped before rehybridisation. Membranes were stripped by immersing in boiling water containing 0.1% SDS (w/v) and shaken for no longer than 1 minute. This process was repeated if necessary until the counts detected from the membrane were less than 5 counts/second.

2.9 Statistical analysis

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

A number of computer programmes were used during data analysis; results were databased using Microsoft EXCEL 2000, and statistical analysis was done using

software written in Microsoft Basic by Y. E. Dubrova and SYSTAT, version 10 (2000), SPSS Inc and STATISTICA, Data analysis software system, Version 6 (2001), Statsoft Inc (both commercially available).

2.10 Sex determination PCR

Sex determination was required to clarify that some of the samples had not been confused. Amplification of the male sex determining Sry region and the Myogenin region present in both males and females was performed to verify that the samples had not been confused during transportation.

DNA was amplified using the Polymerase Chain reaction (PCR). Reactions were performed using a PTC-220 DNA Engine Peltier thermal cycler (MJ Reseach).

Method adapted from McClive and Sinclair (2001).
Sex determining PCRs were performed using 10x reaction buffer (750mM Tris-HCl (pH8.8), 200mM $(NH)_4SO_4$. 0.1% (v/v) Tween20) with $MgCl_2$, supplied with the *Taq* polymerase (ABgene).

Amplification was performed in 10 µl reactions containing 0.2 mM dNTPs, 0.5 µM MyoF primer, 0.5 µM MyoR primer, 0.5 µM SryF1 primer, 0.5 µM SryR1 primer, 0.5 Units of Taq polymerase (ABgene), 1x PCR buffer and 1.5 mM $MgCl_2$. DNA extracted from the tails of mice were used as a template at 1/10 and 1/100 dilutions. Amplification was done in thin-walled 0.2 ml PCR tubes (ABgene). Amplification was performed after by an initial denaturing step at 94°C for 5 mins, then cycling at 94°C for 20 secs, 60 °C for 40 secs and 72 °C for 1 mins for 30 cycles. The PCR products were run on a 2 % (w/v) agarose gel (section 2.5.1) to separate the male (300 bp) and female alleles (245 bp).

Primers

Primers for probe synthesis

Sequence similarities
Ms6-hm G|GGCA|
Hm2 |GGCA|

Loci	Primer	Primer sequence
Ms6-hm	HMA	GGGCAGGGCAGGGCAGGGCA
	HMB	CCGTCCCGTCCCGTCCCGTC
Hm2	HM2FOR	GGCAGGCAGGCAGGCAGGCA
	HM2REV	GTCCGTCCGTCCGTCCGTC

Primer sequences designed by members of the laboratory group.

Primers for Hm2 amplification and sequencing

Primer	Primer sequence
Hm2A	CATGTCCACCTGTCTCTGAC
Hm2B	GGTGTAGCCTGATTAGAGGA
Hm2C	GATGACTGTCAGAGCAGGGA
Hm2D	CCCTCTGCTTTGTGCTTGTG

All primers designed by members of the laboratory group.

Primers for Ms6-hm single molecule analysis

Primer	Sequence
Hm1.1F	AGAGTTTCTAGTTGCTGTGA
Hm1.1R	ATGCCTTAGAACTGACTCTC

Designed by members of the laboratory group.

Primers for genotyping

Strain	Primer	Primer sequence	Designed by
p53	P53I4F	ACACACCTGTAGCTCCAGCAC	Taconic, USA
	P53E5R	AGAGTCTCACGACCTCCGTC	Taconic, USA
	OPT-21	GTGTTCCGGCTGTCAGCGCA	Taconic, USA
XPC	XPCin	TATCTCCTCAAACCCTGCTC	Lisa McDaniel, Dallas, USA
	XPCex	ATTGCGTGCATACCTTGCAC	Lisa McDaniel, Dallas, USA
	XPCneo	CGCATAGCCTTCTATCGCCT	Lisa McDaniel, Dallas, USA

Primers for sex determination

Primer	Sequence	Designed by
SryF1	CCTGTCATGGAGTCAGCCTGTC	Karen Burr
SryR1	GTACTAATGAAGTCCCAGGAGC	Karen Burr
MyoF	TTACGTCCATCGTGGACAGC	McClive &Sinclair (2001)
MyoR	TGGGCTGGGTGTTAGTCTTA	McClive &Sinclair (2001)

3 ANALYSIS OF GERMLINE MUTATION RATES AT EXPANDED SIMPLE TANDEM REPEAT LOCI IN PARP-1 DEFICIENT MICE

3.1 Introduction

3.1.1 General introduction

It has been suggested that radiation induced ESTR instability may be attributed to a genome wide increase in DNA repair. The first DNA repair pathway to be analysed in this study is the repair of single strand breaks (SSBs) by poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is a nuclear enzyme that is involved in the posttranlational modification of various proteins including itself, namely poly(ADP-ribosyl)ation (Friedberg, 1995). PARP-1 is involved in the early DNA damage recognition by binding rapidly to SSBs, blocking accidental homologous recombination and allowing access to other repair enzymes (Herceg et al., 2001).

3.1.2 Characterisation of PARP-1 null mutation in mice

3.1.2.1 Generation of PARP-1 knockout

Three PARP-1 knockout mice have been generated by disrupting different exons in different backgrounds. The knockout mouse used during this study was generated by Menissier de Murcia et al (1997). The inactivation of PARP-1 was achieved by homologous recombination in embryonic stem cells from 129/Sv mice by inserting a PGK-neo cassette in the fourth exon. Interruption of exon 4 disrupts the first four exons that are responsible for the DNA binding activity of the protein and abolishes the expression of any imunodetectable PARP-1. The transformed cells were then injected into C57BL/6 females generating knockouts on a 129Sv/C57BL/6 genetic background.

Although only the exon 4 knockout properties will be discussed in full below, another two knockouts have been generated by disrupting exon 2 in a 129Sv/C57BL/6 background and exon 1 in a 129Sv/ICR background (reviewed in Masutani et al., 2000).

3.1.2.2 PARP-1 null mice phenotype

PARP-1 null mice develop normally and are fertile, suggesting that PARP-1 is not essential for embryonic development, cell proliferation and differentiation (Menissier de Murcia et al., 1997). Null mice displayed no overtly abnormal phenotypes. However, Menissier de Murcia et al. (1997) did show that PARP-$1^{-/-}$ mice produced smaller litter sizes (4.5 pups) compared to PARP-$1^{+/-}$ and PARP-$1^{+/+}$ mice (7.4 pups). They also found that in adulthood PARP null mice weighed significantly less than their wild-type littermates (19g and 23g, respectively).

Initial studies indicated that PARP-1 deficient mice were not cancer prone (Wang et al., 1995; Menissier de Murcia et al., 1997), however a more recent study showed that after 8-10 generations PARP-1 deficient mice have high tumour susceptibility compared to multigeneration wild-type control mice (Tong et al., 2001). They found that PARP-1 null mice developed a wide spectrum of tumours, mainly adenomas and carcinomas between the ages of 12-24 months, indicating that PARP-1 is involved in the late onset of tumour development. It has also been suggested that PARP-1 may work with other nuclear proteins (including p53 and DNA-PK) as a tumour suppressor by minimising aberrant chromosomal recombination (Wong et al., 2001).

3.1.2.3 PARP-1 mice sensitivity to mutagens

PARP-1 null mice have been shown to be hypersensitive to both chemicals and ionising radiation. After exposure to 8 Gy whole body γ-irradiation, all PARP-$1^{-/-}$ mice died within nine days, however over half their wild-type littermates survived at least 8 weeks (Menissier de Murcia et al., 1997). This indicates that PARP-1 plays a role in the recovery after γ-irradiation. Through this study it was also shown that irradiation of PARP-$1^{-/-}$ mice resulted in severe loss and shortening of the villi in the small intestine which could lead to death through systematic dehydration in the mice.

Menissier de Murcia et al. (1997) also studied the effect of alkylating agent, N-methyl-N-nitrosourea (MNU) on PARP null mice. They found that after intraperitoneal injection of MNU, all PARP null mice were dead within 9 days, whereas none of the wild-type mice died within 9 days, although after 15 days half of them were dead. Trucco et al. (1998) examined the sensitivity of PARP-1 null mice to another alkylating agent, methylmethanesulfonate (MMS). They found that cells from

PARP-1 null mice in comparison with the wild-type cells were hypersensitive to MMS.

All three PARP-1 knockouts are sensitive to both ionising radiation and alkylating agents (reviewed in Masutani et al., 2000), therefore indicating that PARP-1 is part of the cellular response to both radiation and chemical induced damage.

PARP-1 deficient cells were analysed using the comet assay, by measuring the tail moment (Trucco et al., 1998). These authors observed that PARP-1 null cells repair DNA breaks induced by MMS, much more slowly than wild-type cells, although the PARP deficient cells do repair all the breaks eventually. This indicates that in PARP-1 knockout mice the repair of SSBs is not impaired but it is delayed possibly due to lack of initial recognition.

3.1.2.4 The effects of PARP-1 on genomic stability

The results of recent publications show that PARP-1 mice exhibit an increased instability. For example, it was observed that PARP-1 null cells show a 4-5 fold increase in the frequency of sister chromatid exchanges (SCE) (Menissier de Murcia et al., 1997). After exposure to MNU the frequency of chromatid breaks was almost five fold higher in PARP-1 null cells. They also showed that exposure to 2 Gy γ-radiation could cause a two fold increase in chromatid and chromosome breaks in the PARP-1 knockout cells. Another study by Trucco et al. (1998) showed that the frequency of micronuclei detected in PARP-1 deficient cells treated with MMS was increased 3 fold compared to wild-type cells. These results are consistent with results obtained from another PARP-1 knockout mouse, which showed higher frequency of SCE and increased frequency of micronuclei (Wang et al., 1997).

Additionally PARP-1 knockout mice have been shown to display telomere shortening and severe chromosomal instability characterised by increased frequencies of chromosome fusions, breaks and aneuploidy (d'Adda di Fagagna et al., 1999).

All of these studies reveal that PARP-1 knockout mice have increased genomic instability and that PARP-1 may an important role in the maintenance of genomic integrity.

3.2 Experimental design

This experiment was designed to determine whether PARP-1 deficiency in mice affects spontaneous and/or radiation induced ESTR instability.

To observe spontaneous ESTR mutation rates, PARP-$1^{-/-}$ and PARP-$1^{+/+}$ male mice were crossed with untreated CBA/J females to obtain control offspring. The use of CBA/J females allowed establishment of parental origin of mutant bands due to difference in maternal and paternal allele size. All the parents and offspring were profiled using two mouse-specific hypervariable single-locus ESTR probes, Ms6-hm and Hm-2.

PARP-$1^{-/-}$ and PARP-$1^{+/+}$ male mice were given whole body acute irradiation of 1 Gy γ-rays at 10-12 weeks of age. Irradiated males were mated with CBA/J untreated females 10 weeks post-irradiation to produce exposed offspring, ensuring litters were derived from irradiated A_s spermatogonia.

3.3 Summary of mutation induction at ESTR loci

Table 3.1 provides a summary of the ESTR mutation data. The number of ESTR mutations observed for both the loci, Ms6-hm and Hm-2 are shown for $PARP^{+/+}$ and $PARP^{-/-}$ control males (non-exposed) and for males for both genotypes after exposure to 1 Gy acute whole body exposure to X-rays. Details of the total number of mutants scored for each locus and the total number of singleton mutations are provided. Scoring of singleton mutants takes into account mutations shared by more than one offspring in a litter, therefore taking into account apparently identical mutations. Mutation clusters may represent a single mutational event and may be scored as such. The data presented in this thesis is based on the total number of mutations observed, as there is no significant difference seen when comparing mutation rates for total number of mutations or for number of singleton mutations.

The summary also presents data on the type of mutations observed for both genotypes, for exposed and non-exposed groups. It can be seen that there is no significant difference in the number of gains or losses between the exposed or non-exposed groups for both genotypes.

Strain, dose	No offspring	No mutations*			Type of mutants†	
		Ms6-hm	*Hm-2*	Total	Gains	Losses
PARP-1$^{+/+}$						
0 Gy	105	9 (6)	8 (8)	17 (14)	10	7
1 Gy	95	22 (20)	24 (17)	46 (37)	25	20
χ^2, *df*=1 (Prob)‡					0.05	(0.8231)
PARP-1$^{-/-}$						
0 Gy	115	22 (18)	25 (20)	47 (38)	26	20
1 Gy	87	25 (21)	16 (12)	41 (33)	20	20
χ^2, *df*=1 (Prob)‡					0.36	(0.5485)

* Number of singleton mutations is given in parenthesis.
† For some mutants the progenitor allele could not be established.
‡ Chi-square test for homogeneity of the type of mutants between control and irradiated males.

Table 3.1: Summary of ESTR mutation data for PARP-1 knockout and wild-type male mice

3.4 Effect of dose and genotype on ESTR mutation rates

ANOVA analysis was performed on the data to determine whether the genotype and/or the dose had an effect on the paternal ESTR mutation rates. It was revealed that both the genotype and the dose had a significant effect on the ESTR mutation rates. A slight interaction between the genotype and dose was also observed.

Source of variation	*df*	F_s	*P*
Genotype	1, 32	7.13	0.0118
Dose	1, 32	13.24	0.0010
Interaction	1, 32	4.88	0.0344
$R^2 = 0.4449$			

F_s, P and R^2 are the sample statistics of F-distribution, its probability and coefficient of determination, respectively.

Table 3.2: ANOVA analysis for effects of the PARP-1 deficiency and radiation on ESTR mutation rate (arc-transformed)

3.5 Spontaneous and radiation-induced ESTR mutation rates in *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ mice

ESTR mutation rates for control and exposed *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ are presented in Table 3.3 and Figure 3.1. The data provides evidence that *PARP-1*$^{+/+}$ mice showed mutation induction after pre-meiotic exposure to ionising radiation. Irradiated *PARP-1*$^{+/+}$ males showed a significant 3-fold increase in germline mutation rate when compared to non-exposed males. Interestingly, *PARP-1*$^{-/-}$ males were found to have a significant 2.5-fold increase in spontaneous ESTR mutation rate when compared to their isogenic counterparts. However, they did not show a detectable elevation in mutation rate after exposure to 1 Gy of ionising radiation.

		Exposed to non-exposed		Non-exposed to *PARP-1*$^{+/+}$	
Strain, dose	Rate	Ratio	P^*	Ratio	P^*
PARP-1$^{+/+}$					
0 Gy	0.0810	-	-	-	-
1 Gy	0.2421	2.99	1.51×10^{-5}	-	-
PARP-1$^{-/-}$					
0 Gy	0.2044	-	-	2.52	0.0003
1 Gy	0.2356	1.15	0.5257		

* Probability of difference (Fisher's exact test, two-tailed).

Table 3.3: Spontaneous and radiation induced ESTR mutation rates in the PARP-1 deficient and wild-type male mice

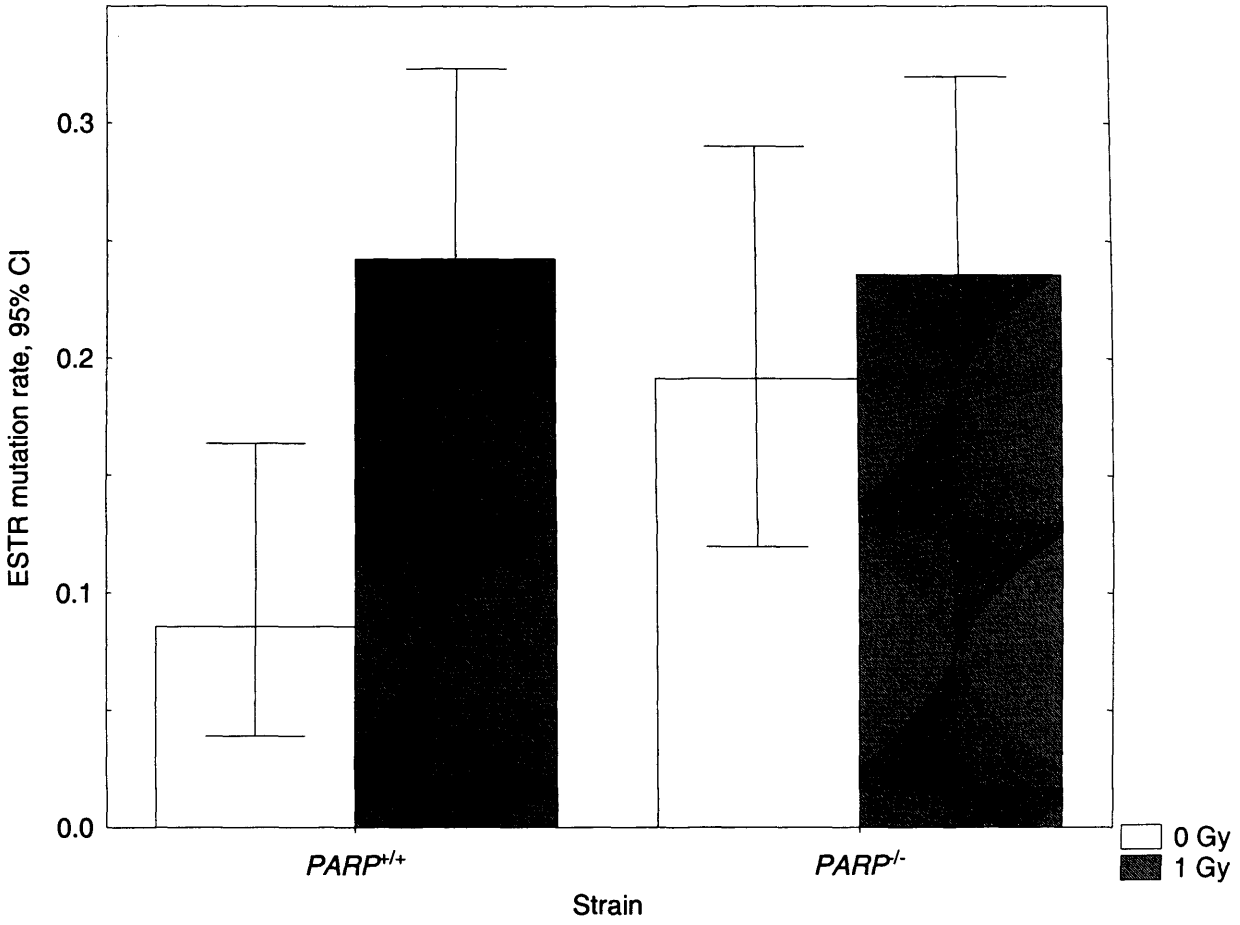


Figure 3.1: Spontaneous and radiation induced ESTR mutation rates in the germline of *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ male mice

3.6 Effect of dose and genotype on litter size

ANOVA analysis of litter size (Table 3.4) indicated that the genotype has no effect on the litter size, however it showed that the dose may have a slight effect on the litter size.

Further analysis of the litter sizes for *PARP-1*$^{-/-}$ and wild-type males (Table 3.5 and Figure 3.2) revealed that non-exposed litter sizes for both genotypes were indistinguishable. Likewise pre-meiotic exposure of *PARP-1*$^{+/+}$ males did not significantly affect the litter size and therefore the fertility of the control males. However, a slight significant decrease was found in litter size in *PARP-1*$^{-/-}$ males after exposure, Suggesting that exposure to ionising radiation affects the fertility of *PARP-1*$^{-/-}$ males.

Source of variation	*df*	F_s	*P*
Genotype	1, 91	0.33	0.5688
Dose	1, 91	5.42	0.0221
Interaction	1, 91	0.49	0.4874
$R^2 = 0.0658$			

F_s, *P* and R^2 are the sample statistics of *F*-distribution, its probability and coefficient of determination, respectively.

Table 3.4: ANOVA analysis for effects of the PARP-1 deficiency and radiation on the litter size

Strain, dose	No litters	Mean ± s.e.m.	*t*	*P*
PARP-1$^{+/+}$				
0 Gy	22	4.86 ± 0.49	-	-
1 Gy	23	4.13 ± 0.41	1.14	0.2568
PARP-1$^{-/-}$				
0 Gy	25	4.92 ± 0.49	-	-
1 Gy	25	3.56 ± 0.40	2.15	0.0368

t and *P* are the Student's test and probability, respectively.

Table 3.5: Mean litter size for the *PARP-1*$^{-/-}$ and wild-type males

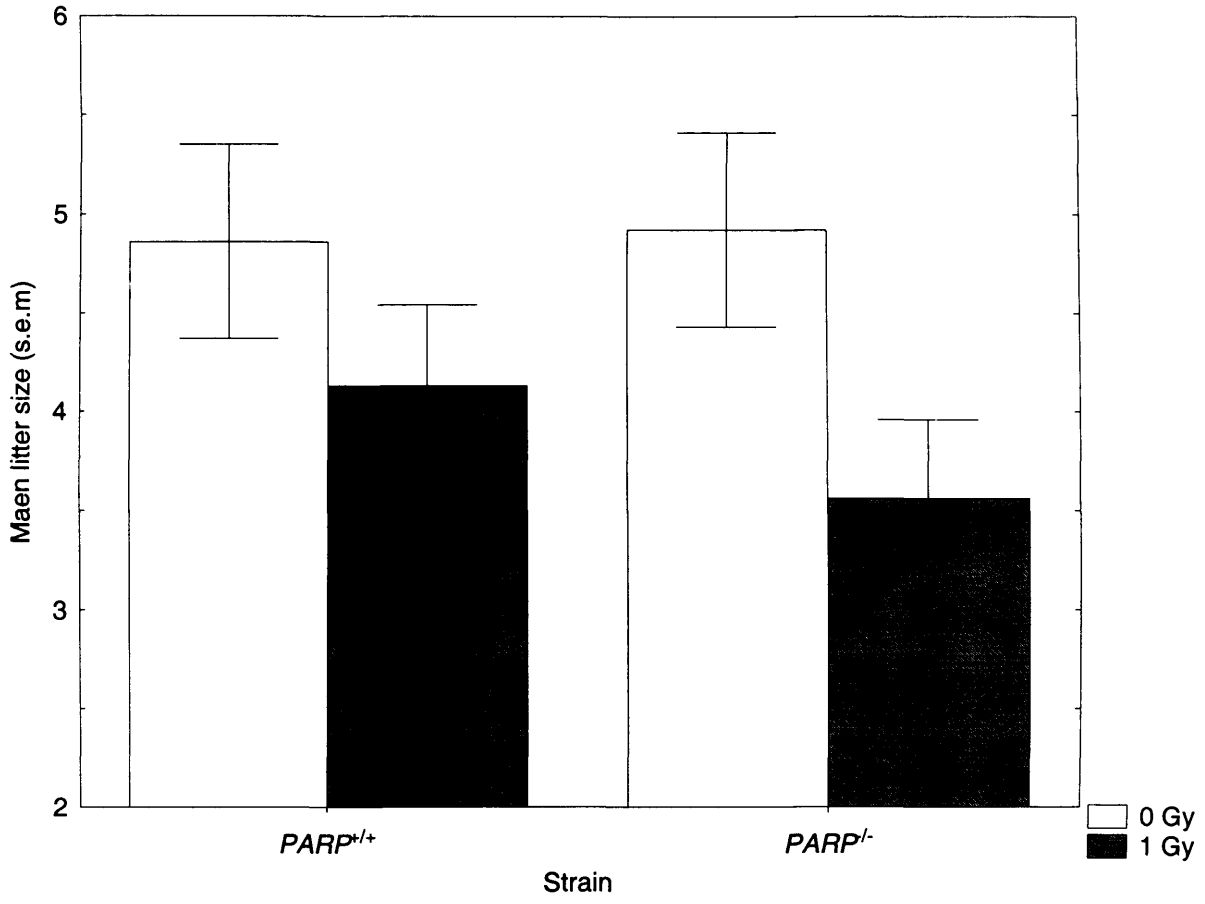


Figure 3.2: *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ litter sizes for exposed and non-exposed male mice

3.7 ESTR mutation spectrum in PARP-1 deficient mice

The mutation spectra for exposed and non-exposed males did not significantly differ; therefore the data was combined for further analysis. Despite the fact that the spontaneous and radiation induced ESTR mutation rates were significantly different between *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ males, the ESTR mutation spectra for *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ were indistinguishable (Figure 3.3). This indicates that although the mutation rates are different that the mechanism of spontaneous and induced mutation is the same.

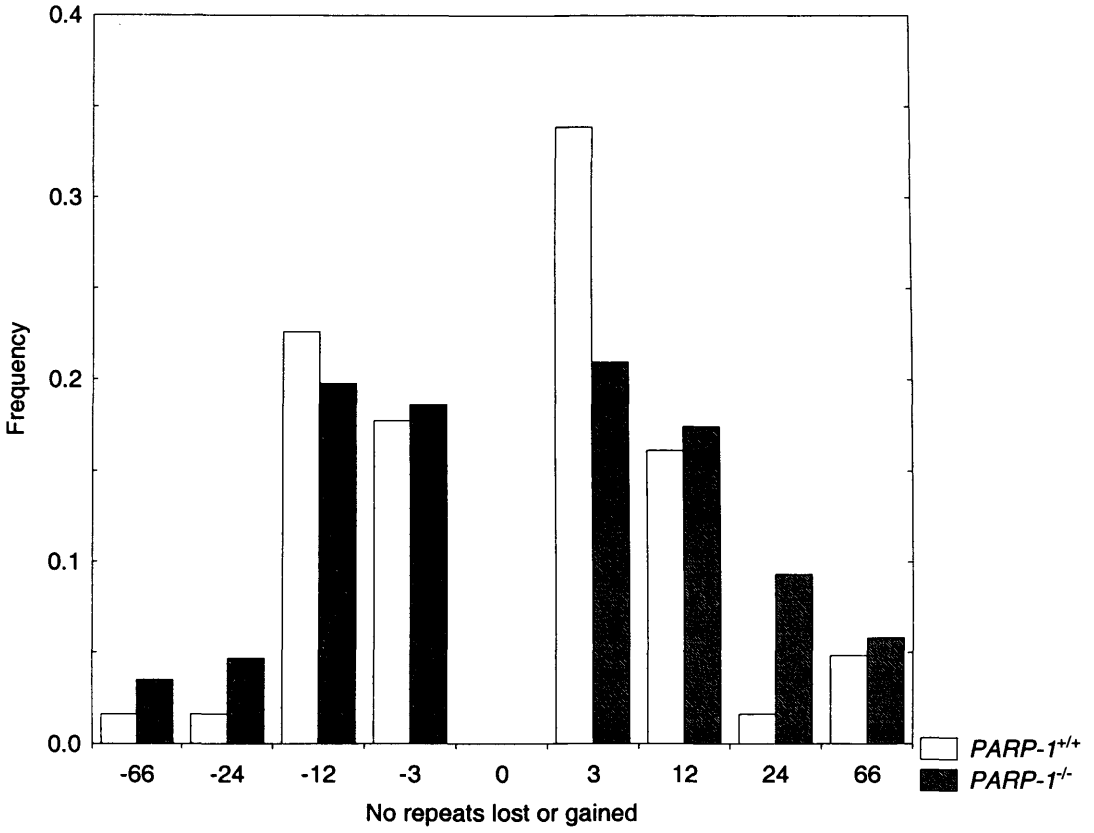


Figure 3.3: ESTR mutation spectrum of *PARP-1*$^{+/+}$ and *PARP-1*$^{-/-}$ male mice (Kolmogorov-Smirnov test, $P>0.10$). Values on the x-axis represent the lower limit of repeat units within bin.

3.8 Discussion

Little is known about the effects of DNA repair deficiency on spontaneous and radiation induced mutation in the germline. The aim of this study was to determine whether *PARP-1* deficiency affected ESTR instability in the mouse male germline. PARP-1 is known to be involved in many important cellular processes including the recognition and repair of DNA single strand breaks. By analysing *PARP-1* deficient mice it was hoped that we could determine whether ESTR instability was possibly a by-product of this DNA repair response gene. It was proposed that through this work it may be possible to shed some light on the mechanisms that act at ESTR loci causing instability.

PARP-1 deficient mice were chosen as they exhibit hypersensitivity to ionising radiation (Menissier de Murcia et al., 1997) and increased genomic instability (Menissier de Murcia., 1997; Wang et al., 1997; Trucco et al., 1998). All previous studies to date have been performed by analysing spontaneous and induced frequencies of genomic instability within somatic cells of PARP-1 null mice. The germline stability of PARP-1 null mice has never been analysed.

The wild-type and PARP-1 deficient mouse strains used were isogenic so all differences observed can be attributed directly to the *PARP-1* gene. The data presented show that *PARP-1*$^{+/+}$ mice have a similar spontaneous mutation rate as previously analysed C57BL/6 strains (data not shown). *PARP-1*$^{+/+}$ mice exhibited a significant increase in ESTR mutation rate after exposure to ionising radiation. In contrast, *PARP*$^{-/-}$ mice show a highly elevated spontaneous mutation rate compared to the isogenic strain, but there was no evidence of mutation induction after exposure to ionising radiation. This data support previous findings that PARP-1 is involved in genomic instability, including the paternal germline.

The increase in spontaneous mutation rate in the germline of *PARP*$^{-/-}$ mice may be explained by the accumulation of unrepaired single strand breaks within the ESTR loci being converted into double strand breaks during replication therefore increasing the instability of ESTRs. This cannot be the case as PARP-1 null mice develop normally although numerous SSB are generated during replication and via other modes, therefore the deficiency of PARP-1 does not significantly affect the repair of SSB lesions (Herceg et al., 2001).

The lack of radiation induced mutation in *PARP*$^{-/-}$ mice could be explained by the high cell killing effects of ionising radiation on the germline of DNA repair deficient mice. PARP-1 null mice are known to be sensitive to ionising radiation, shown by a high lethality following exposure (Menissier de Murcia et al., 1997). Further evidence of a high killing effect of ionising radiation on PARP-1 deficient cells comes from the litter size results. The litter size data show that PARP-1 has no effect on litter size in non-exposed individuals; however PARP-1 deficiency affects the fertility of exposed males. This decrease in fertility indicates that the *PARP*$^{-/-}$ male germline is slightly more radiosensitive than the wild-type germline. PARP-1 is known to be directly involved in the modification of p53 and to mediate apoptosis, *PARP*$^{-/-}$ cells show a decrease in p53 accumulation and activation (Valenzuela et al., 2002). However, Trucco et al., (1998) showed that the sensitivity of PARP-1 deficient cells to mutagens could be attributed to cell death via necrosis after G2/M arrest. It is plausible that the lack of radiation induced ESTR mutation in *PARP*$^{-/-}$ males could be due to preferential killing of irradiated germ cells via necrosis.

The ESTR germline mutation spectra in PARP-1 deficient and wild-type mice were similar, indicating that spontaneous and radiation induced mutation occur via the same mechanism. To get more insight into the mechanism underlining instability in the germline of *PARP-1*$^{-/-}$ mice, the results of this study were compared with the data on ESTR mutation rates in *scid* mice (Barber et al., 2000). *scid* mice carry a nonsense mutation in the catalytic subunit of DNA-protein kinase (DNA-PK_{cs}) (Blunt et al., 1996). These mice are deficient in the recognition and repair of double strand breaks through non-homologous end joining (Biedermann et al., 1991; Blunt et al., 1996). When the results from these two studies were compared it was found that they were very similar. Both DNA repair deficient strains have an elevated spontaneous ESTR mutation and lack ESTR mutation induction after exposure to ionising radiation. The spectra of spontaneous ESTR germline mutations for the deficient and wild-type strain were similar for both groups; therefore the mechanism of mutation may be the same for both strains. Both scid and *PARP-1*$^{-/-}$ mice are deficient in early DNA damage response. Both deficient strains are involved in the early recognition of SSBs or DSBs, this inability to recognise damage may lead to a delay in repair and therefore alter the cell cycle progression. Both strains could therefore display a delay in repair of spontaneous and radiation induced damage.

In *scid* mice, repair of DSBs may occur via homologous recombination which occurs during the late S-G2 phase of the cell cycle, whereas in normal cells most DSB repair would occur via NHEJ during G1-early S phase (Takata et al., 1998). This indicates that in NHEJ deficient mice the repair of DSBs would be delayed. Likewise, there is evidence that the lack of PARP-1 could lead to a delay in SSB repair. A study has shown that the depletion of PARP-1 by antisense RNA results in a considerable delay in the repair of SSBs in mammalian cells (Ding et al.,1992). It is proposed that this delay in repair in both strains could cause an increase in replication fork pausing, which in turn could promote polymerase slippage. Polymerase slippage causes insertions and deletions of DNA, consistent with the type of mutations observed at ESTR loci. This model could also be used to explain the high spontaneous ESTR mutation rates observed in normal mouse strains, as both the length and the complex secondary structure of these loci could promote replication fork pausing and therefore polymerase slippage.

ESTR loci consist of relatively short homologous repeats and therefore may be regarded as highly expanded microsatellite loci. For these loci it has been shown that in microsatellites the longer the repeat array the more replication pausing will occur (Kang et al., 1995). The same group also showed that in the presence of complex secondary structures, such as hairpin loops within expanded triplet repeat microsatellite loci could lead to inhibition of polymerase progression and therefore subsequently promoting polymerase slippage events. It has been found that *Ms6-hm* ESTR locus consists of pentamer repeats that are capable of forming hairpin structures and intrastrand tetraplexes under physiological conditions (Weitzmann et al., 1998). This is evidence that ESTR instability may be due to their size and complex secondary structure causing inhibition of polymerase progression which in turn would lead to polymerase slippage. This model is supported by evidence from recent studies comparing the rates and mutation spectra of ESTR mutation in the germline and somatic tissues of male mice. This comparison led to the conclusion that ESTR instability was a replication based process and possibily including polymerase slippage (Yauk et al., 2002).

If polymerase slippage is responsible for ESTR instability then replication pausing caused by a delay in repair of DNA damage, as in PARP-1 deficient and *scid* mice, could cause further enhancement of the number of polymerase slippage events at the

ESTR loci resulting in the elevated spontaneous mutation rates observed in the PARP-1 deficient and *scid* mice.

From this study a model for the mechanism of mutation at ESTR loci has been proposed. The model suggests that ESTR instability may be due to polymerase slippage further enhanced by replication fork pausing, similar to the model suggested for microsatellite mutation (see Figure 3.4). If this model is correct then the very high spontaneous mutation rates observed at ESTR loci could be attributed directly to the large size of the repeat loci (500-3500 repeats) and their complex secondary structure, which together could lead to replication fork pausing and consequently promoting polymerase slippage events. This proposed model is consistent with the positive correlation observed between the spontaneous mutation rates and the size of the ESTR loci (Bois et al., 2001).

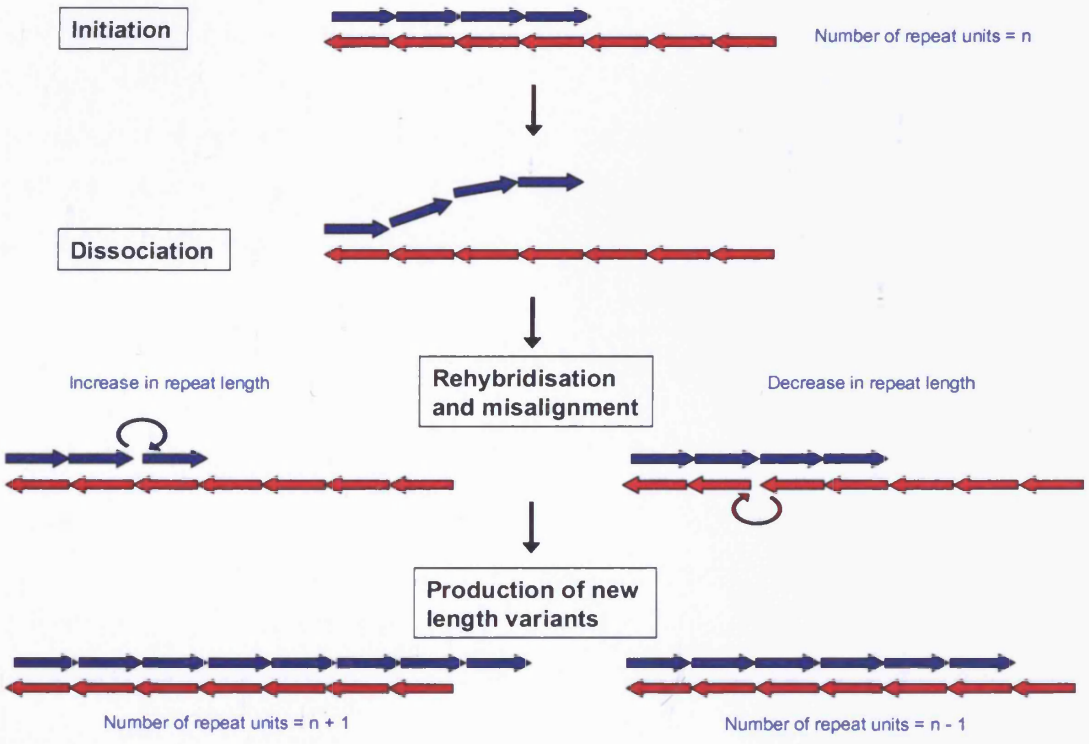


Figure 3.4: Proposed model for ESTR mutation

From this study it has been shown that PARP-1 deficiency causes ESTR instability in the male germline, which has never been observed before. This implies that PARP-1 is involved in the maintenance of germline stability as well as genomic stability as previously reported. The results have revealed that the germline of PARP-1 deficient mice are more sensitive to the high killing effect of radiation than their wild-type littermates. Also from this study a model for the mechanism of mutation at ESTR loci has been proposed.

4 ANALYSIS OF GERMLINE MUTATION RATES AT EXPANDED SIMPLE TANDEM REPEAT LOCI IN P53 DEFICIENT MICE

4.1 Introduction

4.1.1 General introduction

Given the results from the previous chapter indicating that the lack of radiation induced mutations may be due to the preferential killing of irradiated germ cells in both *PARP-1-/-* and *scid* mice, it therefore appears that apoptosis could play an important role in the maintenance of genomic integrity in the germline of exposed mice. The analysis of radiation induced ESTR mutation in mice carrying mutations in genes involved in apoptosis may shed light on whether this high killing effect hypothesis holds true. One such gene involved in apoptosis is the p53 gene. p53 plays a critical role in response to various forms of DNA damage (Vogelstein et al., 2000). The p53 protein is involved in the arrest of the cell cycle to allow adequate repair and the death of cells via apoptosis. Through both these processes p53 prevents the accumulation of mutations (Morris, 2002). In the absence of functional p53, the cell cycle and apoptotic functions of a cell would be lost and genomic instability and cell survival would increase, accelerating oncogenesis.

4.1.2 Characterisation of p53 null mutation in mice

4.1.2.1 Generation of p53 knockout mouse

The frequent association of p53 mutations with both spontaneous and inherited human cancers generated an interest in developing an animal model to study p53-associated cancers. The p53 deficient mouse was the first reported tumour suppressor knockout mouse generated. Donehower et al. (1992) first generated p53-deficient mice by deleting a portion of the p53 gene via recombinant DNA technology. They interrupted exon 5 by inserting a *Pol*II-neo expression cassette. The insertion of the cassette was accompanied by the deletion of 106 nucleotides of exon 5 (including highly conserved p53 domain II) and 350 nucleotides of intron 4. The p53 targeting construct was

electroporated into 129/Sv embryonic stem cells. Stem cells containing the targeted p53 allele were microinjected into 3.5 day old C57BL/6 blastocysts and implanted into pseudopregnant females (F_1 C57BL/6 x CBA) to produce chimaeric mice. The chimaeric males were then mated to C57BL/6 females to produce p53 deficient C57BL/6 x 129/Sv mice.

Protein immunoblotting to assess p53 protein expression showed that only wild-type and heterozygous fibroblasts expressed p53. Southern blotting and RNA-PCR analysis indicated that neither a truncated or a full length p53 protein product was detectable in cells from homozygous mice (Donehower et al., 1992).

Other laboratories have developed different p53 knockout strains via recombination deleting exon 2 through 6 on two different backgrounds; C57BL/6 x 129/Sv and 129/Ola backgrounds (Morris, 2002). However, the knockout containing the deletion of exon 5 was used during this study.

4.1.2.2 p53 null mice phenotype

Mice lacking functional p53 appear to develop normally and lacked any observable gross defects (Donehower et al., 1992). Given that p53 knockout mice not only develop normally but are fertile therefore allowing breeding, provides a useful model to analyse germline mutation induction. Mice deficient in p53 produce normal litter sizes and p53 homozygotes appear normal both morphologically and by histopathology analysis (Donehower et al., 1992).

It has been reported that crossing of heterozygous p53 mice yielded 23% p53 null mice, roughly in keeping with Mendelian ratio of 25% (Donehower et al., 1992). However, subsequent studies have shown that the ratio is smaller, 16% (Jacks et al., 1995). Further studies have shown that a fraction of female null embryos die during embryogenesis due to a neural tube closure defect called exencephaly (Armstrong et al., 1995; Sah et al., 1995). Exencephaly is rarely observed in null males or in their heterozygous or wild-type littermates. This indicates that p53 may have a role in the development during neural closure or that increased mutation rates could be associated with chromosomal events that occur more frequently in p53 null female gametes and embryos.

Although p53 deficient mice develop normally, they have been shown to be susceptible to spontaneous tumour formation (Donehower et al., 1992; Donehower et al., 1996). Donehower et al. (1992) found that all p53 null mice developed tumours by

10 months of age, while mice heterozygous for p53 display a delay in tumour development. Half of the mice heterozygous for p53 developed tumours by 18 months with over 90% incidence by two years, whereas the wild-type littermates do not develop tumours before 18 months with less than 25% developing tumours by the age of two years old. During the studies of tumorgenesis in p53 mice it has been found that p53 null mice develop tumours as early as 6 weeks, while heterozygotes tend to have long tumour free periods usually greater than 9 months (Donehower et al., 1996). The type of tumours observed depends on the genotype and background. p53 null mice tend to have a high incidence of lymphomas, whereas heterozygotes develop osteosarcomas, soft tissue sarcomas and lymphomas (Donehower et al., 1992). The background affects the type of tumour as the absence of p53 exacerbates any prior tumour/cancer predisposition in mice. For example, C57BL/6 mice tend to develop lymphomas at a high incidence at a mean age of 27 months, whereas in p53 deficient C57Bl/6 strains the development of lymphomas is much earlier. Likewise for the 129/Sv background which is susceptible to testicular tumours, p53 deficiency causes tumours to develop sooner than in their wild-type counterparts (Donehower et al., 1996).

The most interesting phenotype of the p53 deficient mouse to human studies is that tumour development in heterozygote mice is similar to that in Li-Fraumeni families, providing a useful model system (Morris, 2002).

4.1.2.3 p53 mice sensitivity to ionising radiation and other mutagenic agents

Cellular levels of p53 are greatly elevated after exposure to ionising radiation and other mutagenic agents. This increase is associated with an arrest in late G1 of the cell cycle (Kuerbitz et al., 1992). This indicates that p53 is required as a response to insult by mutagenic agents.

Cells deficient in active p53 have been shown to be resistant to the lethal effects of ionising radiation (Wang et al., 1996; Alsbeih et al., 2004). However there have been contradicting studies that show cells lacking p53 do not show any resistance to ionising radiation (Slichenmeyer et al., 1993).

After exposure to ionising radiation p53 null and heterozygous mice have been shown to develop tumours more rapidly than wild-type mice and predominantly develop

lymphomas (Kemp et al., 1993). Komarova et al. (2004) have shown that p53 deficient mice can survive doses of ionising radiation that cause lethal haematopoietic syndrome in wild-type animals. Radiation exposed p53 deficient embryos show a higher incidence of malformations in comparison to radiated wild-type mice (70% and 20%, respectively) (reviewed by Donehower et al., 1996).

Mice deficient in p53 have also been shown to be highly susceptible to chemical mutagens. Exposure to chemical mutagens induces various cancers in p53 deficient mice; N-methyl-N-nitrosourea (MNU) induces stomach cancer (Yamamoto et al., 2000), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) increases sensitivity to urinary bladder carcinogenesis (Ozaki et al., 1998), and 1, 2-dimethylhydrazine (DMH) treated p53 deficient mice have an increased incidence of uterine sarcomas (Zhang et al., 2002). p53 deficient mice are also susceptible to teratogenic agents, such as benzo(a)pyrene.

These results suggest that p53 may play an important role as a gatekeeper in carcinogenesis induced by various agents. Wild-type cells undergo prolonged growth arrest after exposure to mutagens whereas p53 deficient cells do not undergo prolonged arrest and exhibit reduced apoptotic ability, therefore allowing mutations to be fixed and passed onto daughter cells more readily (Bielas and Heddle, 2003; Komarova et al., 2004).

4.1.2.4 The effects of p53 on genomic stability

Donehower et al., (1995) first demonstrated that p53 deficiency in mice promoted genomic instability with increased aneuploidy, amplifications and deletions detected by karyotype analysis and comparative genomic hybridisation. It was suggested that p53 deficiency relaxes normal restraints on chromosomal number and organisation during tumourigenesis. Further studies of chromosomal integrity in p53 deficient cells indicated that p53 deficiency allows unrepaired double strand breaks to initiate chromosomal instability (Honma et al., 2000). Cytogenetic techniques have been utilised to analyse the levels of spontaneous and radiation induced stable chromosomal aberrations in heamopoietic tissues of p53 deficient mice (Bouffler et al., 1995). It was found that the spontaneous frequency of aberrations in bone marrow was elevated 20 fold in p53 null mice and 13 fold in p53 heterozygotes compared to wild-type. In the same study the p53 status was seen to have no influence on sister

chromatid exchange or G2 chromatid damage in spleen cells. They also found that exposure to gamma radiation did not affect induced chromosomal aberration in p53 null cells compared to wild-type. However, p53 deficiency did produce a ten fold increase in radiation induced hyperploidy (Bouffler et al., 1995). Wang et al. (1996) also found that the frequency of abnormal bone marrow cells from p53 deficient mice after irradiation was similar in all three genotypes. However, they found that p53 null aberrant cells carried more aberrations than heterozygous or wild-type aberrant cells. All these studies provide evidence that p53 is involved in the maintenance of genome stability.

Several other studies have shown that p53 status affects genomic stability using a method that measures homologous recombination. Pink eyed dilution unstable (p^{un}) mutation has been used to show that p53 influences the reversion to wild-type by deletion of a 70kb duplication via recombination after exposure to radiation (Aubrecht et al., 1999). The recombination frequency after exposure was increased in wild-type and heterozygote but not in null mice, indicating that p53 is involved in DNA damage response following exposure to radiation and p53 deficient mice may process this damage via a different pathway. Another study using the same mutation observed that p53 mutant mice had increased frequency of spontaneous homologous recombination events compared to wild-type (Bishop et al., 2003). This suggests that p53 could be implicated in the maintenance of genomic integrity.

Radiation induced mutation frequencies of the adenine phosphoribosyltransferase (*Aprt*) and hypothanine-guanine phosphoribosyltranferase (*Hprt*) loci were greatly elevated in p53 null mice after exposure to 4 Gy X-rays compared to exposed p53 heterozgotes and wild-type mice (Liang et al., 2002), indicating that the absence of p53 enhances radiation induced mutagenesis. Although there is plenty of evidence that p53 deficiency enhances susceptibility to the effects of ionising radiation at a somatic level there have been reports showing a lack of effect of p53 deficiency in cells on the accumulation of mutations. One such study investigated the spontaneous and radiation induced (1-3 Gy X-rays) thymidine kinase mutation frequencies in p53 null cells (Chuang et al., 1999). This study showed that the lack of p53 protein did not lead to an increase in mutation rate at the *Tk* locus. Another study assessed whether p53 deficiency in cells affected the accumulation of spontaneous and radiation induced point mutations, small deletions and insertions (Sands et al., 1995). It was found that p53 deficient cells have mutation rates that were indistinguishable from

wild-type cells. These results suggest that the role of p53 in tumour suppression does not affect the accumulation of small changes in the genome rather it may have a role in larger changes that cause genomic instability.

All the results show that p53 is involved in genomic instability in one way or another and that increased chromosomal radiosensitivity observed in p53 null mice may be due to reduced cell cycle delay allowing insufficient time for repair and their reduced apoptotic ability.

4.2 Experimental design

To determine whether apoptosis may influence the spontaneous and radiation induced ESTR mutation frequencies p53 deficient mice were analysed.

$p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ males were crossed with untreated CBA/Ca females to obtain control offspring. At reproductive age $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ males were exposed acutely to 1 Gy X-ray whole body irradiation. Irradiated males were then crossed with untreated CBA/Ca females 6 weeks post irradiation.

The use of CBA/Ca females allowed easy allocation of parental origin of mutant bands due to difference in maternal and paternal allele size. All the parents and offspring were profiled using two mouse-specific hypervariable single-locus ESTR probes, Ms6-hm and Hm-2.

Prior to any analysis all males were genotyped using a PCR based assay to verify genotypes of all males.

4.3 Summary of mutation induction at ESTR loci

A summary of the ESTR mutation data can be seen in Table 4.1. The ESTR mutation data for both the *Ms6-hm* and *Hm-2* loci are presented for control $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ male mice (non-exposed) and for $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ male mice that were exposed to 1 Gy of X-rays. The table provides details of total numbers of mutations scored for each locus and the number of singleton mutations. Since there was no significant difference between mutation rates from total mutation data and singleton mutation data, the data presented uses the total number of mutations.

Strain, dose	*Ms6-hm* No mutations*	No bands	*Hm-2* No mutations*	No bands	Total No mutations*	No bands	Type of mutants Gaines	Losses
$p53^{+/+}$								
0 Gy	8 (8)	126	8 (8)	118	16 (16)	244	11	5
1 Gy†	27 (23)	144	16 (14)	119	43 (37)	263	27	15
χ^2, *df*=1 (Prob)‡							0.10	(0.7526)
$p53^{+/-}$								
0 Gy	10 (10)	151	11 (7)	152	21 (17)	303	10	11
1 Gy	23 (19)	113	12 (12)	88	35 (33)	201	17	18
χ^2, *df*=1 (Prob)‡							0.00	(0.9459)
$p53^{-/-}$								
0 Gy	13 (13)	133	10 (7)	125	23 (20)	258	12	11
1 Gy	19 (15)	88	18 (12)	106	37 (27)	194	23	14
χ^2, *df*=1 (Prob)‡							0.56	(0.4524)

* Number of singleton mutations is given in parenthesis.
† For one mutants the progenitor allele could not be established.
‡ Chi-square test for homogeneity of the type of mutants between control and irradiated males.

Table 4.1: Summary of ESTR mutation data for $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ male mice.

Table 4.1 also shows the type of mutations observed in all three genotype before and after exposure. Chi-square analysis shows that there is no significant difference in the numbers of gains and losses in each group.

4.4 Effect of dose and genotype on ESTR mutation rates

ANOVA analysis was performed on the ESTR mutation rate data to compare the variation within the variables (genotype and dose) and between the variables (interaction). The ANOVA results are shown in table 4.2.

It was shown that p53 genotype had no significant effect on the ESTR mutation rate (P=0.7218), however the dose that mice were exposed to did have a significant effect on ESTR mutation rate ($P=2.0\times10^{-5}$). This data suggests that p53 status has no significant effect on ESTR mutation rate, whereas dose does affect ESTR mutation rates.

Source of variation	*df*	F_s	*P*
Genotype	2, 42	0.33	0.7218
Dose	1, 42	23.10	2.0×10^{-5}
Interaction	2, 42	0.95	0.3941
$R^2 = 0.3753$			

F_s, *P* and R^2 are the sample statistics of *F*-distribution, its probability and coefficient of determination, respectively.

Table 4.2: ANOVA analysis for effects of the p53 deficiency and radiation on ESTR mutation rate (arc-transformed)

4.5 Spontaneous and radiation-induced ESTR mutation rates in $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mice

ESTR mutation rates for $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ control and exposed males are given in table 4.3 and Figure 4.1. The frequency of paternal ESTR mutations in $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ after exposure to 1 Gy X-ray increased by 2.49, 2,51 and 2.14 fold respectively, when compared to their non-exposed counterparts. No significant changes were observed when the spontaneous mutation rates of the p53 deficient mice were compared to the spontaneous mutation rate of the $p53^{+/+}$ mice. This confirms the data produced in the ANOVA analysis, that dose does have a significant effect on ESTR mutation rate but the p53 status has no effect on ESTR mutation rates.

		Exposed to non-exposed		Non-exposed to $p53^{+/+}$	
Strains, dose	Rate	Ratio	P^*	Ratio	P^*
$p53^{+/+}$					
0 Gy	0.0656	-	-	-	-
1 Gy	0.1635	2.49	0.0008	-	-
$p53^{+/-}$					
0 Gy	0.0693	-	-	1.06	1
1 Gy	0.1741	2.51	0.0005	-	-
$p53^{-/-}$					
0 Gy	0.0891	-	-	1.36	0.4103
1 Gy	0.1907	2.14	0.0027		

* Probability of difference (Fisher's exact test, two-tailed).

Table 4.3: ESTR mutation rates in the p53 deficient and wild-type male mice

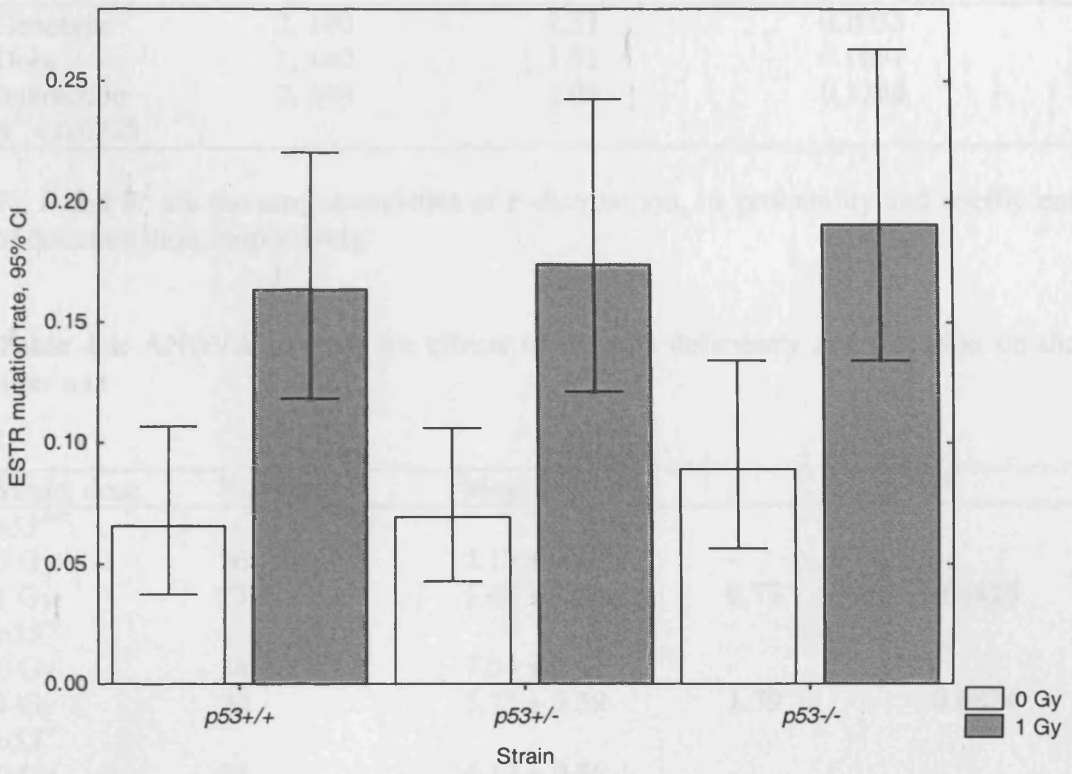


Figure 4.1: Spontaneous and radiation induced ESTR mutation rates in the germline of $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ male mice

4.6 Effect of dose and genotype on litter size

ANOVA analysis was performed on the litter size data to determine whether genotype and/or dose had any effect on the fertility of p53 deficient mice. The ANOVA data is given in table 4.4. The ANOVA analysis shows that neither the genotype or dose had any effect on the litter size. Also the analysis indicated that there was no interaction between the two variables. This data suggests that p53 status has no effect on the fertility whether or not the mice were exposed to ionising radiation. This can be further verified when the raw data is taken into account (Table 4.5). The litter size data shows that there is no significant difference between the litter sizes before or after exposure for all three genotypes. Also it can be seen that there is no difference in litter sizes between the genotypes.

Source of variation	*df*	F_s	*P*
Genotype	2, 140	2.31	0.1033
Dose	1, 140	1.91	0.1691
Interaction	2, 140	2.08	0.1286
$R^2 = 0.0725$			

F_s, *P* and R^2 are the sample statistics of *F*-distribution, its probability and coefficient of determination, respectively.

Table 4.4: ANOVA analysis for effects of the p53 deficiency and radiation on the litter size

Strain, dose	No litters	Mean ± s.e.m.	*t*	*P*
$p53^{+/+}$				
0 Gy	26	5.15 ± 0.50	-	-
1 Gy	27	5.67 ± 0.44	0.77	0.4425
$p53^{+/-}$				
0 Gy	24	7.04 ± 0.42	-	-
1 Gy	20	5.75 ± 0.59	1.79	0.0824
$p53^{-/-}$				
0 Gy	23	6.13 ± 0.36	-	-
1 Gy	26	5.35 ± 0.44	1.37	0.1777

t and *P* are the Student's test and probability, respectively.

Table 4.5: Mean litter size for the p53 deficient and wild-type male mice

Figure 4.2 shows the p53 litter size data, clearly illustrating that there is no significant effect on the litter size due to genotype or dose of exposure.

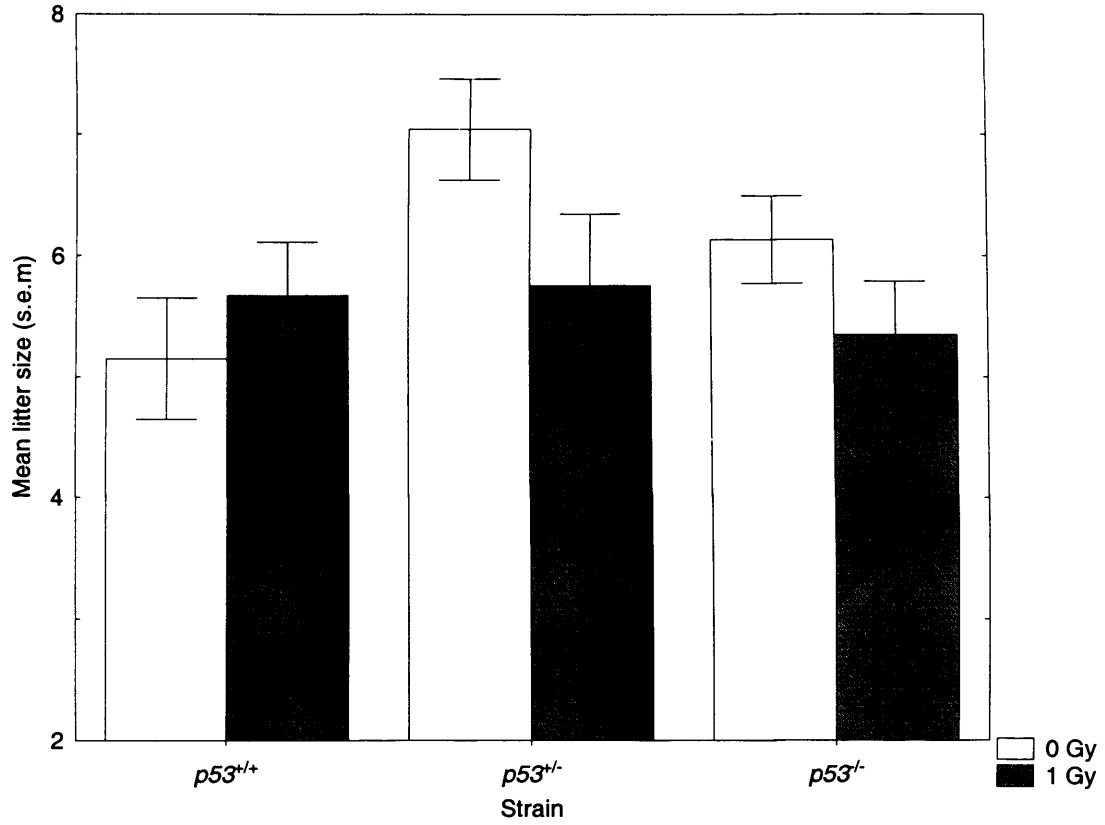


Figure 4.2: $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ litter sizes for exposed and non-exposed male mice

4.7 ESTR mutation spectrum in p53 deficient mice

ESTR mutation spectrum data for exposed and non-exposed mice were combined before statistical analysis as there was no significant difference between the groups. ESTR mutation spectrums for $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mice are shown in Figure 4.3, illustrating that there is no difference between the spectra for all three genotypes. When statistically tested it was found that there was no significant difference between the spectrum of ESTR mutation in $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mice (P=0.1597).

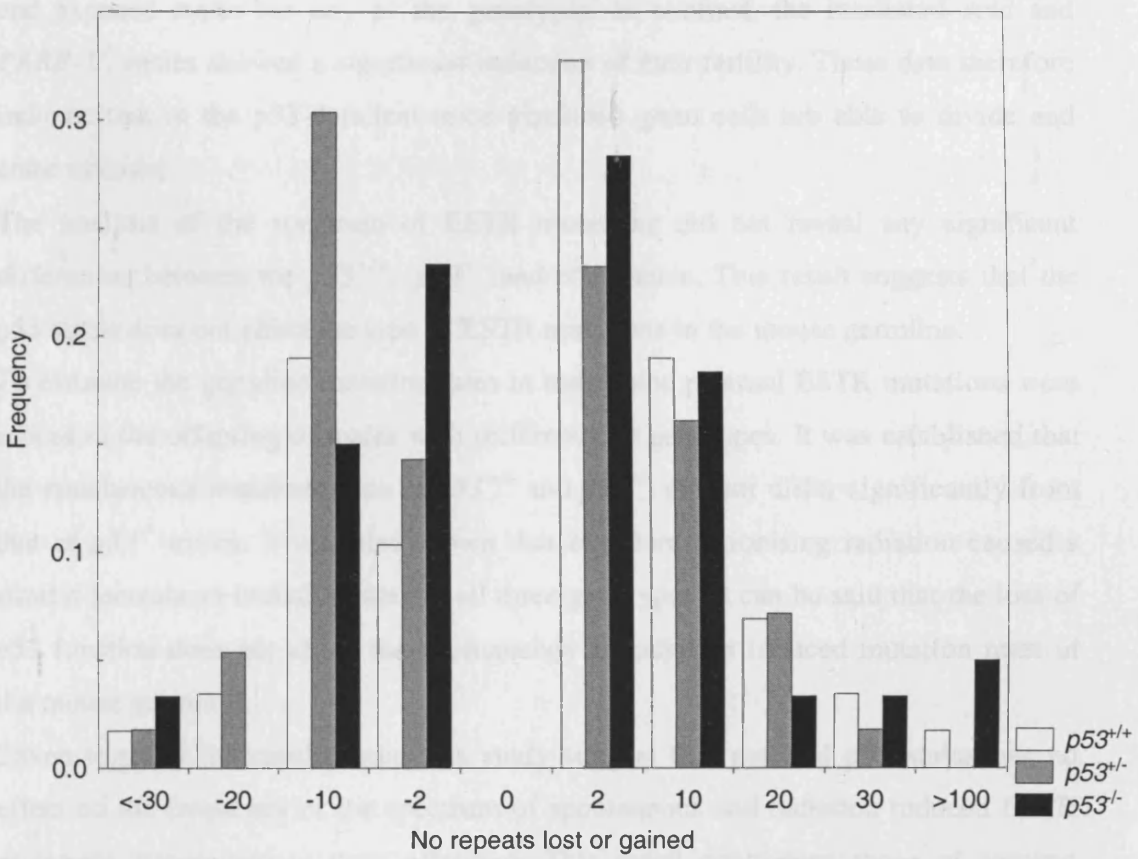


Figure 4.3: Spectrum of paternal ESTR mutations in the wild-type and p53 deficient mice. Kruskal-Wallis test, χ^2=3.67, *df*=2, *P*= 0.1597. Values along the x-axis represent lowest number of repeat units within bin.

4.8 Discussion

As a tumour suppressor protein p53 plays a critical role in the maintenance of genome stability through the regulation of cell cycle arrest and apoptosis (Vogelstein et al., 2000). Mice lacking functional p53 have been used to analyse the *in vivo* effects of p53 deficiency on genomic stability in somatic cells. Little is known about the effects of p53 status on the germline, although it is known that p53 is fully functional during spermatogenesis (Beumer et al., 1998). This study was designed to analyse the germline ESTR mutation rates in p53 knockout mice to determine whether p53 status affected spontaneous and/or radiation induced mutation rates in the germline of male mice.

To evaluate the effects of p53 deficiency of the fertility, litter sizes were compared across all genotypes. No significant differences were observed between the control

and exposed males for any of the genotypes. In contrast, the irradiated *scid* and *PARP-1*$^{-/-}$ males showed a significant reduction of their fertility. These data therefore indicate that in the p53-deficient mice irradiated germ cells are able to divide and enter meiosis.

The analysis of the spectrum of ESTR mutations did not reveal any significant differences between the *p53*$^{+/+}$, *p53*$^{+/-}$ and *p53*$^{-/-}$ mice. This result suggests that the p53 status does not affect the type of ESTR mutations in the mouse germline.

To estimate the germline mutation rates in male mice paternal ESTR mutations were scored in the offspring of males with different p53 genotypes. It was established that the spontaneous mutation rates in *p53*$^{+/+}$ and *p53*$^{+/-}$ did not differ significantly from that of *p53*$^{-/-}$ males. It was also shown that exposure to ionising radiation caused a similar increase in mutation rates in all three genotypes. It can be said that the loss of p53 function does not affect the spontaneous or radiation induced mutation rates in the mouse germline.

Taken together the results from this study suggest that paternal p53 status has no effect on the frequency or the spectrum of spontaneous and radiation induced ESTR mutations transmitted to their offspring. This result contradicts those of somatic instability where p53 knockout mice have been shown to have elevated somatic instability (Review by Morris, 2002). However there are a number of studies that report a lack of accumulation of mutations in p53 deficient cells (Sands et al., 1995; Chuang et al., 1999). Sands et al. (1995) showed that p53 deficient cells have an indistinguishable rate of accumulation of point mutations, small deletions and insertions when compared to wild-type cells. However, the analysis of *Aprt* and *Hprt* protein coding genes showed elevated spontaneous mutation frequencies in p53 deficient mice (Shao et al., 2000; Liang et al., 2002). Laing et al. also showed that the most common type of mutation to occur in p53 deficient mice was large interstitial deletions. p53 deficiency has been shown to result in gross chromosomal abnormalities in the somatic cells of p53 deficient mice (Bouffler et al., 1995) and an increase in mutation frequency at the *hprt* loci has been associated with gross gene arrangements (Wyllie et al., 1994). All the evidence together suggests p53 status affects large chromosomal changes, but not small ones such as point mutations (Liang et al., 2002). Therefore, enhanced mitotic recombination could account for the increased mutation frequencies observed in p53 deficient cells. This is in agreement with the findings that p53 deficient mice show an increased frequency of somatic

homologous recombination (Bishop et al., 2003). This result led to the suggestion that p53 may have a role in suppressing homologous recombination. It seems that mutation detected in p53 deficient somatic cells appears to be as a result of large deletions or other gross chromosomal aberrations. These large aberrations may play an important role in carcinogenesis and contribute to the increased susceptibility to spontaneous tumour development in p53 knockout mice (Donehower et al., 1992). Since it seems that the majority of mutations due p53 deficient cells are large aberrations, this could explain the lack of elevation in the number of ESTR mutations observed in p53 deficient mice. It is possible that the large deletions and other gross chromosomal aberrations caused by p53 deficiency may not be tolerated in the germline or in early development. This could mean that large mutations caused by p53 deficiency may result in prenatal death and would therefore not be transmitted to the offspring. This is consistent with studies that show in humans over 95% of *de novo* chromosomal abnormalities are eliminated in utero by spontaneous abortion (Boue et al., 1985). Since the method used in this study depends on the transmission of mutations to the offspring from parents, it cannot be concluded that p53 deficiency does not affect the mouse germline. A recent study has shown that p53 knockout mice had an elevated rate of radiation induced chromosomal translocation in their sperm compared to wild-type mice (van Buul and van Duijn-Goedhart, 2002). This study indicates that p53 deficiency affects the germline stability, but it is possible that these observed translocations would not be viable and therefore would not be transmitted to the offspring. It should be stressed that the lack of ESTR mutation induction in the irradiated males may be due to the fact that p53 deficient cells only exhibit reduced apoptotic ability as they are able to undergo apoptosis via p53 independent pathways. Cells lacking p53 function have been shown to undergo delayed apoptosis compared to wild-type cells (after 12 hours instead of 6) (Bielas and Heddle, 2003).

Compared to previously studies on deficient mouse strains *scid* and PARP-1 null mice which are deficient in the early recognition and repair of double- or single-strand breaks respectively, the p53 results show a different pattern (shown in Figure 4.4). In contrast to the DNA repair deficient strains which show elevated ESTR mutation rates in the germline of non irradiated males and the lack of radiation induced mutation, the p53 results show no increase in ESTR mutation rates in non-exposed males and an increase in induced mutation no matter the p53 genotype. This data suggests that

pathways involved in the repair of double- or single-strand breaks are important in the maintenance of ESTR stability, whereas p53 deficiency has no effect on ESTR stability. This could be due to a lack of delay in repair as in the other two strains, as p53 deficient cells do not lack the ability to recognise DNA damage as in *scid* and PARP-1 deficient cells. In fact in normal cells p53 retards the cell cycle allowing time to repair damage, whereas in cells lacking p53 function the cell cycle is not halted for as long and allowing less time to repair (Bielas and Heddle, 2003).

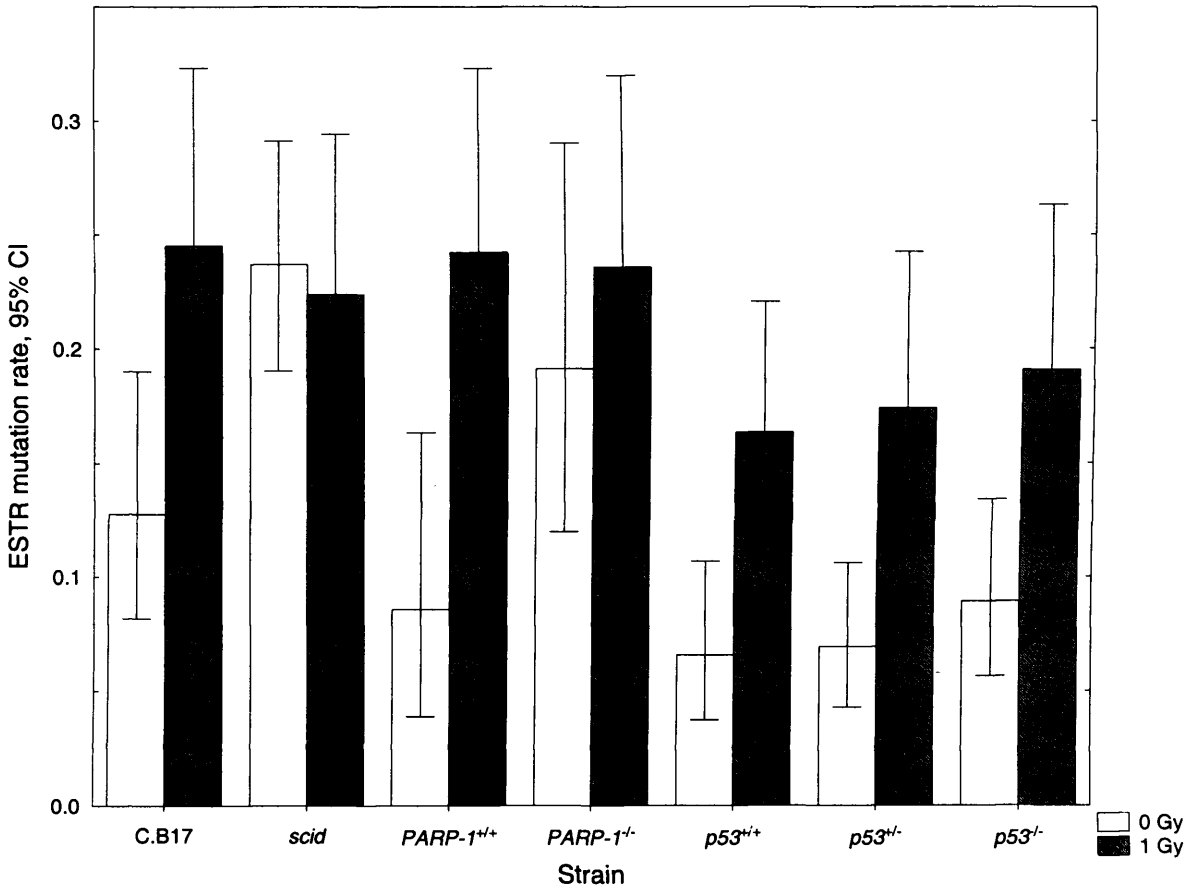


Figure 4.4: Spontaneous and radiation-induced ESTR mutation rates in the germline of wild-type and deficient male mice.

To conclude, it has been shown that paternal p53 status does not affect spontaneous or radiation induced mutation rates or spectrum of mutation at ESTR loci in the mouse male germline, indicating that p53 dependent apoptosis or cell cycle arrest has no role in the mechanism of mutation at ESTR loci.

5 ANALYSIS OF GERMLINE MUTATION RATES AT EXPANDED SIMPLE TANDEM REPEAT LOCI IN XPC DEFICIENT MICE

5.1 Introduction

5.1.1 General introduction

Xeroderma pigmentosum (XP) is a rare autosomal disease characterised by extreme sun sensitivity and increased risk of skin cancer in sun exposed parts of the body in affected individuals (Freidberg, 1995). XP has been directly associated with a defect in nucleotide excision repair (Venema et al., 1990). Nucleotide excision repair (NER) is a fundamental repair pathway that is required for maintaining the integrity of the genome in cells exposed to environmental DNA damage. NER is the most versatile repair pathway as this pathway is able to remove a large variety of structurally unrelated lesions that can cause mutations, cancer or cell death. There are two sub-pathways of NER; global genomic repair (GGR), repairs lesions throughout the genome and transcription coupled repair (TCR), which is selective only repairing the transcribed DNA strand of expressed genes.

There are eight complementation groups in XP. Xeroderma pigmentosum complementation group C (XPC) gene product is responsible for the recognition of lesions for GGR only. Cells from individuals with mutations in the XPC gene are able to efficiently repair UV induced damage in actively transcribed DNA, but are defective in the repair of non-transcribed and transcriptionally silent regions of the genome (Venema et al., 1991).

The generation of NER deficient mice allows the study of the relationship between the DNA repair pathway nucleotide excision repair (NER) and UV induced skin carcinogenesis. Such studies are important as they may aid the assessment of risk of UV exposure along with the risk of exposure to other mutagens for individuals suffering from these disorders.

5.1.2 Characterisation of *XPC* null mutation in mice

5.1.2.1 Generation of XPC mutant mouse

Mutant XPC mice, used in this study, were generated by replacing a *xho*l fragment of the XPC gene containing exon 10 and a portion of each of the flanking introns with a *pol*II-neo selectable marker. The vector was used to disrupt the XPC locus in mouse embryonic stem cells by homologous recombination (Cheo et al., 1997). These cells were then used to generate XPC mutant mice by microinjecting them into C57BL/6 blastocysts, which were transferred in pseudopregnant females. Chimeric mice were then bred to produce XPC heterozygous mice which were used to generate all three genotypes.

Northern blot analysis was used to examine the expression of the wild-type and mutant XPC allele. It was found that XPC mutant mice express truncated transcripts, which is expected to encode a polypeptide shortened by 300 amino acids at the C-terminal with an additional 27 missense amino acids. Loss of the C-terminal region could prevent XPC from interacting with HR23B. The XPC-HR23B complex has a role in the recognition of DNA damage which allows subsequent repair (Matsutani et al., 1994). Another XPC knockout was generated by deleting a large part of the *XPC* gene, spanning from exon 3 to exon 6 (Sands et al., 1995).

5.1.2.2 XPC null mouse phenotype

XPC mutant mice show no apparent development, growth or neurological abnormalities and they were found to be fertile (Cheo et al., 1997). Breeding of heterozygous XPC mice produced offspring of all genotypes in percentages that would be expected if the XPC allele was inherited in strict Mendelian fashion (Cheo et al., 1997). Mice older than 1 year were found through detailed autopsies to have no abnormalities by gross inspection or detailed histological examination of the organs (Choa et al., 1997). Further analysis of XPC mutant mice revealed that there was no evidence for enhanced predisposition to spontaneous internal cancers (Meira et al., 2001).

Cells from XPC mutants have been shown to be abnormally sensitive to ultraviolet (UV) radiation (Sands et al., 1995; Cheo et al., 1996; Cheo et al., 1997). Studies have shown that XPC mutant mice are deficient in the process of NER in the form of GGR

of UV induced DNA damage (Meira et al., 2001). The XPC mouse model mimics the human skin cancer prone disease XP, therefore providing a useful model to analyse the effects of mutagens on GGR deficiency.

5.1.2.3 XPC mice sensitivity to mutagens

In humans mutations in the XPC gene are associated with XP, which is characterised by sensitivity to UV radiation. It is unsurprising that this lead to an interest in the sensitivity of XPC mutant mice to UV radiation. Several studies have reported that XPC mutant mice show an increased sensitivity to UV irradiation compared to their wild-type counterparts. The first study to illustrate that XPC mutations in mice caused a high susceptibility to UV induced damage was performed on mice with disrupted exons 3-6. This study found that animals homozygous for the XPC mutation were highly susceptible to UV induced carcinogenesis compared to heterozygous or wild-type littermates (Sands et al., 1995). They also showed that these homozygous mice displayed pathological skin and eye changes that were consistent with the human form of the disease XP group C. More recently studies have been performed on the XPC mutant with the disruption in exon 10 (Cheo et al., 1997). Mouse embryonic fibroblast (MEF) cells derived from these XPC mutant mice display similar phenotypic characteristics to XPC mutant human cells. MEF from these mice were revealed to be extremely sensitive to killing following exposure to UV irradiation when compared to wild-type and heterozygotes and have been found to be severely defective in global NER, showing a specific defect in the removal of pyrimidine (6-4) pyrimidine photoproducts in the nontranscribed strand of transcriptionally active genes (Cheo et al., 1997; Freidberg et al., 1999, Cheo et al., 2000). Both these defects mimic those observed in human XP-C cells.

Studies of the mutant mice indicated that they were highly predisposed to UV radiation induced skin cancer (Cheo et al 1996; Cheo et al., 2000). Following daily exposure to UV radiation 100% of the *XPC* $^{-/-}$ mice developed skin cancer on the shaved dorsal skin within 25 weeks. In the initial study it was found that no lesions were observed in the *XPC* $^{+/-}$ or *XPC* $^{+/+}$ mice. However, monitoring the *XPC* $^{+/-}$ mice for a longer period revealed that *XPC* $^{+/-}$ animals had an increased risk of developing skin tumours after being exposed to UV radiation. *XPC* $^{+/-}$ animals manifested a 50% incidence of skin cancer after 50 weeks, compared to ~92 weeks in wild-type (Cheo et

al., 2000). This indicates that not only homozygous individuals are at risk, but individuals that are heterozygous for XP-C could also be at risk of cancer associated with exposure to sunlight because of compromised NER.

To date only one study has been performed on the effect of chemical mutagenesis on XPC mutant animals (Cheo et al., 1999). Two chemical carcinogens were used; 2-acetylaminofluorene (2-AAF) and N-OH-2- acetylaminofluorene (N-OH-AAF). It was found that *XPC* $^{-/-}$ mice were more predisposed to 2-AAF and N-OH-AAF induced liver and lung tumours (67% after 15 months compared to 33% in wild-type). In contrast, *XPC* $^{+/-}$ animals showed the lowest incidence of lung and liver cancer (12.5% after 15 months). These quite surprising findings indicate the *XPC* haploinsufficiency may not enhance the predisposition to internal cancers following exposure to some recognised carcinogens.

Since XPC mutant mice mimic the clinical, cellular and molecular phenotypes of the human disease XP, they provide a useful model to examine the risks that various mutagens could pose on affected individuals.

5.1.2.4 The effects of XPC on genomic stability

The effects of XPC mutations on genomic instability have not been directly analysed. However it is known that defects in the NER pathway increase the risk of cancers, which indicates that XPC could be involved in the maintenance genomic stability (reviewed in Sieber et al., 2003). It is widely accepted that carcinogenesis requires several genetic alterations. Most cancers accumulate numerous genetic alterations and it has been suggested that genomic instability is the hallmark of tumourigenesis (Hanahan & Weinberg, 2000). DNA repair decreases genomic instability by reducing the accumulation of mutations which may then go onto cause chromosomal aberration or recombination events, leading to the development of cancers. Therefore it appears possible that deficiencies in DNA repair pathways, such as NER could lead to carcinogenesis and genomic instability.

5.2 Experimental design

The effects of XPC deficiency on the sensitivity to ionising radiation have never been studied. The experiment was therefore designed to analyse the effects of ionising

radiation on spontaneous and radiation induced mutations in the germline of XPC-deficient male mice. We also thought to establish whether the NER repair pathway could influence the stability of ESTR loci.

XPC $^{-/-}$ and *XPC* $^{+/+}$ males were mated with non-exposed CBA/J females to produce the control offspring. At 10-12 weeks of age *XPC* $^{-/-}$ and *XPC* $^{+/+}$ males were acutely exposed to whole body γ-irradiation. The irradiated males were then crossed with non-exposed CBA/J females 8-10 weeks after exposure. All the parents and offspring were analysed by profiling with two hypervariable single-locus ESTR probes, Ms6-hm and Hm-2.

5.3 Summary of mutation induction at ESTR loci

A summary of the ESTR mutation data obtained during the study of the effects of XPC on ESTR stability is presented in Table 5.1. Only the total number of mutations was used for further analysis of the data.

The data for the types of mutations observed for both genotypes indicates that there is no difference in the type of mutation before or after exposure to ionising radiation in either genotype.

Strain, dose	No offspring	No mutations*			Type of mutants	
		Ms6-hm	*Hm-2*	Total	Gains	Losses
$XPC^{+/+}$						
0 Gy	137	9 (9)	3 (3)	12 (12)	5	7
1 Gy	125	15 (15)	9 (5)	24 (20)	9	15
χ^2, *df*=1 (Prob)‡					0.06	(0.8141)
$XPC^{-/-}$						
0 Gy	132	14 (10)	9 (7)	23 (17)	11	12
1 Gy	113	19 (13)	16 (11)	35 (24)	21	14
χ^2, *df*=1 (Prob)‡					0.81	(0.3685)

* Number of singleton mutations is given in parenthesis.
‡ Chi-square test for homogeneity of the type of mutants between control and irradiated males.

Table 5.1: Summary of ESTR mutation data from $XPC^{+/+}$ and $XPC^{-/-}$ males

5.4 Effect of dose and genotype on ESTR mutation rates

To analyse the effects of the genotype and the dose on ESTR mutation rates ANOVA analysis was performed on the mutation data. It was found that ESTR mutation rates are affected by both the XPC genotype and exposure to ionising radiation. Analysis also showed that the interaction between the genotype and exposure was negligible. To further investigate these effects estimates of the mutation rates were calculated from the mutation data.

Source of variation	*df*	F_s	*P*
Genotype	1, 25	8.69	0.0069
Dose	1, 25	6.55	0.0169
Interaction	1, 25	0.42	0.5241
$R^2 = 0.4426$			

F_s, P and R^2 are the sample statistics of F-distribution, its probability and coefficient of determination, respectively.

Table 5.2: ANOVA analysis for effects of the XPC deficiency and radiation on ESTR mutation rate (arc-transformed)

5.5 Spontaneous and radiation-induced ESTR mutation rates in *XPC*$^{+/+}$ and *XPC*$^{+/-}$ mice

The spontaneous and radiation induced ESTR mutation rates for both *XPC* $^{-/-}$ and *XPC*$^{+/+}$ males are shown in Table 5.3 and Figure 5.1. The data shows that after exposure to 1 Gy ionising radiation *XPC* $^{+/+}$ males exhibit a significant 2.1 fold increase in germline induced ESTR mutation rate. The frequency of paternal ESTR mutations in *XPC*$^{-/-}$ after exposure to 1 Gy X-ray increased by 1.8 fold. A slight (2 fold, not significant) increase in the spontaneous mutation rate was observed in XPC deficient mice when compared to wild-type mice, along with a non-significant 1.6 fold increase in induced mutation rate.

		Exposed to non-exposed		Non-exposed to *XPC*$^{+/+}$		Exposed to exposed *XPC*$^{+/+}$	
Strain, dose	Rate	Ratio	P^*	Ratio	P^*	Ratio	P^*
XPC$^{+/+}$							
0 Gy	0.0438	-	-	-	-		
1 Gy	0.0960	2.19	0.0282			-	-
XPC$^{-/-}$							
0 Gy	0.0871	-	-	1.99	0.0616		
1 Gy	0.1549	1.78	0.0298			1.61	0.0706

* Probability of difference (Fisher's exact test, two-tailed).

Table 5.3: ESTR mutation rates in the XPC deficient and wild-type male mice

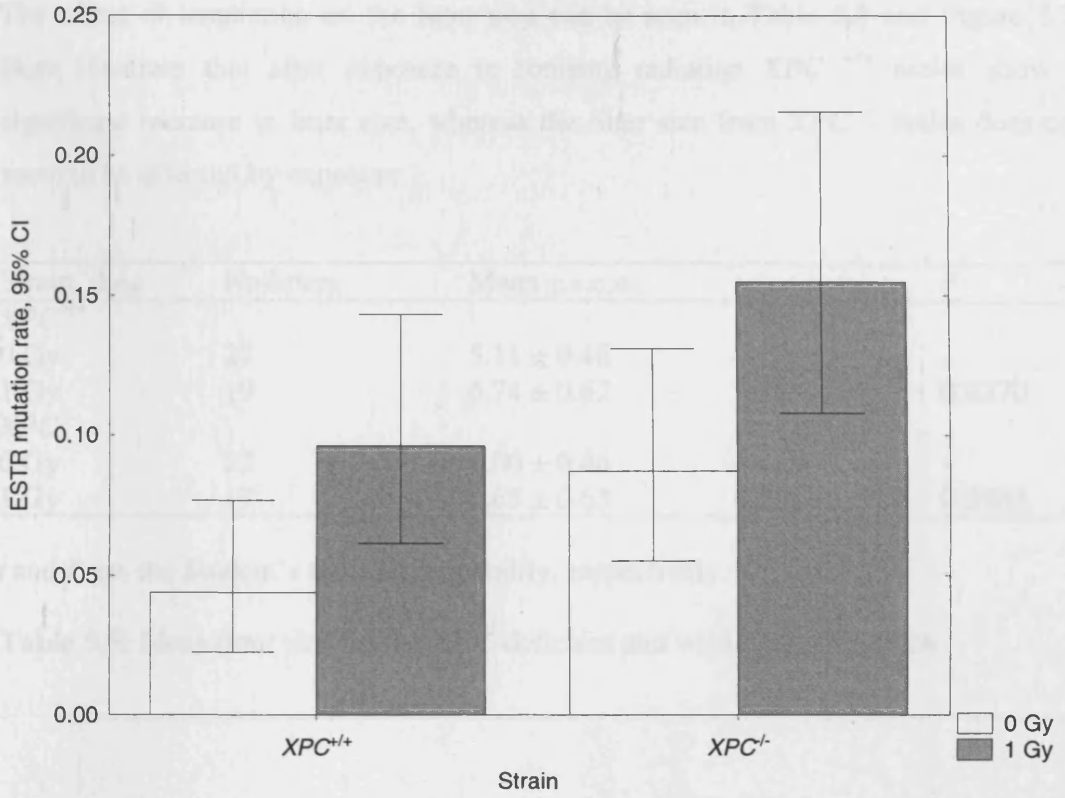


Figure 5.1: Spontaneous and radiation induced ESTR mutation rates in the germline of $XPC^{+/+}$ and $XPC^{-/-}$ male mice

5.6 Effect of dose and genotype on litter size

ANOVA analysis of the litter size data indicate that exposure to ionising radiation has an effect on the litter size, but the XPC genotype does not (Table 5.4).

Source of variation	*df*	F_s	*P*
Genotype	1, 86	0.55	0.4602
Dose	1, 86	4.46	0.0379
Interaction	1, 86	0.83	0.3661
$R^2 = 0.0741$			

F_s, P and R^2 are the sample statistics of F-distribution, its probability and coefficient of determination, respectively.

Table 5.4: ANOVA analysis for effects of the XPC deficiency and radiation on the litter size

The effect of irradiation on the litter size can be seen in Table 5.5 and Figure 5.2. Both illustrate that after exposure to ionising radiation *XPC* $^{+/+}$ males show a significant increase in litter size, whereas the litter size from *XPC* $^{-/-}$ males does not seem to be affected by exposure.

Strain, dose	No litters	Mean ± s.e.m.	*t*	*P*
XPC$^{+/+}$				
0 Gy	27	5.11 ± 0.46	-	-
1 Gy	19	6.74 ± 0.62	2.15	0.0370
XPC$^{-/-}$				
0 Gy	22	6.00 ± 0.46	-	-
1 Gy	17	6.65 ± 0.63	0.85	0.3988

t and *P* are the Student's test and probability, respectively.

Table 5.5: Mean litter size for the XPC-deficient and wild-type male mice

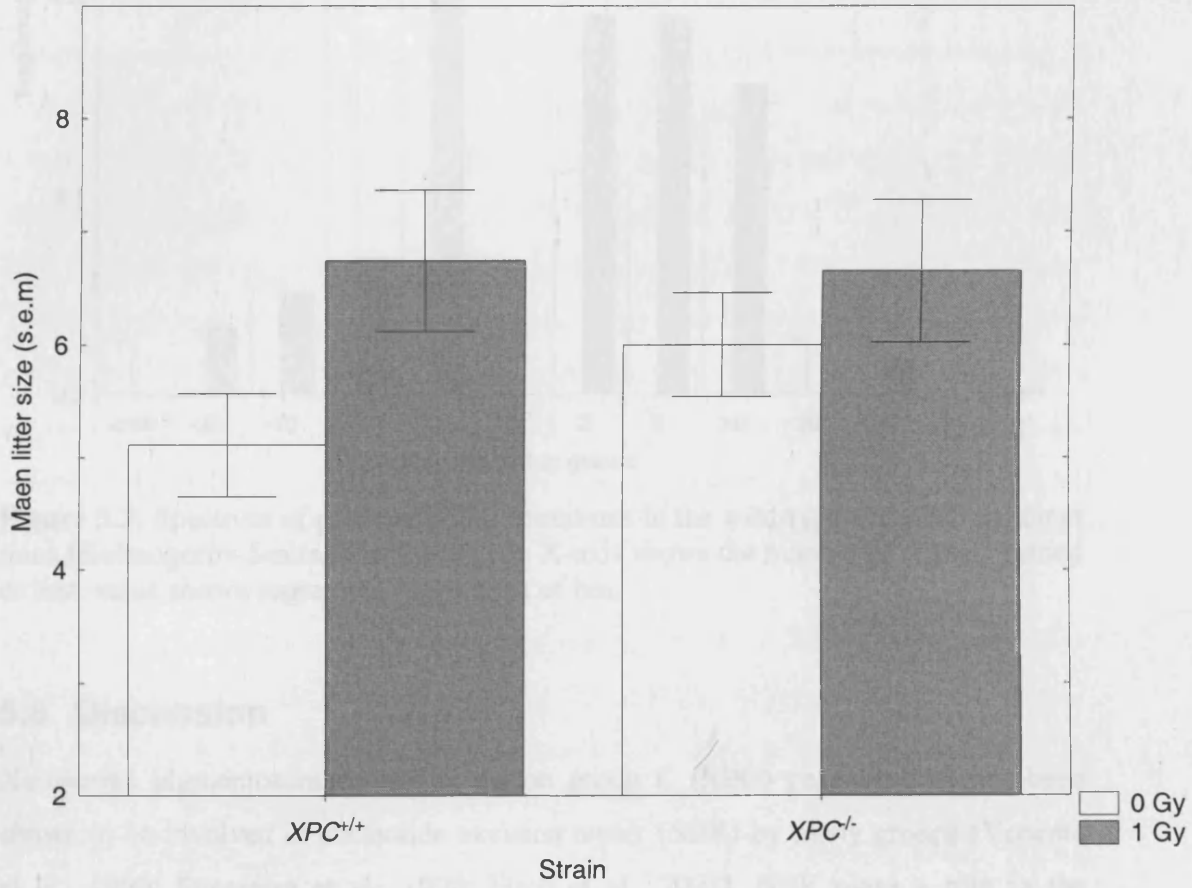


Figure 5.2: Litter sizes for exposed and non-exposed male *XPC*$^{+/+}$ and *XPC*$^{-/-}$ mice

5.7 ESTR mutation spectrum in XPC deficient mice

The control and exposed spectra were indistinguishable and therefore were combined for further analysis. The spectra of both $XPC^{+/+}$ and $XPC^{-/-}$ mice are shown in Figure 5.3. Through statistical analysis it was found that there was no significant difference between the spectra of XPC deficient males compared to their isogenic wild-type counterparts.

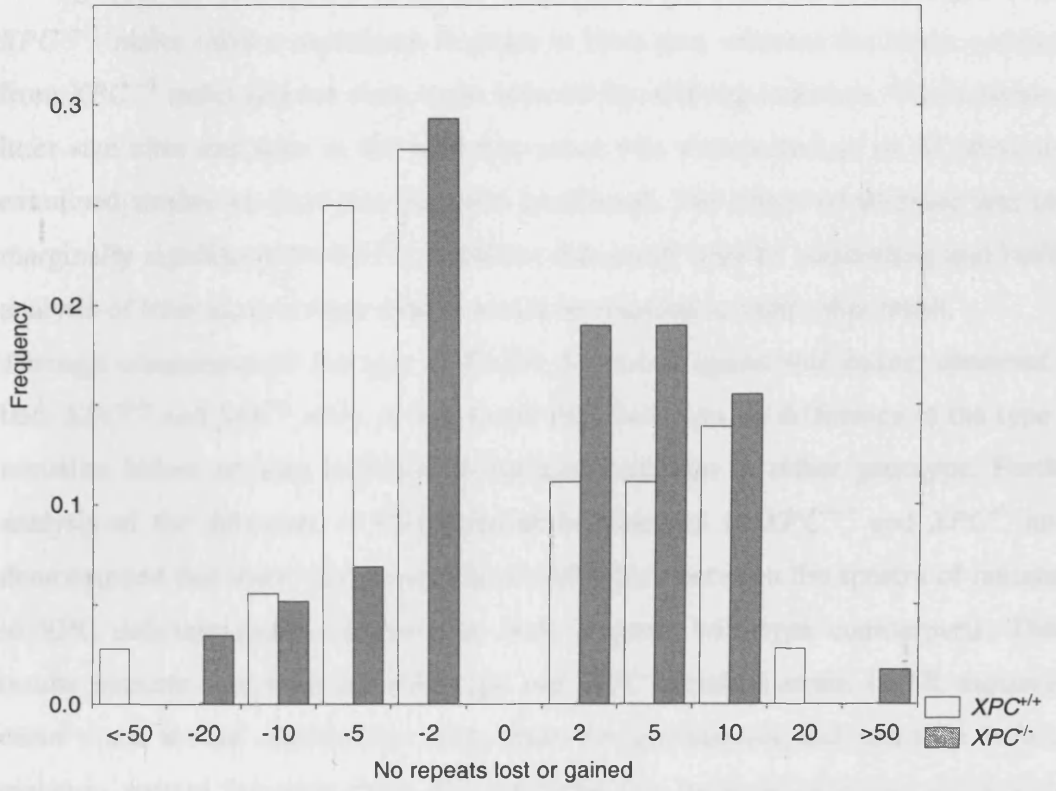


Figure 5.3: Spectrum of paternal ESTR mutations in the wild-type and XPC deficient mice (Kolmogorov-Smirnov test, $P>0.10$). X-axis shows the number of repeats gained or lost, value shown represents lower limit of bin.

5.8 Discussion

Xeroderma pigmentosum complementation group C (XPC) gene product has been shown to be involved in nucleotide excision repair (NER) by many groups (Venema et al., 1990; Sugasawa et al., 1998; Fitch et al., 2003). NER plays a role in the removal of bulky helix-distorting lesions from DNA to allow normal DNA synthesis and cell survival. XP-C patients suffer from a mild form of the rare autosomal disease xeroderma pigmentosum (XP), which is characterised by severe photosensitivity and

the development of skin cancer at a young age (Bootsma et al., 1998). Mice lacking functional XPC have been generated and have provided a useful model for XP in humans as they mimic the symptoms observed in patients (Cheo et al., 1997). To date, nothing is known about the germline instability of XP-C patients and their sensitivity to ionising radiation. This study was carried out in mice to determine whether XPC deficiency affects the germline, increases sensitivity to ionising radiation and whether XPC is involved in the mutation induction at mouse ESTR loci.

During the analysis of these mice it was found that after exposure to ionising radiation *XPC* $^{+/+}$ males show a significant increase in litter size, whereas the litters produced from *XPC* $^{-/-}$ males did not seem to be affected by ionising radiation. The increase in litter size after exposure in the wild-type mice was unexpected as in all previously examined strains the litter size has been unaffected. The observed increase was only marginally significant (P=0.037), therefore this result may be misleading and further analysis of litter sizes in these strains would be required to verify this result.

Through comparison of the type of ESTR mutations (gains and losses) observed in both *XPC*$^{+/+}$ and *XPC*$^{-/-}$ mice, it was found that there was no difference in the type of mutation before or after exposure to ionising radiation in either genotype. Further analysis of the spectrum of ESTR mutation observed in *XPC*$^{+/+}$ and *XPC*$^{-/-}$ mice demonstrated that there was no significant difference between the spectra of mutation in XPC deficient males compared to their isogenic wild-type counterparts. These results indicate that both the wild-type and XPC deficient strain ESTR mutations occur via a similar mechanism. Also since the spontaneous and radiation induced mutation spectra for each strain did not differ this indicates that like the *PARP-1* deficient mice, radiation induced and spontaneous ESTR mutations occur through the same mechanism in each strain.

Estimates of spontaneous and radiation induced germline ESTR mutation rates were obtained for *XPC*$^{+/+}$ and *XPC*$^{-/-}$ males by profiling their offspring. The data indicates that after exposure to 1 Gy ionising radiation *XPC* $^{+/+}$ males display a significant 2.1 fold increase in germline induced ESTR mutation rate. The frequency of paternal ESTR mutations in *XPC*$^{-/-}$ after exposure to 1 Gy X-ray was elevated by 1.8 fold. A non-significant increase (2 fold) in the spontaneous mutation rate was observed in XPC deficient mice when compared to wild-type mice.

The increase in radiation induced ESTR mutation rates are as expected when compared to data from normal mice strains (data not shown). The increase in mutation

rate in both strains indicates that both wild-type and XPC deficient strains are sensitive to ionising radiation. The data indicates that *XPC*$^{-/-}$ mice are more susceptible to exposure to ionising radiation than their wild-type counter parts, as they have a higher mutation rate after exposure (a 1.6 fold non-significant difference). The increased sensitivity to ionising radiation compared to wild-type can be explained by the elevated spontaneous ESTR mutation rates observed in *XPC*$^{-/-}$ mice, as both strains showed almost equal levels of mutation induction after exposure to ionising radiation (2.1 x in wild-type and 1.8 x *XPC*$^{-/-}$). It may be said that, the *XPC*$^{-/-}$ male germline shows a slightly higher spontaneous frequency of mutation than wild-type, therefore rendering the *XPC*$^{-/-}$ male germline more radiosensitive.

It is possible that the elevation in spontaneous ESTR mutation rate observed in *XPC*$^{-/-}$ males may be due to the mechanism proposed in chapter 3 for *PARP-1* deficient and *scid* mice which are also deficient in DNA repair. Both previously analysed DNA repair deficient strains are defective in the early recognition of SSBs or DSBs, this inability to recognise damage may lead to a delay in repair and therefore alter the cell cycle progression. It was proposed that delay in repair could cause an increase in replication fork pausing, which in turn could promote polymerase slippage. To investigate this hypothesis the role of XPC has to be explored.

As already noted XPC is involved in the GGR sub-pathway of NER. NER is a complex process which is able to eliminate a wide variety of DNA damage (Freidberg et al., 1995). There are two distinct sub-pathways of NER, global genomic repair (GGR) and transcription coupled repair (TCR). XPC has been shown to only be involved in GGR, which is involved in the elimination of lesions across the genome, whereas TCR only removes lesions from transcribed regions (reviewed in Bernstein et al., 2002). GGR contributes to reducing the risk of damage induced mutagenesis, which could cause long term effects such as carcinogenesis, cell malfunction and aging (Sugasawa et al., 2002). XPC initiates NER as part of a complex (XPC-hHR23B complex). It was found that the XPC-hHR23B complex was the earliest damage detector to initiate NER (Sugasawa et al., 1998). It was shown that the XPC-hHR23B complex senses and binds tightly to distortions in damaged DNA, locally modifying the DNA duplex to allow the repair machinery to enter the site of damage. XPC-hHR23B complex has a strong specific affinity for damaged DNA, binding directly to the lesion (Sugasawa et al., 1998). Further analysis showed that the complex recognises specific DNA structures that prevent the maintenance of the

normal Watson-Crick structure (Sugasawa et al., 2002). It was later found that XPC is not only involved in the recognition of lesions but it also plays a role in the recruitment of the repair machinery, as it interacts with the TFIIH transcription factor an essential component of NER (Yokoi et al., 2000). All this evidence shows that XPC is involved in the early recognition of DNA damage, as in the previously studied DNA repair deficient strains. The inability to recognise damage may lead to a delay in repair. In fact lack of XPC shows a marked reduction in the number of dimers repaired in the nontranscribed strand *in vitro* (Venema et al., 1990; Venema et al., 1991). The initial study showed that 18% of dimers were removed from inactive chromatin after 4 hours and 52% after 24 hours in XP-C human fibroblast compared to 45% and 90%, respectively from transcribed regions in wild-type fibroblasts (Venema et al., 1990). The second experiment showed that XPC deficient cells were capable of removing lesions from transcribed regions, although they were less efficient at removing lesions from non-transcribed regions (Venema et al., 1991). In non-transcribed regions only 8% compared to 33% was removed and after 24 hours only 14% had been removed compared with 71% in wild-type cells. These results indicate that XPC deficiency severely restricts the repair of lesions by GGR, indicating that only a small percentage of damage otherwise repaired via XPC and GGR is repaired via alternative pathways.

It is known that the presence of DNA adducts may severely affect the progression of DNA polymerase, thus triggering cellular checkpoints to allow the repair of damage before replication resumes (Osborn et al., 2002). Therefore, it is possible the delay in repair of bulky lesions by NER or that the remaining unrepaired bulky lesions could lead to replication pausing and promote polymerase slippage.

The data from this study has been used to corroborate the model for the mechanism of mutation at ESTR proposed from previous studies, that mutation at ESTR loci are due to polymerase slippage. This model could be used to explain the elevated spontaneous ESTR mutation rates observed in XPC deficient mice, as XPC is involved in early recognition of DNA damage and that repair of lesions is in some cases delayed, but in other instances absent. This could then in turn lead to polymerase slippage as result of prolonged replication fork pausing at the damage site.

To conclude, the data presented in this chapter suggests that XPC deficiency does increase an individual's sensitivity to ionising radiation. The results of this study

therefore provide the first experimental evidence for the effects of XPC deficiency on spontaneous and radiation induced mutation rates in the mouse germline.

6 ANALYSIS OF GERMLINE MUTATION RATES AT EXPANDED SIMPLE TANDEM REPEAT LOCI IN POLκ DEFICIENT MICE

6.1 Introduction

6.1.1 General introduction

The recently discovered the Y family of DNA polymerases includes numerous enzymes which promote replication through DNA lesions (Ohmori et al., 2001). These enzymes have been implicated in copying of DNA templates containing lesions that distort the DNA structure and block synthesis via other DNA polymerases. The Y family polymerases are able to bypass lesions through a process called translesion synthesis (TLS). TLS is a DNA damage response which allows the bypass of DNA damage and extension of replication forks through the damage site without repairing the damage. It has been proposed that the majority of spontaneous mutations in humans arise from mis-copying of a damaged DNA template before repair has had time to occur or that the inaccuracy of these enzymes in copying undamaged templates is responsible (Lindahl and Woods, 1999). This would suggest that the Y family of DNA polymerases has a large role in the generation of spontaneous mutations in humans.

Polymerase kappa (polκ) is one of the specialised error-prone DNA polymerases belonging to the Y family of DNA polymerases (Ohmori et al., 2001). Polκ has no proof-reading activity and has a high error rate *in vitro* (Ohashi et al., 2000).

6.1.2 Characterisation of polκ null mutation in mice

6.1.2.1 Generation of polκ deficient mice

Two independent groups have generated polκ deficient mice (Schenten et al., 2002; Shimizu et al., 2003). The strain used during this study was the one described by Schenten et al. (2002). Using homologous recombination, this group generated polκ deficient mice by gene targeting. 129/Ola derived embryonic stem cells were targeted with a vector containing *lox*P flanked exon 6 (making the locus susceptible to Cre

recombinase-mediated deletion) and a neomycin resistance cassette for selection. ES cells were then used to generate chimeric mice (CB.20 foster mothers). Chimeric mice were mated with C57BL/6 Cre-deleter mice for germline transmission and deletion of exon 6. Exon 6 was chosen as it contains two essential catalytic residues and replacement of these results in a complete loss of the DNA polymerase activity *in vitro* and mRNA splicing from exon 5 to 7 leads to a frameshift mutation (Ohashi et al., 2000; Gerlach et al., 2001). The mice generated by Schenten et al. were on a 129/Ola and C57BL/6 mixed background.

To ensure inactivation of the polκ gene RT-PCR was used to amplify the polκ transcript using primers spanning exon 6. Polκ deficient mice gave rise to a shorter product than the wild-type indicating the lack of exon 6. Northern blot analysis revealed that the intensity of the expression of polκ in mutant mice was five times less than in wild-type. Schenten et al. suggest that the frameshift mutation leading to a premature stop codon renders mRNA lacking exon 6 less stable than the wild-type mRNA.

Shimizu et al. (2003) generated polκ deficient mice using a targeting construct to replace exons 5 and 6 with a neomycin resistant gene (as described by Ogi et al., 2002) resulting in mice in a C57BL/6 x CBA background.

6.1.2.2 Polκ null mice phenotype

Mice homozygous for the deletion of exon 6 or exons 5 and 6 were found to be viable (Schenten et al., 2002; Shimizu et al., 2003). Neither of the mutant strains shows any obvious abnormalities and they are both fertile. When heterozygotes for the mutant allele were mated it was found that offspring genotypes were present at the expected Mendelian ratio (Schenten et al., 2002). It was also found that both males and females were fertile and the genotype did not affect the litter size.

6.1.2.3 Polκ mice sensitivity to mutagens

To date there have been limited studies on the effects of mutagens on polκ deficiency. It has been reported that the sensitivity of polκ mutant mouse embryonic stem cells to ionising radiation is similar to that in the wild-type cells, whereas they show moderately elevated sensitive to the effects of UV irradiation (Ogi et al., 2002). This

result was expected as *in vitro* studies had shown that polκ is unable to bypass certain forms of UV induced damage (Ohashi et al., 2000; Zhang et al., 2000). Further analysis of the sensitivity of polκ deficient mice to killing by UV showed that mouse embryonic fibroblasts (MEF) from homozygous mutant mice are sensitive to UV radiation (Schenten et al., 2002). During this study the sensitivity of polκ deficient MEF were compared to the sensitivity of XPC deficient MEF. It was found that polκ deficient cells were as sensitive to UV radiation as MEF from mice homozygous for mutant XPC. This indicates that since XPC mutants are only partially deficient in NER, that the deficiency of polκ only leads to cell killing after the induction of some forms of UV damage (possibly thymine glycol) (Schenten et al., 2002). Polκ may be required for the bypass of one or more types of base damage caused by UV radiation. This notion is supported by independent observations that polκ is able to bypass thymine glycol photoproducts, but not major photoproducts such as thymine dimers and (6-4) photoproducts *in vitro* (Ohashi et al., 2000; Zhang et al., 2000).

Benzo(a)pyrene (B(a)P) is an environmental carcinogen that is present in cigarette smoke and air pollutants. Since polκ has been shown to bypass benzo(a)pyrene (B(a)P)-guanine adducts a major DNA lesion generated by cigarette smoke (Zhang et al., 2002), studies were designed to determine whether polκ was involved in the bypass of these lesions in mice. It was shown that polκ mutant embryonic stem cells are hypersensitive to both the lethal and the mutagenic effects of B(a)P compared to their wild-type counterparts (Ogi et al., 2002). Another study using polκ deficient MEF showed similar results. They demonstrated that polκ was responsible for two thirds of the B(a)P guanine adducts lesion bypass events and that polκ causes fewer mutagenic events than bypass of these lesions by other polymerases (Avkin et al., 2004). Together these studies provide strong evidence that polκ plays an important role in the accurate bypass of DNA lesions induced by B(a)P in mammalian cells, therefore protecting cells from the genotoxic effects of B(a)P.

6.1.2.4 The effects of polκ on genomic stability

The ability of polκ to increase the rates of single base substitutions and deletions leading to frameshift mutations using template misalignment (Wolfe et al., 2003),

suggests that once recruited polκ could play a role in genomic integrity through the introduction of lesion triggered and spontaneous mutations during replication.

Other evidence that polκ may be involved in genomic instability is through its possible involvement in tumourgenesis. It is widely accepted that genomic instability is involved in cancer development. Overexpression in lung cancer tumours indicates that polκ may contribute to lung cancer development by the acceleration of the accumulation of mutations (O-Wang et al., 2001). Another study showed that overexpression of polκ induces breaks and stimulates DNA exchanges as well as aneuploidy (Bavoux et al., 2005). It is possible that the accumulation of mutations caused by polκ may increase genomic instability.

6.2 Experimental design

To date nothing is known about the spontaneous germline mutation rates of polκ deficient mice. To analyse the effect of polκ deficiency on the spontaneous ESTR mutation rates, *polκ* $^{-/-}$ and *polκ* $^{+/+}$ males were mated with non-exposed BALB/c females to produce the control offspring. All the parents and offspring were analysed by profiling with two hypervariable single-locus ESTR probes, Ms6-hm and Hm-2.

6.3 Summary of spontaneous mutation at ESTR loci

A summary of ESTR mutation data is presented in Table 6.1. The ESTR mutation data for both the single locus probes is presented for *polκ* $^{-/-}$ and *polκ* $^{+/+}$ male mice (non-exposed). The table contains the numbers of total mutations and singleton mutations scored. There was no significant difference between the total mutation data and the singleton data, therefore only the total mutation numbers were used to analyse the data.

The table also presents the numbers of gain and loss mutants observed for both genotypes. It was found that there was no significant difference in the type of mutations observed between the wild-type and polκ deficient mice.

	Ms6-hm		*Hm-2*		Total		Type of mutants	
Strain	No mutations*	No bands	No mutations*	No bands	No mutations*	No bands	Gains	Losses
polK$^{+/+}$	10 (8)	94	6 (6)	126	16 (14)	220	7	9
polK$^{-/-}$	25 (23)	104	7 (5)	106	32 (28)	210	15	17
χ^2, *df*=1, (Prob)‡							0.04	(0.8377)

* Number of singleton mutations is given in parenthesis.
‡ Chi-square test for homogeneity of the type of mutants between the *polK*$^{+/+}$ and *polK*$^{-/-}$ males.

Table 6.1: Summary of ESTR mutation data, showing total number of bands scored and number of mutations

6.4 Spontaneous ESTR mutation rates in *Polκ*$^{+/+}$ and *Polκ*$^{-/-}$ mice

The estimates of the spontaneous ESTR mutations rates for both *polκ*$^{-/-}$ and *polκ*$^{+/+}$ males are presented in Table 6.2. A significant 2.1 fold increase in spontaneous ESTR mutation rate was observed in *polκ*$^{-/-}$ males when compared to *polκ*$^{+/+}$ males.

Strain	No mutations	No bands	Rate per Locus	Ratio *Polκ*$^{-/-}$ to *Polκ*$^{+/+}$	Prob.*
polK$^{+/+}$	16	220	0.0727	-	
polK$^{-/-}$	32	210	0.1524	2.10	0.0131

* Probability of difference from the wild-type strain (Fisher's exact test, two-tailed).

Table 6.2: ESTR mutation rates in the *polκ*$^{+/+}$ and *polκ*$^{-/-}$ male mice

6.5 ESTR mutation spectrum in polκ deficient mice

The ESTR mutation spectrum of *polκ*$^{-/-}$ males was compared to *polκ*$^{+/+}$ isogenic males and the results can be seen in Figure 6.1 and illustrate that there was no difference between the mutation spectra for the two genotypes. Statistical analysis indicates that there was no significant difference between the spectra of mutation of *polκ*$^{-/-}$ males when compared to the spectra of *polκ*$^{+/+}$ males.

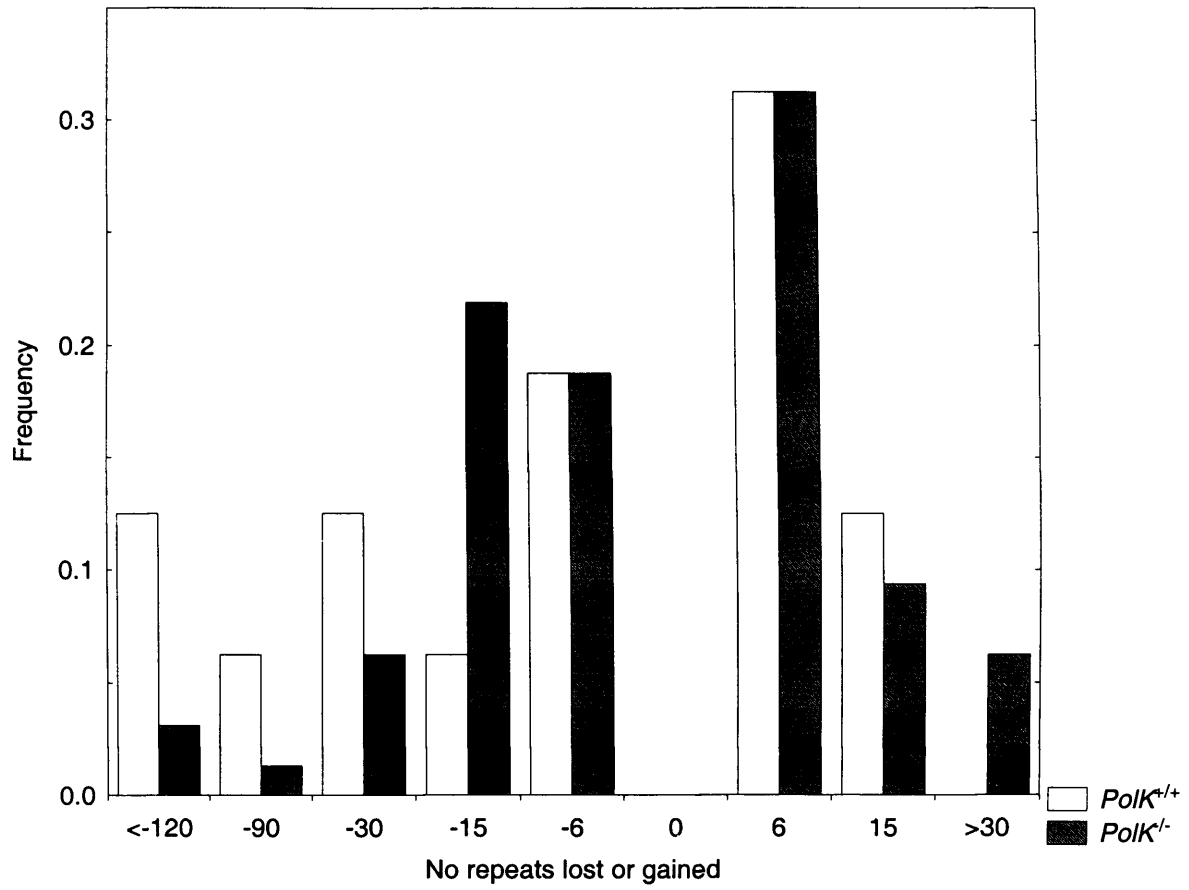


Figure 6.1: Spectrum of paternal ESTR mutations in the the $polκ^{+/+}$ and $polκ^{-/-}$ male mice (Kolmogorov-Smirnov test, $P>0.10$). The values on the x-axis represent the loer limit of number of repeats included in each bin.

6.6 Discussion

The aim of this study was to establish whether polκ may influence the spontaneous ESTR mutation rate in the mouse male germline. Given that this issue has never been analysed, the data presented in this chapter illustrates for the first time that polκ deficiency affects the spontaneous ESTR mutation in the germline of male mice.

During the analysis of ESTRs in polκ deficient mice it was found that there was no difference in the types of mutations observed in the polκ mutant mice compared to wild-type mice. This indicates that the mechanism of mutation in the two strains is the same. Further evidence of a similar mutation mechanism at the ESTR loci came from the analysis of the spectra of ESTR mutation in both strains, showing no significant difference between the $polκ^{-/-}$ and $polκ^{+/+}$ males.

Statistics showed that there was no significant difference between the spectra of mutations for *polκ*$^{-/-}$ males when compared to the spectra of *polκ*$^{+/+}$ males.

Mutation rates in the germline of polκ deficient and wild-type males were evaluated by scoring ESTR mutations in their offspring. A significant 2 fold increase in spontaneous ESTR mutation rate was found in *polκ*$^{-/-}$ males when compared to *polκ*$^{+/+}$ males. Therefore it can be inferred from the data that the lack of polκ enhances spontaneous ESTR mutation. Another conclusion that can be drawn from the data is that the mechanism of ESTR mutation in both strains is the same, but is enhanced in polκ deficient mice. We know that the mechanism of ESTR mutation is due to a non targeted event that probably involves a replication based process. This lead to the theory that the mechanism could involve polymerase slippage due to replication fork pausing. To determine whether the same mechanism that has been proposed for PARP-1 deficient and scid mice holds true for the polκ mice the data needs to be compared (see Figure 6.2) and the function of polκ must be considered.

Figure 6.2 presents the comparison of the spontaneous ESTR mutation rates in the germline of DNA repair deficient mice. When compared to their isogenic wild-type counterparts, all deficient strains show a significant increase in spontaneous ESTR mutation rates. This indicates that the increase in ESTR mutation rates in these strains may be due to their deficiency in DNA repair, supporting the hypothesis that DNA repair may be involved in the non-targeted event that leads to ESTR mutations.

To investigate whether the polymerase slippage hypothesis proposed during the PARP-1 study could account for the increased ESTR mutation rate observed in polκ deficient males, the function of polκ has to be taken into account. Polymerase kappa is known to belong to a superfamily of proteins, the Y-family of DNA polymerases (Ohmori et al., 2001). Polκ is a specialised DNA polymerase that may be used by cells to bypass sites of base damage via a mechanism called translesion synthesis (TLS). It is known that when normal replicative polymerases encounter DNA lesions during DNA replication, the replication fork is stalled. To resume DNA replication a polymerase, such as polκ, with translesion synthesis ability is required (Toueille and Hubscher, 2004). Bergoglio et al. (2002) found that polκ localised in the nuclei at replication forks in a proportion of undamaged cells examined. This suggests that polκ may function as part of the replicative machinery itself. They also showed that when replication forks were arrested using an antireplicative agent that there was a

dramatic increase in the number of cells containing polκ within the nucleus. This demonstrated that polκ is associated with both functional and chemical-mediated stalled replication forks. The association of polκ with stalled replication forks suggests that polκ is recruited to resume replication stalled by blocking lesions. Other Y family polymerases have been shown to accumulate at stalled DNA replication forks (Kannouche et al., 2002). Since all the Y family polymerases can bypass a variety of damage bases *in vitro*, each having different affinities to different types of lesions, this suggests that they all may be present in the nucleus awaiting recruitment to resume replication forks.

Since polκ is required to resume replication at stalled replication forks, the lack of polκ may enhance the number of stalled replication forks within the genome and/or prolong the delay before lesions may be bypassed by other polymerases or alternatively, repaired by repair machinery. An increase in the number of stalled replication forks would increase the level of cell death, as replication pausing allows time for repair to occur and may lead to cell death if not dealt with (Velasco-Miguel *et al.*, 2003). A prolonged delay in replication before repair or bypass of lesion could lead to enhanced polymerase slippage as suggested in the PARP-1 and *scid* studies. It is possible that an increase in the number of delayed replication forks in polκ deficient mice could account for the enhanced spontaneous mutation rate observed in the $pol\kappa^{-/-}$ males when compared to $pol\kappa^{+/+}$ males.

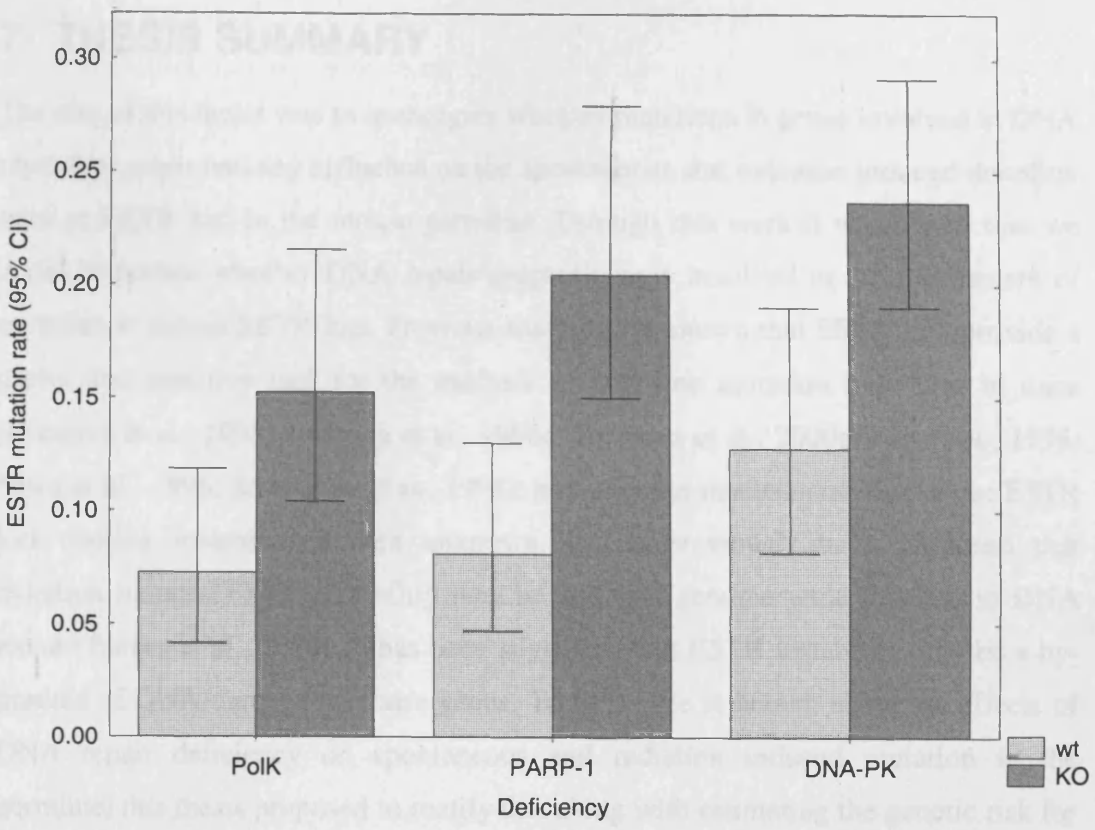


Figure 6.2: ESTR mutation rates in the germline of wild type (wt) and deficient (KO) male mice. The 95% confidence intervals, CI for mutation rate, estimated from the Poisson distribution are shown.

7 THESIS SUMMARY

The aim of this thesis was to investigate whether mutations in genes involved in DNA repair/apoptosis had any influence on the spontaneous and radiation induced mutation rates at ESTR loci in the mouse germline. Through this work it was hoped that we could determine whether DNA repair/apoptosis were involved in the mechanism of mutation at mouse ESTR loci. Previous studies have shown that ESTR loci provide a useful and sensitive tool for the analysis of germline mutation induction in mice (Dubrova et al., 1993; Dubrova et al., 1998a; Dubrova et al., 2000a; Fan et al., 1995; Niwa et al., 1996; Sadamoto et al., 1994); however the mechanisms that act at ESTR loci causing instability remain unknown. It has previously been proposed that radiation induced ESTR instability may be due to a genome wide increase in DNA repair (Barber et al., 2000). It has been suggested that ESTR instability may be a by-product of DNA damage response genes. To date little is known about the effects of DNA repair deficiency on spontaneous and radiation induced mutation in the germline; this thesis proposed to rectify this along with estimating the genetic risk for a cohort of individuals with mutations at DNA repair genes. All of the above factors lead to the initiation of this study.

During the work for this thesis four DNA damage response gene deficiencies in mice were analysed; 3 genes involved in DNA repair and one involved in apoptosis. All four genes are involved in essential pathways of maintaining the stability of the genome.

The first part of my work was to analyse whether poly(ADP-ribose) polymerase-1 (PARP-1) deficiency in mice effects spontaneous and radiation induced ESTR mutation rates in the male germline. PARP-1 is known to be involved in the early DNA damage recognition and repair of single strand breaks (Herceg et al., 2001). The assessment of PARP-1 deficient and isogenic PARP-1 wild-type males was carried out by assessing spontaneous and radiation induced ESTR mutation rates. The data showed that PARP-1 deficient mice had a significant increase in spontaneous ESTR mutation rate when compared to isogenic wild-type mice. PARP-1 wild-type mice showed a significant increase in ESTR mutation rate after exposure to 1Gy of acute irradiation. In contrast, PARP-1 deficient mice showed no increase in ESTR mutation rate in the germline after exposure to ionising radiation. These results indicate that

PARP-1 is involved in the maintenance of genomic stability at ESTR loci in the germline of non-exposed male mice.

To gain more insight into the mechanisms that act at ESTR loci causing instability in the germline, the PARP-1 results were compared to those obtained through a similar study preformed on *scid* mice (Barber et al., 2004). The results from these studies were very similar; both DNA repair deficient strains have an elevated spontaneous ESTR mutation rate and lack ESTR mutation induction after exposure to ionising radiation. Both scid and PARP-1 deficient mice are deficient in early DNA damage recognition and it has been suggested that an inability to recognise damage may lead to a delay in repair and alter the cell cycle progression. Through these results it has been proposed that PARP-1 and *scid* mice show an elevation in germline ESTR mutation rates due to a delay in repair which could enhance replication fork pausing and therefore enhancing replication slippage. This provides the first model for the mechanism of mutation at ESTR loci.

The lack of radiation induced ESTR mutation and reduction in fertility in the DNA repair deficient strains studied led to the hypothesis that this phenomenon may be due to the high killing effect of radiation. To test this hypothesis the second part of my study involved the analysis the effects of p53 deficiency on spontaneous and radiation induced ESTR instability. The tumour suppressor, p53 plays a critical role in the arrest of the cell cycle to allow adequate time for repair or cell death via apoptosis (Vogelstein et al., 2000). To analyse whether p53 status had any affect on ESTR instability the spontaneous and radiation induced ESTR mutations were assessed in p53 deficient and isogenic wild-type male mice. The data obtained through this study showed that the p53 status did not affect the spontaneous or radiation induced ESTR mutation rates in the mouse male germline. From this study it can be concluded that p53 has no involvement in ESTR stability.

The third part of my work involved a gene which is associated with the rare autosomal disease xeroderma pigmentosum (XP), xeroderma pigmentosum complementation group C (XPC). The XPC gene product has been shown to initiate global genomic repair (GGR), one of the nucleotide excision repair (NER) pathways, by sensing and binding to bulky DNA lesions (Sugasawa et al., 1998). To determine the effect of XPC deficiency on ESTR mutation rates both the spontaneous and radiation induced mutation rates in XPC deficient and isogenic wild-type male mice were analysed and compared. From this study it was found that unlike the other DNA

repair deficient strains both the wild-type and the XPC deficient males showed an elevation of ESTR mutation after exposure to 1 Gy of ionising radiation. This increase in mutation rates in both strains indicates that both XPC deficient and wild-type mice are sensitive to ionising radiation.

XPC deficient mice were observed to be more susceptible to exposure to ionising radiation than their wild-type counterparts, as they are more susceptible to spontaneous mutations. This study indicates that XPC patient may be more radiosensitive than normal patients.

A non-significant increase in spontaneous mutation rates was found in XPC deficient males compared to wild-type. This slight increase may be explained by the fact that XPC deficiency only partially impairs NER as GGR alone is affected and transcription coupled repair can still occur (Venema et al., 1991). It is plausible that the elevated ESTR mutation rates observed in XPC deficient mice may be due the mechanism proposed through the PARP-1 study. It is known that XPC is involved in the recognition of bulky lesions and the recruitment of repair machinery, therefore is involved in the early response to DNA damage (Sugasawa et al., 1998; Yokoi et al., 2000). Studies have shown that the lack of XPC severely restricts the repair of bulky lesions by GGR and indicate that only a small percentage are repaired via other pathways (Venema et al., 1990; Venema et al., 1991). The presence of bulky lesions affects polymerase progression (Osborn et al., 2002), therefore it is possible that the delay in repair of bulky lesions by NER or unrepaired bulky lesions could lead to prolonged replication fork pausing and promote polymerase slippage, as proposed in chapter 3.

This study also presented the first experimental evidence for the effects of XPC deficiency on spontaneous and radiation induced mutation rates in the mouse germline.

The final part of work presented in this thesis analysed the effect of polymerase κ (polκ) deficiency on ESTR stability. Polκ is a member of the Y-family DNA polymerases that are able to bypass lesions via a process called translesion synthesis (TLS) (Ohmori et al., 2001). TLS is a DNA damage response that allows the bypass of DNA lesions and extension of replication forks through damaged sites without repairing the lesion (Lindahl and Woods, 1999). To determine whether polκ influenced ESTR stability, mice deficient in polκ and wild-type mice were analysed

and their spontaneous mutation rates observed. The data obtained from this study showed that polκ deficient mice had an elevated spontaneous mutation rate when compared to wild-type. Therefore polκ deficiency enhances spontaneous ESTR mutations in the mouse male germline. It is known that polκ along with other Y-family polymerases are required to resume replication at stalled replication forks (Toueille and Hubscher, 2004). Polκ has been associated with stalled replication forks and it has been suggested that polκ is recruited to resume replication stalled by blocking lesions (Bergoglio et al., 2002). Since polκ is required to resume stalled replication forks, it is possible that the lack of polκ may enhance the number of stalled replication forks within the genome or prolong the delay before lesion may be bypassed by other polymerases or repaired. This could lead to enhanced polymerase slippage as suggested through the PARP-1 study. Therefore the increase in spontaneous mutation rate in polκ deficient males could be due to an increase in delayed forks.
Little was known about polκ *in vivo*; this study shows that polκ may be involved in germline instability.

This study has shown for the first time that DNA repair deficiencies cause ESTR instability in the male germline. This implies that DNA repair is involved in the maintenance of germline stability. The data showed that the spontaneous ESTR mutation rate is elevated in the germline of DNA repair deficient male mice This raises the possibility that DNA-repair deficiencies could affect the germline mutation rates throughout the genome and therefore could be regarded as an important genetic risk factor, therefore highlighting a cohort of individuals that could be more sensitive to mutagens.

During this thesis a model for the mechanism of mutation at mouse ESTR loci has been proposed. It has been suggested that the lack or delay of DNA repair could cause replication fork pausing following by polymerase slippage. This model suggests that ESTR instability may be due to polymerase slippage which may be further enhanced by replication fork pausing, similar to the model proposed for microsatellite instability. The high spontaneous mutation rates at ESTR loci could be explained by this model through their large size and their complex secondary structure both of

which could lead to replication fork pausing and therefore promote polymerase slippage events. This model could also be used to explain elevated radiation and chemical induced ESTR mutation rates. It is possible that exposure to DNA damaging agents could result in global replication fork pausing, therefore polymerase slippage. This theory is supported by the data that indicate that the spontaneous and radiation induced ESTR mutation spectra are similar and ESTR mutation only occurs in replication proficient diploid germ cells (Dubrova et al., 1998a).

The work presented in this thesis has shown for the first time that DNA repair deficiencies can cause germline instability in mice and suggested a model for the mechanism of mutation at ESTR loci.

8 FUTURE WORK

The work presented in this thesis provides initial information for a model for the characterisation of the mutation mechanism at ESTR loci. Also the results presented show that DNA repair deficiencies increase ESTR instability. To continue this work it may be possible to carry out the analysis of other deficient mouse strains in association with ESTR instability, the effects of mice heterozygous for DNA repair deficiencies and the effects of chemical mutagens on ESTR stability.

8.1 Effects of DNA repair deficiency on ESTR stability

DNA repair is essential for genomic integrity. Several DNA repair pathways have been studied during the work presented in this thesis. To analyse the effects of DNA repair on ESTR stability further, the influence of other DNA repair genes should be analysed. Genes involved in essential pathways involved in maintaining the stability of the genome should be studied as well as genes that provide good models for human disorders.

8.1.1 Ataxia Telangiectasia Mutated

Ataxia telangiectasia mutated (ATM) is responsible for an autosomal recessive disorder called ataxia telangiectasia (AT). AT is characterised by an early onset progressive cerebrallar ataxia, telangiectasia, immunodeficiency, chromosomal instability, hypersensitivity to ionising radiation and an increased incidence in cancers (Lavin and Shiloh, 1997).

ATM is a protein kinase central to all DNA maintenance responses and when absent results in the disease AT (Bernstein et al., 2002). ATM plays a central role in signalling DNA damage, particularly double strand breaks and is activated on recognitions of damage. ATM acts as a cellular gatekeeper and is the key initiation factor in the cascade of events leading to at least six DNA damage responsive pathways (Figure 8.1). Mice lacking ATM would not only provide a good model for a known human disorder but also they lack a gene that has a key role in DNA repair. The knockout (*Atm*-ΔSRI) harbouring a mutation that is common in people suffering with ataxia telangiectasia (the 7636del9 deletion, Spring et al., 2001; Spring et al.,

2002) represents an attractive model for this analysis. In contrast to two well known Atm knockouts, where the truncating mutations produce a highly unstable and undetectable protein (Elson et al., 1996; Barlow et al., 1999), the ΔSRI mutant expresses relatively stable protein with abolished ATM kinase activity (Spring et al., 2002). The ΔSRI missense mutation has a dominant-negative effect and mice carrying this mutation have a higher risk of cancer (Spring et al., 2002), therefore providing a better experimental model for ATM deficiency in humans.

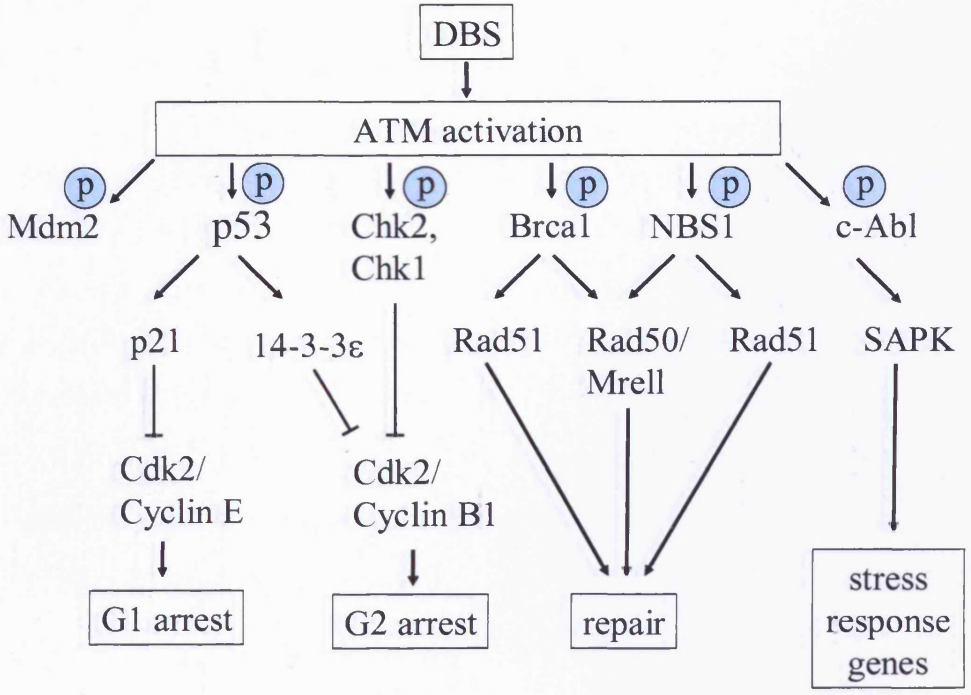


Figure 8.1: ATM signalling pathway (adapted from Bernstein et al., 2002)

8.1.2 Msh2

Msh2 is involved in mismatch repair through the initial recognition of mispairs (Jiricny and Nystrom-Laliti, 2000). Mismatch repair (MMR) eliminates base-base mismatches and insertion-deletion loops which arise as a consequence of DNA polymerase slippage during DNA replication, which in turn leads to microsatellite instability in MMR deficient mice. Defects in mismatch repair in humans give rise to microsatellite instability and a predisposition to cancer, particularly hereditary non-polyposis colorectal cancer (HNPCC) syndrome (Lynch et al., 1996).

8.1.3 BRCA1

An estimated 50% of inherited cases of breast cancer are likely due to mutations in the breast cancer tumour suppressor gene, BRCA1. Mutations in BRCA1 have also been implicated in almost all families with histories of both ovarian and breast cancers (Gayther et al., 1998).

The BRCA1 protein acts as part of a large multimeric complex referred to as the BRCA1 associated genome surveillance complex (BASC) thought to act as a DNA damage sensor and BRCA1 is known to interact with a number of proteins that have roles in multiple cellular processes (Wang et al., 2000; Deng and Brodie, 2000).

Mutations in BRCA1 have been shown to cause a reduction in homologous recombination of DSBs and increased genomic instability (Moynahan, 1999). Loss of BRCA1 leads to defective DNA damage repair, abnormal centrosome duplication. G2-M cell cycle checkpoint defect, growth retardation, increased apoptosis, genetic instability and tumourigenesis (Deng and Brodie, 2000). Although the exact role of BRCA1 remains unknown it is thought to play a role in the processing of DNA damage. BRCA1 has been associated with mismatch repair, homologous recombination and transcription coupled repair (Bernstein et al., 2002).

8.2 Effects of mice heterozygous for DNA repair genes on ESTR instability

This study has highlighted that DNA repair deficiencies increases ESTR instability, however, the effects on ESTR instability in mice heterozygous for mutations at genes involved in DNA repair remains unclear. Also it is possible that exposure to ionising radiation may affect mutation rates in the germline of mice heterozygous for mutations in DNA repair genes. Various studies have shown that mice heterozygous for some DNA repair genes have elevated risk of tumour development (Fodde and Smits, 2002). In a human population heterozygous individuals are more common; therefore the analysis of mice heterozygous for mutations in DNA repair genes is more relevant to the human situation and would highlight a cohort of individuals that may be more sensitive to spontaneous and/or induced mutations.

8.3 Effects of DNA repair deficiencies on somatic tissues

A novel, single molecule PCR (SM-PCR) based approach using, the *Ms6-hm* locus has been developed to monitor spontaneous and radiation induced ESTR instability in mouse somatic and germ cells (Yauk et al., 2002). SM-PCR has been shown to give robust estimates of both spontaneous and radiation induced germline mutation rates in mice when compared to ESTR pedigree data. SM-PCR reduces the numbers of mice needed for the measurement of mutation frequencies considerably and allows detailed analysis of the spectrum of spontaneous and radiation induced ESTR mutation.

Future work involving SM-PCR should establish whether DNA repair deficiency has any affect on ESTR mutation rates in the somatic tissue as well as in the germline in mice. During the work for this thesis it was been found that the *Hm-2* locus in some of the deficient strains is particularly small and could therefore be used alone or alongside the *Ms6-hm* locus for single molecule analysis.

8.4 Effects of chemical mutagens on ESTR instability in DNA repair deficient mice

Just like radiation, exposure to chemical mutagens affects ESTR mutation rates in the germline of normal mouse strains (Vilarino-Guell et al., 2003). It remains to be established whether exposure to chemical mutagens may affect ESTR mutation rates in the germline of DNA repair deficient mice. The analysis of the effects of chemical mutagens on ESTR instability could aid in the verification of the mutation mechanism at ESTR loci. These types of studies could also highlight individuals which might exhibit increased sensitivity to exposure to certain chemical mutagens.

8.5 Maternal effect on ESTR instability

All studies involving ESTR instability to date have been designed to analyse the effects of exposure and/or DNA repair deficiency on the paternal germline. It would be interesting and important to investigate the maternal effect of DNA repair deficiency and exposure to ionising radiation on ESTR instability.

9 References

Aaltonen, L. A., Peltomaki, P., Leach, F. S., Sistonen, P., Pylkkanen, L., Mecklin, J. P., Jarvinen, H., Powell, S. M., Jen, J., Hamilton, S. R., and et al. (1993). Clues to the pathogenesis of familial colorectal cancer. *Science* **260**, 812-816.

Adler, I. D. (1996). Comparison of the duration of spermatogenesis between male rodents and humans. *Mutat Res* **352**, 169-172.

Alsbeih, G., Torres, M., Al-Harbi, N., and Alsubael, M. (2004). Loss of wild-type Trp53 protein in mouse fibroblasts leads to increased radioresistance with consequent decrease in repair of potential lethal damage. *Radiat Res* **161**, 185-192.

Amarger, V., Gauguier, D., Yerle, M., Apiou, F., Pinton, P., Giraudeau, F., Monfouilloux, S., Lathrop, M., Dutrillaux, B., Buard, J., and Vergnaud, G. (1998). Analysis of distribution in the human, pig, and rat genomes points toward a general subtelomeric origin of minisatellite structures. *Genomics* **52**, 62-71.

Andersen, T. H., and Nilsson-Tillgren, T. (1997). A fungal minisatellite. *Nature* **386**, 771.

Archibald, A. L., Haley, C. S., Brown, J. F., Couperwhite, S., McQueen, H. A., Nicholson, D., Coppieters, W., Van de Weghe, A., Stratil, A., Wintero, A. K., and et al. (1995). The PiGMaP consortium linkage map of the pig (*Sus scrofa*). *Mamm Genome* **6**, 157-175.

Armour, J. A.; Povey, S.; Jeremiah, S. and Jeffreys, A. J. (1990). Systematic cloning of human minisatellites from ordered array charomid libraries. *Genomics* **8**, 501-512.

Armour, J. A. (1999). Minisatellites and mutation processes in tandemly repetitive DNA. In Microsatellites: Evolution and Applications, D. B. Goldstein, and C. Schlotterer, eds. (Oxford, Oxford University Press), pp. 24-34.

Armstrong, J. F., Kaufman, M. H., Harrison, D. J., and Clarke, A. R. (1995). High-frequency developmental abnormalities in p53-deficient mice. *Curr Biol* **5**, 931-936.

Ashley, T. (1994). Mammalian meiotic recombination: a re-examination. *Hum Genet* **94**, 587-593.

Asghari, V.; Schoots, O.; van Kats, S.; Ohara, K.; Jovanovic, V.; Guan, H. C.; Bunzow, J. R.; Petronis, A. and Van Tol, H. H. (1994). Dopamine D4 receptor repeat: analysis of different native and mutant forms of the human and rat genes. *Mol Pharmacol* **46**, 364-373.

Aubrecht, J., Secretan, M. B., Bishop, J. R., and Schiestl, R. H. (1999). Involvement of p53 in X-ray induced intrachromosomal recombination in mice. *Carcinogenesis* **20**, 2229-2236.

Avkin, S., Goldsmith, M., Velasco-Miguel, S., Geacintov, N., Friedberg, E. C., and Livneh, Z. (2004). Quantitative analysis of translesion DNA synthesis across a benzo[a]pyrene-guanine adduct in mammalian cells. *J Biol Chem* **279**, 53298-53305.

Bacon, A. L., Dunlop, M. G., and Farrington, S. M. (2001). Hypermutability at a poly(A/T) tract in the human germline. *Nucleic Acids Res* **29**, 4405-4413.

Baker, S. M.; Bronner, C. E.; Zhang, L.; Plug, A. W.; Robatzek, M.; Warren, G.; Elliott, E. A.; Yu, J.; Ashley, T.; Arnheim, N.; Flavell, R. A. and Liskay, R. M. (1995). Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell* **82**, 309-319.

Baker, S. M.; Plug, A. W.; Prolla, T. A.; Bronner, C. E.; Harris, A. C.; Yao, X.; Christie, D. M.; Monell, C.; Arnheim, N.; Bradley, A.; Ashley, T. and Liskay, R. M. (1996). Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. *Nat Genet* **13**, 336-342.

Balazs, I., Baird, M., Clyne, M., and Meade, E. (1989). Human population genetic studies of five hypervariable DNA loci. *Am J Hum Genet* **44**, 182-190.

Barber, R., Plumb, M., Smith, A. G., Cesar, C. E., Boulton, E., Jeffreys, A. J., and Dubrova, Y. E. (2000). No correlation between germline mutation at repeat DNA and meiotic crossover in male mice exposed to X-rays or cisplatin. *Mutat Res* **457**, 79-91.

Barber, R., Plumb, M. A., Boulton, E., Roux, I., and Dubrova, Y. E. (2002). Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice. *Proc Natl Acad Sci U S A* **99**, 6877-6882.

Barber, R. C., Miccoli, L., van Buul, P. P. W., Burr, K. L-A., van Duyn-Goedhart, A., Angulo, J. F., and Dubrova, Y. E. (2004). Germline mutation rates at tandem repeat loci in DNA-repair deficient mice. *Mutat Res* **554**, 287-295.

Barlow, C., Eckhaus, M. A., Schaffer, A. A., and Wynshaw-Boris, A. (1999). Atm haploinsufficiency results in increased sensitivity to sublethal doses of ionising radiation in mice. *Nat Genet* **21**, 359-360.

Batchelor, A. L., Phillips, R. J., and Searle, A. G. (1966). A comparison of the mutagenic effectiveness of chronic neutron- and gamma-irradiation of mouse spermatogonia. *Mutat Res* **3**, 218-229.

Batchelor, A. L., Phillips, R. J. L., and Searle, A. G. (1967). The reversed dose-rate effect with fast neutron irradiation of mouse spermatogonia. *Mutat Res* **4**, 229-231.

Batty, D. P., and Wood, R. D. (2000). Damage recognition in nucleotide excision repair of DNA. *Gene* **241**, 193-204.

Bavoux, C., Leopoldino, A. M., Bergoglio, V., O-Wang, J., Ogi, T., Bieth, A., Judde, J. G., Pena, S. D., Poupon, M. F., Helleday, T., Tagawa, M., Machado, C., Hoffmann, J. S., and Cazaux, C. (2005). Up-regulation of the error-prone DNA polymerase (kappa) promotes pleiotropic genetic alterations and tumourigenesis. *Cancer Res* **65**, 325-330.

Beckman, J. S. and Weber, J. L. (1992). Survey of human and rat microsatellites. *Genomics* **12**, 627-631.

BEIR VI. (1999). Health effects of exposure to RADON. (National Academy Press, Washington, D.C.).

Bergoglio, V., Bavoux, C., Verbiest, V., Hoffman, J-S., and Cazaux, C. (2002). Localisation of human DNA polymerase κ to replication foci. *J Cell Sci* **115**, 4413-4418.

Bergthorsson, J. T., Eiriksdottir, G., Barkardottir, R. B., Egilsson, V., Arason, A., and Ingvarsson, S. (1995). Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3. *Hum Genet* **96**, 437-443.

Bernstein, C., Bernstein, H., Payne, C. M., and Garewal, H. (2002). DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe prptection against carcinogenesis. *Mutat Res* **511**, 145-178.

Beumer, T. L., Roepers-Gajadien, H. L., Gademan, I. S., van Buul, P. P. W., Gil-Gomez, G., Rutgers, D. H., and de Rooij, D. G. (1998). The role of the tumor suppressor p53 in spermatogenesis. *Cell Death Diff* **5**, 669-677.

Biedermann, K. A., Sun, J. R., Giaccia, A. J., Tosto, L. M., and Brown, J. M. (1991). *Scid* mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. *Proc Natl Acad Sci U S A* **88**, 1394-1397.

Bielas, J. H., and Heddle, J. H. (2003). Elevated mutagenesis and decreased DNA repair at a transgene are associated with proliferation but not apoptosis in p53-deficient cells. *Proc Natl Acad Sci U S A* **100**, 12853-12858.

Bishop, A. J. R., Hollander, M. C., Kosaras, B., Sidman, R. L., Fornace, A. J. Jr, and Schiestl, R. H. (2003). Atm-, p53- and gadd45a-deficient mice show an increased frequency of homologous recombination at different stages during development. *Cancer Res* **63**, 5335-5343.

Blunt, T., Finnie, N. J., Taccioli, G. E., Smith, G. C., Demengeot, J., Gottlieb, T. M., Mizuta, R., Varghese, A. J., Alt, F. W., Jeggo, P. A., and Jackson, S. P. (1995). Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine *scid* mutation. *Cell* **80**, 813-823.

Blunt, T., Gell, D., Fox, M., Taccioli, G. E., Lehmann, A. R., Jackson, S. P., and Jeggo, P. A. (1996). Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse. *Proc Natl Acad Sci U S A* **93**, 10285-10290.

Bois, P., Stead, J. D. H., Bakshi, S., Williamson, J., Neumann, R., Moghadaszadeh, B., and Jeffreys, A. J. (1998a). Isolation and characterization of mouse minisatellites. *Genomics* **50**, 317-330.

Bois, P., Williamson, J., Brown, J., Dubrova, Y. E., and Jeffreys, A. J. (1998b). A novel unstable mouse VNTR family expanded from SINE B1 elements. *Genomics* **49**, 122-128.

Bois, P., and Jeffreys, A. J. (1999). Minisatellite instability and germline mutation. *Cell Mol Life Sci* **55**, 1636-1648.

Bois, P. R., Southgate, L., and Jeffreys, A. J. (2001). Length of uninterrupted repeats determines instability at the unstable mouse expanded simple tandem repeat family MMS10 derived from independent SINE B1 elements. *Mamm Genome* **12**, 104-111.

Bois, P. J., Grant, G. R., and Jeffreys, A. J. (2002). Minisatellites show rare and simple intra-allelic instability in the mouse germ line. *Genomics* **80**, 2-4.

Bohr, V. A., Smith, C. A., Okumoto, D. S., and Hanawalt, P. C. (1985). DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. *Cell* **40**, 359-369.

Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature* **301**, 527-530.

Bosma, G. C., Davisson, M. T., Ruetsch, N. R., Sweet, H. O., Shultz, L. D., and Bosma, M. J. (1989). The mouse mutation severe combined immune deficiency (*scid*) is on chromosome 16. *Immunogenetics* **29**, 54-57.

Boue, A., Boue, J., and Gropp, A. (1985). Cytogenetics of pregnancy wastage. *Adv Hum Genet* **14**, 1-57.

Boudsocq, F., Ling, H., Yang, W., and Woodgate, R. (2002). Structure-based interpretation of missense mutations in Y-family DNA polymerases and their implications for polymerase function and bypass. *DNA Repair* **1**, 343-358.

Bouffler, S. D., Kemp, C. J., Balmain, A., and Cox, R. (1995). Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. *Cancer Res* **55**, 3883-3889.

Bridges, B. A. (2001). Radiation and germline mutation at repeat sequences: Are we in the middle of a paradigm shift? *Radiat Res* **156**, 631-641.

Brinkmann, B.; Klintschar, M.; Neuhuber, F.; Huhne, J. and Rolf, B. (1998). Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. *Am J Hum Genet* **62**, 1408-1415.

Buard, J., and Vergnaud, G. (1994). Complex recombination events at the hypermutable minisatellite CEB1 (D2S90). *EMBO J* **13**, 3203-3210.

Buard, J., and Jeffreys, A. J. (1997). Big, bad minisatellites. *Nat Genet* **15**, 327-328.

Buard, J., Bourdet, A., Yardley, J., Dubrova, Y. E., and Jeffreys, A. J. (1998). Influences of array size and homogeneity on minisatellite mutation. *EMBO J* **17**, 3495-3502.

Buard, J., Collick, A., Brown, J., and Jeffreys, A. J. (2000a). Somatic versus germline mutation processes at minisatellite CEB1 (D2S90) in humans and transgenic mice. *Genomics* **65**, 95-103.

Buard, J., Shone, A. C., and Jeffreys, A. J. (2000b). Meiotic recombination and flanking marker exchange at the highly unstable human minisatellite CEB1 (D2S90). *Am J Hum Genet* **67**, 333-344.

Budowle, B., Moretti, T. R., Keys, K. M., Koons, B. W., and Smerick, J. B. (1997). Validation studies of the CTT STR multiplex system. *J Forensic Sci* **42**, 701-707.

Burke, T., and Bruford, M. W. (1987). DNA fingerprinting in birds. *Nature* **327**, 149-152.

Cardis, E., Gilbert, E. S., Carpenter, L., Howe, G., Kato, I., Armstrong, B. K., Beral, V., Cowper, G., Douglas, A., Fix, J., Fry, S. A., Kaldor, J., Lave, C., Salmon, L., Smith, P. G., Voelz, G. L., and Wiggs, L. D. (1995). Effects of low doses and low dose rates of external ionizing radiation: cancer mortality among nuclear industry workers in three countries. *Radiat Res* **142**, 117-132.

Carvalho, H., Augusto da Costa, R. M., Chigancas, V., Weinlich, R., Brumatti, G., Amarante-Mendes, G. P., Sarasin, A., and Menck, C. F. M. (2003). Effect of cell confluence on ultraviolet light apoptotic responses in DNA repair deficient cells. *Mutat Res* **544**, 159-166.

Chaillet, J. R., Bader, D. S., and Leder, P. (1995). Regulation of genomic imprinting by gametic and embryonic processes. *Genes Dev* **9**, 1177-1187.

Chalmers, A. J. (2004). Poly(ADP-ribose) polymerase-1 and ionizing radiation: Sensor, signaller and therapeutic target. *Clin Oncol* **16**, 29-39.

Charles, D. J., and Pretsch, W. (1986). Enzyme-activity mutations detected in mice after paternal fractionated irradiation. *Mutat Res* **160**, 243-248.

Charles, D. R. (1950). Radiation-induced mutations in mammals. *Radiology* **55**, 579-581.

Cheo, D. L., Meira, L. B., Hammer, R. E., Burns, D. K., Doughty, A. T., and Friedberg, E. C. (1996). Synergistic interaction between XPC and p53 mutations in double-mutant mice: neural tube abnormalities and accelerated UV radiation-induced skin cancer. *Curr Biol* **6**, 1691-1694.

Cheo, D. L., Ruven, H. J. T., Meira, L. B., Hammer, R. E., Burns, D. K., Tappe, N. J., van Zeeland, A. A., Mullenders, L. H. F, and Friedberg, E. C. (1997). Characterisation of defective nucleotide excision repair in XPC mutant mice. *Mutat Res* **374**, 1-9.

Cheo, D. L., Burns, D. K., Meira, L. B., Houle, J. F., and Friedberg, E. C. (1999). Mutational inactivation of the Xeroderma pigmentosum group C gene confers predisposition to 2-acetylaminoflurene-induced liver and lung cancer and to spontaneous testicular cancer in *Trp53*$^{-/-}$ mice. *Cancer Res* **59**, 771-775.

Cheo, D. L., Meira, L. B., Burns, D. K., Reis, A. M., Issac, T., and Friedberg, E. C. (2000). Ultraviolet B radiation-induced skin cancer in mice deficient in the *Xpc*, *Trp53* and *Apex* (HAP1) genes: genotype-specific effects on cancer predisposition and pathology of tumors. *Cancer Res* **60**, 1580-1584.

Chaung, Y-Y, E., Chen, Q., and Liber, H. L. (1999). Radiation-induced mutations at the autosomal thymidine kinase locus are not elevated in p53-null cells. *Cancer Res* **59**, 3073-3076.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci U S A* **81**, 1991-1995.

Cleaver, J. E., and Kraemer, K. H. (1995). Xeroderma pigmentosum and Cockayne syndrome. In: Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D. (Eds), The metabolic basis of inherited diseases, Seventh ed., McGraw-Hill, New York, pp. 4393-4419.

Cummings, C. J., and Zoghbi, H. Y. (2000). Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum Mol Genet* **9**, 909-916.

d'Adda di Fagagna, F., Hande, M. P., Tong, W-M., Lansdorp, P.M, Wang, Z-Q., and Jackson, S. P. (1999). Functions of poly(ADP-ribose) polymerase in controlling telomere length and chromosomal stability. *Nat Genet* **23**, 76-80.

Deng, C. X., and Brodie, S. G. (2000). Roles of BRCA1 and its interacting proteins. *Bioessays* **22**, 728-737.

Dietrich, W.; Katz, H.; Lincoln, S. E.; Shin, H. S.; Friedman, J.; Dracopoli, N. C. and Lander, E. S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. *Genetics* **131**, 423-447.

Ding, R., Pommier, Y., Kang, V. H., and Smulson, M. (1992). Depletion of poly (ADP-ribose) polymerase by antisense RNA expression results in a delay in DNA strand break rejoining. *J Biol Chem* **267**, 12804-12812.

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C, A Jr., Butel, J. S., and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221.

Donehower, L. A., Godley, L. A., Aldaz, C. M., Pyle, R., Shi, Y. P., Pinkel, D., Gray, J., Bradley, A., Medina, D., and Varmus, H. E. (1995). Deficiency of p53 accelerates mammary tumourigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. *Genes Dev* **9**, 882-895.

Donehower, L. A. (1996). The p53-deficient mouse: a model for basic and applied cancer studies. *Semin Cancer Biol* **7**, 269-278.

Doyle, P., Maconochie, N., Roman, E., Davies, G., Smith, P. G., and Beral, V. (2000). Fetal death and congenital malformation in babies born to nuclear industry employees: report from the nuclear industry family study. *Lancet* **356**, 1293-1299.

Dronkert, M. L., and Kanaar, R. (2001). Repair of DNA interstrand cross-links. *Mutat Res* **486**, 217-247.

Dubrova, Y. E., Jeffreys, A. J., and Malashenko, A. M. (1993). Mouse minisatellite mutations induced by ionizing-radiation. *Nat Genet* **5**, 92-94.

Dubrova, Y. E., Nesterov, V. N., Krouchinsky, N. G., Ostapenko, V. A., Neumann, R., Neil, D. L., and Jeffreys, A. J. (1996). Human minisatellite mutation rate after the Chernobyl accident. *Nature* **380**, 683-686.

Dubrova, Y. E., Nesterov, V. N., Krouchinsky, N. G., Ostapenko, V. A., Vergnaud, G., Giraudeau, F., Buard, J., and Jeffreys, A. J. (1997). Further evidence for elevated human minisatellite mutation rate in Belarus eight years after the Chernobyl accident. *Mutat Res* **381**, 267-278.

Dubrova, Y. E., Plumb, M., Brown, J., Fennelly, J., Bois, P., Goodhead, D., and Jeffreys, A. J. (1998a). Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation. *Proc Natl Acad Sci U S A* **95**, 6251-6255.

Dubrova, Y. E., Plumb, M., Brown, J., and Jeffreys, A. J. (1998b). Radiation-induced germline instability at minisatellite loci. *Int J Radiat Biol* **74**, 689-696.

Dubrova, Y. E., Plumb, M., Brown, J., Boulton, E., Goodhead, D., and Jeffreys, A. J. (2000a). Induction of minisatellite mutations in the mouse germline by low-dose chronic exposure to gamma-radiation and fission neutrons. *Mutat Res* **453**, 17-24.

Dubrova, Y. E., Plumb, M., Gutierrez, B., Boulton, E., and Jeffreys, A. J. (2000b). Genome stability - Transgenerational mutation by radiation. *Nature* **405**, 37-37.

Dubrova, Y. E., Bersimbaev, R. I., Djansugurova, L. B., Tankimanova, M. K., Mamyrbaeva, Z., Mustonen, R., Lindholm, C., Hulten, M., and Salomaa, S. (2002a). Nuclear weapons tests and human germline mutation rate. *Science* **295**, 1037.

Dubrova, Y. E., Grant, G., Chumak, A. A., Stezhka, V. A., and Karakasian, A. N. (2002b). Elevated minisatellite mutation rate in the post-Chernobyl families from Ukraine. *Am J Hum Genet* **71**, 801-809.

Dubrova, Y. E., and Plumb, M. A. (2002). Ionising radiation and mutation induction at mouse minisatellite loci. The story of the two generations. *Mutat Res* **499**, 143-150.

Dubrova, Y. E. (2005). Radiation-induced mutation at tandem repeat DNA loci in the mouse germline: Spectra and doubling doses. *Radiat Res* **163**, 200-207.

Ehling, U. H., Favor, J., Kratochvilova, J., and Neuhauser-Klaus, A. (1982). Dominant cataract mutations and specific-locus mutations in mice induced by radiation or ethylnitrosourea. *Mutat Res* **92**, 181-192.

Ehling, U. H. (1991). Genetic risk assessment. *Annu Rev Genet* **25**, 255-280.

El-Khamisy, S. F., Masutani, M., Suzuki, H., and Caldecott, K. W. (2003). A requirement for PARP-1 for the assembly of stability of XRCC1 nuclear foci at sites od oxidative DNA damage. *Nucleic Acids Res* **31**, 5526-5533.

Ellegren, H., Lindgren, G., Primmer, C. R., and Moller, A. P. (1997). Fitness loss and germline mutations in barn swallows breeding in Chernobyl. *Nature* **389**, 593-596.

Ellegren, H. (2000). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends Genet* **16**, 551-558.

Ellegren, H. (2004). Microsatellites: simple sequences with complex evolution. *Nat Rev Genet* **5**, 435-445.

Elson, A., Wang, Y., Daugherty, C. J., Morton, C. C., Zhou, F., Campos-Torres, J., and Leder, P. (1996). Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. *Proc Natl Acad Sci USA* **93**, 13084-13089.

Fan, Y. J., Wang, Z., Sadamoto, S., Ninomiya, Y., Kotomura, N., Kamiya, K., Dohi, K., Kominami, R., and Niwa, O. (1995). Dose-response of a radiation induction of a germline mutation at a hypervariable mouse minisatellite locus. *Int J Radiat Biol* **68**, 177-183.

Farber, R. A., Petes, T. D., Dominska, M., Hudgens, S. S., and Liskay, R. M. (1994). Instability of simple sequence repeats in a mammalian cell line. *Hum Mol Genet* **3**, 253-256.

Favor, J., Neuhauser-Klaus, A., and Ehling, U. H. (1987). Radiation-induced forward and reverse specific locus mutations and dominant cataract mutations in treated strain BALB/c and DBA/2 male mice. *Mutat Res* **177**, 161-169.

Field, D., and Wills, C. (1996). Long, polymorphic microsatellites in simple organisms. *Proc R Soc Lond B Biol Sci* **263**, 209-215.

Fischhaber, P. L., Gerlach, V. L., Feaver, W. J., Hatahet, Z., Wallace, S. S., and Friedberg, E. C. (2002). Human DNA polymerase κ bypasses and extends beyond thymine glycols during translesion synthesis *in vitro*, preferentially incorporating correct nucleotides. *J Biol Chem* **277**, 37604-37611.

Fitch, M. E., Cross, I. V., and Ford, J. M. (2003). p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradaition-induced DNA damage, *in vivo*. *Carcinogenesis* **24**, 843-850.

Fodde, R., and Smits, R. (2002). A matter of dosage. *Science* **298**, 761-763.

Frankenberg-Schwager, M. (1990). Induction, repair and biological relevance of radiation-induced DNA lesions in eukaryotic cells. *Radiat Environ Biophys* **29**, 273-292.

Friedberg, E. C., Walker, G. C., and Siede, W. (1995). DNA repair and mutagenesis. (ASM Press, Washington, D.C.).

Friedberg, E. C. (1996). Relationship between DNA repair and transcription. *Annu Rev Biochem* **65**, 15-42.

Friedberg, E. C., Cheo, D. L., Meira, L. B., and Reis, A. M. (1999). Cancer predisposition in mutant mice defective in the XPC DNA repair gene. *Prog Exp Tumor Res* **35**, 37-52.

Freidberg, E. C., and Meira, L. B. (2003). Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage. Version 5. *DNA Repair* **2**, 501-530.

Freudenreich, C. H., Kantrow, S. M., and Zakian, V. A. (1998). Expansion and length-dependent fragility of CTG repeats in yeast. *Science* **279**, 853-856.

Gardner, M. J., Snee, M. P., Hall, A. J., Powell, C. A., Downes, S., and Terrell, J. D. (1990). Results of case-control study of leukaemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. *Br Med J* **300**, 423-429.

Gatti, R. A., Becker-Catania, S., Chun, H. H., Sun, X., Mitui, M., Lai, C., Khanlou, N., Babaei, M., Cheng, R., Clark, C., *et al.* (2001). The pathogenesis of Ataxia-Telangiectasia. *Clin Rev Allergy Immunol* **20**, 87-107.

Gayther, S. A., Pharoah, P. D., and Ponder, B. A. (1998). The genetics of inherited breast cancer. *J Mammary Gland Biol Neoplasm* **3**, 365-376.

Gerlach, V. L., Aravind, L., Gotwat, G., Schultz, R. A., Koonin, E. V., and Friedberg, E. C. (1999). Human and mouse homologs of *Escherichia coli* DinB (DNA polymerase IV), members of the UmuC/DinB superfamily. *Proc Natl Acad Sci U S A* **96**, 11922-11927.

Gibbs, M., Collick, A., Kelly, R. G., and Jeffreys, A. J. (1993a). A tetranucleotide repeat mouse minisatellite displaying substantial somatic instability during early preimplantation development. *Genomics* **17**, 121-128.

Gill, P., Jeffreys, A. J., and Werrett, D. J. (1985). Forensic application of DNA 'fingerprints'. *Nature* **318**, 577-579.

Goodhead, D. T. (1988). Spatial and temporal distribution of energy. *Health Phys* **55**, 231-240.

Gordenin, D. A., Kunkel, T. A., and Resnick, M. A. (1997). Repeat expansion--all in a flap? *Nat Genet* **16**, 116-118.

Gradwohl, G., Menissier de Murcia, J. M., Molinete, M., Simonin, F., Koken, M., Hoeijmakers, J. H., and de Murcia, G. (1990). The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity of single-strand breaks in DNA. *Proc Natl Acad Sci U S A* **87**, 2990-2994.

Graw, J., Favor, J., Neuhauser-Klaus, A., and Ehling, U. H. (1986). Dominant cataract and recessive specific locus mutations in offspring of X-irradiated male mice. *Mutat Res* **159**, 47-54.

Griffin, C. S., and Tease, C. (1988). Gamma-ray-induced numerical and structural chromosome anomalies in mouse immature oocytes. *Mutat Res* **202**, 209-213.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**, 6-13.

Feinberg, A. P., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Addendum Anal Biochem* **137**, 266-267.

Fleck, O., and Schar, P. (2004). Translesion DNA synthesis: little fingers teach tolerance. *Curr Biol* **14**, 389-391.

Hagelberg, E., Gray, I. C., and Jeffreys, A. J. (1991). Identification of the skeletal remains of a murder victim by DNA analysis. *Nature* **352**, 427-429.

Haines, G. A., Hendry, J. H., Daniel, C. P., and Morris, I. D. (2001). Increased levels of comet-detected spermatozoa DNA damage following in vivo isotopic- or X-irradiation of spermatogonia. *Mutat Res* **495**, 21-32.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100**, 57-70.

Hancock, J. M. (1996). Simple sequences in a "minimal' genome. *Nat Genet* **14**, 14-15.

Hanneman, W. H., Legare, M. E., Sweeney, S., and Schimenti, J. C. (1997). Cisplatin increases meiotic crossing-over in mice. *Proc Natl Acad Sci U S A* **94**, 8681-8685.

Haracska, L. Prakash, L., and Prakash, S. (2002). Role of human DNA polymerase κ as an extender in translesion synthesis. *Proc Natl Acad Sci U S A* **99**, 16000-16005.

Hartley, K. O., Gell, D., Smith, G. C., Zhang, H., Divecha, N., Connelly, M. A., Admon, A., Lees-Miller, S. P., Anderson, C. W., and Jackson, S. P. (1995). DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* **82**, 849-856.

Honma, M., Momose, M., Tanabe, H., Sakamoto, H., Yu, Y., Little, J. B., Sofuni, T., and Hayashi, M. (2000). Requirement of wild-type p53 protein for maintenance of chromosomal integrity. *Mol Carcinog* **28**, 203-214.

Hoyes, K. P., Wadeson, P. J., Sharma, H. L., Hendry, J. H., and Morris, I. D. (1998). Mutation studies in *lacI* transgenic mice after exposure to radiation or cyclophosphamide. *Mutagenesis* **13**, 607-612.

Hearne, C. M., Ghosh, S., and Todd, J. A. (1992). Microsatellites for linkage analysis of genetic traits. *Trends Genet* **8**, 288-294.

Hedenskog, M.; Sjogren, M.; Cederberg, H. and Rannug, U. (1997). Induction of germline-length mutations at the minisatellites PC-1 and PC-2 in male mice exposed to polychlorinated biphenyls and diesel exhaust emissions. *Environ Mol Mutagen* **30**, 254-259.

Henderson, S. T. and Petes, T. D. (1992). Instability of simple sequence DNA in *Saccharomyces cerevisae*. *Mol Cell Biol* **12**, 2749-2757.

Herceg, Z., and Wang, Z-Q. (2001). Functions of poly (ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res* **477**, 97-110.

Heyer, E., Puymirat, J., Dieltjes, P., Bakker, E., and de Knijff, P. (1997). Estimating Y chromosome specific microsatellite mutation frequencies using deep rooting pedigrees. *Hum Mol Genet* **6**, 799-803.

Imai, H., Nakagama, H., Komatsu, K., Shiraishi, T., Fukuda, H., Sugimura, T., and Nagao, M. (1997). Minisatellite instability in severe combined immunodeficiency mouse cells. *Proc Natl Acad Sci U S A* **94**, 10817-10820.

Imbert, G.; Saudou, F.; Yvert, G.; Devys, D.; Trottier, Y.; Garnier, J. M.; Weber, C.; Mandel, J. L.; Cancel, G.; Abbas, N.; Durr, A.; Didierjean, O.; Stevanin, G.; Agid, Y. and Brice, A. (1996). Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. *Nat Genet* **14**, 285-291.

Ingvarsson, S., Finnsdottir, V., Sigurdsson, A., and Geirsson, G. (2000). Population studies and validation of paternity determinations by six microsatellite loci. *J Forensic Sci* **45**, 692-695.

Ito, J., and Braithwaite, D. K. (1991). Compilation and alignment of DNA polymerase sequences. *Nucleic Acids Res* **19**, 4045-4057.

Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. (1994). Tumour spectrum analysis in p53-deficient mice. *Curr Biol* **4**, 1-7.

Jarman, A. P., Nicholls, R. D., Weatherall, D. J., Clegg, J. B., and Higgs, D. R. (1986). Molecular characterisation of a hypervariable region downstream of the human alpha-globin gene cluster. *EMBO J* **5**, 1857-1863.

Jeffreys, A. J., Brookfield, J. F., and Semeonoff, R. (1985a). Positive identification of an immigration test-case using human DNA fingerprints. *Nature* **317**, 818-819.

Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985b). Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**, 67-73.

Jeffreys, A. J., Wilson, V., and Thein, S. L. (1985c). Individual-specific 'fingerprints' of human DNA. *Nature* **316**, 76-79.

Jeffreys, A. J., and Morton, D. B. (1987). DNA fingerprints of dogs and cats. *Anim Genet* **18**, 1-15.

Jeffreys, A. J., Wilson, V., Kelly, R., Taylor, B. A., and Bulfield, G. (1987a). Mouse DNA 'fingerprints': analysis of chromosome localization and germ- line stability of hypervariable loci in recombinant inbred strains. *Nucleic Acids Res* **15**, 2823-2836.

Jeffreys, A. J., Wilson, V., Wong, Z., Royle, N., Patel, I., Kelly, R., and Clarkson, R. (1987b). Highly variable minisatellites and DNA fingerprints. *Biochem Soc Symp* **53**, 165-180.

Jeffreys, A. J., Royle, N. J., Wilson, V., and Wong, Z. (1988a). Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. *Nature* **332**, 278-281.

Jeffreys, A. J., Wilson, V., Neumann, R., and Keyte, J. (1988b). Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res* **16**, 10953-10971.

Jeffreys, A. J., Neumann, R., and Wilson, V. (1990). Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell* **60**, 473-485.

Jeffreys, A. J., MacLeod, A., Tamaki, K., Neil, D. L., and Monckton, D. G. (1991a). Minisatellite repeat coding as a digital approach to DNA typing. *Nature* **354**, 204-209.

Jeffreys, A. J., Royle, N. J., Patel, I., Armour, J. A., MacLeod, A., Collick, A., Gray, I. C., Neumann, R., Gibbs, M., Crosier, M., and et al. (1991b). Principles and recent advances in human DNA fingerprinting. *EXS* 58, 1-19.

Jeffreys, A. J., Allen, M. J., Hagelberg, E., and Sonnberg, A. (1992). Identification of the skeletal remains of Josef Mengele by DNA analysis. *Forensic Sci Int* **56**, 65-76.

Jeffreys, A. J., Tamaki, K., MacLeod, A., Monckton, D. G., Neil, D. L., and Armour, J. A. (1994). Complex gene conversion events in germline mutation at human minisatellites. *Nat Genet* **6**, 136-145.

Jeffreys, A. J. (1997). Spontaneous and induced minisatellite instability in the human genome. *Clin Sci* **93**, 383-390.

Jeffreys, A. J., and Neumann, R. (1997). Somatic mutation processes at a human minisatellite. *Hum Mol Genet* **6**, 129-132; 134-126.

Jeffreys, A. J., Bois, P., Buard, J., Collick, A., Dubrova, Y., Hollies, C. R., May, C. A., Murray, J., Neil, D. L., Neumann, R., *et al.* (1997). Spontaneous and induced minisatellite instability. *Electrophoresis* **18**, 1501-1511.

Jeffreys, A. J., Murray, J., and Neumann, R. (1998a). High-resolution mapping of crossovers in human sperm defines a minisatellite-associated recombination hotspot. *Mol Cell* **2**, 267-273.

Jeffreys, A. J., Neil, D. L., and Neumann, R. (1998b). Repeat instability at human minisatellites arising from meiotic recombination. *EMBO J* **17**, 4147-4157.

Jeffreys, A., Barber, R., Bois, P., Buard, J., Dubrova, Y. E., Grant, G., Hollies, C. R. H., May, C. A., Neumann, R., Panayi, M., Ritchie, A. E., Shone, A. C., Signer, E., Stead, J. D. H., and Tamaki, K. (1999). Human minisatellites, repeat DNA instability and meiotic recombination. *Electrophoresis* **20**, 1665-1675.

Jeffreys, P. D., Gorina, S., and Paveletich, N. P. (1995). Crystal structure of the tetramerization domain of the p53 tumour suppressor at 1.7 angstroms. *Science* **267**, 1498-1502.

Jimenez, G. S., Bryntesson, F., Torres-Arzayus, M. I., Priestley, A., Beeche, M., Saito, S., Sakaguchi, K., Appella, E., Jeggo, P. A., Taccioli, G. E., Wahl, G. W., and Hubank, M. (1999). DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage. *Nature* **400**, 81-83.

Jiricny, J., and Nystrom-Lahti, M. (2000). Mismatch repair defects in cancer. *Curr Opin Genet Dev* **10**, 157-161.

Jobling, M. A., Bouzekri, N., and Taylor, P. G. (1998). Hypervariable digital DNA codes for human paternal lineages: MVR-PCR at the Y-specific minisatellite, MSY1 (DYF155S1). *Hum Mol Genet* **7**, 643-653.

Kang, S., Ohshima. K., Shimizu, M., Amirhaeri, S., and Wells, R. D. (1995). Pausing of DNA synthesis *in vitro* at specific loci in CTG and CGG triplet repeats from human hereditary disease genes. *J Biol Chem* **270**, 27014-27021.

Kannouche, P., Fernandez de Henestrosa, A. R., Coull, B., Vidal, A. E., Gray, C., Zicha, D., Woodgate, R., and Lehmann, A. R. (2002). Localization of DNA polymerases η and ι to the replication machinery is tightly co-ordinated in human cells. *EMBO J* **21**, 6246-6256.

Katoh, M., Inomata, T., Horiya, N., Suzuki, F., Shida, T., Ishioka, K., and Shibuya, T. (1994). Studies on mutations in male germ cells of transgenic mice following exposure to isopropyl methanesulfonate, ethylnitrosourea or X-ray. *Mutat Res* **341**, 17-28.

Katti, M. V., Ranjekar, P. K., and Gupta, V. S. (2001). Differential distribution of simple sequence repeats in eukaryotic genome sequences. *Mol Biol Evol* **18**, 1161-1167.

Kawaguchi, Y.; Okamoto, T.; Taniwaki, M.; Aizawa, M.; Inoue, M.; Katayama, S.; Kawakami, H.; Nakamura, S.; Nishimura, M.; Akiguchi, I.; Kimura, J.; Narumiya, S. and Kakizuka, A. (1994). CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. *Nat Genet* **8**, 221-228.

Kellerer, A. M. (2000). Risk estimates for radiation-induced cancer--the epidemiological evidence. *Radiat Environ Biophys* **39**, 17-24.

Kelly, R., Bulfield, G., Collick, A., Gibbs, M., and Jeffreys, A. J. (1989). Characterization of a highly unstable mouse minisatellite locus: evidence for somatic mutation during early development. *Genomics* **5**, 844-856.

Kelly, R., Gibbs, M., Collick, A., and Jeffreys, A. J. (1991). Spontaneous Mutation At the Hypervariable Mouse Minisatellite Locus *Ms6-hm* - Flanking DNA-Sequence and

Analysis of Germline and Early Somatic Mutation Events. *Proc R Soc Lond* **B245**, 235-245.

Kelly, R. G. (1994). Similar origins of 2 mouse minisatellites within transposon-like LTRs. *Genomics* ***24***, 509-515.

Kemp, C. J., Donehower, L. A., Bradley, A., and Balmain, A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumours. *Cell* **74**, 813-822.

Kirk, K. M., and Lyon, M. F. (1984). Induction of congenital malformations in the offspring of male mice treated with X-rays at pre-meiotic and post-meiotic stages. *Mutat Res* **125**, 75-85.

Kiuru, A., Auvinen, A., Luokkamaki, M., Makkonen, K., Veidebaum, T., Tekkel, M., Rahu, M., Hakulinen, T., Servomaa, K., Rytomaa, T., and Mustonen, R. (2003). Hereditary minisatellite mutations among the offspring of Estonian Chernobyl cleanup workers. *Radiat Res* **159**, 651-655.

Kodaira, M., Satoh, C., Hiyama, K., and Toyama, K. (1995). Lack of effects of atomic bomb radiation on genetic instability of tandem-repetitive elements in human germ cells. *Am J Hum Genet* **57**, 1275-1283.

Kodaira, M., Izumi, S., Takahashi, N., and Nakamura, N. (2004). No evidence of radiation effect on mutation rates at hypervariable minisatellite loci in the germ cells of atomic bomb survivors. *Radiat Res* **162**, 350-356.

Komarova, E. A., Kondratov, R. V., Wang, K., Christov, K., Golovkina, T. V., Goldblum, J. R., and Gudkov, A. V. (2004). Dual effect of p53 on radiation sensitivity *in vivo*: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. *Oncogene* **23**, 3265-3271.

Koschinsky, M. L.; Beisiegel, U.; Henne-Bruns, D.; Eaton, D. L. and Lawn, R. M. (1990). Apolipoprotein(a) size heterogeneity is related to variable number of repeat sequences in its mRNA. *Biochemistry* **29**, 640-644.

Kovalchuk, O., Dubrova, Y. E., Arkhipov, A., Hohn, B., and Kovalchuk, I. (2000). Wheat mutation rate after Chernobyl. *Nature* **407**, 583-584.

Kratochvilova, J. (1981). Dominant cataract mutations detected in offspring of gamma-irradiated male mice. *J Hered* **72**, 302-307.

Kratochvilova, J., and Favor, J. (1988). Phenotypic characterization and genetic analysis of twenty dominant cataract mutations detected in offspring of irradiated male mice. *Genet Res* **52**, 125-134.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following radiation. *Proc Natl Acad Sci U S A* **89**, 7491-7495.

La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., and Fischbeck, K. H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* **352**, 77-79.

Lalioti, M. D., Scott, H. S., Genton, P., Grid, D., Ouazzani, R., M'Rabet, A., Ibrahim, S., Gouider, R., Dravet, C., Chkili, T., Bottani, A., Buresi, C., Malafosse, A., and Antonarakis, S. E. (1998). A PCR amplification method reveals instability of the dodecamer repeat in progressive myoclonus epilepsy (EPM1) and no correlation between the size of the repeat and age at onset. *Am J Hum Genet* **62**, 842-847.

Lalioti, M. D.; Scott, H. S. and Antonarakis, S. E. (1999). Altered spacing of promoter elements due to the dodecamer repeat expansion contributes to reduced expression of the cystatin B gene in EPM1. *Hum Mol Genet* **8**, 1791-1798.

Lancaster, C. A., Peat, N., Duhig, T., Wilson, D., Taylor-Papadimitriou, J., and Gendler, S. J. (1990). Structure and expression of the human polymorphic epithelial mucin gene: an expressed VNTR unit. *Biochem Biophys Res Commun* **173**, 1019-1029.

Lane, D. P. (1992). p53, guardian of the genome. *Nature* **358**, 15-16.

Lavin, M. F., and Shiloh, Y. (1997). The genetic defect in Ataxia-Telangiectasia. *Annu Rev Immunol* **15**, 177-202.

Leonard, A., and Deknudt, G. (1967). Relation between the x-ray dose and the rate of chromosome rearrangements in spermatogonia of mice. *Radiat Res* **32**, 35-41.

Levine, A. J., Finlay, C. A., and Hinds, P. W. (2004). P53 is a tumour suppressor gene. *Cell* **S116**, 67-69

Levinson, G., and Gutman, G. A. (1987). Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol Biol Evol* **4**, 203-221.

Li, L., Peterson, C., and Legerski, R. (1996). Sequence of the mouse XPC cDNA and genomic structure of the human XPC gene. *Nucleic Acids Res* **24**, 1026-1028.

Liang, L., Shao, C., Deng, L., Mendonca, M. S., Stambrook, P. J., and Tischfield, J. A. (2002). Radiation-induced genetic instability *in vivo* depends on p53 status. *Mutat Res* **502**, 69-80.

Lichter, J. B. Barr, C. L., Kennedy, J. L., Van Tol, H. H., Kidd, K. K., and Livak, K. J. (1993). A hypervariable segment in the human dopamine receptor D4 (DRD4) gene. *Hum Mol Genet* **2**, 767-773.

Lieber, M. R. (1997). The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. *Bioessays* **19**, 233-240.

Lindahl, T., and Wood, R. D. (1999). Quality control by DNA repair. *Science* **286**, 1897-1905.

Ling, H., Boudsocq, F., Woodgate, R., and Yang, W. (2001). Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication. *Cell* **107**, 91-102.

Liquori, C. L., Ricker, K., Moseley, M. L., Jacobsen, J. F., Kress, W., Naylor, S. L., Day, J. W., and Ranum, L. P. (2001). Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. *Science* **293**, 864-867.

Little, J. (1993). The Chernobyl accident, congenital anomalies and other reproductive outcomes. *Paediatr Perinat Epidemiol* **7**, 121-151.

Little, J. B. (2000). Radiation carcinogenesis. *Carcinogenesis* **21,** 397-404.

Livshits, L. A., Malyarchuk, S. G., Kravchenko, S. A., Matsuka, G. H., Lukyanova, E. M., Antipkin, Y. G., Arabskaya, L. P., Petit, E., Giraudeau, F., Gourmelon, P., *et al.* (2001). Children of Chernobyl cleanup workers do not show elevated rates of mutations in minisatellite alleles. *Radiat Res* **155**, 74-80.

Luning, K. G., (1971). Testing for recessive lethals in mice. *Mutat Res* **11**, 125-132.

Luning, K. G., and Searle, A. G. (1971). Estimates of the genetic risks from ionizing irradiation. *Mutat Res* **12**, 291-304.

Luning, K. G., and Eiche, A. (1975). X-ray-induced recessive lethal mutations in the mouse. *Mutat Res* **34**, 163-174.

Lynch, H. T., Smyrk, T., and Lynch, J. F. (1996). Overview of natural history, pathology, molecular genetics and management of HNPCC (Lynch syndrome). *Int J Cancer* **69**, 38-43.

Lyon, M. F., Phillips, R. J. S., and Searle, A. G. (1964). The overall rates of dominant and recessive lethal and visible mutation induced by spermatogonial x-irradiation of mice. *Genet Res* **5**, 448-467.

Lyon, M. F., and Morris, T. (1966). Mutation rates at a new set of specific loci in the mouse. *Genet Res* **7**, 12-17.

Lyon, M. F., Papworth, D. G., and Phillips, R. J. (1972a). Dose-rate and mutation frequency after irradiation of mouse spermatogonia. *Nat New Biol* **238**, 101-104.

Lyon, M. F., Phillips, R. J., and Bailey, H. J. (1972b). Mutagenic effects of repeated small radiation doses to mouse spermatogonia. I. Specific-locus mutation rates. *Mutat Res* **15**, 185-190.

Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F. Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., *et al.* (1990). Germ line p53 mutations in familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* **250**, 1233-1238.

Masse, R. (2000). Ionizing radiation. *C R Acad Sci III 323*, 633-640.

Masutani, C., Sugasawa, K., Yanagisawa, J., Sonoyama, T., Ui, M., Enomoto, T., Takio, K., Tanaka, K., van der Spek, P. J., Bootsma, D., *et al.* (1994). Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23. *EMBO J* **13**, 1831-1843.

Masutani, C., Araki, M., Sugasawa, K., van der Spek, P. J., Yamada, A., Uchida, A., Maekawa, T., Bootsma, D., Hoeijmakers, J. H., and Hanaoka, F. (1997). Identification and characterisation of XPC-binding domain of hHR23B. *Mol Cell Biol* **17**, 6915-6923.

Masutani, C., Nozaki, T., Nakamoto, K., Nakagama, H., Suzuki, H., Kusuoka, O., Tsutsumi, M., and Sugimura, T. (2000). The response of Parp knockout mice against DNA damaging agents. *Mutat Res* **462**, 159-166.

Matsuura, T., Yamagata, T., Burgess, D. L., Rasmussen, A., Grewal, R. P., Watase, K., Khajavi, M., McCall, A. E., Davis, C. F., Zu, L., Achari, M., Pulst, S. M., Alonso, E., Noebels, J. L., Nelson, D. L., Zoghbi, H. Y., and Ashizawa, T. (2000). Large expansion of the ATTCT pentanucleotide repeat in spinocerebellar ataxia type 10. *Nat Genet* **26**, 191-194.

May, C. A., Jeffreys, A. J., and Armour, J. A. (1996). Mutation rate heterogeneity and the generation of allele diversity at the human minisatellite MS205 (D16S309). *Hum Mol Genet* **5**, 1823-1833.

May, C. A., Tamaki, K., Neumann, R., Wilson, G., Zagars, G., Pollack, A., Dubrova, Y. E., Jeffreys, A. J., and Meistrich, M. L. (2000). Minisatellite mutation frequency in human sperm following radiotherapy. *Mutat Res* **453**, 67-75.

McMurray, C. T. (1999). DNA secondary structure: a common and causative factor for expansion in human disease. *Proc Natl Acad Sci U S A* **96**, 1823-1825.

Meira, L. B., Reis, A. M. C., Cheo, D. L., Nahari, D., Burns, D. K., and Friedberg, E. C. (2001). Cancer predisposition in mutant mice defective in multiple pathways: uncovering important genetic interactions. *Mutat Res* **477**, 51-58.

Menissier de Murcia, J., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Mark, M., oliver, F. J., Masson, M., Dierich, A., LeMeur, M., Walztinger, C., Chambon, P., and de Murcia, G. (1997). Requirement of poly (ADP-ribose) polymerase in recovery from DNA damage in mice and cells. *Proc Natl Acad Sci U S A* **94**, 7303-7307.

Miller, R. D., Hogg, J., Ozaki, J. H., Gell, D., Jackson, S. P., and Riblet, R. (1995). Gene for the catalytic subunit of mouse DNA-dependent protein kinase maps to the *scid* locus. *Proc Natl Acad Sci U S A* **92**, 10792-10795.

Mirkin, S. M., and Smirnova, E. V. (2002). Positioned to expand. *Nat Genet* **31**, 5-6.

Mitchell, D. L., and Nairn, R. S. (1989). The biology of the (6-4) photoproduct. *Photochem Photobiol* **49**, 805-819.

Monckton, D. G., Wong, L. J., Ashizawa, T., and Caskey, C. T. (1995). Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses. *Hum Mol Genet* **4**, 1-8.

Morris, S. M. (2002). A role for p53 in frequency and mechanism of mutation. *Mutat Res* **511**, 45-62.

Moynahan, M. E., Chiu, J. W., Koller, B. H., and Jasin, M. (1999). Brca1 controls homology-directed DNA repair. *Mol Cell* **4**, 511-518.

Muller, H. J. (1927). Artificial transmission of the gene. *Science* **66**, 84.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E., and White, R. (1987). Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* **235**, 1616-1622.

Neel, J. V., Schull, W. J., Awa, A. A., Satoh, C., Otake, M., Kato, H., and Yoshimoto, Y. (1989). Implications of the Hiroshima-Nagasaki genetic studies for the estimation of the human "doubling dose" of radiation. *Genome* **31**, 853-859.

Nelson, S. L., Giver, C. R., and Grosovsky, A. J. (1994). Spectrum of X-ray-induced mutations in the human hprt gene. *Carcinogenesis* **15**, 495-502.

Niwa, O., Fan, Y. J., Numoto, M., Kamiya, K., and Kominami, R. (1996). Induction of a germline mutation at a hypervariable mouse minisatellite locus by ^{252}Cf radiation. *J Radiat Res* **37**, 217-224.

Ogi, T., Shinkai, Y., Tanaka, K., and Ohmori, H. (2002). Polκ protects mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene. *Proc Natl Acad Sci U S A* **99**, 15548-15553.

Ohashi, E., Bebenek, K., Matsuda, T., Feaver, W. J., Gerlach, V. L., Friedberg, E. C., Ohmori, H., and Kunkel, T. A. (2000a). Fidelity and processivity of DNA synthesis by DNA polymerase κ, the product of the human *DINB1* gene. *J Biol Chem* **275**, 39678-39684.

Ohashi, E., Ogi, T., Kusumoto, R., Iwai, S., Masutani, C., Hanaoka, F., and Ohmori, H. (2000b). Error-prone bypass of certain DNA lesions by the human DNA polymerase κ. *Gens Dev* **14**, 1589-1594.

Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawerence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001). The Y-family of DNA polymerases. *Mol Cell* **8**, 7-8.

Orr, H. T.; Chung, M. Y.; Banfi, S.; Kwiatkowski, T. J., Jr.; Servadio, A.; Beaudet, A. L.; McCall, A. E.; Duvick, L. A.; Ranum, L. P. and Zoghbi, H. Y. (1993). Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nat Genet* **4**, 221-226.

Osborn, A. J., Elledge, S. J., and Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway. *Trends Cell Biol* **12**, 509-516.

Otake, M., Schull, W. J., and Neel, J. V. (1990). Congenital malformations, stillbirths, and early mortality among the children of atomic bomb survivors: a reanalysis. *Radiat Res* **122**, 1-11.

O-Wang, J., Kawamura, K., Tada, Y., Ohmori, H., Kimura, H., Sakiyama, S., and Tagawa, M. (2001). DNA polymerase κ, implicated in spontaneous and DNA damage-induced mutagenesis, is overexpressed in lung cancer. *Cancer Res* **61**, 5366-5369.

Pampfer, S., Streffer, C., and Muller, W. U. (1989). Micronucleus formation in 2-cell embryos after in vitro X-irradiation of mouse spermatozoa. *Mutat Res* **210**, 191-196.

Papiha, S. S., and Sertedaki, A. (1995). Oligonucleotide (CAC)5 fingerprinting: validity and reliability in paternity testing. *Electrophoresis* **16**, 1624-1626.

Pearson, C. E. (2003). Slipping while sleeping? Trinucleotide repeat expansion in germ cells. *Trends Mol Med* **9**, 490-495.

Phelan, C. M., Rebbeck, T. R., Weber, B. L., Devilee, P., Ruttledge, M. H., Lynch, H. T., Lenoir, G. M., Stratton, M. R., Easton, D. F., Ponder, B. A., *et al.* (1996). Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. *Nat Genet* **12**, 309-311.

Phillips, R. J. S., and Searle, A. G. (1964). The effect of dose-rate on the yield of translocations and dominant lethals following spermatogonial irradiation of mice. *Genet Res* **5**, 468-472.

Pitkevitch, V. A., Ivanov, V. K., Tsyb, A. F., Maksyoutov, M. A., Matiash, V. A., and Shchukina, N. V. (1997). Exposure levels for persons involved in recovery operations after the Chernobyl accident. Statistical analysis based on the data of the Russian National Medical and Dosimetric Registry (RNMDR). *Radiat Environ Biophys* **36**, 149-160.

Poirier, G. G., de Murcia, G., Jongstra-Bilen, J., Niedergang, C., and Mandel, P. (1982). Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure. *Proc Natl Acad Sci U S A* **79**, 3423-3427.

Pretsch, W., Favor, J., Lehmacher, W., and Neuhauser-Klaus, A. (1994). Estimates of the radiation-induced mutation frequencies to recessive visible, dominant cataract and enzyme-activity alleles in germ cells of AKR, BALB/c, DBA/2 and (102xC3H)F1 mice. *Mutagenesis* **9**, 289-294.

Pretsch, W., Neuhauser-Klaus, A., and Favor, J. (2000). A comparison of enzyme activity mutation frequencies in germ cells of mice (*Mus musculus*) and golden hamsters (*Mesocricetus auratus*) after exposure to 2 + 2 Gy gamma-irradiation. *Mutagenesis* **15**, 39-43.

Prives, C., and Hall, P. A. (1999). The P53 pathway. *J Pathol* **187**, 112-126.

Pupko, T., and Graur, D. (1999). Evolution of microsatellites in the yeast *Saccharomyces cerevisiae*: role of length and number of repeated units. *J Mol Evol* **48**, 313-316.

Reardon, J. T., Mu, D., and Sancer, A. (1996). Overproduction, purification, and characterisation of the XPC subunit of the human DNA repair excision nuclease. *J Biol Chem* **32**, 19451-19456.

Richards, R. I., and Sutherland, G. R. (1994). Simple repeat DNA is not replicated simply. *Nat Genet* **6**, 114-116.

Russell, W. L. (1951). X-ray-induced mutation in mice. *Cold Spring Harb Symp Quant Biol* **16**, 327-336.

Russell, W. L., Russell, L. B., and Kelly, E. M. (1958). Radiation dose rate and mutation frequency. *Science* **128**, 1546-1550.

Russell, W. L. (1965). Studies in mammalian radiation genetics. *Nucleonics* **23**, 53-62.

Russell, W. L., and Kelly, E. M. (1982a). Mutation frequencies in male mice and the estimation of genetic hazards of radiation in men. *Proc Natl Acad Sci U S A* **79**, 542-544.

Russell, W. L., and Kelly, E. M. (1982b). Specific-locus mutation frequencies in mouse stem-cell spermatogonia at very low radiation dose rates. *Proc Natl Acad Sci U S A* **79**, 539-541.

Sachs, L. (1982). Applied Statistics (New York, Springer).

Sadamoto, S., Suzuki, S., Kamiya, K., Kominami, R., Dohi, K., and Niwa, O. (1994). Radiation induction of germline mutation at a hypervariable mouse minisatellite locus. *Int J Radiat Biol* **65**, 549-557.

Sah, V. P., Attardi, L. D., Mulligan, G. J., Williams, B. O., Bronson, R. T., and Jacks, T. (1995). A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* **10**, 175-180.

Sambrook, J., and Russell, D. W. (2001). Molecular Cloning; A Laboratory Manual, 3rd Edition (New York, Cold Spring Harbour Laboratory Press).

Sands, A. T., Abuin, A., Sanchez, A., Conti, C. J., and Bradley, A. (1995a). High susceptibility to ultraviolet-induced carcinogenesis in mice lacking XPC. *Nature* **377**, 162-165.

Sands, A. T., Suraokar, M. B., Sanchez, A., Marth, J. E., Donehower, L. A., and Bradley, A. (1995b). p53 deficiency does not affect the accumulation of point mutations in transgene target. *Proc Natl Acad Sci U S A* **92**, 8517-8521.

Sankaranarayanan, K. (1998). Ionizing radiation and genetic risks IX. Estimates of the frequencies of mendelian diseases and spontaneous mutation rates in human populations: a 1998 perspective. *Mutat Res* **411**, 129-178.

Satoh, C., Takahashi, N., Asakawa, J., Kodaira, M., Kuick, R., Hanash, S. M., and Neel, J. V. (1996). Genetic analysis of children of atomic bomb survivors. *Environ Health Perspect* **104** Suppl 3, 511-519.

Schenten, D., Gerlach, V. L., Guo, C., Velasco-Miguel, S., Hladik, C. L., White, C. L., Friedberg, E. C., Rajewsky, K., and Esposito, G. (2002). DNA polymerase κ deficiency does not affect somatic hypermutation in mice. *Eur J Immunol* **32**, 3152-3160.

Schindewolf, C., Lobenwein, K., Trinczek, K., Gomolka, M., Soewarto, D., Fella, C., Pargent, W., Singh, N., Jung, T., and de Angelis, M. H. (2000). Comet assay as a tool to screen for mouse models with inherited radiation sensitivity. *Mamm Genome* **11**, 552-554.

Schlotterer, C., and Tautz, D. (1992). Slippage synthesis of simple sequence DNA. *Nucleic Acids Res* **20**, 211-215.

Schonian, G., Meusel, O., Tietz, H. J., Meyer, W., Graser, Y., Tausch, I., Presber, W., and Mitchell, T. G. (1993). Identification of clinical strains of *Candida albicans* by DNA fingerprinting with the polymerase chain reaction. *Mycoses* **36**, 171-179.

Searle, A. G. (1974). Mutation induction in mice. *Adv Radiat Biol* **4**, 131-205.

Searle, A. G., and Beechey, C. V. (1981). The effects of radiation dose-rate and quality on the induction of dominant lethals in mouse spermatids. *Mutat Res* **81**, 403-410.

Searle, A. G., and Edwards, J. H. (1986). The estimation of risks from the induction of recessive mutations after exposure to ionising radiation. *J Med Genet* **23**, 220-226.

Selby, P. B. (1979). Induced skeletal mutations. *Genetics* **92**, s127-133.

Shall, S., and de Murcia, G. (2000). Poly (ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? *Mutat Res* **460**, 1-15.

Shao, C., Deng, L., Henegariu, O., Liang, L., Stanbrook, P., and Tischfield, J. A. (2000). Chromosome instability contributes to loss of heterozygosity in mice lacking p53. *Proc Natl Acad Sci U S A* **97**, 7405-7410.

Shimizu, T., Shinkai, Y., Ogi, T., Ohmori, H., and Azuma, T. (2003). The absence of DNA polymerase κ does not affect somatic hypermutation of the mouse immunoglobin heavy chain gene. *Immunol Lett* **86**, 265-270.

Sieber, O. M., Heinimann, K., and Tomlinson, I. P. M. (2003). Genomic instability-the engine of tumourigenesis? *Nat Rev Cancer* **3**, 701-708.

Smith, J. C., Newton, C. R., Alves, A., Anwar, R., Jenner, D., and Markham, A. F. (1990). Highly polymorphic minisatellite DNA probes. Further evaluation for individual identification and paternity testing. *J Forensic Sci Soc* **30**, 3-18.

Soldani, C., and Scovassi, A. I. (2002). Poly (ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* **7**, 321-328.

Somers, C. M., Yauk, C. L., White, P. A., Parfett, C. L. J., and Quinn, J. S. (2002). Air pollution induces heritable DNA mutations. *Proc Natl Acad Sci U S A* **99**, 15904-15907.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503-517.

Spring, K., Cross, S., Li, C., Watters, D., Ben-Senior, L., Waring, P., Ahangari, F., Lu, S. L., Chen, P., Misko, I., Paterson, C., Kay, G., Smorodinsky, N. I., Shiloh, N. I., and Lavin, M. F. (2001). *Atm* knock-in mice harbouring an in-frame deletion corresponding to the human *ATM* 7636del9 common mutation exhibit a variant phenotype. *Cancer Res* **61**, 4561-4568.

Spring, K., Ahangari, F., Scott, S. P., Waring, P., Purdie, D. M., Chen, P. C., Hourigan, K., Ramsay, J., McKinnon, P. J., Swift, M., and Lavin, M. F. (2002). Mice heterozygous for mutation in Atm, the gene involved in ataxia-telangiectasia, have heightened susceptibility to cancer. *Nat Genet* **32**, 185-190.

Stallings, R. L. (1994). Distribution of trinucleotide microsatellites in different categories of mammalian genomic sequence: implications for human genetic diseases. *Genomics* **21**, 116-121.

Strand, M., Prolla, T. A., Liskay, R. M., and Petes, T. D. (1993). Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* **365**, 274-276.

Sugasawa, K., Ng, J. M. Y., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P. M., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. J. (1998). Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol Cell* **2**, 223-232.

Sugasawa, K., Okamoto, T., Shimizu, Y., Masutani, C., Iwai, S., and Hanaoka, F. (2001). A multistep damage recognition mechanism for global genomic nucleotide excision repair. *Genes Dev* **15**, 507-521.

Sugasawa, K., Shimizu, Y., Iwai, S., and Hanaoka, F. (2002). A molecular mechanism for DNA damage recognition by the xeroderma pigmentosum group C protein complex. *DNA Repair* **1**, 95-107.

Suzuki, S., Mitani, K., Kuwabara, K., Takahashi, Y., Niwa, O., and Kominami, R. (1993). Two mouse hypervariable minisatellites: chromosomal location and simultaneous mutation. *J Biochem* **114**, 292-296.

Sybenga, J. (1999). What makes homologous chromosomes find each other in meiosis? A review and an hypothesis. *Chromosoma* **108**, 209-219.

Takata, M., Sasaki, M. S., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinhara, A., and Takeda, S. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have

overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J* **17**, 5497-5508.

Tamaki, K., May, C. A., Dubrova, Y. E., and Jeffreys, A. J. (1999). Extremely complex repeat shuffling during germline mutation at human minisatellite B6.7. *Hum Mol Genet* **8**, 879-888.

Tautz, D., and Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Res* **12**, 4127-4138.

Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res* **17**, 6463-6471.

Tease, C., and Fisher, G. (1996). Cytogenetic and genetic studies of radiation-induced chromosome damage in mouse oocytes. II. Induced chromosome loss and dominant visible mutations. *Mutat Res* **349**, 155-162.

The Ionising Radiations Regulations (1999). (London, The Stationery Office Limited).

Tong, W-M., Cortes, U., and Wang, Z-Q. (2001). Poly (ADP-ribose) polymerase: a guardian angel protecting the genome and suppressing tumorigenesis. *Biochim Biophys Acta* **1552**, 27-37.

Toth, G., Gaspari, Z., and Jurka, J. (2000). Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Res* **10**, 967-981.

Toueille, M., and Hubscher, U. (2004) Regulation of the DNA replication fork: a way to fight genomic instability. *Chromosoma* **113**, 113-125.

Trepicchio, W. L., and Krontiris, T. G. (1993). IGH minisatellite suppression of USF-binding-site- and E mu-mediated transcriptional activation of the adenovirus major late promoter. *Nucleic Acids Res* **21**, 977-985.

Trucco, C., Oliver, F. J., de Murcia, G., and Menissier-de Murcia, J. (1998). DNA repair defect in poly (ADP-ribose) polymerase-deficient cell lines. *Nucleic Acids Res* **26**, 2644-2649.

Turri, M. G., Cuin, K. A., and Porter, A. C. (1995). Characterisation of a novel minisatellite that provides multiple splice donor sites in an interferon-induced transcript. *Nucleic Acids Res* **23**, 1854-1861.

Tyrrell, R. M., and Amaudruz, F. (1987). Evidence for two independent pathways of biological effective excision repair from its rate and extent in cells cultured from sun-sensitive humans. *Cancer Res* **47**, 3725-3728.

Uchida, A., Sugasawa, K., Masutani, C., Dohmae, N., Araki, M., Yokoi, M., Ohkuma, Y., and Hanaoka, F. (2002). The carboxy-terminal domain of the XPC protein plays a critical role in nucleotide excision repair through interactions with transcription factor IIH. *DNA Repair* **1**, 449-461.

Uchida, M., Hanai, S., Uematsu, N., Sawamoto, K., Okano, H., Miwa, M., and Uchida, K. (2001). Genetic and functional analysis of PARP, a DNA strand break-binding enzyme. *Mutat Res* **477**, 89-96.

Uljon, S. N., Johnson, R. E., Edwards, T. A., Prakash, S., Prakash, L., and Aggarwal, A. K. (2004). Crystal structure of the catalytic core of human DNA polymerase kappa. *Structure* **12**, 1395-1404.

Umar, A., Boyer, J. C., Thomas, D. C., Nguyen, D. C., Risinger, J. I., Boyd, J., Ionov, Y., Perucho, M. and Kunkel, T. A. (1994). Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. *J Biol Chem* **269**, 14367-14370.

UNSCEAR (2000). The United Nations Scientific Committee on the Effects of Atomic Radiation. *Health Phys* **79**, 314.

Urquhart, A., Kimpton, C. P., Downes, T. J., and Gill, P. (1994). Variation in short tandem repeat sequences--a survey of twelve microsatellite loci for use as forensic identification markers. *Int J Legal Med* **107**, 13-20.

Valenzuela, M. T., Guerrero, R., Nunez, M. I., Ruiz De Almodovar, J. M., Sarker, M., de Murcia, G., and Oliver, F. J. (2002). PARP-1 modifies the effectiveness of p53-mediated DNA damage response. *Oncogene* **21**, 1108-1116.

van Buul, P. P. W. (1983). Induction of Chromosome Aberrations by Ionizing Radiation in Stem Cell Spermatogonia of Mammals. In Radiation-Induced Chromosomal Damage in Man (New York, Alan R. Liss, Inc.), pp. 369-400.

Van Buul, P. P. W., and van Duijn-Goedhart, A. (2002). Enhanced recovery of radiation-induced translocations from spermatogonial stem cells of p53 null mice. *Mutat Res* **508**, 29-32.

Van Hoffen, A., Balajee, A. S., van Zeeland. A. A., and Mullenders, L. H. F. (2003). Nucleotide excision repair and its interplay with transcription. *Toxicology* **193**, 79-90.

Velasco-Miguel, S., Richardson, J. A., Gerlach, V. L., Lai, W. C., Gao, T., Russell, L. D., Hladik, C. L., White, C. L. III., and Friedberg, E. C. Constitutive and regulated expression of the mouse *Dinb* (*Polκ*) gene encoding DNA polymerase kappa. *DNA Repair* **2**, 91-106.

Venema, J., van Hoffen, A., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. (1990). The residual repair capacity of xeroderma pigmentosum complementary group C fibroblasts is highly specific for transcriptionally active DNA. *Nucleic Acids Res* **18**, 443-448.

Venema, J., van Hoffen, A., Karcagi, V., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. F. (1991). Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strands of active genes. *Mol Cell Biol* **11**, 4128-4134.

Vergnaud, G., Gauguier, D., Schott, J. J., Lepetit, D., Lauthier, V., Mariat, D., and Buard, J. (1993). Detection, cloning, and distribution of minisatellites in some mammalian genomes. *EXS* **67**, 47-57.

Vilarino-Guell, C., Smith, A. G., and Dubrova, Y. E. (2003). Germline mutation induction at mouse repeat DNA loci by chemical mutagens. *Mutat Res* **526**, 63-73.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. *Nature* **408**, 307-310.

Wakefield, R. (2004). The cancer epidemiology of radiation. *Oncogene* **23**, 6404-6428.

Wang, L., Cui, Y., Lord, B. I., Roberts, S.A., Potten, C. S., Hendrey, J. H., and Scott, D. (1996). Gamma-ray-induced cell killing and chromosome abnormalities in the bone marrow of p53-deficient mice. *Radiat Res* **146**, 259-266.

Wang, W., Fukuda, M., Kishida, T., and Tamaki, Y. (1996). Quintuplex PCR-amplification of microsatellites. *Nippon Hoigaku Zasshi* **50**, 231-236.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. (2000). BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of abberant DNA structures. *Genes Dev* **14**, 927-939.

Wang, Z-Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M., and Wagner, E. F. (1995). Mice lacking ADPRT and poly (ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Dev* **9**, 509-520.

Wang, Z-Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K., and Wagner, E. F. (1997). PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev* **11**, 2347-2358.

Washington, M. T., Johnson, R. E., Prakash, L., and Prakash, S. (2002). Human DINB1-encoded DNA polymerase κ is a promiscuous extender of mispaired promer termini. *Proc Natl Acad Sci U S A* **99**, 1910-1914.

Weber, J. L., and Wong, C. (1993). Mutation of human short tandem repeats. *Hum Mol Genet* **2**, 1123-1128.

Weitzmann, M. N., Woodford, K. J., and Usdin, K. (1998). The mouse Ms6-hm hypervariable microsatellite forms a hairpin and two unusual tetraplexes. *J Biol Chem* **46**, 30742-30749.

Wierdl, M., Dominska, M., and Petes, T. D. (1997). Microsatellite instability in yeast: dependence on the length of the microsatellite. *Genetics* **146**, 769-779.

Wolfe, W. T., Washington, M. T., Prakash, L., and Prakash, S. (2003). Human DNA polymerase κ uses template-primer misalignment as a novel means for extending mispaired termini and for generating single-base deletions. *Genes Dev* **17**, 2191-2199.

Wong, L. J., Ashizawa, T., Monckton, D. G., Caskey, C. T., and Richards, C. S. (1995). Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent. *Am J Hum Genet* **56,** 114-122.

Wong, Z., Wilson, V., Jeffreys, A. J., and Thein, S. L. (1986). Cloning a selected fragment from a human DNA 'fingerprint': isolation of an extremely polymorphic minisatellite. *Nucleic Acids Res* **14**, 4605-4616.

Wong, Z., Wilson, V., Patel, I., Povey, S., and Jeffreys, A. J. (1987). Characterization of a panel of highly variable minisatellites cloned from human DNA. *Ann Hum Genet* **51**, 269-288.

Woodgate, R. (1999). A plethora of lesion-replicating DNA polymerases. *Genes Dev* **13**, 2191-2195.

Wright, W. E., and Shay, J. W. (2000). Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat Med* **6**, 849-851.

Wyllie, A. H., Carder, P. J., Clarke, A. R., Cripps, K. J., Gledhill, S., Greaves, M. F., Griffiths, S., Harrison, D. J., Hooper, M. L., Morris, R. G., *et al.* (1994). Apoptosis in carcinogenesis: the role of p53. *Cold Spring Harb Symp Quant Biol* **59**, 403-409.

Yamamoto, M., Tsukamoto, T., Sakai, H., Shirai, N., Ohgaki, H, Furihata, C., Donehower, L. A., Yoshida, K., and Tatematsu, M. (2000). p53 knockout mice (-/-) are more susceptible than (+/-) or (+/+) mice to N-methyl-N-nitrosourea stomach carcinogenesis. *Carcinogenesis* **21**, 1891-1897.

Yamauchi, M., Nishimura, M., Tsuji, S., Terada, M., Sasanuma, M., and Shimada, Y. (2002). Effect of SCID mutation on the occurrence of mouse Pc-1 (Ms6-hm) germline mutations. *Mutat Res* **503**, 43-49.

Yauk, C. L., and Quinn, J. S. (1996). Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site. *Proc Natl Acad Sci U S A* **93,** 12137-12141.

Yauk, C. (1998). Monitoring for induced heritable mutations in natural populations: application of minisatellite DNA screening. *Mutat Res* **411,** 1-10.

Yauk, C. L., Fox, G. A., McCarry, B. E., and Quinn, J. S. (2000). Induced minisatellite germline mutations in herring gulls (*Larus argentatus*) living near steel mills. *Mutat Res* **452,** 211-218.

Yauk, C. L., Dubrova, Y. E., Grant, G. R., and Jeffreys, A. J. (2002). A novel single molecule analysis of spontaneous and radiation-induced mutation at a mouse tandem repeat locus. *Mutat Res* **500**, 147-156.

Yokoi, M., Masutani, C., Maekawa, T., Sugasawa, K., Ohkuma, Y., and Hanaoka, F. (2000). The xeroderma pigmentosum group C protein complex XPC-HR23B plays n

important role in the recruitment of transcription factor IIH to damaged DNA. *J Biol Chem* **275**, 9870-9875.

Zhang, Y., Yuan, F., Xin, H., Wu, X., Rajpal, D. K., Yang, D., and Wang, Z. (2000a). Human DNA polymerase κ synthesizes DNA with extraordinarily low fidelity. *Nucleic Acids Res* **28**, 4147-4156.

Zhang, Y., Yuan, F., Wu, X., Wang, M., Olga, R., Taylor, J-S., Geacintov, N. E., and Wang, Z. (2000b). Error-free and error-prone lesion bypass by human DNA polymerase κ *in vitro*. *Nucleic Acids Res* **28**, 4138-4146.

Zhang, Y., Wu, X., Guo, D., Rechkoblit, O., and Wang, Z. (2002). Activities of human DNA polymerase κ in response to the major benzo[a]pyrene DNA adducts: error-free lesion bypass and extension synthesis from opposite the lesion. *DNA Repair* **1**, 559-569.

Zhang, Z., Li, L., Lantry, L. E., Wang, Y., Wiseman, R. W., Lubet, R. A., and You, M. (2002b). p53 transgenic mice are highly susceptible to 1, 2-dimethylhydrazine-induced uterine sarcomas. *Cancer Res* **62**, 3024-3029.

Zhou, B. S., and Elledge, S. J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-439.