Analysis of Germline Mutations Induced by Chemicals

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by

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Dedicated to Snoopy on her birthday (29th October) and my family, especially to Raquel who died of leukaemia at the age of sixteen on the 9th of December 1999.

Dedicado a Snoopy en el día de su cumpleaños (29 de Octubre) y a mi familia, especialmente a Raquel que falleció de leucemia a los dieciséis años el 9 de Diciembre de 1999.

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Abstract

The high abundance of chemical pollutants in the environment represents a genetic risk to humans. The development of reliable and sensitive tests for the analysis of the genetic effects of exposure to chemical mutagens is required. Previous work has shown that expanded simple tandem repeat (ESTR) loci provide a sensitive system for monitoring radiation-induced mutation in the mouse germline.

Here, the results of the first systematic study on germline mutation induction at mouse ESTR loci by chemical mutagens are presented. Mutation rates at two ESTR loci were studied in the germline of male mice exposed to two monofunctional alkylating agents, ethyl-nitrosourea (ENU) and isopropyl methanesulfonate (iPMS), as well as to the topoisomerase-II inhibitor, etoposide (ET). Pre-meiotic exposure to alkylating agents resulted in a highly significant increase in ESTR mutation rate, but did not alter post-meiotically exposed cells. Pre-meiotic mutation induction by ENU and iPMS was linear within the interval of doses from 12.5 mg/kg to 25 mg/kg and reached a plateau at higher concentrations. Paternal exposure to etoposide resulted in ESTR mutation induction at meiotic stages but did not affect post- or pre-meiotic cells. The pattern of ESTR mutation induction after pre-meiotic and meiotic exposure to chemical mutagens was similar to that previously obtained by various traditional approaches for monitoring germline mutation in mice.

Using microarrays, the analysis of the pattern of changes in gene expression in the testis of male mice exposed to ENU was studied. This analysis revealed that exposure to this chemical mutagen does not result in detectable changes in gene expression.

The results of this study show that ESTR loci provide a new and efficient biomonitoring system for assessing the genetic effects of chemical mutagens, capable of detecting increases in mutation rates at very low doses and in small sample sizes.

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Abbreviations

bp	Base pair
CA	Chromosomal aberration
CP	Cyclophosphamide
DLT	Dominant lethality test
DNA	Deoxyribonucleic acid
DSB	Double-strand break
ENU	Ethyl nitrosourea
EST	Expressed sequence tag
ESTR	Expanded simple tandem repeat
ET	Etoposide
GGR	Global genome repair
Gy	Gray
HR	Homologous recombination
IDL	Insertion/deletion loop
iPMS	Isopropyl methanesulfonate
kb	Kilobase
LET	Linear energy transfer
M	Molar
ml, mg, mM	Millilitre, milligram, millimolar
MIC	Methylisocyanate
MMR	Mismatch repair
MN	Micronuclei
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RPA	Replication protein A
SC	Synaptonemal complex
SCE	Sister chromatid exchange
SLT	Specific locus test
SM-PCR	Single molecule-PCR
SSB	Single-strand break
STR	Simple tandem repeat
TCR	Transcription coupled repair
Topo-II	Topoisomerase-II
TRCF	Transcription repair coupling factor
UDS	Unscheduled DNA synthesis
VNTR	Minisatellite, variant number of tandem repeat
XP	Xeroderma pigmentosum
µl, µg, µM	Microlitre, microgram, micromolar

1 Introduction

We live in a world where hazardous chemicals are prevalent, either for a specific purpose such as herbicides and insecticides, or as a by-product such as vehicle emissions and welding fumes.

Some of these hazardous agents are genotoxic and may cause mutations in exposed somatic and germ cells. Somatic mutations occurring in proto-oncogenes or tumour suppressor genes could lead to the development of cancer in the exposed tissues. On the other hand, exposure to the germline can result in miscarriages, birth defects, physical and mental growth development problems, and a broad range of diseases in the offspring of exposed parents. It is therefore important to be aware of the mutagenic properties and threat to health caused by the chemical agents present in the environment.

To date, in several epidemiological studies the genetic effects of human exposure to environmental chemicals have been addressed (Doll and Peto, 1981; Mauderly, 1994; McClellan, 1987; Stayner *et al.*, 1998). However, these studies have failed to generate a reliable risk assessment due to the genetic variation within a population, the inability to assess the dose of exposure, the difficulty to find an analogous control population and the inability to assess a specific agent.

The use of model systems overcomes the problems of epidemiological analysis. Model systems use genetically homogeneous populations under identical environmental conditions exposed to specific doses of chemical mutagens.

Currently, the mouse model provides the only reliable source of experimental data for the evaluation of genetic risk for human populations exposed to chemical mutagens. Despite the benefits of mouse models for the analysis of mutagenic agents, there is still no reliable and powerful system to analyse the mutagenicity of chemical agents in the germline. The most reliable system used to analyse germline mutations is the specific locus test (SLT) (see 1.3.1), which needs tens of thousands of offspring to obtain statistically significant results. Another commonly used system is the dominant lethality test (DLT) (see 1.3.2). Although it is not as reliable as the SLT, it only requires the analysis of a few hundred offspring. Transgenic mouse constructs (see 1.3.3) have also been used for the analysis of germline mutagenicity. This system has the advantage

of being the only available *in vivo* system that allows the analysis of mutation induction in different tissues.

1.1 Human exposure to chemical mutagens

Ideally, regulations and safety measures for the handling of chemical agents should prevent human exposure to these agents. However, there are numerous examples illustrating that these regulations are not efficient or stringent enough to avoid such an exposure. Efforts need to be made to minimise the risk of exposure to chemical mutagens; it is essential to detect and/or identify genotoxic agents, locate the sources of contamination and find the route of exposure for a specific mutagen.

It is also important to analyse the genetic risks of human exposure to chemical mutagens. It is known that exposure to chemical agents can induce genetic diseases, including cancer and can also increase miscarriage, genetic defects and congenital malformations among children conceived by parents who have previously been exposed to chemical mutagens.

1.1.1 Occupational exposure

As a result of the prevalence of genotoxic chemicals in the environment, there is a large range of workers potentially exposed to these agents.

Due to its abundance in the environment, only second to oxygen, crystalline silica is responsible for the potential exposure of 3.2 million workers in the USA alone (NIOSH, 1974; NIOSH, 1983). It is present in mining-related milling operations, iron and steel milling, quarrying, construction, glass and cement-making, ceramics, silicon and ferro-silicon foundry, metal manufacturing, manufacture of machinery and in agriculture.

Exposure to crystalline silica produces significant increases in the levels of micronuclei (MN), chromosomal aberrations (CA) and sister chromatid exchanges (SCE). Epidemiological studies have provided sufficient evidence to certify crystalline silica as genotoxic and carcinogenic when inhaled (IARC, 1977; IARC, 1987).

Diesel emission particles are responsible for the occupational exposure of approximately 1.35 million workers in the USA alone; exposure is common in

transportation workers, operators of diesel-powered equipment, railroad workers, truck drivers and others (Keshava and Ong, 1999).

Diesel emissions are mostly composed of carbon compounds formed through the incomplete combustion of fuel, many of which are mutagenic or carcinogenic. Exposure to these compounds induce MN, CA, SCE (McClellan, 1987; NIOSH, 1988) and increase the risk of mortality from lung cancer and non-cancer pulmonary disease (Mauderly, 1994; Muscat and Wynder, 1995; Stayner *et al.*, 1998).

Organic chemicals such as tetrachloroethylene are used in the cleaning industry. Exposure to tetrachloroethylene occurs through inhalation, skin absorption, and ingestion. Exposure to this chemical has been observed to increase the risk of urinary bladder, oesophagus, lung and cervical cancer (IARC, 1995).

Genotoxic inorganic chemicals are also used in industry. For example, beryllium and beryllium compounds are used in ceramics, foundries, smelters, electronics, welding, metal plating and others. It has been shown that exposure to beryllium induces SCE and DNA single-strand breaks (SSBs) (IARC, 1993; Larramendy *et al.*, 1981) and also increases the incidence of mortality from lung cancer (Wagoner *et al.*, 1980).

It has been estimated that occupational exposure accounts for 4% of all human cancers (Doll and Peto, 1981); in addition, according to some estimates, germline exposure could account for 50% of foetal deaths, 30% of mental retardation, 20% of congenital defects and 2% of male infertility (Hook, 1982).

The list of genotoxic chemicals, to which workers can be exposed, is almost endless and includes insecticides, pesticides, coal dust, wood dust, asphalt fumes, welding fumes, glass fibres, asbestos, vinyl chloride, benzene, cadmium, nickel, arsenic, etc.

1.1.2 Accidental exposure

Another situation in which people can be exposed to chemical mutagens is in accidents. One of the most striking examples is the accident that occurred in the early hours of the 3rd of December 1984 in Bhopal. On that night, in The Union Carbide Plant (Figure 1-1), water entered a tank containing over 40 metric tones of a pesticide called methylisocyanate (MIC). The water caused a runaway chemical reaction resulting in a rapid increase of pressure and temperature. This reaction led to the formation of a chemical cloud of MIC and other reaction products that escaped from the pesticide plant into the surrounding crowded neighbourhood, causing the sudden deaths of thousands of people (Sathyamala, 1996). Eighteen years later, the disaster continues with 120,000-150,000 survivors still chronically ill. Due to somatic exposure to the chemical mutagen, the number of cancer incidences in the affected population is increasing, 10 to 15 people die every month due to exposure-related illness, making a total of over 20,000 people to date. The effects of exposure in the germline have led to the high incidence of spontaneous abortions; however, the rate of still birth and congenital malformation were not altered (Bhandari *et al.*, 1990; Kapoor, 1991).

Figure 1-1 The Bhopal Plant. The Union Carbide Plant, Bhopal, India. In December 1984, the release of poisonous chemicals killed and injured thousands.

Photo by Chris Rainier, Copyright Corbis.

Bhopal is not an isolated event, other large scale accidental exposures have occurred as a result of mercury and dioxin leaks in Minimata (Japan) and Seveso (Italy) respectively.

These accidents highlight the potential risk of exposure to chemical mutagens. Although unlikely to be exposed to such a high concentration, we are chronically exposed to low doses of a high variety of chemical agents.

1.1.3 Medical exposure

Chemotherapy is the main source of human exposure to the very high doses of chemical mutagens. Some of the most commonly used anticancer drugs are actinomycin D, adriamycin, amsacrine, bleomycin, busulfan, carmustine, chlorambucil, cisplatin, cyclophosphamide, etoposide, fluorouracil, mechlorethamine, melphalan, mercaptopurine, mitomycin C, mitoxantrone, procarbazine, thioTEPA, trophosphamide, vinblastine and vincristine.

The same anticancer drugs that are life-saving in the short term are known to have serious late-occurring side effects, including the induction of secondary tumours (Linassier *et al.*, 2000; Yokoyama *et al.*, 2000). These secondary cancers are attributed to the mutational effects of the anticancer drugs in somatic cells. The possible effect of these chemicals on the germline is not well understood. It is known that some of these agents generate DNA damage in germ cells (Witt and Bishop, 1996), which could possibly affect the children conceived by parents who have previously undergone chemotherapy. Despite the fact that the results of some studies do not provide experimental evidence for increases in mortality and malformation frequencies among the offspring of chemotherapy patients (Arnon *et al.*, 2001; Byrne, 1999; Green *et al.*, 1997), additional larger studies are clearly warranted.

Similarly, people employed in the manufacture, preparation and administration of these drugs to the patients can potentially be exposed. Cyclophosphamide (CP) is one of the most commonly used anticancer drugs. This drug is an alkylating agent known to induce mutations in the mouse germline (Ehling and Neuhauser-Klaus, 1988b). It has also been shown that hospital workers, pharmacy technicians, and animal caretakers are exposed during the handling of CP (Sessink *et al.*, 1993; Sessink *et al.*, 1994; Sessink *et al.*, 1997). Sessink *et al.* reported the finding of CP in the urine of workers at concentrations of 0.2-19 micrograms/24 hr. These authors also detected CP in urine samples of three people not directly involved in the preparation of CP.

1.1.4 Conclusions

Given the currently extensive exposure to chemical mutagens, the effort to predict the consequences of such an exposure to humans certainly remains one of the most important issues of toxicology and genetics. To date very little is known about potential genetic effects of exposure to chemical mutagens in humans, and even less is known about their effect on germline cells. Systems used to date are not sensitive enough for the analysis of low doses of chemicals to which populations are more commonly exposed. There is evidently a need for a powerful, reliable and reproducible model system for the analysis of the potentially mutagenic agents present in the environment.

The most commonly used systems for the analysis of germline mutations induced by chemicals typically target male mouse germline, allowing the evaluation of dose-response and stage-specificity of mutation induction across different stages of mouse spermatogenesis. The analyses of chemical agents, performed in different cellular stages of the male mouse germline, have revealed the particular mutagenic action of some chemical agents for a specific spermatogenic stage. For this reason, prior to the description of the most relevant mechanisms for the analysis of germline mutations, it was considered relevant to have an overview of mouse spermatogenesis.

1.2 Mouse spermatogenesis

Spermatogenesis is the production of mature sperm cells from the primordial cells (Figure 1-2). Primordial cells divide to form type A_1 spermatogonia. These cells differ from the primordial cells by their smaller size and the presence of ovoid nucleus with the chromatin associated to the nuclear membrane. Type A_1 spermatogonia are stem cells that can undergo self-renewal or differentiation to type A_2 spermatogonia. Type A_2 cells divide to generate type A_3 spermatogonia. These three cellular stages are difficult to differentiate and they are characterised by a more rounded nucleus, an overall lighter nuclear staining and prominent nucleoli. Type A_3 spermatogonia divide mitotically to generate type A_4 spermatogonia. This new cell type can follow three different pathways: it can undergo self-renewal generating new type A_4 spermatogonia, apoptosis or differentiation into intermediate spermatogonia (Gilbert *et al.*, 2000).

Intermediate spermatogonia are of the first stem cell type committed to become mature sperm. They divide mitotically to generate the type B spermatogonia, which are the last stem cell type to undergo mitotic division generating primary spermatocytes, which is the cell type that enters meiosis (see 1.2.1).

At the end of the meiotic division I, each primary spermatocyte generates a pair of secondary spermatocytes. These cells undergo meiotic division II, which results in formation of two haploid spermatids. Spermatids differentiate into spermatozoa, the mature sperm cells (Gilbert *et al.*, 2000). In mice, the progression from spermatogonial stem cell to mature sperm takes 42 days (Searle, 1974).

1.2.1 Meiosis

Meiosis is the process of two successive cell divisions, following one single phase of replication, used to create haploid cells from the originally diploid (Figure 1-3). The first cell division, called meiotic division I consist of four phases:

Prophase I – This phase is the longest and most complicated of the process of meiosis. It is subdivided into five different stages:

Leptotene. During this stage the chromatin of the chromatids is very thin and it is not possible to distinguish individual chromosomes. Some thickening can be found at regular intervals, like beads on a string.

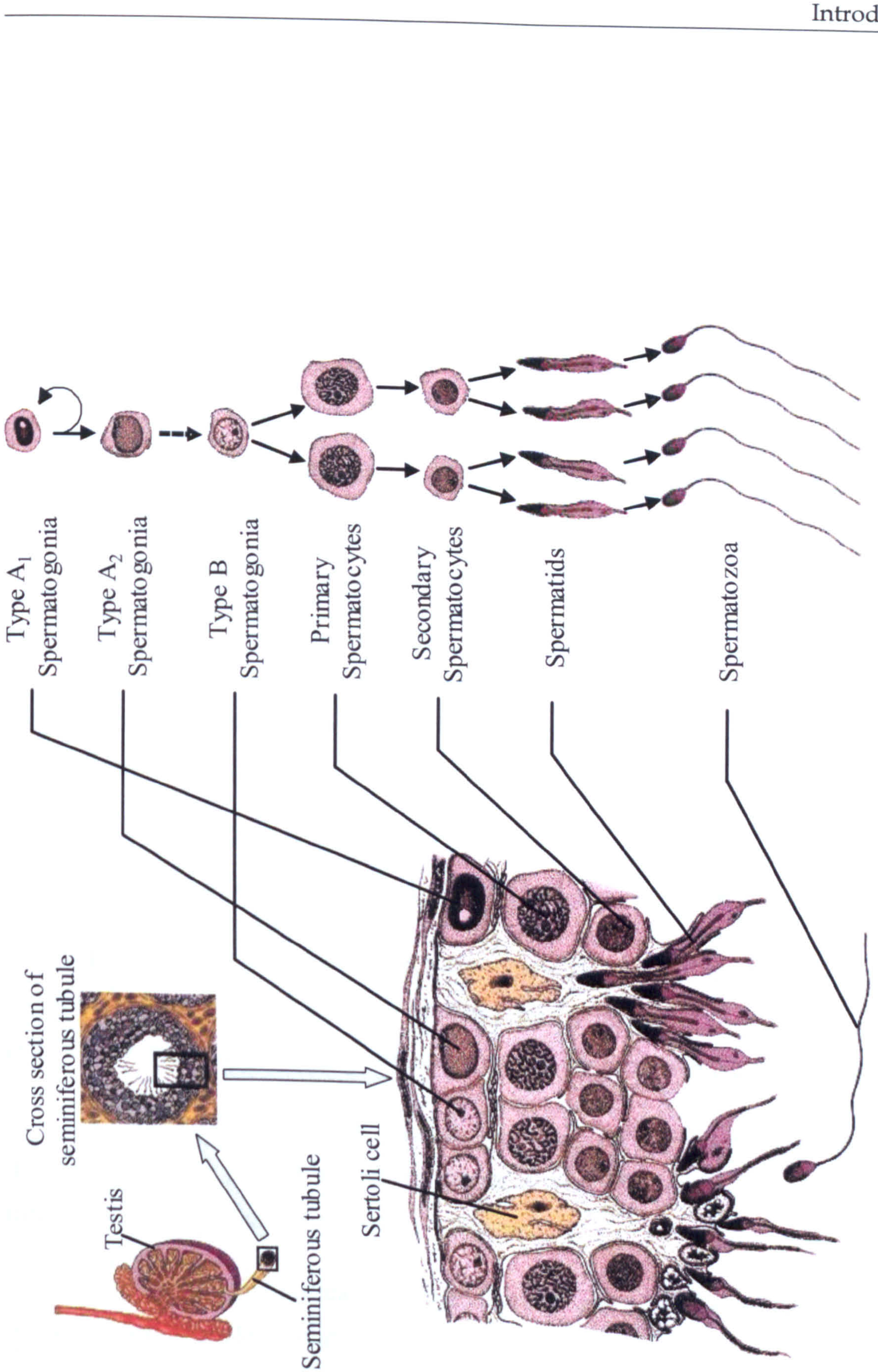


Figure 1-2 Stages of spermatogenesis. Adapted from Gilbert *et al.*, 2000.

Zygotene. At this stage the synapsis of homologous chromosomes begins. Synapsis requires the formation of a proteinaceous structure called the synaptonemal complex. The new DNA structure created is called bivalent or tetrad.

Pachytene. This stage starts when synapsis is complete. It is recognised by the shortening and thickening of the chromatids. At this stage crossing-over takes place, although on some occasions it can continue into the next stage.

Diplotene. During this stage the chromosomes start the process of desynapsis, and move apart allowing the chiasmata, the region where crossing-over is occurring, to be seen for the first time.

Diakinesis. This stage represents the continuation of diplotene; chromosomes stay connected and continue moving apart.

Metaphase I – At this phase the nuclear membrane is completely dissolved and the meiotic spindle is fully developed. Metaphase I is recognised by the alignment of the bivalents in the metaphase plate.

Anaphase I – During this phase the fibres of the meiotic spindle contract, pulling each chromosome of a bivalent towards opposite poles.

Telophase I – The meiotic spindle continues separating the chromosomes. Cytokinesis takes place at this stage, dividing the initial cell to produce two daughter cells, each with a single set of chromosomes in a newly formed nuclear envelope.

At the end of the first meiotic division, each primary spermatocyte is transformed into a pair of secondary spermatocytes. This cellular stage undergoes meiotic division II which consists of four phases:

Prophase II - The nuclear envelope dissolves and a new set of spindle fibres is formed. The chromosomes start to move towards the metaphase plate.

Metaphase II – All chromosomes align in the metaphase plate preparing for the chromosomal division.

Anaphase II – The centromere of the chromosomes splits and each chromatid of a chromosome is pulled apart toward one pole of the cells.

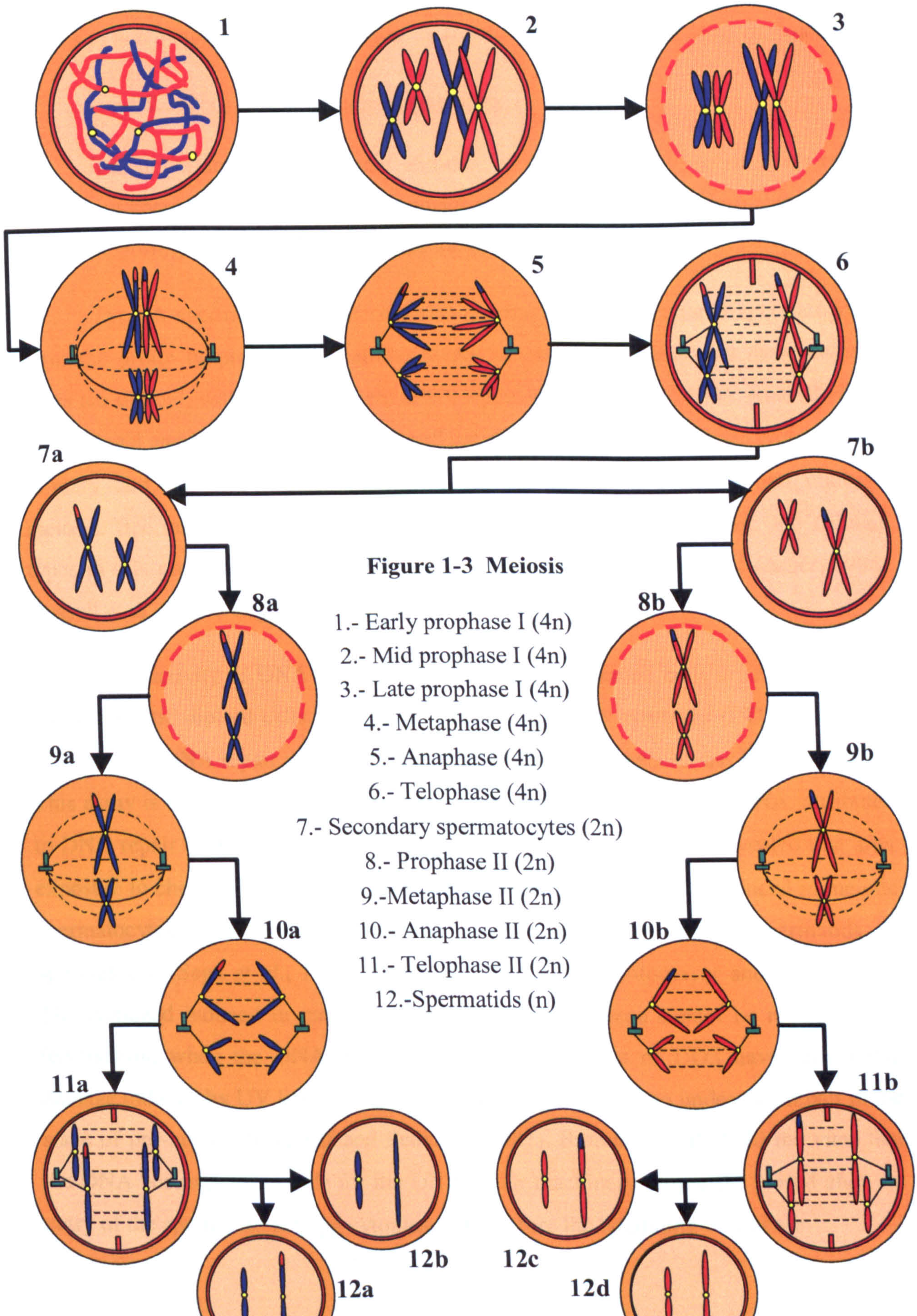


Figure 1-3 Meiosis

1.- Early prophase I (4n)
2.- Mid prophase I (4n)
3.- Late prophase I (4n)
4.- Metaphase (4n)
5.- Anaphase (4n)
6.- Telophase (4n)
7.- Secondary spermatocytes (2n)
8.- Prophase II (2n)
9.-Metaphase II (2n)
10.- Anaphase II (2n)
11.- Telophase II (2n)
12.-Spermatids (n)

Telophase II – Chromatids are pulled towards the poles of the cell, the initial cell divides in two daughter cells by cytokinesis and the nuclear envelope is reconstructed.

1.2.2 DNA repair and spermatogenesis

It is important, for subsequent sections, to discuss the activity of DNA repair throughout spermatogenesis. All spermatogenic cellular stages can be grouped in three major categories, namely pre-meiotic, meiotic and post-meiotic.

Type A spermatogonia, intermediate spermatogonia and type B spermatogonia form the group of pre-meiotic cellular stages of spermatogenesis. All these cellular types are diploid, with fully proficient DNA repair activity (Adler, 1996; Russell *et al.*, 1958; Vogel and Natarajan, 1995). The cellular stages forming the meiotic group are the primary and secondary spermatocytes. These are the cellular stages that undergo meiosis. Spermatids and spermatozoa compose the post-meiotic stages; the cellular types in this group are haploid where DNA repair is greatly suppressed (Adler, 1996; Russell *et al.*, 1958; Vogel and Natarajan, 1995).

The activity of DNA repair in meiotic and post-meiotic cells is lower than that on pre-meiotic diploid cells. Using the unscheduled DNA synthesis (UDS) assay, the efficiency of DNA repair across different stages of spermatogenesis has been evaluated. This assay is based in the detection of newly synthesised fragments of DNA, attributed to DNA repair of damaged sites in the exposed cells. This assay has revealed that after exposure to chemical agents, X-rays or UV radiation, DNA repair is induced in meiotic spermatocytes through early spermatid stages but not in mature spermatids or spermatozoa (Sega *et al.*, 1976; Sotomayor *et al.*, 1999; Sotomayor and Sega, 2000). The damaged induced in mature spermatids or spermatozoa cannot be repaired until fertilisation, when the DNA repair is reactivated. Studies of UDS have shown that damage induced by UV light in male gametes before fertilisation undergoes repair in the stage of pro-nuclei (Brandriff and Pedersen, 1981). Brandriff *et al.*, hold the view that the DNA becomes accessible for the DNA repair machinery during the period after the entry of the sperm into the egg cytoplasm and before the beginning of S phase.

1.3 Traditional systems to analyse germline mutations

1.3.1 Specific locus test

To date, the specific locus test (SLT) is considered the most reliable system for the analysis of germline mutations induced by chemicals or radiation. The SLT, also known as the Russell-7-locus test, was developed in the 1950s (Russell, 1951; Searle, 1975). This system was initially used to analyse male germ cells; however, it has also been used to analyse female germline mutation (Russell *et al.*, 1996). It is based on the analysis of phenotypical changes in offspring from treated mice. These changes in morphological traits, detected with this assay, are indicative of specific gene mutations.

Treated homozygous mice, carrying dominant wild-type alleles (Table 1-1), are mated to homozygous test partners with recessive alleles at the same loci. All offspring produced from this mating scheme will show either dominant phenotype or mutations arising in the treated wild-type parents (Figure 1-4).

The mating of treated male mice at different time points allows the analysis of mutation induction at different cellular stages of spermatogenesis. All different cellular stages are affected when the animal is exposed, although the damage induced to different types of sperm cells can only be analysed in the offspring. As offspring can only be derived from mature sperm cells (spermatozoa), the analysis of a specific cellular stage is performed by mating treated mice as many days after the treatment as needed for the desired stage to reach spermatozoa (Figure 1-5).

The main advantage of this system is the ability to detect clear-cut phenotypic changes in the offspring, unambiguously attributable to mutation at the specific loci in the germline of control and treated animals. However, due to its low spontaneous mutation rate (7.95×10^{-6} per male gamete (Russell *et al.*, 1982)), the detection of increases in mutation rate normally requires the profiling of very large numbers of offspring, ranging from tens of tens of thousands to up to millions.

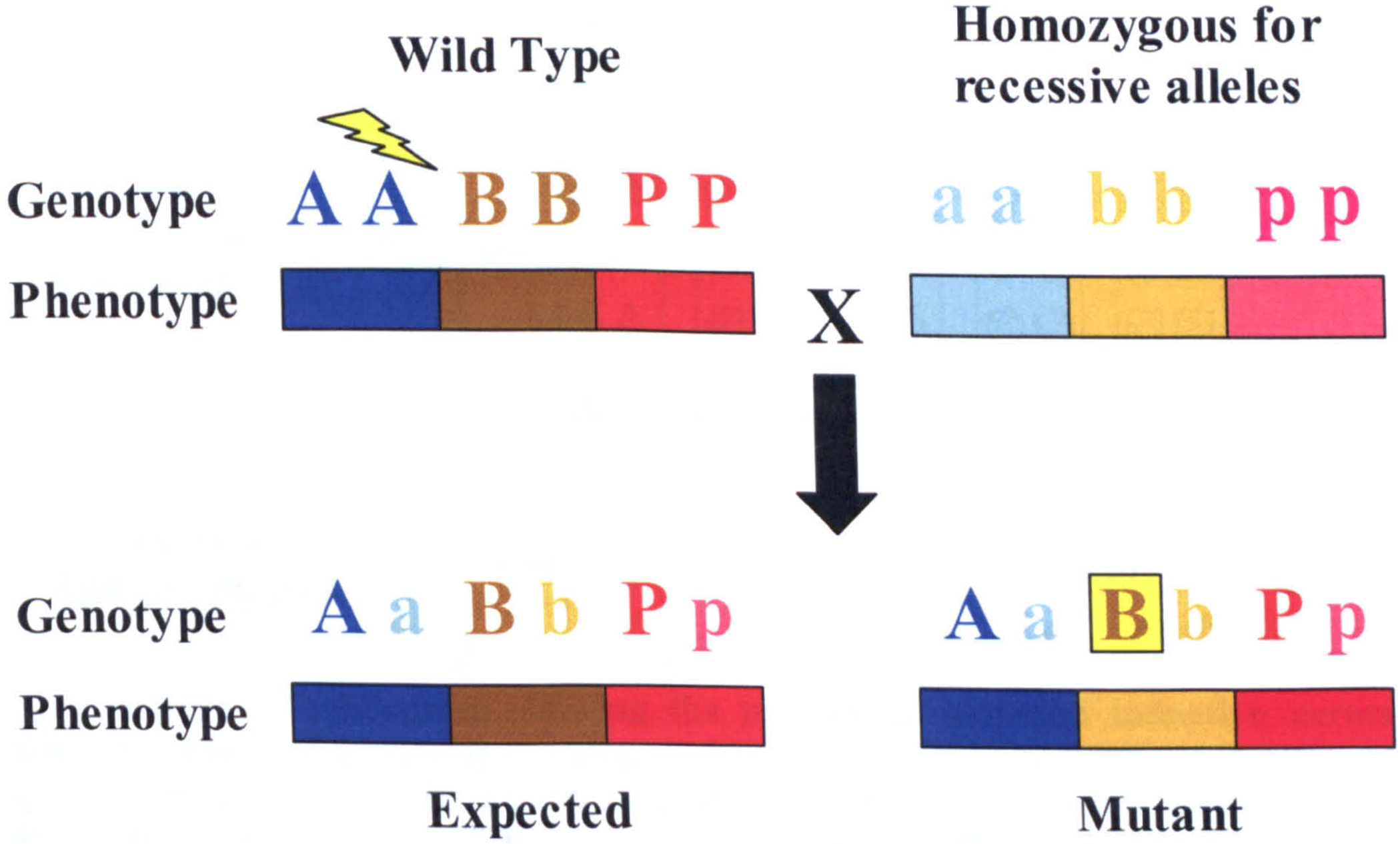
To date, using the SLT, the mutagenicity of many chemicals has been analysed, including adriamycin, bleomycin, busulfan, chlorambucil, cisplatin, cyclophosphamide, ethyl-methanesulfonate, ethyl nitrosourea, etoposide, fluorouracil, holoxan, isopropyl methanesulfonate, mechlorethamine, melphalan, mercaptopurine, methyl-methanesulfonate, mitomycin C, procarbazine, propyl-methanesulfonate,

trophosphamide, vincristine and others. Some of these results will be presented in section 1.4.

Table 1-1 Specific locus test loci.

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

Figure 1-4 Mutation detection by specific locus test.



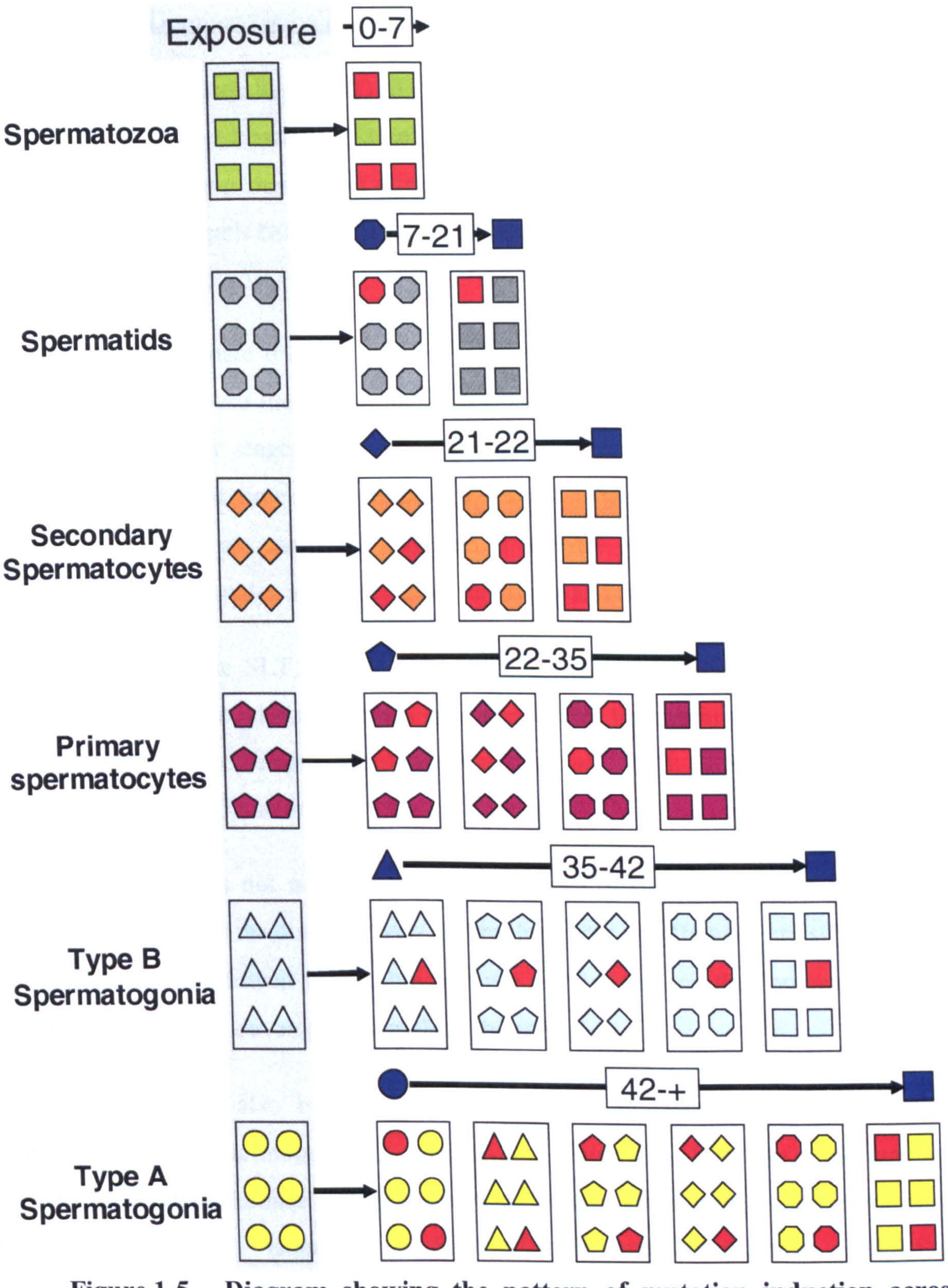


Figure 1-5 Diagram showing the pattern of mutation induction across different stages of mouse spermatogenesis. Each shape corresponds to a different spermatogenic cell type: ■, spermatozoa; ●, spermatid; ◆, secondary spermatocytes; ⬟, primary spermatocytes; ▲, type B spermatogonia; ●, type A spermatogonia. Mutated cells are given in red. Each other colour corresponds to a specific cell type at the time of exposure: green, spermatozoa; grey, spermatid; orange, secondary spermatocytes; purple, primary spermatocytes; blue, type B spermatogonia and yellow, type A spermatogonia. Framed numbers correspond to the period of time, in days, necessary for a specific cellular stage, exposed to a chemical mutagen, to become spermatozoa.

1.3.2 Dominant lethality test

The dominant lethality test (DLT) is based on the detection of *in utero* deaths in the offspring of exposed parents. These increases in lethality are mainly attributed to chromosomal aberrations that do not affect the capacity of the germ cell to fertilise and result in embryonic death before and after implantation (Bishop *et al.*, 1983; Ehling *et al.*, 1978).

In this assay male mice are treated with a mutagen and sequentially mated to virgin females to analyse the mutagenic effect of the chemical agent. Similarly to the SLT, different cellular stages of spermatogenesis can be analysed by mating treated male mice at different times after exposure (Figure 1-5). Approximately 12-15 days after mating, females are sacrificed and uterine analysis is performed to establish the number of pregnant females and the total of the living and dead implants.

Similarly to the SLT, the DLT was initially used for the analysis of mutation induction in male mice, although it was later applied for the investigation of dominant-lethal mutations in the female germline (Bishop *et al.*, 1983; Generoso *et al.*, 1971; Katoh *et al.*, 1990; Sudman *et al.*, 1992).

The DLT does not need to examine as many offspring as the SLT to obtain statistically significant results; however, several hundreds of offspring are still required. The DLT is not considered as reliable because only mutations that prevent implants from becoming a fully developed animal can be scored.

This test has also been used to analyse the mutagenic potential of many chemicals including actinomycin D, adriamycin, bleomycin, busulfan, chlorambucil, cisplatin, cyclophosphamide, ethyl-methanesulfonate, ethyl nitrosourea, etoposide, fluorouracil, holoxan, isopropyl methanesulfonate, mechlorethamine, melphalan, mercaptopurine, methyl-methanesulfonate, mitomycin C, mitoxantrone, nitrogen mustard, procarbazine, propyl-methanesulfonate, thioTEPA, triethylenemelamine, trophosphamide, vincristine, and others. Some data obtained from these analyses are presented in section 1.4.

1.3.3 Transgenic mouse constructs

Transgenic mouse constructs are engineered to detect mutations *in vivo* in any tissue. There are two different transgenic mouse constructs commercially available, known as Muta™Mouse and Big Blue mice.

The Muta™Mouse contains a concatamer of approximately 40 copies of a bacterial *lacZ* gene, incorporated by lambda phage, at a single site on both chromosomes of a homologous pair (Gossen *et al.*, 1989). The mutation analysis is performed by isolation of genomic DNA from a tissue, followed by excision and packaging of the lambda genomes in phage particles and mutation scoring by phage plating on a *lacZ*⁻ *E. coli* strain in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Mutations at the *lacZ* gene are identified as clear plaques on the blue background of non-mutant plaques. The Big Blue mice contain *lacI* gene inserted in their genome and the mutation scoring in these mice is similar to that for Muta™Mouse.

The analysis of mutations using transgenic mouse constructs has the advantage of being able to compare spontaneous and induced mutation rates in the germline with those in other tissues. On the other hand, due to the lambda phage shuttle vector system and the requirements for successful rescue from genomic DNA, detection of mutants characterized by relatively large deletions of 50 bp to 500 bp are expected to be limited (Putman *et al.*, 1997).

1.4 Classification of chemical mutagens

Chemical agents have similar properties based on their chemical structure. Such chemicals belong to the same chemical class and frequently have similar toxicological modes of action. This section gives brief descriptions of the different types of chemical mutagens and provides representative chemical structures for each group.

1.4.1 Alkylating agents

Alkylating agents were the first effective non-hormonal drugs used to treat cancer (Adair and Bogg, 1931). This group contains a wide range of chemicals, many of them proved or suspected to be carcinogenic (Lawley, 1989).

Alkylating agents are electrophilic compounds with affinity for electron-rich atoms in biologic molecules with which they form covalent bonds (Lawley, 1966; Singer, 1975; Singer and Kusmierek, 1982). Alkylating agents are monofunctional or bifunctional depending on their mode of action. Monofunctional alkylating agents have a single reactive group that interacts with a single nucleophilic centre in DNA, while bifunctional agents react with two sites in DNA, the latter known as cross-linking agents (see 1.4.2). Some examples of monofunctional alkylating agents are given (Figure 1-6).

Most alkylating agents enter cells by diffusion and react with different oxygen and nitrogen positions in DNA, predominantly with O^6 and N^7 positions of guanine. The affinity of a particular alkylating agent towards oxygen and nitrogen radicals of DNA is referred to as the Swain-Scott constant (s) (Swain and Scott, 1953). Low s values indicate higher affinity for less nucleophilic groups like O^6 position of guanine and the phosphodiester groups of the DNA backbone, whereas high values indicate higher affinity for more nucleophilic groups like N^7 position of guanine (Roberts, 1978).

It has been demonstrated that the major DNA repair mechanisms involved in the repair of the cytotoxicity of monofunctional alkylating agents are the mismatch repair (MMR) (see 1.7.1) and nucleotide excision repair (NER) pathways (see 1.7.2) (Duckett *et al.*, 1996; Kat *et al.*, 1993). When damaged DNA template enters replication, alkylated bases mispair, generating base substitution; for example, O^6-ethylguanine

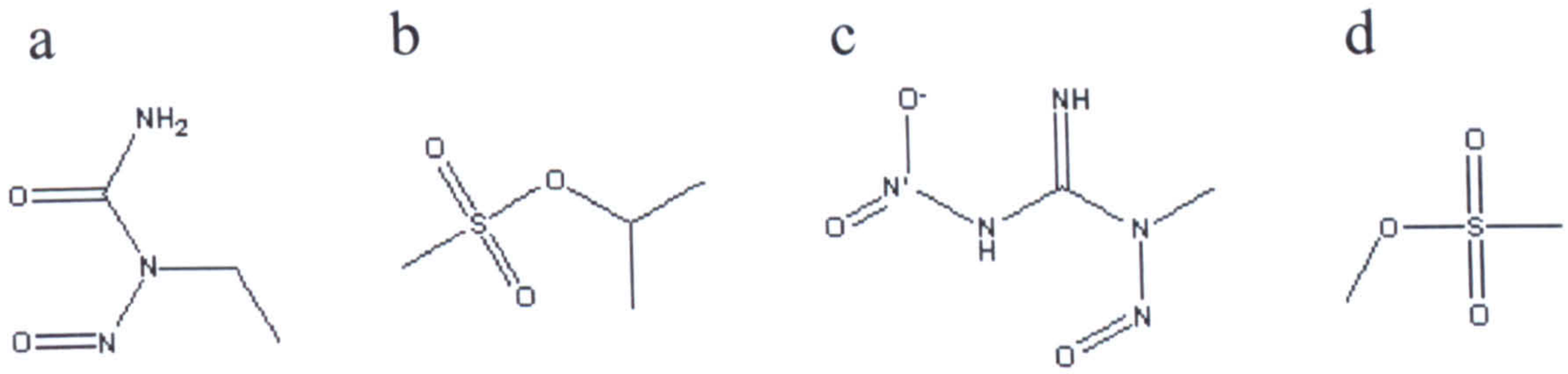


Figure 1-6 Monofunctional alkylating agents. a, ethyl nitrosourea; b, isopropyl methanesulfonate; c, methyl-nitro-nitrosoguanidine; d, methyl-methanesulfonate.

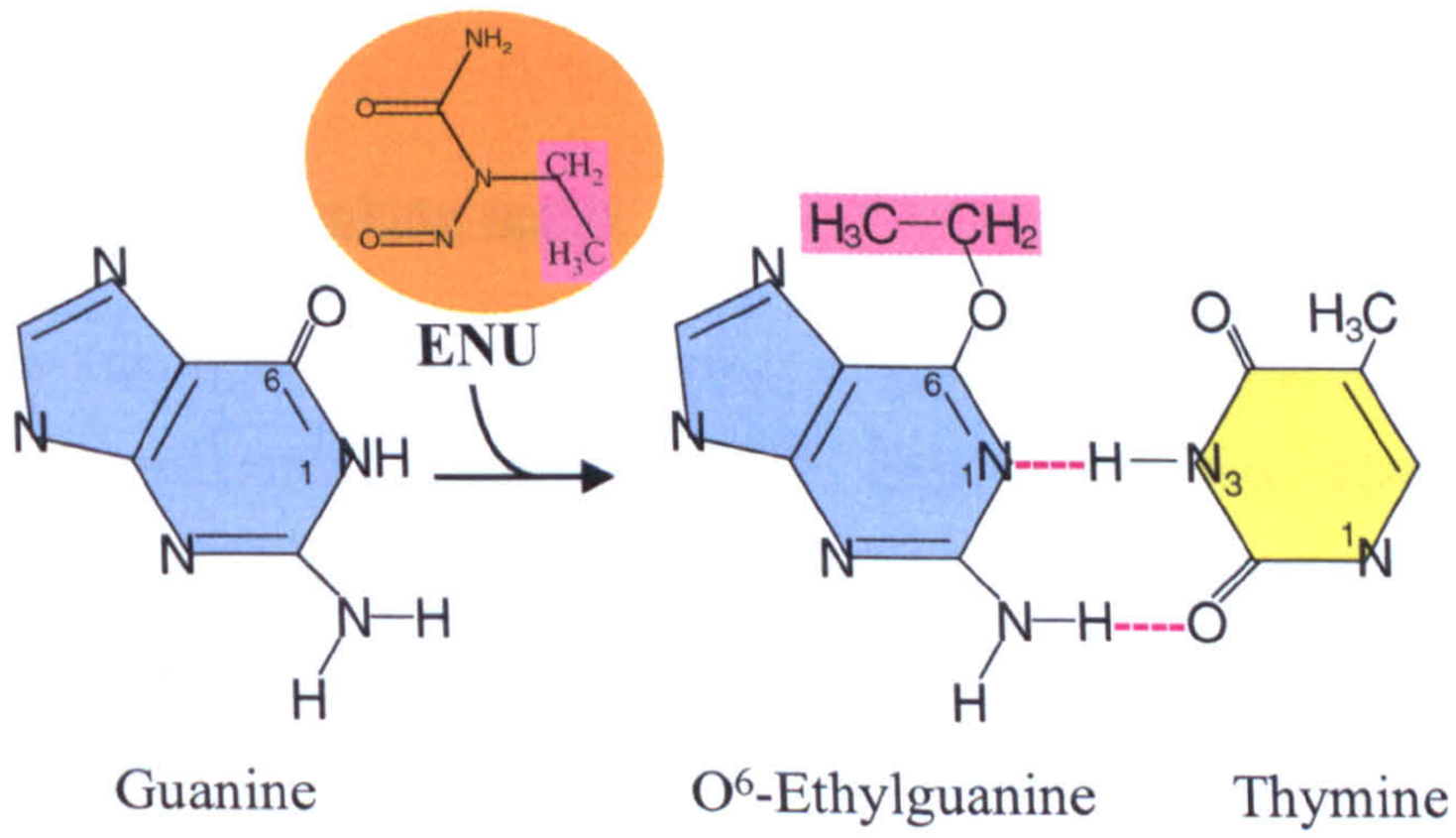


Figure 1-7 The alkylation of the O^6 position of guanine by ENU leads to mispairing with a thymine.

directly mispairs with thymine (Figure 1-7) and O^4-ethylthymine directly mispairs with guanine (Griffiths, 2000). In addition, DNA alkylation can also result in DNA SSBs (Verly and Paquette, 1972; Verly, 1974).

Using the SLT, germline mutagenicity of several alkylating agents has been analysed. The results of these studies show clear differences in the pattern and stage-specificity of mutation induction by alkylating agents. For example, ethyl nitrosourea (ENU) at doses of 50-400 mg/kg, has proved to be a very powerful mutagen across all stages of spermatogenesis (Favor, 1998; Favor, 1999; Justice *et al.*, 1999; Russell *et al.*, 1979; Russell and Hunsicker, 1984). On the other hand, methyl methanesulfonate has only been shown to be mutagenic in post-meiotic stages (Ehling and Neuhauser-Klaus, 1990) (Table 1-2).

For some chemicals inconclusive results have been obtained. For example, the analysis of dominant-lethal mutations suggested that exposure to iPMS only affects post-meiotic stages of mouse spermatogenesis (Ehling and Neuhauser-Klaus, 1995) (Table 1-2). On the other hand, the results of the same study also indicated that iPMS could as well be regarded as a pre-meiotic mutagen by the SLT test (Ehling and Neuhauser-Klaus, 1995).

1.4.2 Cross-linking agents

Cross-linking agents are bifunctional alkylating agents that have the capacity to react with two nucleophilic centres in DNA. Exposure to these chemicals results in intrastrand or interstrand cross-links, depending on whether the two linked nucleotides are on the same or opposite strands respectively (Eastman, 1987; Kohn *et al.*, 1966). Cross-linking agents do not exclusively react with DNA, they can also create cross-links between DNA, RNA and proteins.

There are different families of cross-linking agents: mustards (e.g. cyclophosphamide, melphalan, nitrogen mustard), platinum derivatives (e.g. cisplatin), some photoactivated psoralens; and other specific alkylating compounds like the antibiotic mitomycin C, busulfan and procarbazine (Figure 1-8).

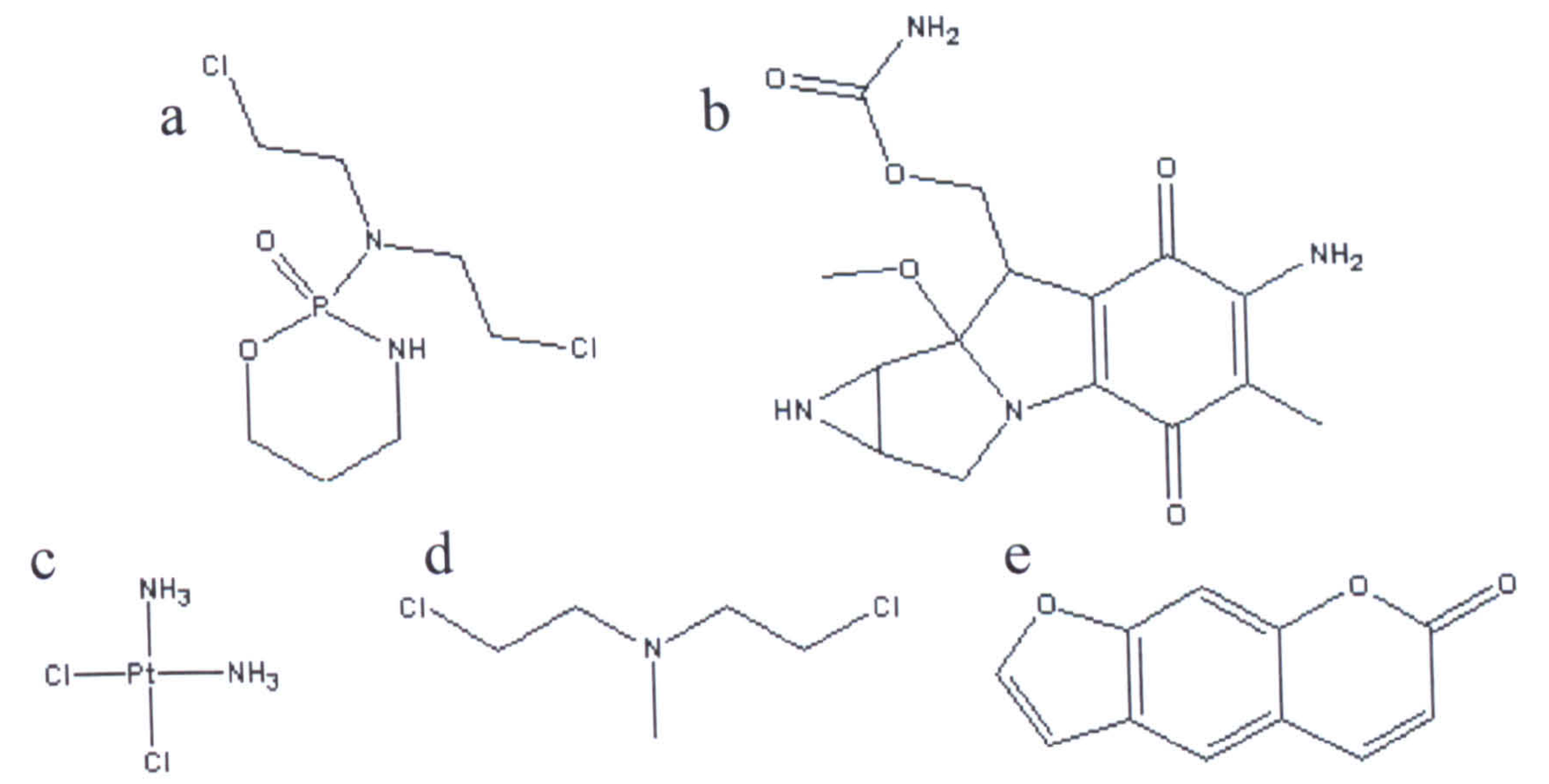


Figure 1-8 Cross-linking agents. a, cyclophosphamide; b, mitomycin C; c, cisplatin; d, nitrogen mustard; e, psoralens.

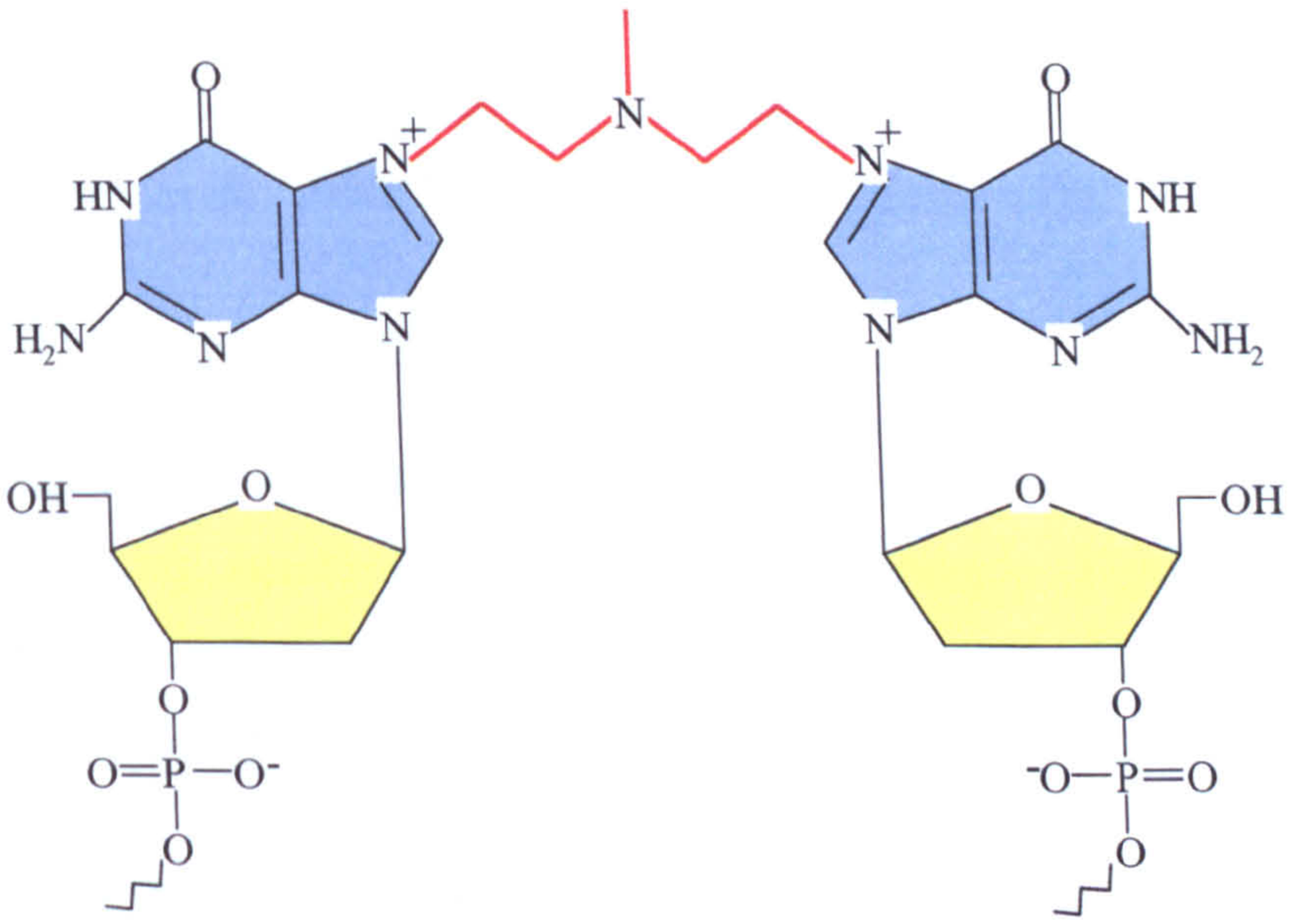


Figure 1-9 Mustard-induced DNA interstrand cross-link followed by a modification at N^7 position of guanine (red).

Interstrand DNA cross-links (Figure 1-9) are a severe type of damage as they inhibit DNA strand separations and therefore affect DNA transcription and replication. DNA cross-links lead to DNA double-strand breaks (DSBs). Interstrand cross-links are repaired by NER (see 1.7.2), homologous recombination (HR) (see 1.7.3) or non-homologous end-joining (NHEJ) (see 1.7.4) (Essers *et al.*, 2000; Panasci *et al.*, 2002). Intrastrand cross-links are less damaging to DNA and are repaired by the NER pathway (see 1.7.2) (Moggs *et al.*, 1996; Zamble *et al.*, 1996).

Germline mutagenicity of a cross-linking agent melphalan has been analysed with the DLT, which has revealed that it only affects post-meiotic stages of spermatogenesis (Generoso *et al.*, 1995). However, the same analyses performed using the SLT has found that melphalan can affect all stages of spermatogenesis, including stem cells (Russell *et al.*, 1990). On the other hand, exposure to chlorambucil, which is structurally related to melphalan, and cyclophosphamide only results in germline mutation induction in post-meiotic cells (Ehling and Neuhauser-Klaus, 1988b; Russell *et al.*, 1989) (Table 1-2).

Prior to this work, ESTR loci had been used, only once, for the analysis of germline mutations induced by a cross-linking agent cisplatin (Barber *et al.*, 2000). The results of this study showing the lack of mutation induction by cisplatin across all stages of mouse spermatogenesis are consistent with the data previously obtained by either SLT or DLT (Katoh *et al.*, 1990; Russell *et al.*, 1990) (Table 1-2).

1.4.3 Blocking agents

Blocking agents are chemicals whose action blocks cellular processes. Some examples of blocking agents are adriamycin and etoposide (Figure 1-10); these two agents are anticancer drugs that inhibit the DNA-rejoining activity of the essential enzyme topoisomerase-II (topo-II).

Topo-II is an endonuclease that removes torsional stress in DNA and creates transient staggered breaks in both strands of DNA; these double-stranded cleavages allow the passage of one double helix through another (Liu, 1989). Due to the requirement for DNA strand passage activity in a number of critical nuclear processes, including replication, recombination, and chromosome segregation (Berger and Wang, 1996; Wang, 1985), topo-II is essential for the survival of proliferating eukaryotic cells.

The genetic damage induced through the poisoning of topo-II is DNA DSB (Ferguson, 1998). It has been observed that, similar to DSB induced by ionising radiation, those induced by blocking agents are repaired by the NHEJ (see 1.7.4) and HR pathways (Chen *et al.*, 1996; Nitiss and Wang, 1988) (see 1.7.3).

It has been observed that treatment of mitotic cells with etoposide generates anaphase cells with enlarged chromosomes. This abnormality leads to numerical and structural aberration in daughter cells (Cimini *et al.*, 1997). The effect on the germline has been analysed using the SLT (see 1.3.1) and it has been proved that doses of 70-80 mg/kg of etoposide exhibit significant mutagenicity in primary spermatocytes (Russell *et al.*, 1998; Russell *et al.*, 2000a). In contrast, adriamycin, that has the same effect as etoposide on topo-II, fails to induce germline mutations (Russell *et al.*, 1990; Sudman *et al.*, 1992). The specific mutagenic effect of etoposide has been associated with decreases in meiotic crossing-over, which is explained by the involvement of topo-II in meiotic recombination (Russell *et al.*, 2000a).

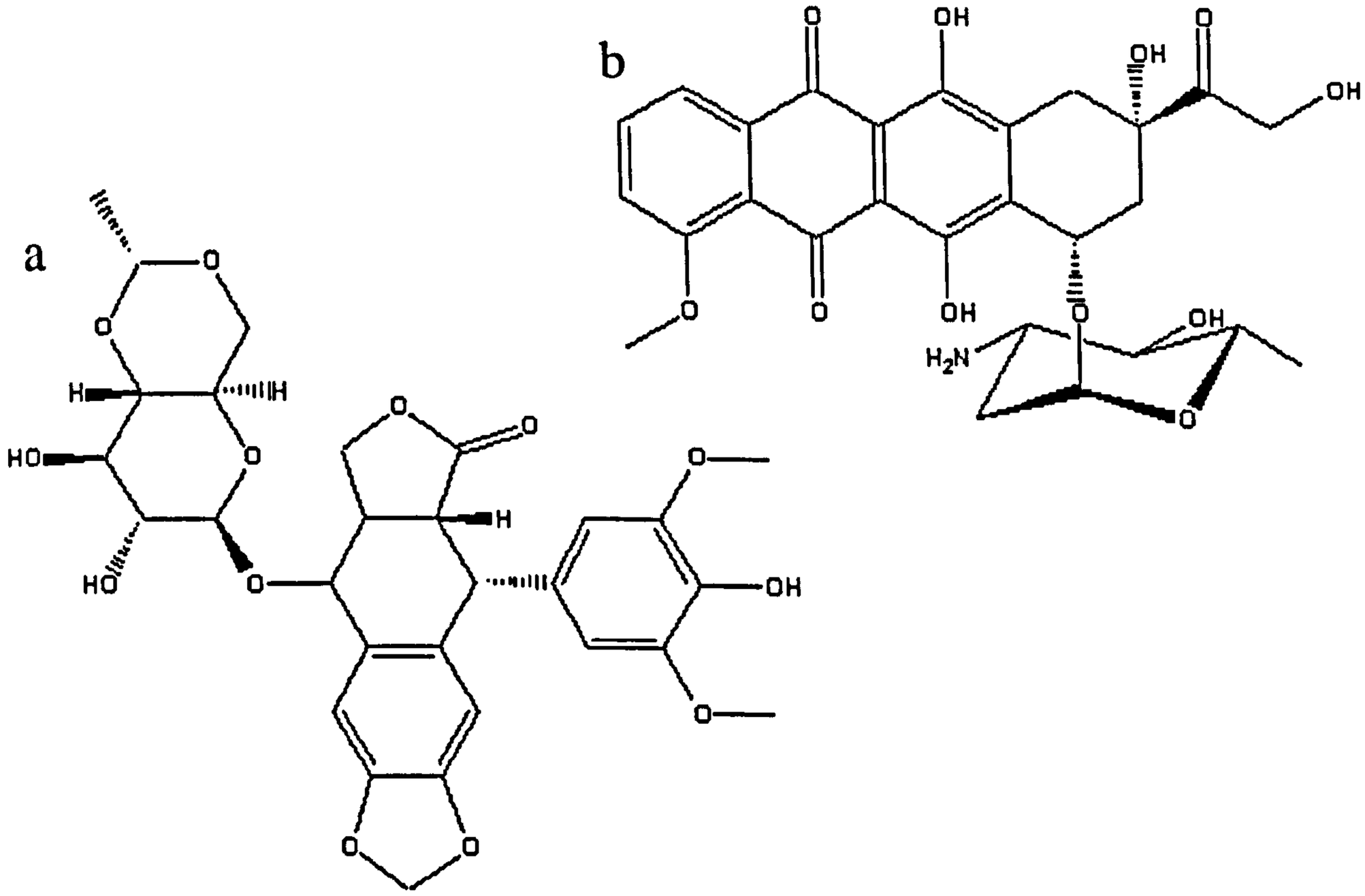


Figure 1-10 Blocking agents. a, etoposide; b, adriamycin.

1.4.4 Radiomimetic agents

Radiomimetic agents are chemicals with damaging capabilities similar to those produced by ionising radiation. Although not unique, the best known radiomimetic agent is bleomycin (Figure 1-11). Bleomycin causes DNA damage through the generation of oxygen radicals as well as through direct intercalation and binding to DNA. As a result of these interactions bleomycin leads to DNA single and blunt-ended DSBs (Charles and Povirk, 1998; Povirk, 1996). Similar to radiation-induced DSBs, those induced by bleomycin are repaired by the NHEJ (see 1.7.4) and HR (see 1.7.3) repair pathways. Repair by HR should be, in theory, error-free, however misrepair by NHEJ leads to deletions and translocation (Povirk, 1996; Wang *et al.*, 1997).

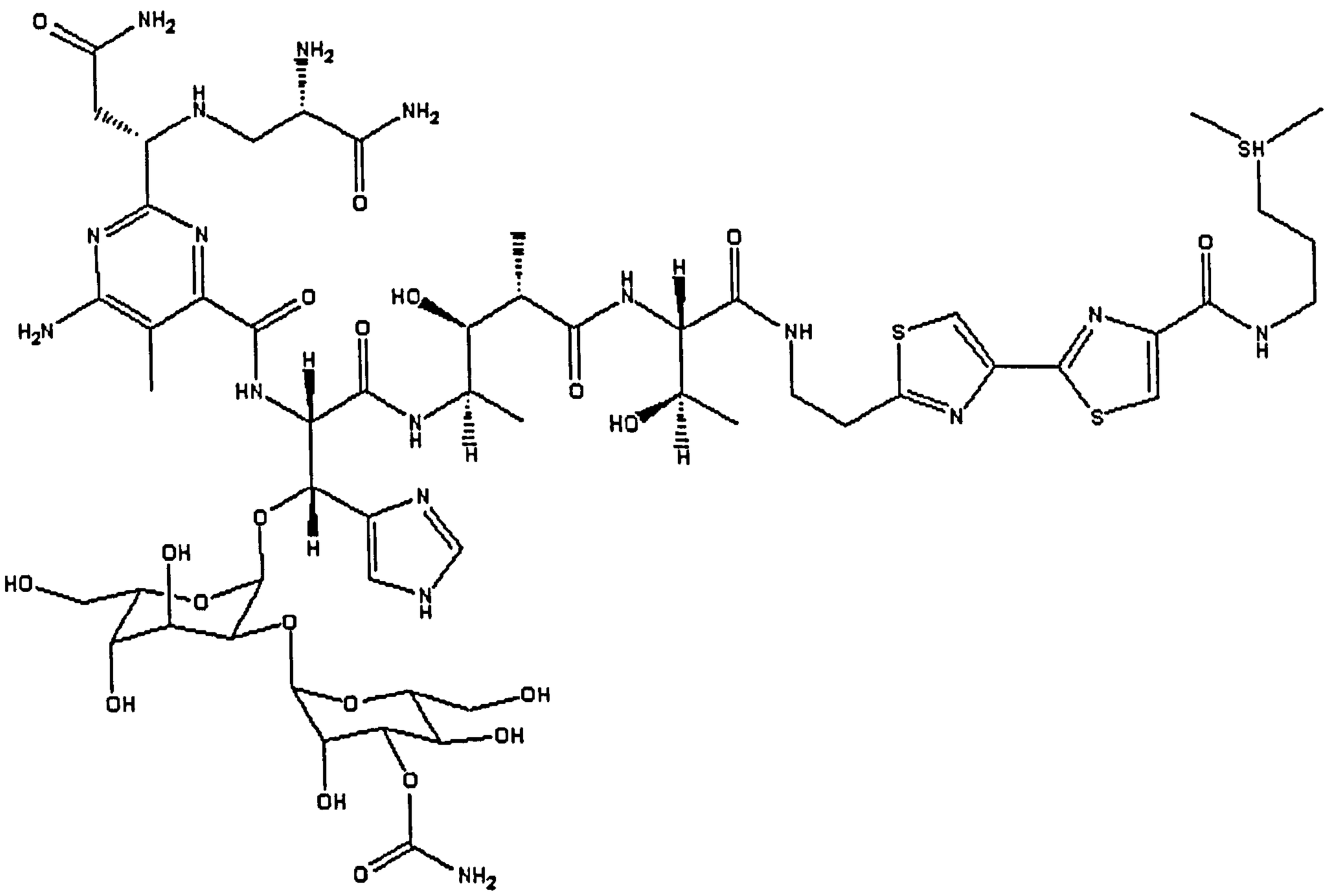


Figure 1-11 Radiomimetic agent. Bleomycin.

Doses of 10-35 mg/kg of bleomycin were used for the analysis of germline mutations induction in mice. Bleomycin was initially considered a female germline specific mutagen (Sudman *et al.*, 1992); however, further studies have shown that it is also a male germline mutagen, albeit specific to spermatogonia (Russell *et al.*, 2000b) (Table 1-2). Using the SLT, bleomycin has been proved to be a very unusual mutagen, showing mutation induction in spermatogonia, but not in post-spermatogonial stages, most of which are represented by multilocus deletions (Russell *et al.*, 2000b).

1.4.5 Aneuploidy inducers

This type of chemical does not have a direct effect on DNA. The targets of aneuploidy inducers are microtubules, tubulin or integral components of the cytoskeleton. Some examples of aneuploidy inducers are *Vinca* alkaloids (e.g. vincristine and vinblastine (Figure 1-12 a, b)) and taxanes (e.g. paclitaxel and docetaxel (Figure 1-12 c, d)). Some of these chemicals are currently used as anticancer drugs.

Vinca alkaloids are plant derivatives; they are dimeric compounds with complex ring systems. *Vinca* alkaloids have several effects on the exposed cells, including disruption of microtubules, inhibition of synthesis of proteins and nucleic acids, lipid metabolism alteration and modification of membrane lipid content (Cline, 1968; Schellenberg and Gillespie, 1980; Schroeder *et al.*, 1981; Wilson *et al.*, 1974; Wilson, 1975).

The only well documented effect is the disruption of microtubules due to the binding of these alkaloids to tubulin, the subunit protein of microtubules. Microtubules are involved in many cellular processes including the formation of the mitotic and meiotic spindle. The disruption of microtubules causes dissolution of the spindle leading to arrest of the dividing cells, aneuploidy and cell death (Bruchovsky *et al.*, 1965; Kallio *et al.*, 1995; Krishan, 1968; Malawista *et al.*, 1968; Parvinen *et al.*, 1978).

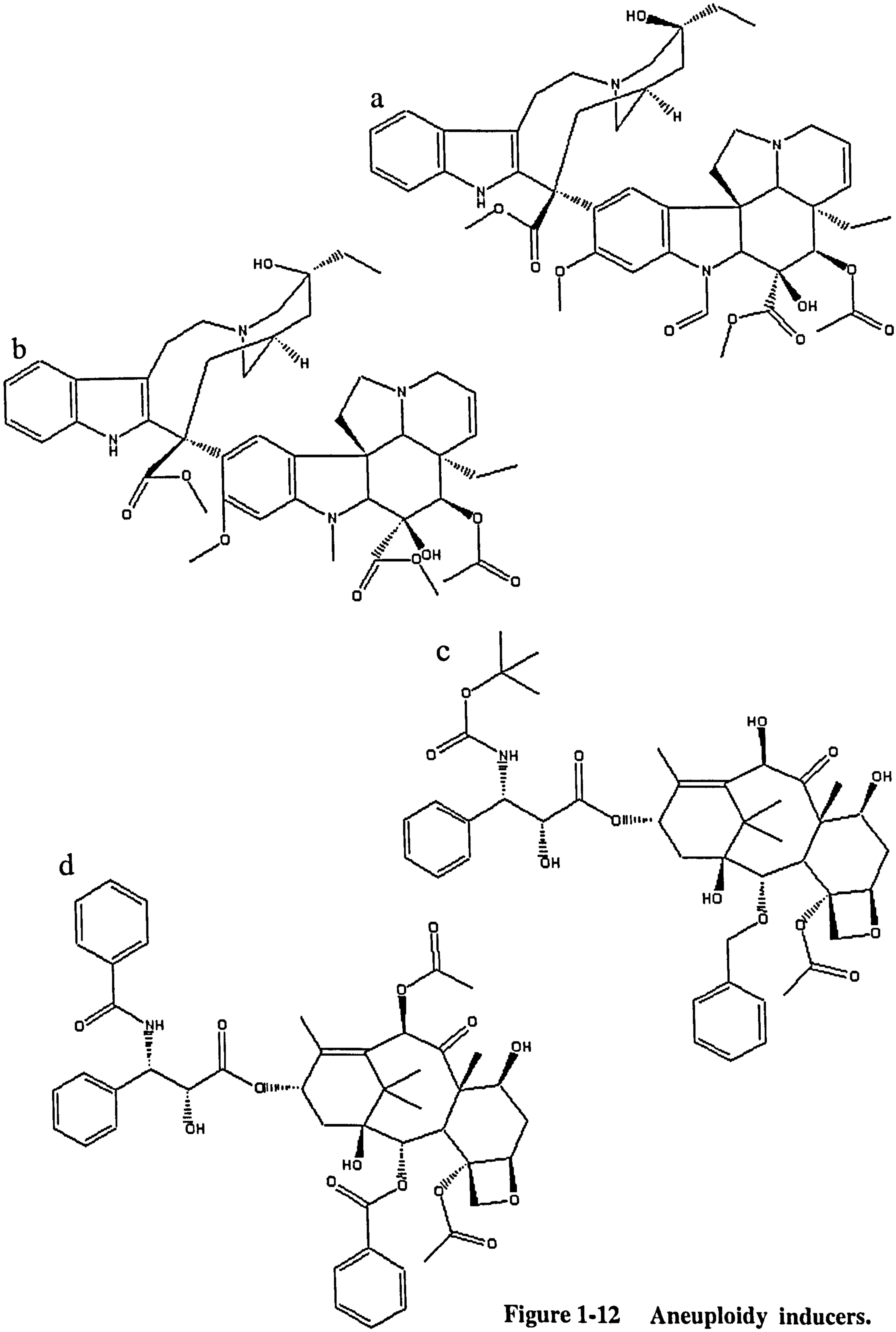


Figure 1-12 Aneuploidy inducers. a, vincristine; b, vinblastine; c, docetaxel; d, paclitaxel.

Taxanes are typified by paclitaxel and its semisynthetic analogue docetaxel. Paclitaxel was initially found in yew trees and other members of the yew family. They consist of an oxetane ring attached to a derivative of taxane. The interaction of these drugs with microtubules creates stable microtubule structures due to the disruption of the equilibrium between soluble and polymerised tubulin (Kumar, 1981; Schiff and Horwitz, 1980). Stable microtubules induce the loss of a variety of cellular structures and cell functions including cellular morphology, secretion and cell division (Green and Goldman, 1983; Mole-Bajer and Bajer, 1983; Santibanez *et al.*, 1995; Sukhacheva *et al.*, 2001).

The effect on the germline is characterised by elevated numbers of aneuploid cells (Kallio *et al.*, 1995; Mailhes *et al.*, 1999; Miller and Adler, 1992; Sukhacheva *et al.*, 2001). Increases in hyperploidy were reported in meiotic metaphase II cells 6, 24 and 48 hours after exposure to 0.25-0.75 mg/kg (Sheu *et al.*, 1992). However, the analysis of the transmissibility of unbalanced DNA to the offspring of parents exposed to the aneuploidy inducers vincristine has generated negative results for the dose range of 0.5-3 mg/kg (Ehling *et al.*, 1988) (Table 1-2). Because of their typical mode of action, aneuploidy inducers do not alter the mutation rate in the germline when analysed with the SLT or the DLT.

1.4.6 Antimetabolites

Antimetabolites are chemicals showing a close structural resemblance to metabolites that are required for normal physiological functioning; they affect cells by interfering with the utilization of the essential metabolite. Several purine and pyrimidine analogues are used in cancer chemotherapy (e.g. mercaptopurine and fluorouracil (Figure 1-13)).

Pyrimidine and purine analogues block the synthesis of nucleotides. The lack of nucleotides strongly inhibits DNA replication, transcription and repair (Boumah *et al.*, 1984). The blockage of these processes results in the generation of DNA SSBs and cell arrest (Halloran and Fenton, 1998; Pieper *et al.*, 1988).

The analysis of mutation induction in the germline of mice exposed to mercaptopurine and fluorouracil using both SLT and DLT has so far generated conflicting results (Table 1-2). Thus, it has been shown that the pre-meiotic exposure to

mercaptopurine results in elevated rate of dominant-lethal mutations (Russell and Hunsicker, 1987), whereas no increase in SLT mutations was detected at this stage (Generoso *et al.*, 1975). These differences are probably attributed to the damage caused by the chemical, which appear to be lethal and non-heritable.

Preliminary data on the mutagenicity of fluorouracil show the lack of mutation induction by this chemical, though they were obtained by profiling too few offspring to give considered reliable results (James and Smith, 1982; Russell *et al.*, 1981).

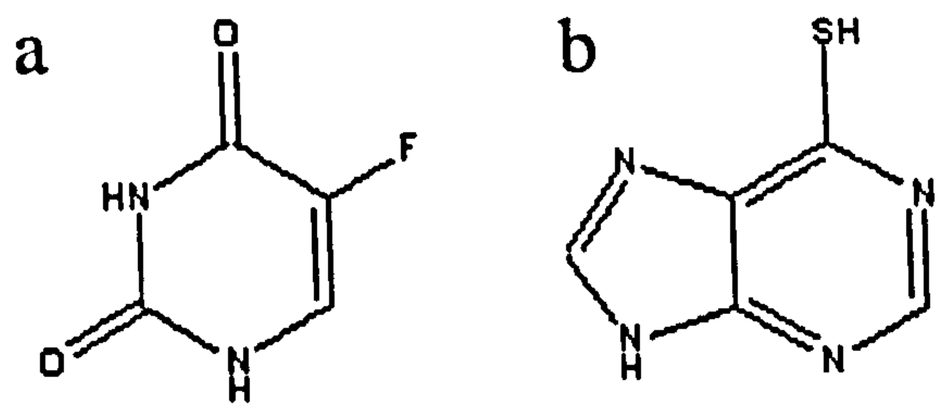


Figure 1-13 Antimetabolites. a, fluorouracil; b, mercaptopurine.

1.4.7 Conclusion

To date, the SLT and DLT has been considered as the most reliable for the analysis of germline mutation induction by chemical mutagens. These tests have provided very important data on the germline mutagenicity of chemical agents; however, some of these results still remain inconclusive. The failure of conventional systems for monitoring germline mutation in mice to generate a cohesive set of results on the mutagenicity of some chemical is primarily attributed to the low spontaneous mutation rate, which, in turn, allows robust mutation detection at very high doses of exposure. The high-dose exposure frequently does not reflect the dose-range of environmental, occupational or medical exposure for humans. In addition, these systems also require the profiling of very large numbers of offspring to obtain statistically significant results, implying large amounts of time, effort and economics resources to be invested in the analysis.

Table 1-2 Stage-specificity of mutation induction in the germline of male mice exposed to chemical mutagens.

Chemical	Test	Stages affected	Reference
Alkylating agents			
ENU	SLT	All stages	(Shelby and Tindall, 1997)
	DLT	Post-meiotic	(Generoso *et al.*, 1984)
iPMS	SLT	All stages	(Ehling and Neuhauser-Klaus, 1995)
	DLT	Post-meiotic (inconclusive)	(Ehling and Neuhauser-Klaus, 1995)
MMS	SLT	Post-meiotic	(Ehling and Neuhauser-Klaus, 1990)
	DLT	Post-meiotic	(Ehling and Neuhauser-Klaus, 1990)
Cross-linking agents			
Melphalan	SLT	All stages	(Russell *et al.*, 1990)
	DLT	Post-meiotic	(Generoso *et al.*, 1995)
Cyclophosphamide	SLT	Post-meiotic	(Ehling and Neuhauser-Klaus, 1988b)
	DLT	Post-meiotic	(Ehling and Neuhauser-Klaus, 1988b)
Chlorambucil	SLT	Post-meiotic	(Russell *et al.*, 1989)
	DLT	Post-meiotic	(Generoso *et al.*, 1995)
Cisplatin	SLT	Negative	(Russell *et al.*, 1990)
	DLT	Negative	(Katoh *et al.*, 1990)
	ESTR	Negative	(Barber *et al.*, 2000)
Blocking agents			
Etoposide	SLT	Meiotic	(Russell *et al.*, 1998)
Adriamycin	SLT	Negative	(Russell *et al.*, 1990)
	DLT	Negative	(Sudman *et al.*, 1992)
Radiomimetics			
Bleomycin	SLT	Pre-meiotic	(Russell *et al.*, 2000b)
	DLT	Negative	(Sudman *et al.*, 1992)
Aneuploidy inducers			
Vincristine	SLT	Negative	(Ehling *et al.*, 1988)
	DLT	Negative	(Ehling *et al.*, 1988)
Antimetabolites			
Mercaptopurine	SLT	Negative	(Russell and Hunsicker, 1987)
	DL	Pre-meiotic	(Generoso *et al.*, 1975)
Fluorouracil	SLT	Inconclusive	(Russell *et al.*, 1981)
	DL	Negative	(James and Smith, 1982)

A new model system for the analysis of germline mutation induction by chemicals is therefore clearly needed. This system should ideally include a set of loci with high spontaneous mutation rates, which would allow the detection of low-dose exposure to chemical mutagens by profiling relatively small number of offspring.

The tandem repeat DNA loci are thought to fulfil the criteria required for the analysis of chemical mutagens in the germline. Generally they have a high spontaneous mutation rate, are found in the genome of most mammalian organisms, they have been used for the analysis of germline mutations in humans, and mutations at these loci, result mostly in insertions or deletions which are easily detectable by standard genetic techniques.

The repetitive DNA families commonly used for genomic analysis are microsatellites, minisatellites and expanded simple tandem repeats.

1.5 Tandem repeat DNA loci

1.5.1 Microsatellites

Microsatellites, also known as simple tandem repeats (STRs), are ubiquitous in both prokaryotic and eukaryotic genome (Field and Wills, 1996; Hancock, 1995). STRs are found in both coding and non-coding regions of the genomes (Katti *et al.*, 2001; Tautz and Renz, 1984), although they are more frequent in the latter (Toth *et al.*, 2000). They are represented by short arrays (less than 1 kb) of mostly invariable short repeats (1-6 bp) (Toth *et al.*, 2000) (Table 1-3). The distribution of STR array lengths is species-specific, for example 43% of rat STRs have array sizes greater than or equal to 40 bp compared to only 12% in humans (Beckman and Weber, 1992). Different taxa also exhibit different abundance of repeat types (Hancock, 1995); in humans, the most abundant repeat types are A, AC, AAAN, AAN, and AG (in decreasing order of abundance), while in the rat the most common STR motifs are AC, AG, A, AAAN, and AAGG (Beckman and Weber, 1992).

STRs are highly polymorphic. In *Mus musculus* approximately 50% of loci are variable, and up to 90% of them differ from those in *Mus spretus* or *Mus casteneus* (Hearne *et al.*, 1992). Estimates of spontaneous mutation rates at STR loci differ substantially between loci, ranging from 10^{-5} to 10^{-3} per locus per generation (Dietrich *et al.*, 1992; Heyer *et al.*, 1997; Weber and Wong, 1993). This difference in mutation rate has been found to be correlated with STR array length (Pupko and Graur, 1999; Wierdl *et al.*, 1997). About 90% of mutations at STR loci are thought to involve the gain or loss of a single repeat unit (Brinkmann *et al.*, 1998).

Despite the lack of direct experimental evidence, it is generally accepted that mutation at STR loci is mainly attributed to replication slippage (Levinson and Gutman, 1987). Slippage implies the displacement of strands of a replication duplex, followed by the mispairing of complementary bases within the STR array. The circumstantial evidence that supports the existence of this mechanism is based on the difference between spontaneous mutation rates observed in males and females as well as between younger and older males (Brinkmann *et al.*, 1998). These differences may be related to the number of mitotic divisions undergone in male and female gametogenesis. In humans, oogonia undergo approximately 22 cellular divisions prior to meiosis, whereas sperm cells from a 29 year old man have undergone approximately 350 cell divisions,

which increases with age (Vogel and Motulsky, 1997), therefore providing a plausible explanation for a 6-fold difference between STR mutation rate in the male and female germline (Brinkmann *et al.*, 1998).

The hypothesis explaining STR mutation by replication slippage is also supported by the lack of flanking marker exchanges in the new mutant alleles (Levinson and Gutman, 1987) and by the similarities in mutation rates between autosomal and Y-linked STR loci (Mahtani and Willard, 1993). The latter observation clearly shows that STR mutation cannot be explained either by intra-allelic exchanges, or by any other recombination-like events, which are absent across most of Y chromosome.

Another hypothesis explaining STR mutation is based in the capacity of some repeat units under physiological conditions to form hairpins, and probably triplexes and tetraplexes (Mitas, 1997). It has been proposed that the formation of these secondary structures is array length-dependent, and that they interfere in DNA replication, creating instability or promoting DNA recombination (Mitas, 1997). One model of hairpin-formation mutagenesis suggests that the crucial role in this process belongs to flap endonuclease-1 (FEN-1) protein. Mutations in yeast *RAD27* gene (*FEN-1* homologue) have been shown to increase trinucleotide repeat expansion (Freudenreich *et al.*, 1998). FEN-1 is an endo- and exonuclease, involved in the replacement of Okazaki fragments. Synthesis of DNA replacing an Okazaki fragment displaces the 5' end of the downstream strand, creating a flap which is cleaved by the endonuclease activity of FEN-1 (Lieber, 1997). Hairpin structures are resistant to FEN-1 activity, which is hypothesised to increase the frequency of replication errors and strand slippage (Harrington and Lieber, 1994).

The function of STR is not known; however, a number of human genetic diseases have been found to be caused by dynamic expansions of STR, mainly of trinucleotides repeats (Djian, 1998; Mitas, 1997). A large group of disorders, mostly neurodegenerative, are attributed to genes encoding large polyglutamine tracts due to the expansion of CAG repeats in coding regions. These expansions have been observed in diseases such as Huntington's disease (The Huntington's Disease Collaborative Research Group, 1993), 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 (David *et al.*, 1997; Imbert *et al.*, 1996; Orr *et al.*, 1993; Pulst *et al.*, 1996;

Sanpei *et al.*, 1996; Zhuchenko *et al.*, 1997). It is thought that large tracts of polyglutamine may cause neurodegenerative diseases by activating pro-apoptotic proteases such as apopain, which induces programmed cell death, apoptosis. (Djian, 1998; Goldberg *et al.*, 1996)

Some trinucleotide repeat expansions, associated with diseases, are present in non-protein-coding regions of the genes. For example, fragile X syndrome is a disease causing mental retardation; it is caused by an expansion of up to 3000 repeats of a CCG motif present in the 5'UTR of the *FMR1* gene. This expansion results in the loss of gene function due to hypermethylation and alteration of the chromatin structure (Green and Djian, 1998).

Although most STR repeat expansions are trinucleotides, some diseases are caused by the expansion of other motif lengths. For example, an expansion of a pentanucleotide repeat in the intron 9 of the *SCA10* gene results in spinocerebellar ataxia type 10 (Matsuura *et al.*, 2000).

Due to their relatively high mutation rate and abundance in the human genome, STR have been intensively used for the construction of genome-wide linkage maps (Dib *et al.*, 1996), the identification of individuals on paternity testing or forensic analyses (Budowle *et al.*, 1997; Caglia *et al.*, 1998; Gill *et al.*, 1994; Ingvarsson *et al.*, 2000; Papiha and Sertedaki, 1995; Urquhart *et al.*, 1994), mapping genes involved in diseases (Bergthorsson *et al.*, 1995; Machado *et al.*, 2000), studies on evolution of populations (Ellegren, 2000; Tautz, 1989) and the analysis of association of STR instability with human diseases (Stallings, 1994). In the most of these studies, STR loci are detected by PCR amplification, followed by gel electrophoresis.

However, it should be noted that, because of the frequently low spontaneous mutation rate in the mouse germline, STR loci do not represent the most suitable experimental model for the analysis of mutation induction. This level of mutation rate would require profiling of large number of offspring for the analysis of the mutagenicity of a specific chemical agent and would not allow the analysis of low doses of exposure in a realistic sample size.

Table 1-3 Characteristics of tandem repeat DNA loci.

		Array size	Repeat size (bp)	Repeat variation	Position in the genome	Mutation Mechanism
Microsatellites		10 bp-1 kb	1-6	No	Across the genome	Replication slippage
Minisatellites		0.5-50 kb	10-100	Yes	Sub-telomeric	Gene conversion Crossing-over
ESTRs	**MMS10**	18bp-8kb	6, 9	No	Across the genome	?
	Ms6-hm (Pc-1)	1-16 kb	5	No	Chromosome 4	?
	Hm-2	2-25 kb	4	No	Chromosome 9	?
	Pc-2	0.1-20 kb	7	No	Chromosome 6	?

1.5.2 Minisatellites

Minisatellites, also known as variable number of tandem repeats (VNTRs), have been found in the genome of most higher eukaryotes including humans, mice, rat, cats, dogs, birds and pigs (Andersen and Nilsson-Tillgren, 1997; Archibald *et al.*, 1995; Burke and Bruford, 1987; Jeffreys *et al.*, 1985b; Jeffreys and Morton, 1987; Schonian *et al.*, 1993; Vergnaud *et al.*, 1993).

There are an estimated 1500 minisatellites in both the mouse and human genomes (Bois *et al.*, 1998a; Jeffreys, 1987). They are generally GC-rich and composed of tandemly repeated units of between 10-100 bp within arrays of 0.5-50 kb (Armour *et al.*, 1990; Wong *et al.*, 1987) (Table 1-3).

The chromosomal distribution of minisatellites differs in different organisms. For example; in humans, the vast majority of minisatellites are clustered in the proterminal regions of chromosome arms (~90%) (Amarger *et al.*, 1998; Vergnaud *et al.*, 1993), while pig, rat and mouse minisatellites are sequentially more dispersed and less preferentially associated with centromeres or telomeres (Amarger *et al.*, 1998; Jeffreys *et al.*, 1987).

Many minisatellites are highly polymorphic due to variation in the number of repeat units between alleles (length polymorphism), and small differences in repeat unit sequence within alleles (Bois and Jeffreys, 1999; Jeffreys *et al.*, 1985b). The repeat elements in a subset of human minisatellites share a common 10-15 bp 'core' sequence, similar to the generalized recombination signal (chi) of *E. coli*, which might act as a recombination signal in the generation of hypervariability (Jeffreys *et al.*, 1985a). Alternative cores, which may be associated with other sets of minisatellites such as AT-rich minisatellites, have also been reported (Jarman *et al.*, 1986; Nakamura *et al.*, 1987).

The mutational processes generating minisatellite hypervariability dramatically differ in somatic and germ cells. Somatic mutations are rare and involve only relatively simple intra-allelic events arising by replication slippage or unequal crossing-over between sister chromatids (Jeffreys and Neumann, 1997; May *et al.*, 1996). On the other hand, germline mutations are mostly inter-allelic, and arise by complex gene conversion-like events which involve recombination-based repair of meiotic DSB, most

probably taking place during meiosis (Bois and Jeffreys, 1999; Buard *et al.*, 2000; Jeffreys *et al.*, 1990; Jeffreys *et al.*, 1995; May *et al.*, 1996).

Germline mutation rates are loci specific and have been extensively studied at the human minisatellites MS32, MS205, B6.7, and CEB1 which display germline mutation rates of 0.8%, 0.4%, 5%, and 13% respectively (Armour *et al.*, 1999; Buard *et al.*, 1998; Jeffreys *et al.*, 1994; May *et al.*, 1996; Tamaki *et al.*, 1999).

The function of the majority of minisatellites is not known; however a few minisatellites, found within coding sequences of the human genome, show associations with various human diseases. For example, variation in the size of the minisatellite within the *apo(a)* gene results in changes in molecular weight of the protein coded by this locus, which range from 400 to 800 kDa (Koschinsky *et al.*, 1990). Higher levels of *apo(a)* protein in plasma, which are inversely correlated to the repeat number of the enclosed minisatellite, lead to coronary vessel disease, artery disorders and brain vessel disorders (Bliskovskii, 1994). Variation in the number of repeats at minisatellites located within the mucin gene family and the D4 dopamine receptor (DRD4) also affects the size of proteins coded by these genes (Asghari *et al.*, 1994; Gendler *et al.*, 1990; Lancaster *et al.*, 1990; Lichter *et al.*, 1993).

Regulatory functions, associated with susceptibility to disease, have also been attributed to minisatellites located in putative gene regulatory regions. For example, the proto-oncogene *HRAS1* has a minisatellite located 1kb downstream from the start of its transcription site. Each repeat of the HARS1 minisatellite has homology to a transcription factor binding site. Individuals who have rare alleles of the minisatellite have an increased risk of certain types of cancers (Krontiris, 1995; Nakamura *et al.*, 1998; Phelan *et al.*, 1996). Other examples of minisatellites which may influence gene expression are found upstream of the *CSTB* gene (Lalioti *et al.*, 1997; Lalioti *et al.*, 1998; Lalioti *et al.*, 1999) within the D_H-J_H interval of the human immunoglobulin heavy chain (Trepicchio and Krontiris, 1993) and in the donor site of the exon 2 of the human interferon-inducible gene 6-16 (Turri *et al.*, 1995).

The characteristically high polymorphism present in minisatellites, has allowed these loci to be exploited as a very important source of information. Minisatellites can be easily analysed by Southern blot hybridisation of genomic DNA with either single

locus or multilocus probes. Single locus probes are unique for a specific minisatellite within a genome (Wong *et al.*, 1986), while multilocus probes consist of tandem repeats of the core sequence allowing simultaneous detection of many minisatellites to produce a DNA fingerprint completely specific to an individual (Jeffreys *et al.*, 1985a).

Analyses with multilocus probes have been used for the identification of individuals for paternity testing (Jeffreys *et al.*, 1992; Smith *et al.*, 1990) or forensic analyses (Gill *et al.*, 1985). Single locus probes have been used for population studies (Balazs *et al.*, 1989), human historical migrations (Jobling *et al.*, 1998) and the analysis of increased germline mutation in radiation contaminated areas (Dubrova *et al.*, 1996; Dubrova *et al.*, 1997; Dubrova *et al.*, 2002).

Minisatellite variant repeat typing by PCR (MVR-PCR) and small-pool PCR (SP-PCR) have been used to analyse in detail the mechanisms of minisatellite mutation. MVR-PCR detects patterns of variant interspersed repeat units along a minisatellite, revealing the spectrum of mutations (Buard and Vergnaud, 1994; Jeffreys *et al.*, 1993; Jeffreys *et al.*, 1998a; Jeffreys *et al.*, 1998b; Jobling *et al.*, 1998; May *et al.*, 1996; Tamaki *et al.*, 1999). SP-PCR is used to isolate a practically unlimited number of *de novo* minisatellite mutations from the sperm DNA, which are further characterised by MVR-PCR (Armour *et al.*, 1999; Buard *et al.*, 2000; May *et al.*, 1996; May *et al.*, 2000; Monckton *et al.*, 1995; Tamaki *et al.*, 1999).

In contrast to the well-characterised human minisatellites, currently little is known about the true mouse minisatellites. The only attempt to isolate them has revealed a set of relatively stable loci with the mutation rate well below 10^{-3} per gamete and with most of the mutations attributed to intra-allelic events (Bois *et al.*, 1998a). Given that the known mouse minisatellites show a relatively low spontaneous mutation rate, these loci have not been considered as a useful tool for monitoring mutation induction in the mouse germline.

1.5.3 Expanded Simple Tandem Repeats

To date, expanded simple tandem repeats (ESTRs) have only been identified in the mouse genome. They were identified by DNA fingerprinting and were initially considered to be mouse minisatellites (Jeffreys *et al.*, 1987). However, further analysis has revealed a number of fundamental differences between these tandem repeats and minisatellites, which led to the reclassification of them as ESTRs (Bois and Jeffreys, 1999). First of all, these loci consist of homogeneous relatively short repeats, ranging from 4 to 9 bp (Bois *et al.*, 1998b; Gibbs *et al.*, 1993; Jeffreys *et al.*, 1987; Kelly *et al.*, 1989; Kelly *et al.*, 1991), whereas minisatellite repeats are longer (10-100 bp) and show a substantial variation in their sequence within the arrays (Armour *et al.*, 1990; Bois and Jeffreys, 1999; Jeffreys *et al.*, 1985b; Wong *et al.*, 1987). Another fundamental difference between these two types of repeat loci is that the true human minisatellite almost exclusively mutates in the germline, while ESTR loci show a significant instability in somatic cells (Barber *et al.*, 2000; Bois *et al.*, 1998a; Bois and Jeffreys, 1999; Buard *et al.*, 2000; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Fan *et al.*, 1995; Jeffreys *et al.*, 1990; Jeffreys *et al.*, 1995; Jeffreys and Neumann, 1997; May *et al.*, 1996; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994).

To date, a number of mouse ESTR loci have been isolated, including *Ms6-hm* (also known as *Pc-1*), *Hm-2*, *Pc-2* and numerous ESTR loci belonging to the MMS10 repeat family. The summary of recent studies analysing these loci is given below.

The *Ms6-hm* locus, located on chromosome 4, consists of expanded perfect pentameric repeats (GGGCA), which form arrays of 1-16 kb in length (Kelly *et al.*, 1989; Kelly, 1990) (Table 1-3). This locus has a high spontaneous mutation rate in the germline of different inbred strains of mice ranging from 2.5% to 15% (Barber *et al.*, 2002; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a; Kelly *et al.*, 1991). *Ms6-hm* is flanked by a mammalian retrotransposon-like (MaLR) long terminal repeat (LTR) sequence of the mouse transcript (MT) family from which it originated (Kelly *et al.*, 1989).

The *Hm-2* locus consists of identical tetrameric sequences (GGCA), expanding for a total length of 2-25 kb in chromosome 9 (Gibbs *et al.*, 1993) (Table 1-3). Similar to *Ms6-hm*, *Hm-2* originated within a MaLR LTR sequence, however, from the ORR1

family. The *Hm-2* spontaneous mutation rate in the germline of different inbred strains of mice varies from 2% to 14% (Barber *et al.*, 2002; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a; Gibbs *et al.*, 1993).

The *Pc-2* locus, located on the chromosome 6, consists of heptanucleotide repeat units (GGCAGGA), which form arrays of 0.1-20 kb in length (Table 1-3). It originated by expansion within the B1 family of short interspersed elements (SINE). The germline mutation rate observed at *Pc-2* is 3.3% (Suzuki *et al.*, 1993).

The large family of mouse ESTRs called MMS10 is composed of GGCAGA (80%) and GGCAGAGGA (20%) repeat units, expanded from within B1 elements throughout the mouse genome (Bois *et al.*, 1998b). The array is 18 bp-8 kb in length, and it has a 1.7% average spontaneous mutation rate in the mouse germline (Bois *et al.*, 2001) (Table 1-3).

The very high spontaneous mutation rates at ESTR loci potentially make them useful for monitoring germline mutation in mice. Indeed, the results of numerous publications presented below show that exposure to ionising radiation results in elevated ESTR mutation rate in the germline of irradiated male mice (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994).

1.6 Mutation induction at mouse ESTR loci

It has recently been established that ESTR loci provide a very powerful tool for the analysis of radiation-induced germline mutation in mice (Barber *et al.*, 2000; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a; Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994). It should be noticed that these studies have generated conflicting results on the stage-specificity of mutation induction at mouse ESTR loci. Thus, the data obtained in Leicester show that the effects of radiation in the germline can only be detected at ESTR loci in pre-meiotic stages of spermatogenesis, whereas exposure to ionising radiation does not affect ESTR mutation rates in post-meiotic cells (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova and Plumb, 2002). In contrast, the results of the Japanese group suggest that exposure to ionising radiation affects both pre-meiotic and post-meiotic stages of spermatogenesis (Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994). In addition, the latter group has also reported an elevated mutation rate in the germline of non-exposed females mated to the exposed males (Fan *et al.*, 1995; Niwa and Kominami, 2001; Sadamoto *et al.*, 1994).

Dubrova *et al.* have also analysed a dose-response for radiation-induction mutation at ESTR loci in pre-meiotic stages of spermatogenesis. The analysis has shown that acute and chronic exposure to low- and high-LET sources of ionising radiation results in a linear increase in ESTR mutation rate (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a). In addition, these studies evaluated the doubling dose for radiation-induced ESTR mutation in the mouse germline, which represents the measure of sensitivity of a given system for mutation monitoring. Doubling dose is the dose of radiation exposure which results in an elevated mutation rate that is equal to the double of the spontaneous mutation rate. The doubling dose obtained at ESTR loci was 0.33 Gy (Dubrova *et al.*, 1998a), almost identical to the doubling dose obtained by the SLT approach (0.34 Gy) (Luning and Searle, 1971; Russell *et al.*, 1982), suggesting that the sensitivity of both systems for mutation induction by ionising radiation is similar. Table 1-4 presents a comparison of doubling doses obtained with different systems for the analysis of germline mutations. The results of these studies generated reasonably similar estimates of the doubling dose.

Table 1-4 Estimates of spontaneous mutation rates and doubling dose for acute irradiation of spermatogonia in mice.

System	Spontaneous mutation rate	Doubling dose, Gy‡	Exposure, Gy	Total tested*	Reference
SLT	7.95×10^{-6}	0.34 (0.22, 0.50)	3,6,6.7	1,051,869	(Luning and Searle, 1971; Russell *et al.*, 1982)
Dominant visibles	8.11×10^{-6}	0.17 (0.00, 0.59)	6, 12	225,017	(Luning and Searle, 1971)
Dominant cataract	7.38×10^{-7}	0.56 (-0.14, 3.75)	1.5-6	107,369	(Favor, 1989)
Enzyme activity	2.85×10^{-6}	0.44 (-0.09, 2.68)	6	36,422	(Pretsch *et al.*, 1994)
Skeletal malformation	2.88×10^{-4}	0.27 (-0.07, 1.67)	6	2,493	(Luning and Searle, 1971)
Semisterility	1.04×10^{-3}	0.31 (0.03, 0.95)	12	2,124	(Luning and Searle, 1971)
ESTR loci	5.56×10^{-2}	0.33 (0.06, 0.75)	0.5, 1	252	(Dubrova *et al.*, 1998a)
Mean for 7 systems	–	0.35 (0.20, 0.95)	–	–	

‡The lower and upper 95% confidence limits computed from the Poisson distribution are given in parentheses
*Including offspring from the control and irradiated parents.

The results of these studies highlight important advantages of ESTR loci for monitoring radiation-induced mutation in the mouse germline. First of all, the very high spontaneous and radiation-induced mutation rates at these loci allow mutation detection in very small samples (Table 1-4). Secondly, the increases in ESTR mutation rates can be detected after exposure to much lower doses of radiation than those by traditional approaches for mutation detection in mice (Table 1-4).

The main shortcoming of the system for mutation detection by ESTR loci is that the mechanisms of spontaneous and radiation-induced mutation at these loci still remain unknown. Nevertheless, the data obtained from radiation studies support the hypothesis that ESTR loci themselves are not the direct target of irradiation (Dubrova *et al.*, 1998a; Fan *et al.*, 1995). Thus the analysis of germline mutation induction at ESTR loci revealed a 4-fold increase in mutation rate after exposure of spermatogonia and stem cells to 1 Gy X-ray (Dubrova *et al.*, 1998a). It is known that exposure to 1 Gy of irradiation produces no more than 70 DSBs, 1,000 SSBs and 2,000 damaged bases in the eukaryotic cells (Frankenberg-Schwager, 1990; Ward, 1990). Therefore, assuming that ESTR are random targets, 45,000 extra points of damage per haploid genome would be necessary to generate the 4-fold increase in ESTR mutation rate observed (Dubrova *et al.*, 1998a).

The results of two recent transgenerational studies suggest the possibility that the mechanisms of germline mutation at ESTR loci may be even more complicated. They show that ESTR mutation rates in the germline of first- and second-generation non-exposed offspring of irradiated male mice are remarkably elevated (Barber *et al.*, 2002; Dubrova *et al.*, 2000b). The data demonstrate the existence of transgenerational radiation-induced genomic instability in the germline of offspring of irradiated parents and rule out the possibility that this phenomenon may be attributed to radiation-induced mutation at any specific set of genes in the exposed males (Barber *et al.*, 2002). From these results, the authors conclude that the generation of genomic instability is attributed to an epigenetic mechanism.

It may be assumed that elevated ESTR mutation rate detected in pre-meiotic stages of spermatogenesis cannot be explained by mutation induction in the diploid spermatogonia and stem cells, but instead could result from DNA damage accumulated on these stages, subsequently leading to activation of some processes during meiosis.

Alternatively, mutation induction at these loci could also reflect recombination-like events occurring in diploid germ cells (Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b). Elevated frequency of somatic recombination after exposure to radiation or chemicals has been reported in yeast (Galli and Schiestl, 1999), *Drosophila* (Frei and Wurgler, 1996; Rodriquez-Arnaiz *et al.*, 1996; Vogel, 1992) and human fibroblasts (Li *et al.*, 1997). Similarly, induction of recombination has been observed in germ cells, since it is found to occur in *Xenopus laevis* oocytes in response to UV and X-rays (Hays *et al.*, 1990; Hellgren, 1992; Sweigert and Carroll, 1990).

From these data, it was hypothesised that the recombination-like process involved in the increased mutation rate at ESTR loci could be attributed to genome-wide increase in meiotic crossing-over. However, no correlation was found between ESTR mutation rate and frequency of crossing-over in unexposed mice and those exposed to X-rays or cisplatin (Barber *et al.*, 2000). Cisplatin was the first and only chemical agent analysed using ESTR loci prior to this project. This analysis revealed, in accord with the SLT (Russell *et al.*, 1990) and the DLT (Katoh *et al.*, 1990), that cisplatin does not induce increases in germline mutation rate at any stage of spermatogenesis (Barber *et al.*, 2000).

The mechanism of mutation at ESTR loci has also been hypothesised to be a by-product of DNA repair activity in response to, most probably, DNA DSB. DNA repair proteins are not only involved in the recognition of the damage introduced by the mutagenic agent; some of its components and structurally related proteins are also or exclusively involved in recombinational and meiotic processes (Baker *et al.*, 1995; Baker *et al.*, 1996; Hollingsworth *et al.*, 1995; Ross-Macdonald and Roeder, 1994). In addition, DNA repair knock-out mice have revealed microsatellite instability which could be linked to instability at ESTR loci (Baker *et al.*, 1995; Baker *et al.*, 1996; Umar *et al.*, 1994).

The lack of active DNA repair in post-meiotic stages of spermatogenesis (see 1.2.2) could explain the lack of induction at ESTR loci, observed by Dubrova *et al.* at this stage (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova and Plumb, 2002). However, the reactivation of DNA repair after fertilisation (the stage of pro-nuclei, see 1.2.2), could potentially explain the data showing mutation induction in post-meiotic germ cells and at ESTR alleles derived from non-irradiated mothers (Fan *et al.*, 1995;

Niwa *et al.*, 1996; Niwa and Kominami, 2001; Sadamoto *et al.*, 1994). Given the fact that the activation of DNA-repair pathways after exposure to ionising radiation and other mutagens could affect the stability of ESTR loci, the next section covers the main DNA repair pathways in eukaryotes.

1.7 DNA Repair pathways

Alterations affecting the structure and integrity of DNA molecules can arise spontaneously through intrinsic DNA damage (e.g. deamination, depurination, etc.), DNA replication errors and after exposure to environmental agents such as chemical mutagens and ionising radiation. If these changes are left uncorrected, cells could accumulate enough mutations to become functionally unviable. To prevent harmful consequences of DNA damage, cells have developed a complex set of DNA repair mechanisms.

1.7.1 Mismatch repair

The mismatch repair (MMR) pathway repairs mismatched bases and short insertion/deletion loops (IDLs) which can arise from several processes, the most important of them being replication errors. However, these type of errors can also arise from the formation of a heteroduplex between two homeologous DNA sequences during recombination, hairpins from imperfect palindromes or deamination of 5-methylcytosine (Friedberg *et al.*, 1995).

The conceptual problem of the mismatch repair system is how to distinguish the correct and incorrect bases in the mismatch; this cannot be achieved by enzymatic scanning of the structure of DNA as both bases are normal components of DNA.

The best-known system to distinguish between parental and daughter strands of a newly divided cell is that of *E. coli*. The strands are distinguished by the amount of methylated bases (Figure 1-14). The recognition is based on the lack of methylation of a newly-synthesised strand immediately after replication (Wagner and Meselson, 1976). The mechanism for distinguishing parental and daughter strands in eukaryotes must be different from prokaryotes since some eukaryote cells do not methylate DNA. Newly synthesised DNA strands are known to be preferentially nicked after replication. It is therefore suggested that these nicks are the signals recognised by the eukaryote

mismatch repair to discriminate between parental template and daughter strand (Alberts, 2002).

In *E. coli,* the mismatch repair process starts with the homodimer of the protein MutS, which detects mismatched bases and small deletions/insertions that escaped proof-reading by the replicating polymerase (Modrich, 1991; Modrich and Lahue, 1996) (Figure 1-14). The discrimination between the correct and incorrect base is carried out by MutH, which specifically binds to the methylated sequences and distinguishes between parental and daughter strands. Binding of MutS to the mismatched base triggers binding of a homodimer of MutL, which creates a link between MutS and MutH, activating the endonuclease activity of MutH (Au *et al.*, 1992; Ban and Yang, 1998a; Ban and Yang, 1998b; Hall and Matson, 1999). Active MutH cleaves the phosphodiester bond between two nucleotides in the daughter strand. MutL is recognised by MutU which binds at the site of the nick. MutU is a helicase which facilitates the excision of a segment of DNA from the daughter strand, containing the mispaired base, by DNA-specific exonucleases, RecJ, ExoVII, ExoI, or ExoX (Modrich and Lahue, 1996; Viswanathan and Lovett, 1998). Finally DNA polymerase III holoenzyme resynthesises the excised fragment, and DNA ligase seals the gap.

Several MutS and MutL homologs exist in yeast and mammals (Table 1-5). Msh1 in *S. cerevisiae* is essential for correct mitochondrial function and protects against base substitutions (Chi and Kolodner, 1994; Reenan and Kolodner, 1992). Msh4 and Msh5 are meiosis-specific and have a role in recombination and chromosome segregation but are not involved in mismatch repair (Hollingsworth *et al.*, 1995; Ross-Macdonald and Roeder, 1994). Msh2, Msh3 and Msh6 form two heterodimers, MutSα (Msh2/Msh6) and MutSβ (Msh2/Msh3), which recognise mispairs (Acharya *et al.*, 1996; Drummond *et al.*, 1995; Habraken *et al.*, 1996; Hughes and Jiricny, 1992; Marsischky *et al.*, 1996) and IDLs (Genschel *et al.*, 1998; Jiricny, 1998; Kolodner and Marsischky, 1999; Marra *et al.*, 1998). Similarly, multiple MutL homologs have been found (Table 1-5). Mlh2, only identified in *S. cerevisiae*, is not involved in DNA repair. Mlh1, Pms2 (Pms1 in yeast) and Pms1 (Mlh3 in yeast) interact to form three heterodimers; MutLα (Mlh1/Pms2) has the major MutL activity (Harfe and Jinks-Robertson, 2000) while MutLβ (Mlh1/Pms1) and Mlh1/Mlh3 play minor roles in mismatch repair (Kato *et al.*, 1998; Raschle *et al.*, 1999).

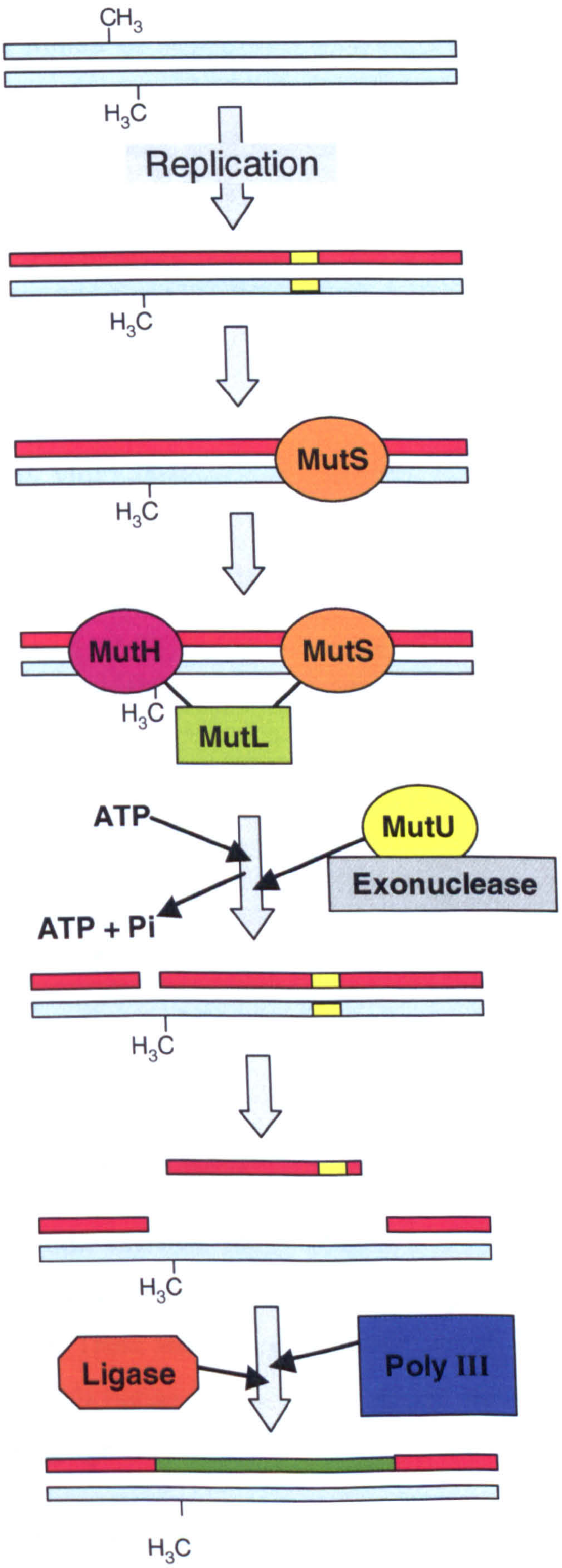


Figure 1-14 ***E. coli*** **mismatch repair.** Parental methylated strands are represented in blue, daughter non-methylated strands are represented in red. Mispair/IDL induced after replication represented in yellow rectangles.

In eukaryote cells, the mismatch repair process is initiated by the binding of the bacterial MutS homologs MutSα or MutSβ to mismatched base pairs or IDLs. The process follows with the recruitment of a MutL complex by MutSα or MutSβ. The nuclease, equivalent to MutH in *E. coli*, that generates the nick in the daughter strand is not known. Following the cleavage at 3' or 5' to the mismatch, an exonuclease removes a fragment of 100 to 200 nucleotides containing the mispaired base. The generated gap is filled by DNA polymerase δ and sealed with a ligase.

Table 1-5 *E. coli* Mut homologs in *S. cerevisiae* and *H. sapiens*.

E. coli	*S. cerevisiae*	*H. sapiens*
MutS	Msh1	Not identified
	Msh2, Msh3, Msh4, Msh5, Msh6	Msh2, Msh3, Msh4, Msh5, Msh6
MutL	Mlh1	Mlh1
	Mlh2, Mlh3	Pms1
	Pms1	Pms2
MutH	Not identified	Not identified
MutU	Not identified	Not identified

1.7.2 Nucleotide excision repair

The DNA nucleotide excision repair (NER) is the system by which cells enzymatically remove a region of DNA containing chemically modified bases, also known as chemical adducts. These adducts can be modifications such as methylations or ethylations introduced by alkylating agents or interstrand and intrastrand cross-links introduced by cross-linking agents.

These adducts locally alter the normal conformation of DNA. The key to this system is the capacity of some enzymes to detect these alterations on the structure of DNA.

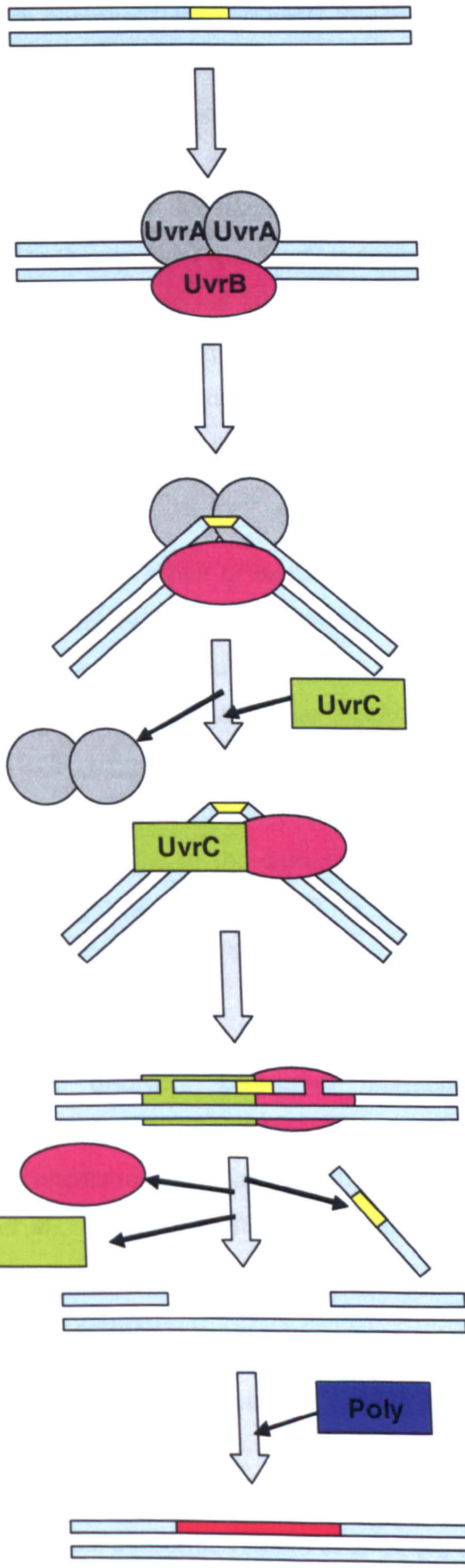


Figure 1-15 ***E.coli*** **nucleotide excision repair.**

In *E. coli* the protein complex $(UvrA)_2(UvrB)_1$ binds to undamaged DNA and slides along the surface of the DNA double helix until it finds an adduct (Figure 1-15). At this stage an ATP-dependent change in conformation occurs generating a bend in the DNA at the position of the damaged base. Then the two subunits of UvrA dissociate from the complex, leaving UvrB on the damaged site. After the dissociation, an endonuclease, UvrC, binds to the damaged site. UvrC induces a conformational change which enables UvrB to cleave the damaged strand at a position 3' from the lesion. Following this incision, UvrC catalyzes nicking of the damaged DNA strand at a position 5' from the lesion. Following the incisions, the nicked section of DNA, containing the adduct, is removed by helicases and the gap left is resynthesised (Friedberg *et al.*, 1995).

It has been observed that the repair of damaged bases in DNA is preferentially repaired when located in an actively transcribed gene. The protein called transcription-repair coupling factor (TRCF) is considered responsible for the preference of NER for actively transcribed genes. Damaged DNA causes RNA polymerase to stall at the position of the damage. TRCF recognises and interacts with the ternary complex formed by stalled RNA polymerase-mRNA-damaged DNA. This interaction displaces the complex leaving TRCF alone, bound to the damaged DNA. TRCF has shown high affinity for UvrA, suggesting that TRCF bound to damaged DNA could recruit $(UvrA)_2(UvrB)_1$ starting the NER process (Friedberg *et al.*, 1995).

The importance of NER for human health is illustrated by the occurrence of a rare autosomal recessive disorder, xeroderma pigmentosum (XP). Patients characteristically show severe photosensitivity and abnormal pigmentation, often accompanied by mental retardation, and they usually develop skin cancer at a very young age (Bootsma *et al.*, 1998). Cells from these patients have a defect in NER. Complementation studies have revealed that eight genes are involved in XP: XPA through XPG and XPV (XP-Variant).

Similarly to the prokaryote NER, mammalian NER has two repair branches, the global genome repair (GGR) (Figure 1-16 a) which repairs DNA lesions across the genome and transcription coupled repair (TCR) (Figure 1-16 b) which specifically removes DNA lesions from the transcribed strand of an active gene.

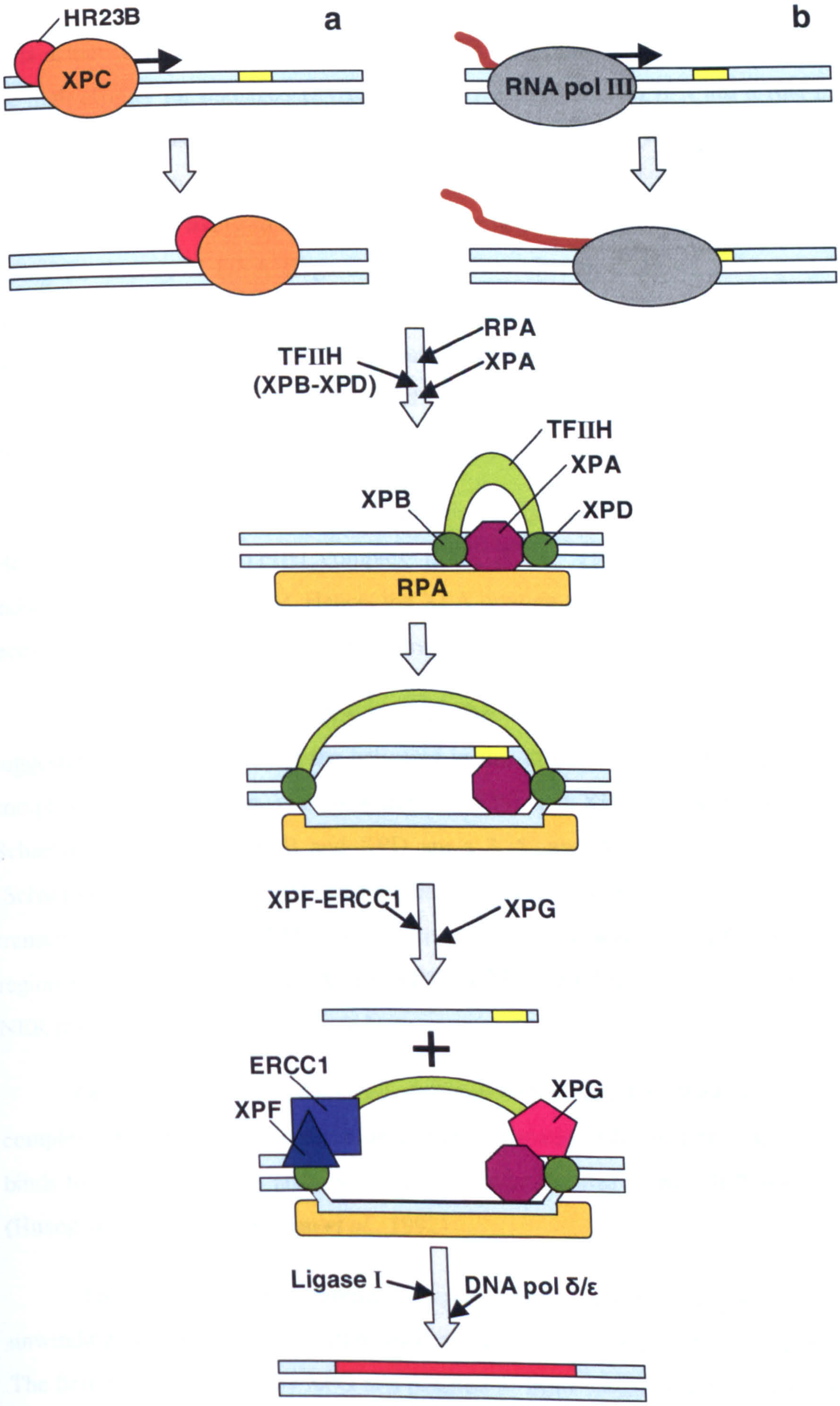


Figure 1-16 Mammalian nucleotide excision repair. a, global genome repair; b, transcription coupled repair.

The initial step of the mammalian NER is the recognition of the damage. This step is achieved with the proteins XPA and XPC. The protein XPE has been found to have high affinity for damaged DNA; however, it is still unclear if it has a role in the recognition of the damage. XPA binds to replication protein A (RPA) which enhances the affinity of XPA for damaged DNA; XPC forms a complex with HR23B. The process of repair is likely to start with the binding of XPC-HR23B to a DNA lesion causing unwinding of DNA (Figure 1-16 a). This alteration allows XPA protein to access the damaged site. The complex XPC-HR23B is not required for TCR, as in the transcriptional process the DNA is unwound because of a stalled RNA polymerase II allowing binding of XPA to the damaged base without assistance from XPC-HR23B (Figure 1-16 b).

XPA interacts with other NER components, such as replication protein A (RPA) (He *et al.*, 1995), the TFIIH complex (Park *et al.*, 1995) and the XPF-ERCC1 endonuclease (Li *et al.*, 1994). Hence, the XPA binding to the damaged site may imply recruitment of the entire NER protein pathway.

The opening of the DNA around the lesion is ATP-dependent, strongly suggesting the involvement of the helicases in the TFIIH complex. TFIIH is a protein complex of nine subunits, two of which are XPB and XPD (Schaeffer *et al.*, 1993; Schaeffer *et al.*, 1994). XPB and XPD are a 3'-5' and 5'-3' helicases, respectively (Schaeffer *et al.*, 1994). TFIIH has a similar role in transcription initiation and NER. In transcription initiation, TFIIH unwinds the promoter site while in NER it unwinds the region recognised by XPA-RPA and XPC-HR23B, enabling the accumulation of the NER machinery around the damaged site.

Opening of the DNA damaged area may be facilitated by RPA, a heterotrimeric complex involved in NER, replication and recombination (Wold, 1997). RPA optimally binds to a 30 nucleotides area, which is similar to the size of the NER open complex (Huang and Sancar, 1994; Kim *et al.*, 1992).

The incision of the damaged strand occurs after its recognition and DNA unwinding. This process is accomplished by the endonucleases XPG and XPF-ERCC1. The first incision is made by XPG at a position 3' from the damaged site (O'Donovan *et al.*, 1994), followed by a 5'-incision by XPF-ERCC1 (Sijbers *et al.*, 1996). The specific

endonuclease activity of XPG and XPF-ERCC1 on the damaged strand is provided by RPA, which binds with defined polarity to the undamaged DNA strand (de Laat *et al.*, 1998). These two incisions release a 24-32 nucleotides long fragment containing the damaged base. The final step of the NER repair is the resynthesis of the created gap by polymerase δ and ε (Shivji *et al.*, 1995), followed by the ligation of the newly synthesised DNA strand by, most probably, ligase I.

1.7.3 Homologous recombination

DNA DSBs can be repaired by either the homologous recombination (HR) or the non-homologous end-joining (NHEJ) (see 1.7.4) pathways. In *S. cerevisiae*, screens for mutants sensitive to ionising radiation have produced many mutant alleles of genes involved in HR (Friedberg *et al.*, 1995). In contrast, equivalent screens in mammalian cells have yielded mutant alleles for NHEJ but not HR genes. Thus, when DNA chromosomal structures allow DSBs to be repaired by either HR or NHEJ, *S. cerevisiae* preferentially uses HR, while mammalian cells preferentially repair DSBs by NHEJ (Godwin *et al.*, 1994; Jeong-Yu and Carroll, 1992; Lukacsovich *et al.*, 1994; Sargent *et al.*, 1997). DNA DSBs can be introduced by ionising radiation, radiomimetic chemicals such as bleomycin; and blocking agents such as etoposide.

There are various models of HR based on analysis in different organisms under various biological circumstances (Cromie *et al.*, 2001) (Figure 1-17). The initial step of HR is the generation of a 3'-ended single-stranded DNA by nucleolytic resection of the DSB by a complex containing Rad50p, Mre11p and Xrs2p (NBS1 in humans). The resulting 3'-ends are then bound to Rad51p in a process influenced by RPA, Rad52p, Rad54p and others proteins. Rad51p can interact with a homologous sequence located in a sister chromatid, homologous chromosome or at an ectopic location, catalysing strand-exchange events, in which the damaged strand invades the homologous sequence, displacing one strand as a D-loop. This event is also influenced by members of the RAD50 group of proteins, RPA and other factors (Petukhova *et al.*, 1998).

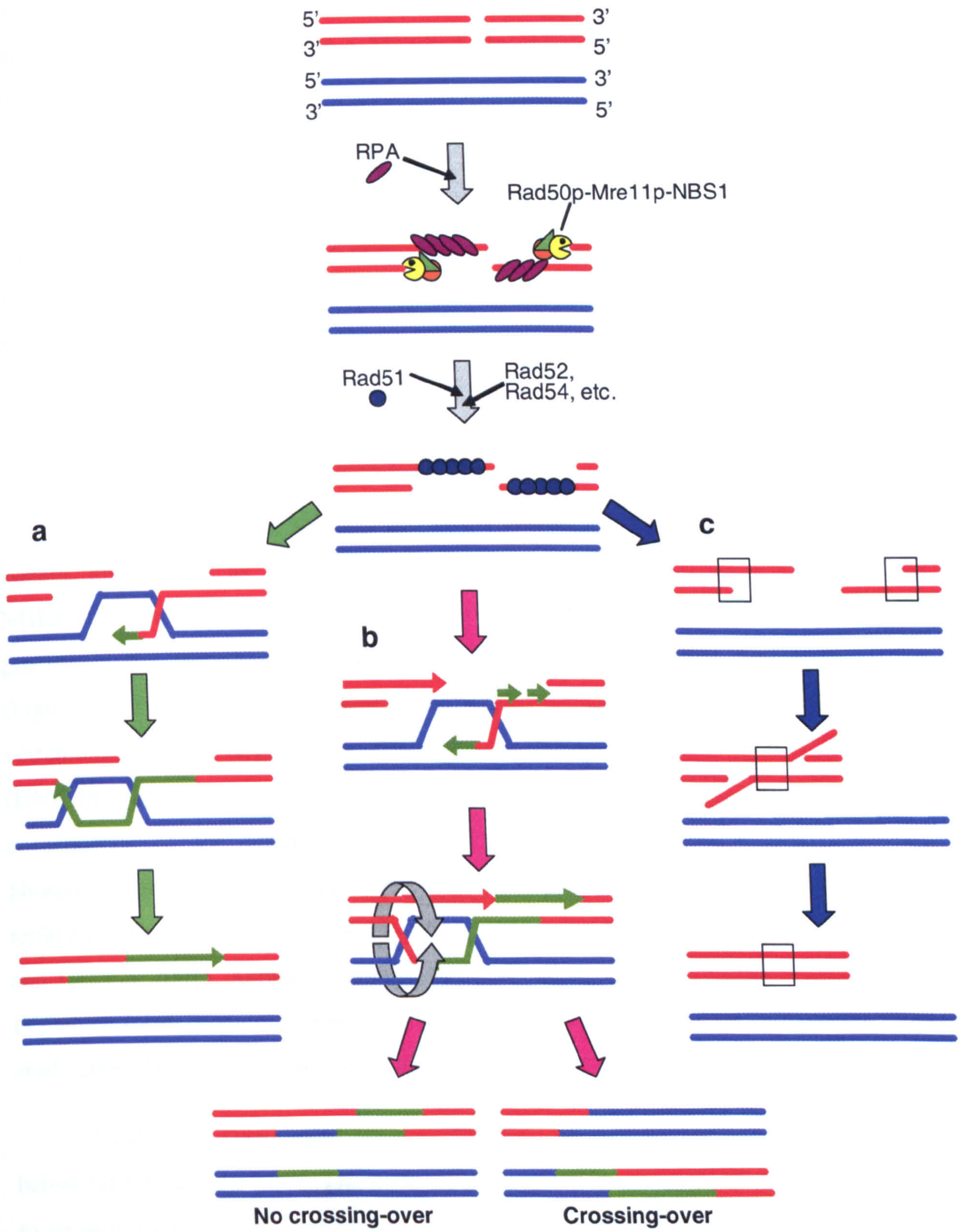


Figure 1-17 Homologous recombination repair. a, single strand invasion; b, double strand invasion; c, single strand annealing (homologous sequences shown as black boxes).

If both 3'-ends of the damaged DNA molecules are extended copying the information from the undamaged homologous sequence and then are subsequently ligated, the result is creation of a Holliday junction (Figure 1-17 b). This Holliday junction is then resolved, with or without crossing-over, by cleavage and ligation, resulting in two undamaged DNA molecules (Haber, 2000; Hoeijmakers, 2001).

HR can also occur by the pathway of single-strand annealing (Figure 1-17 c). This is a simpler process that can occur when direct repeats flank the 3'-ended single-stranded DNA originated from the DSB. The homologous region can be as small as 30 bp. This system resolves DSBs by annealing these single-strand regions of homology losing the genetic material between repeats.

1.7.4 Non-homologous end-joining

Similar to the HR system, the non-homologous end-joining (NHEJ) repair system, also known as illegitimate recombination repair, resolves DSBs; however, NHEJ does not require homology between the DNA ends. The initial step of this process is the recognition of the DSB by protein Ku (Dynan and Yoo, 1998) (Figure 1-18). Protein Ku is a heterodimer of two subunits called Ku70 and Ku80. In vertebrates, Ku is targeted by DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme (Smith and Jackson, 1999). As a consequence of the binding to the DSB, DNA-PK is activated producing the phosphorylation of several substrates including XRCC4 (Leber *et al.*, 1998) and replication factor A2 (RFA2) (Wang *et al.*, 2001) facilitating NHEJ repair (Durocher and Jackson, 2001). Another factor thought to be associated with NHEJ is DNA ligase IV, which forms a complex with XRCC4 and physically religates the DNA ends (Chen *et al.*, 2000; McElhinny *et al.*, 2000; Ramsden and Gellert, 1998).

Most DNA DSBs need some processing, which generally introduce deletions, before NHEJ can take place. The mammalian complex Rad50-Mre11-NBS1 is believed to be responsible for the nucleolytic processes necessary for the repair of DSBs. This complex has been shown to localize to sites of DNA DSBs (Maser *et al.*, 1997; Nelms *et al.*, 1998) and has endonuclease, exonuclease and unwinding activities (Paull and Gellert, 1999; Trujillo *et al.*, 1998). Other proteins like FEN-1 (Wu *et al.*, 1999) and

Artemis (Moshous *et al.*, 2001) have also been related to the processing of DSBs prior repair by NHEJ.

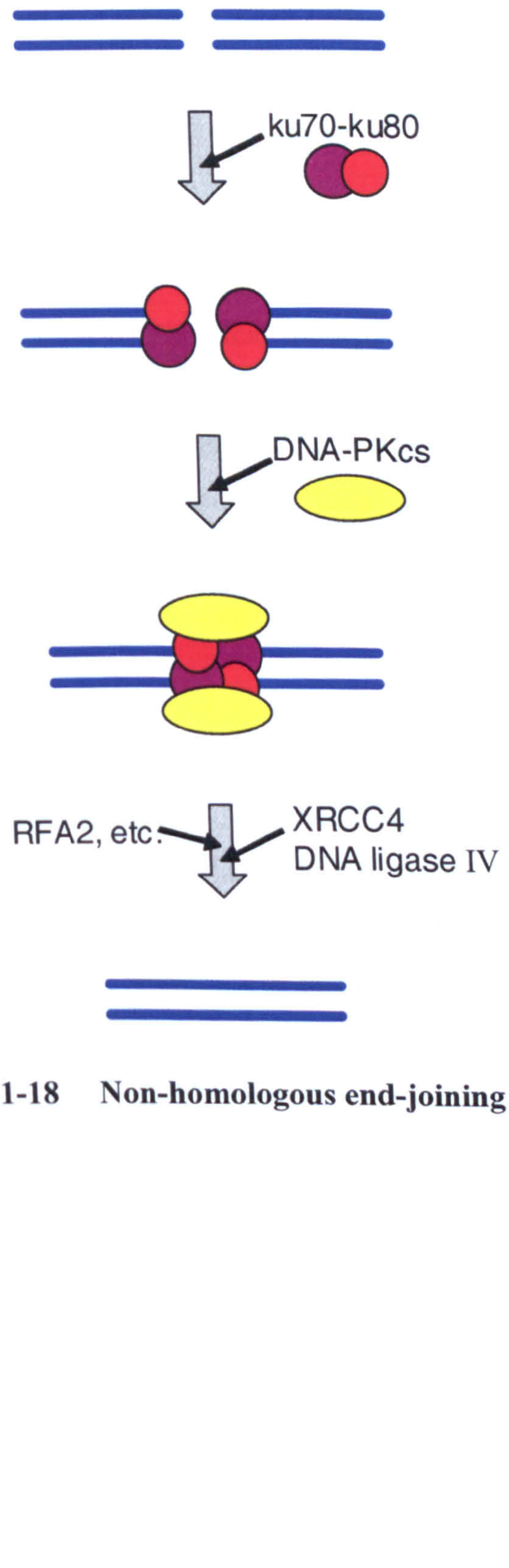


Figure 1-18 Non-homologous end-joining repair.

1.8 Conclusions

Chemical agents are highly prevalent in our society and they present a serious risk factor to human health. As stated in section 1.1, human exposure of chemical mutagens, especially to low doses, occurs frequently. The mutational effects of these exposures have to be analysed, particularly those on the germline since the damage could be transmitted through generations. To date, the best source of experimental data to evaluate the risk of chemical mutagens in germ cells is based on mouse models (see 1.3). However, these systems are not sensitive enough for the analysis of low doses of exposure, to which populations are more likely exposed.

The results of studies on mutation induction at ESTR loci in the germline of male mice exposed to ionising radiation suggest that this system may also be useful for the analysis of germline mutation by chemical mutagens. However, to date there has been little knowledge on whether exposure to chemical mutagens could affect ESTR mutation rates in the germline of treated parents. The only study specifically designed to address this issue failed to detect ESTR mutation induction in male mice exposed to a cross-linking agent cisplatin (Barber *et al.*, 2000). Further studies on mutation induction at mouse ESTR loci by chemical mutagens are therefore warranted.

The main goal of my work is to investigate the suitability of ESTR loci for the monitoring of germline mutation induction by chemical mutagens, including some well-known chemical mutagens and anticancer drugs and to gain insight into mechanisms of spontaneous and induced germline instability at ESTR loci. Furthermore, it is intended to analyse whether exposure to chemical mutagens can affect the expression of genes involved in DNA repair, apoptosis or cell cycle checkpoints and other essential cellular functions in the germline of treated male mice. Since the mutational process at ESTR loci is not known, the analysis of the changes in gene expression in the male mouse germline, might reveal relevant genes involved in the process and therefore shed light on the mechanisms of mutagenesis at ESTR loci.

It is hoped that this survey will enable me to establish whether exposure to chemicals could enhance ESTR mutation rate and to determine whether ESTR loci could be used as a bio-monitoring system for assessing chemical mutagenicity in the mammalian germline.

2 Materials and methods

MATERIALS

2.1 Chemical reagents

Chemicals were obtained from Amersham Biosciences (Little Chalfont, UK), Fisher Scientific (Manchester, UK), Fisons (Loughborough, UK), Flowgen (Ashby de la Zouch, UK), FMC Bioproducts (Rockland, USA), and Sigma Biochemical Company (Poole, UK). Molecular biology reagents were obtained from ABGene (Epsom, UK), Ambion, Inc. (Austin, USA), Amersham Biosciences (Little Chalfont, UK), Bio-Rad (Hemel Hemstead, UK), Invitrogen UK (Paisley, UK), Millipore (Watford, UK), NEN Life Sciences (Hounslow, UK), New England Biolabs (Hitchin, UK), PE Biosystems (Division of Perkin-Elmer, Warrington, UK), Qiagen Ltd. (Crawley, UK), Sigma Biochemical Company (Poole, UK), and Stratagene (Amsterdam, The Netherlands). Specialised equipment was obtained from Axon Instruments (California, USA), Bio-Rad (Hemel Hempstead, UK), Cecil Instruments (Cambridge, UK), Fisher Scientific (Manchester, UK), Genetic Research Instrumentation (Braintree, UK), Helena Biosciences (Sunderland, UK), Heraeus Instruments (Hanau, Germany), Hybaid (Teddington, UK), MJ Research (Waltham, USA), PE Biosystems (Division of Perkin-Elmer, Warrington, UK), Shandon Southern (Runcorn, UK), and UVP Life Sciences (Cambridge, UK).

2.2 Enzymes

Restriction enzymes and React™ buffers were supplied by Invitrogen. A cloned version of the Klenow fragment of DNA polymerase I of *E. coli* was supplied by Amersham Biosciences (produced by USB, USA). *Taq* DNA polymerase was obtained from ABGene. Superscript II reverse transcriptase was supplied by Gibco/BRL. Proteinase K was supplied by Sigma-Aldrich.

2.3 Molecular weight markers

1 kb DNA ladder was supplied by Invitrogen.

0.24-9.5 kb RNA ladder was supplied by Gibco/BRL

2.4 Oligonucleotides

Oligonucleotides for PCR amplification were synthesised in-house (Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, UK). Hexadeoxyribonucleotides or Pd6 random hexamers were obtained from Amersham Biosciences.

2.5 Mice

7-8 week old C57BL/6J and CBA/Ca inbred mice were purchased from Harlan-Olac Ltd. (Bicester, UK) and housed in the Biomedical Services of the Centre for Mechanisms of Human Toxicity at Leicester University.

2.6 Mutational agents

Ethyl nitrosourea (ENU) (CAS No. 759-73-9) and etoposide (ET) (CAS No. 9041-93-4) were supplied by Sigma-Aldrich, isopropyl methanesulfonate (iPMS) (CAS No. 926-06-7) was supplied by Fisher Scientific.

2.7 Standard solutions

Southern blot solutions (depurinating solution, denaturing solution, and neutralising solution), 20xSodium Chloride-Sodium Citrate (SSC) buffer and 10xTris-borate/EDTA (TBE) electrophoresis buffer were supplied by the media kitchen, Department of Genetics, University of Leicester. 11.1xPCR buffer was supplied by R. Neumann.

2.8 Computers

This thesis was produced using a Pentium III PC and a UMAX Vista-S6E scanner. It was printed on an HP4000 6MP LaserJet printer. Data were stored, analysed, and presented using the software packages Adobe Acrobat, Adobe Photoshop, Clarisdraw, EndNote, Microsoft Word, Microsoft Excel, Microsoft Access, Microsoft PowerPoint, Statistica, Systat and software written in Microsoft Basic by Y. E. Dubrova. Internet searches were performed using Microsoft Internet Explorer, and Netscape Navigator.

METHODS

2.9 Mice

2.9.1 Housing

Mice were housed at 22°C and 50% humidity in plastic isolators in the Biomedical Services of Centre for Mechanisms of Human Toxicity at Leicester University. The mouse colony was maintained under a 12 hr light/12 hr dark cycle. Food and water was provided ad libitum.

2.9.2 Identification

Mice were individually identified by ear punch.

2.9.3 Procedures

2.9.3.1 Mutation analysis

CBA/Ca male mice, 7-8 weeks old, were given a single intraperitoneal injection of the chemical dissolved in phosphate buffered saline (PBS) for ENU and iPMS, or in DMSO for ET. Solutions were prepared as concentrations that allowed injection of the dose required using 0.2 ml when dissolved in PBS and 0.1 ml when dissolved in DMSO for a 25 g mouse. Each dosed mice was mated for four days with two or four C57BL/6J female mice at different time points after the administration of the drug to analyse mutation induction at different stages of spermatogenesis (Figure 1-5).

All animals were culled by a schedule 1 method. Males were culled when mating schedules were completed. Offspring were culled approximately at two weeks of age; female mice were culled as the same time as their offspring. Tail and liver samples were taken for genetic analyses.

2.9.3.2 Microarrays

CBA/Ca male mice, 7-8 weeks old, were given a single intraperitoneal injection of ENU at a dose of 50 mg/kg dissolved in PBS. Mice were culled either 6 or 24 hr after exposure by a schedule 1 method. Testes were extracted, placed in a 2 ml cryovial tube, immediately frozen in liquid nitrogen and stored at -80°C.

2.9.4 DNA preparation

Approximately half of the tissue was finely chopped with a scalpel and suspended with 1 ml of Lysis Buffer A (0.1 M NaCl, 25 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0), 1ml of Lysis Buffer B (1% SDS, 12.5 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0) and 30 µl of Proteinase K (25 mg/ml) in a 15 ml Eppendorf Phase Lock Gel™ Light tube. The contents were mixed by inversion and incubated at 55°C for 5 hr to overnight with occasional mixing. DNA was separated from proteins and bone by one or two phenol/chloroform and one chloroform extractions (see 2.10.1). DNA was precipitated from the aqueous layer by the addition of 100% ethanol with 10% 3 M NaAc. DNA was transferred to a 1.5 ml eppendorf tube, rinsed with 80% ethanol and suspended in an appropriate amount of distilled water (see 2.10.2).

2.9.5 RNA preparation

One testis was disrupted on ice using Tri Reagent Buffer (Sigma-Aldrich, UK) (see 2.15.1). The lysate was homogenised and the phases separated by centrifugation. RNA was precipitated from the upper colourless phase in 1 volume of isopropanol, washed in 70% ethanol and dissolved in RNAse-free water (see 2.15.2). A further purification with Tri Reagent Buffer was then performed followed by precipitation of RNA in 100% ethanol with 10% 3 M NaAc. RNA was air-dried and dissolved in an appropriate amount of RNAse-free water (see 2.15.3).

2.10 General methods for handling DNA

2.10.1 Phenol/Chloroform extraction

Phenol/chloroform and chloroform were used to purify DNA from proteins after an initial digestion with Proteinase K. 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 mixture was emulsified by repeated inversion. To simplify the separation of the different phases, Phase Lock Gel™ Light tubes were used. Phase Lock Gel™ Light tubes physically separate the upper aqueous phase containing DNA from the phenol and proteinaceous interface. Additional phenol/chloroform extractions were occasionally needed to obtain a clear aqueous layer. A final extraction with chloroform was used to remove traces of phenol. The DNA was then precipitated in ethanol.

2.10.2 Ethanol precipitation

In order to recover DNA following manipulation, ethanol precipitation was used. The aqueous layer was transferred by decantation into a 15 ml polypropylene tube containing two volumes of 100% ethanol and 1/10 volume of 3 M NaAc (pH 5.5). The solution was gently mixed by inversion to precipitate the DNA, which was removed into a 1.5 ml eppendorf containing 80% ethanol. DNA was pelleted by centrifugation in a bench top microcentrifuge for 1 min at 13,000 rpm. The ethanol was removed with a pipette and the pellet left to air dry, and finally dissolved in the required amount of distilled water.

2.10.3 Restriction endonuclease digestion

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

2.10.4 Evaluation of DNA concentration

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

2.10.5 Electrophoresis

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

2.10.6 Southern blotting

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

2.11 Synthesis of synthetic repeat probes

ESTR probes were synthesised by PCR (see 2.14.2) using the pair of primers MMS10F and MMS10R for MMS10, HMA and HMB for Ms6-hm, and HM2FOR and HM2REV for Hm-2.

ESTR	Primers	Sequence
MMS10	MMS10F	$^{5'}$GGCAGAGGCAGAGGCAGA$^{3'}$
	MMS10R	$^{5'}$TCTGCCTCTGCCTCTGCC$^{3'}$
Ms6-hm	HMA	$^{5'}$GGGCAGGGCAGGGCAGGGCA$^{3'}$
	HMB	$^{5'}$CCCTGCCCTGCCCTGCCCTG$^{3'}$
Hm-2	HM2FOR	$^{5'}$GGCAGGCAGGCAGGCAGGCA$^{3'}$
	HM2REV	$^{5'}$TGCCTGCCTGCCTGCC$^{3'}$

2.11.1 Purification of probes

PCR products were purified using QIAquick PCR Purification Kits (Qiagen) as recommended by the manufacturer.

2.12 DNA hybridisation

2.12.1 Random oligonucleotide labelling of DNA fragments

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

2.12.2 Hybridisation

Membranes were pre-hybridised for at least 15 min at 65°C in 7% SDS, 0.5 M Na_2PO_4 pH 7.2, 1 mM EDTA (modified from Church and Gilbert, 1984). Hybridisation was carried out at 65°C for 5-24 hr in a Maxi 14, or Mini 10 hybridisation oven (Hybaid).

2.12.3 Post-hybridisation washing

After hybridisation, the membrane was washed at 65°C once in phosphate wash solution (40 mM Na_2HPO_4, 0.5% SDS) for 10 min and in 2-5 changes of high stringency wash solution (0.1xSSC, 0.01% SDS), for 10 min each.

2.12.4 Autoradiography

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

2.12.5 Stripping filters for rehybridisation

Nylon filters required for hybridisation with a different probe were stripped by immersing in boiling 0.1% SDS, and shaken for 15 min.

2.13 Statistical Analysis

Most statistical procedures are described by Sokal and Rohlf (1995). The 95% confidence limits for mutation rate were derived from the Poisson distribution (Sachs, 1982). The doubling dose, D was estimated as $D=m_0/ind$, where m_0 is spontaneous mutation rate and *ind* is the rate of mutation induction averaged over the interval of doses for each mutagen (Luning and Searle, 1971). The 95% confidence limits for doubling dose were also derived from the Poisson distribution (Sachs, 1982).

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

2.14 PCR

2.14.1 PCR buffer

11.1xPCR buffer (Jeffreys *et al.*, 1988) was produced in Leicester by R. Neumann with the components in the table below. dNTPs (sodium salt) were supplied by Promega. Ultra pure (non-acetylated) BSA was supplied by Ambion.

Component	Concentration of Stock Solution	Volume (arbitrary units)	Final concentration in PCR reaction
Tris-HCl pH 8.8	2 M	167	45 mM
Ammonium Sulphate	1 M	83	11 mM
$MgCl_2$	1 M	33.5	4.5 mM
2-mercaptoethanol	100%	3.6	6.7 mM
EDTA pH 8.0	10 mM	3.4	4.4 µM
dATP	100 mM	75	1 mM
dCTP	100 mM	75	1 mM
dGTP	100 mM	75	1 mM
dTTP	100 mM	75	1 mM
BSA	10 mg/ml	85	113 µg/ml
Total Volume		676	

2.14.2 Synthesis of ESTRs probes

DNA was amplified using the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988) on a Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Cetus, Connecticut, USA) or a PTC-255 DNA Engine Tetrad Peltier thermal cycler (MJ Research, USA).

PCR reactions for Ms6-hm were performed in 20 µl reactions with 2 µl of 11.1xPCR buffer as described in 2.14.1, plus 1 µM of each primer (see 2.11), and 2.5 U/µl *Taq* polymerase. For Hm-2 and MMS10 the PCR reactions were performed in a total volume of 7 µl containing 0.63 µl of 11.1xPCR buffer, 0.4 µM of each primer (see 2.11) and 0.07 U/µl *Taq* polymerase. Note that no input DNA was required as the repeat specific primers also act as a template, producing various lengths of pure repeat sequence.

2.15 General methods for handling RNA

2.15.1 RNA isolation

RNA was extracted from one whole testis. Each sample was transferred to a 1.5 ml pellet pestle tub (Anachem, UK) and disrupted on ice with 100 µl of Tri Reagent buffer (Sigma-Aldrich, UK) using a pellet pestle® motor kontes gun (Anachem, UK) with a PK/100 disposable pellet pestle (Anachem, UK). The lysate was homogenized with 900 µl of Tri Reagent buffer on ice using the motor gun. The phases were separated by centrifugation in a bench top microcentrifuge at 14,000 rpm for 10 min at 4°C after the addition of 200 µl of chloroform to the lysate.

2.15.2 Isopropanol precipitation and purification

The upper colourless aqueous phase was transferred to a new tube with 1 volume of isopropanol. RNA was precipitated by centrifugation of the samples at 14,000 rpm at 4°C for 10 min. .The pellet was washed twice with 70% ethanol, left to air-dry and dissolved in 100 µl of RNAse-free water. Further purification of RNA was performed adding 1000 µl of Tri Reagent buffer and repeating the procedure of extraction.

2.15.3 Ethanol precipitation

The pellet was then dissolved in 200 µl of RNAse-free water and re-precipitated by spinning at 14,000 rpm at 4°C for 10 min in 0.1 volumes of 3 M NaAc (pH 5.2) and 3 volumes of 100% ethanol. The new RNA pellet was washed in 70% ethanol, air-dried and dissolved in the required amount (6-20µl) of RNAse-free water.

2.15.4 RNA gel electrophoresis

Quality of RNA was assessed by running 2 µg of each sample in a 1.4% non-denaturing agarose gel (wide range/standard 3:1, Sigma) in an 8.3 cm horizontal submarine format with 1xTBE (44.5 mM Tris-borate pH 8.3, 1 mM EDTA) buffer. 3 µl of RNA loading dye/buffer was added to each sample (30% glycerol, 0.2% Bromophenol Blue, 0.2% Xylene Cyanol FF). RNA samples were run alongside 2 µg of RNA ladder (0.24-9.5 kb; Gibco/BRL) at 60 V for approximately 45 min. Gels were stained in 50 ml of TBE buffer containing 50 µg of EtBr for 20 min. Gels were scanned in a Bio-Rad Imager (Hemel Hempstead, UK).

2.15.5 Estimation of RNA concentration

RNA concentration was assayed by the measurement of absorbance at a wavelength of 260 nm in a spectrophotometer (Perkin/Elmer Spectometrer UV/VIS Lambda 2) using 1 µl of RNA sample in 999 µl of DEPC water.

2.16 Production of the microarray

Microarrays were built from 4000+ EST clones obtained from Research Genetics (Human known gene set) (http://www.resgen.com) and GenBank (IMAGE collections held at the MRC Human Gene Mapping Project (http://www.hgmp.mrc.ac.uk/)). These clones were sequence verified prior to arraying. All of the plasmid inserts were amplified by use of a universal primer set and a standard PCR mix (ABGene) employed. All PCR products that failed in initial amplification or produced multiple bands were re-amplified using plasmid specific primers. The products were all electrophoresed on an agarose gel and size of the insert sequence determined by comparison with the ladder using MultiAnalyst software (Bio-Rad). After PCR the reaction products were precipitated and prepared for array using the methods described by Eisen and DeRisi (DeRisi *et al.*, 1997; Eisen and Brown, 1999).

Arrays were printed on poly-L-lysine coated slides and UV cross-linked and blocked prior to use as previously described (DeRisi *et al.*, 1997). The arrays were printed using an arrayer built essentially according to the Stanford designs and can be seen at http://www.le.ac.uk/cmht/microarray_lab/Microarray_Facilities/Microarray_Facilities.htm. The centre to centre distance of the features was 210 μm and each feature was 90-100 μm in diameter.

2.17 cDNA synthesis and labelling

The synthesis and labelling of cDNA was carried out essentially as described by DeRisi (DeRisi *et al.*, 1997) and Eisen (Eisen and Brown, 1999). Priming was achieved using 50 μg of total RNA with 4 μg of oligo $dT_{(25)}$. After denaturation at 70°C for 8 min annealing was allowed to occur as the temperature decreased to 42°C over a period of 30 min in a PTC-255 Peltier Tetrad thermal cycler (MJ Research, USA). At this point dNTPs (Pharmacia, USA) were added to final concentrations of 0.5 mM with the exception of dTTP which was at 0.2 mM. Cy3 or Cy5 labelled dUTP (Amersham Bioscience, UK) was added to a final concentration of 0.1 mM. 1x first strand buffer (Gibco/BRL) and 20 U of RNAsin were added to the reaction. Reverse transcription was initiated by the addition of 100 U of Superscript II (Gibco/BRL) and allowed to proceed for 2 h at 42°C. 100 U of Superscript II were added 1 h after the initiation of the reaction. RNA was removed from the synthesized cDNA by hydrolysis by addition of 0.195 M NaOH, 10 mM EDTA and 0.22% SDS to the solution and incubation at 70°C for 10 min. The reaction was neutralized by addition of 3 M HCl and 1 M of Tris-HCl pH 7.5.

2.17.1 Purification of cDNA

PCR products were purified by passage through a Centrisep column (Princeton Separation, USA) as recommended by the manufacturer.

2.18 Hybridisation

2.18.1 Pre-hybridisation

Arrays were denatured at 100°C for 1 minute followed by an immediate decrease to 20°C in a PTC-255 Peltier Tetrad thermal cycler (MJ Research, USA).

Arrays were pre-hybridised under a coverslip using 20 µl of pre-hybridisation buffer (6x SSPE, 0.5% SDS, 2 µl Denhardts and 4 µg bovine serum albumin) at 42°C for 45 to 60 min. After the pre-hybridisation, arrays were washed as described in 2.18.3 and scanned for printing defects in GenePix 4000A scanner (see 2.19).

2.18.2 Hybridisation

Hybridization was carried out in humidified chambers at a temperature of 42°C for approximately 16 h. The hybridization buffer consisted of 50% deionised formamide, 0.5% SDS, 6x SSPE and 2.5x Denhardts, 0.06 µg/µl poly $A_{(80)}$, 0.66 µg/µl Mouse Cot 1 DNA (Gibco/BRL), 0.27 µg/µl yeast tRNA (Gibco/BRL).

Following hybridisation, arrays were washed and analysed as described in sections 2.18.3 and 2.19 respectively.

2.18.3 Washing

Following hybridisation, arrays were washed at room temperature, in 1.0xSSC, 0.03% SDS. Arrays were initially plunged in several times until the coverslip fell off. After 10 min, arrays were transferred into a 0.2xSSC solution for 10 min, followed by a final wash in 0.05xSSC for 10 min.

2.19 Analysis of microarrays

Microarrays were scanned in a GenePix 4000A (Axon Instruments, CA) and the fluorescence measured using the GenePix 3.0 (version 3.0.5) software (Axon Instruments, CA). Feature sizes were initially determined using the inbuilt automated parameters and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined and the median fluorescence of these pixel measurements taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default parameters of GenePix 3.0.

Data processing was initially carried out using ConvertData version 3.3.3a (latest version available http://www.le.ac.uk/cmht/microarray_lab/Microarray_Downloads/Microarray_Downloads.htm), subsequently imported into Microsoft Access where tables were created and finally transferred to Microsoft Excel. Built in facilities were used to calculate mean values and standard deviation.

3 Germline mutations at ESTR loci

When this project began, there was strong evidence demonstrating that ESTR loci provide a very powerful technique for the analysis of radiation-induced mutation in the mouse germline (Barber *et al.*, 2000; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a). However, there was no relevant experimental data showing that exposure to chemical mutagens could also result in elevated mutation rate at ESTR loci. The only germline evidence for mutation induction at hypervariable tandem repeat loci came from two pilot studies of male mice exposed to polychlorinated biphenyls (Hedenskog *et al.*, 1997) and urban herring gulls exposed to environmental chemical pollutants (Yauk and Quinn, 1996; Yauk *et al.*, 2000). Barber *et al.* analysed ESTR mutation induction in the germline of male mice exposed to the anticancer drug cisplatin but failed to detect any changes in mutation rate across different stages of spermatogenesis (Barber *et al.*, 2000).

Data on radiation-induced ESTR mutation suggest that ESTR loci themselves are not the direct target of irradiation (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Fan *et al.*, 1995). Ionising radiation generates damaged bases, SSBs and DSBs in DNA from exposed cells (Frankenberg-Schwager, 1990; Ward, 1990). Radiation-induced DNA damage accumulated elsewhere in the genome or activation of DNA repair are believed to trigger some yet unknown mechanisms leading to mutation induction at ESTR loci (see 1.6). Somatic recombination after exposure to radiation or chemicals has been reported using yeast (Galli and Schiestl, 1999), *Drosophila* (Frei and Wurgler, 1996; Rodriquez-Arnaiz *et al.*, 1996; Vogel, 1992) and human fibroblasts (Li *et al.*, 1997). Induction of recombination has also been observed in germ cells, since it is found to occur in *Xenopus laevis* oocytes in response to UV light and X-rays (Hays *et al.*, 1990; Hellgren, 1992; Sweigert and Carroll, 1990). From these data it is possible to expect that exposure to chemical mutagens, capable of induction of a variety of DNA lesions, may also result in elevated recombination rate and subsequently in the mutation induction at ESTR loci.

3.1 Mutation scoring

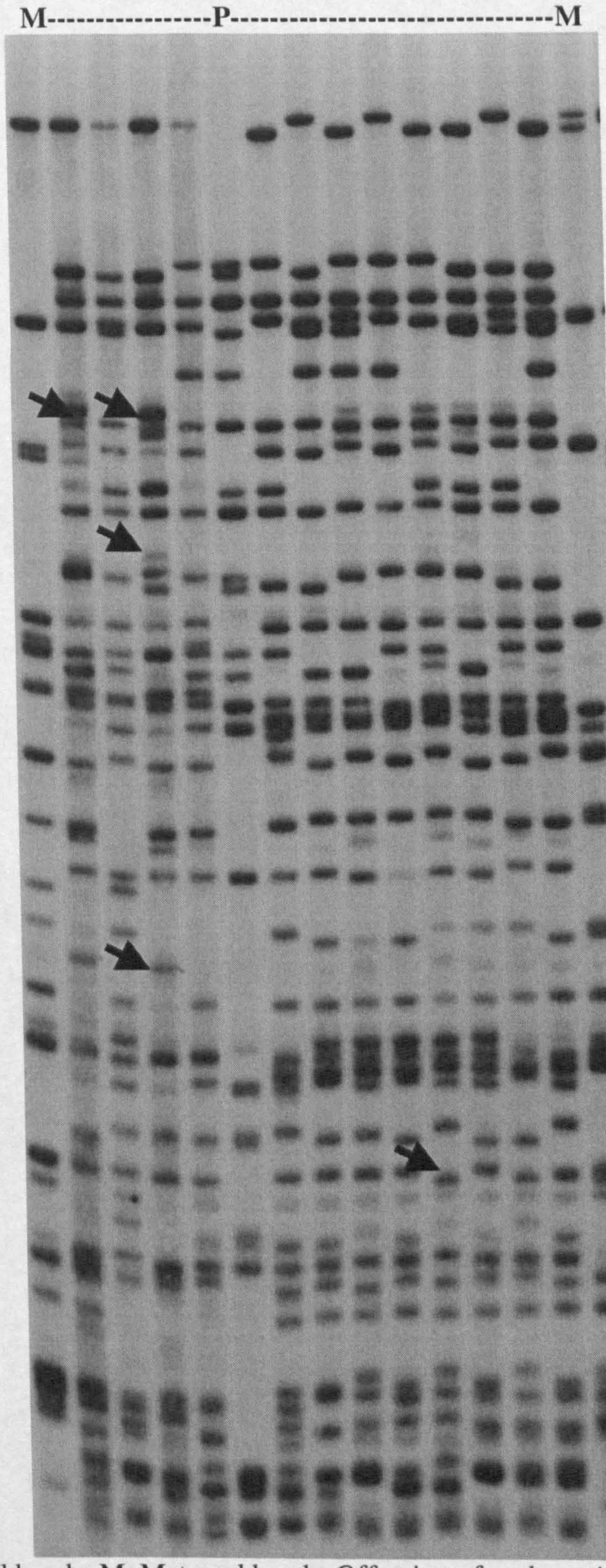
Fingerprinting analyses of the mouse germline were generated by profiling offspring with one multilocus probe (MMS10) (Bois *et al.*, 1998b) and two mouse-specific hypervariable single-locus probes (Ms6-hm (Kelly *et al.*, 1989) and Hm-2 (Gibbs *et al.*, 1993)).

The fingerprinting by probe MMS10 resulted in a complex pattern of DNA fragments, shared by parents and offspring and did not allow the parental origin of new alleles to be determined. This probe was therefore used to verify the correct parentage for the offspring of non-exposed and treated males (Figure 3-1 a).

ESTR mutations were scored using two mouse-specific hypervariable single-locus probes, Ms6-hm and Hm-2 over the well-resolved region between 2.5 and 22 kb (Figure 3-1 b, c). ESTR mutants were identified as a novel DNA fragment present in the offspring that could not be ascribed to either parent. Similar to previous studies on radiation–induced mutations at ESTR loci (Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a), some cases of germline mosaicism with *de novo* mutations, shared by more than one offspring in the litter, were observed. All cases of germline mosaicism were recorded and treated separately.

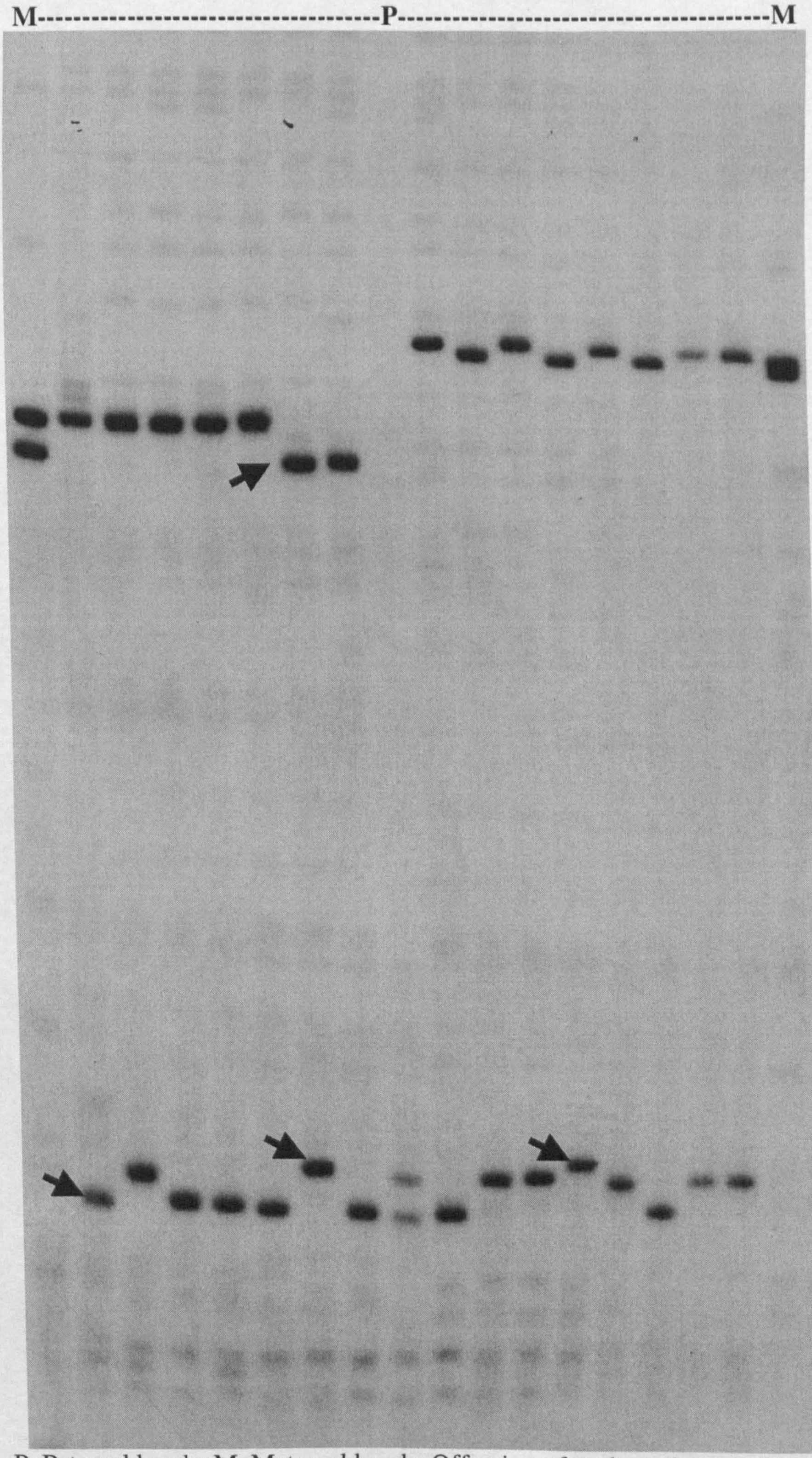
This study was performed on the offspring conceived from CBA/Ca male and C57BL/6J female inbred strains of mice. These strains were chosen because they have a specific non-overlapping size range of alleles at both ESTR loci. This considerably facilitated detection of mutant bands and establishment of the parental origin of mutant bands (Figure 3-1 b, c).

Figure 3-1 a MMS10 DNA fingerprinting.



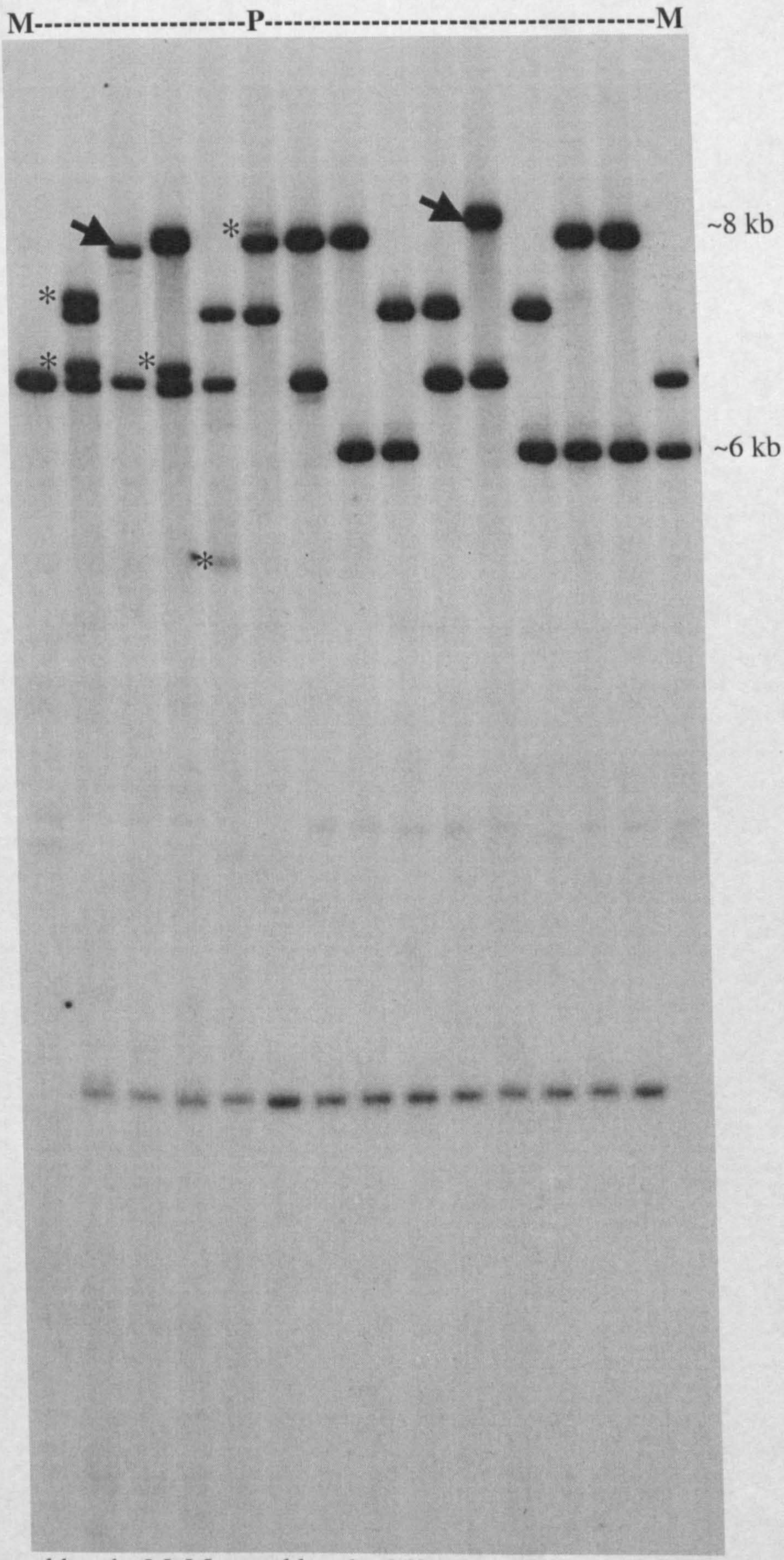
P, Paternal bands; M, Maternal bands. Offspring of each mating are fitted between the parents. Arrows indicate some bands of mutant offspring.

Figure 3-1 b ***Hm-1*** **loci DNA fingerprinting.**



P, Paternal bands; M, Maternal bands. Offspring of each mating are fitted between the parents. Arrows indicate bands of mutant offspring.

Figure 3-1 c ***Hm-2*** **loci DNA fingerprinting.**



P, Paternal bands; M, Maternal bands. Offspring of each mating are fitted between the parents. Arrows indicate bands of mutant offspring. Asterisks indicate somatic mutations originated in the offspring.

3.2 Spontaneous mutation rate

3.2.1 Introduction

Prior to the analysis of ESTR mutation induction by chemical mutagens, it was essential to evaluate ESTR mutation rates in the germline of non-exposed CBA/Ca males and C57BL/6J females. ESTR mutation rate in the non-exposed C57BL/6 females were previously evaluated (Barber *et al.*, 2000). On the other hand, the CBA/Ca inbred males from Harlan were used in the current study, whereas all data on radiation-induced mutations at ESTR loci have been obtained using CBA/H male mice from the Harwell colony (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a). Despite the fact that both male strains belong to the same substrain of CBA mice (Festing, 1996), their spontaneous and induced mutation rates at ESTR loci may vary. It has recently been shown that three different mouse inbred strains have statistically different spontaneous mutation rate at ESTR loci (Barber *et al.*, 2002).

3.2.2 Results

The control group was generated by mating 10 non-exposed CBA/Ca male to 20 untreated C57BL/6J females. Table 3-1 presents a summary of mutation data obtained by profiling all offspring and their parents by two single-locus ESTR probes Ms6-hm and Hm-2. Spontaneous mutation rates at these two loci were 0.050 and 0.070 for CBA/Ca male and C57BL/6J female mice, respectively. Both estimates are very close to those previously reported for CBA/H males (Dubrova *et al.*, 2000a; 0.054 per locus, P=1, Fisher's exact test) and C57BL/6 females (Barber et al., 2002; 0.071 per locus, P=1). This apparent similarity in ESTR mutation rates suggests that mutation scoring in my study was nearly identical to that in the above-mentioned publications. Most importantly, it will also allow cross-comparison between the results on mutation induction at mouse ESTR loci by chemical mutagens presented in this section and previous data on radiation-induced mutation (Barber *et al.*, 2000; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a; Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994).

Table 3-1 Summary of spontaneous ESTR mutation data.

Cross	No. Litters	No. offs.	Number of mutations scored with different probes*							
			Ms6-hm		Hm-2		Ms6-hm + Hm2		Mutation rates	
			Paternal	Maternal	Paternal	Maternal	Paternal	Maternal	Paternal	Maternal
CBA/Ca x C57BL[†]	19	121	5 (5)	9 (9)	7 (7)	8 (7)	12 (12)	17 (16)	0.0496	0.0702
CBA/H x CBA/H[‡]	12	74	5 (4)	5 (4)	3 (3)	4 (4)	8 (7)	9 (8)	0.0540	0.0608
F1(CBA/HxC57BL)xCBA/H[§]	19	119	11 (7)	11 (10)	6 (3)	6 (5)	17 (10)	17 (15)	0.0714	0.0714

* Numbers of singleton mutations are given in parentheses.

[†]This thesis, [‡]Dubrova *et al.*, 2000a, [§]Barber *et al.*, 2000

3.3 ESTR mutation induction by alkylating agents

3.3.1 Introduction

The first two chemicals selected in this project were the alkylating agents ethyl nitrosourea (ENU) and isopropyl methanesulfonate (iPMS). Exposure to these chemicals results in a wide spectrum of well-characterised DNA lesions (Fujun *et al.*, 1990; Sega *et al.*, 1986; van Zeeland, 1996) and they belong to a group of powerful mutagens, showing a very high mutagenicity across different stages of mouse spermatogenesis (Ehling *et al.*, 1972; Ehling and Neuhauser-Klaus, 1995; Favor, 1998; Katoh *et al.*, 1994; Liegibel and Schmezer, 1997; Renault *et al.*, 1997; Russell *et al.*, 1979; Russell *et al.*, 1982; Russell and Hunsicker, 1984; Shelby and Tindall, 1997; Suzuki *et al.*, 1997b). Most importantly, using the SLT and the DLT (see 1.3), extensive data on the germline mutagenicity of these alkylating agents have been accumulated, which should greatly facilitate a cross-comparison between the results of my study and those obtained by traditional approaches for monitoring germline mutation in mice. For example, it has been demonstrated that ENU affects all stages of spermatogenesis when tested with the SLT (Favor, 1998; Russell *et al.*, 1979; Russell *et al.*, 1982; Russell and Hunsicker, 1984), while inconclusive results have been found for iPMS, although it is believed to affect only post-meiotic stages of spermatogenesis (Ehling *et al.*, 1972; Ehling and Neuhauser-Klaus, 1995; Shelby and Tindall, 1997). The different stage-specificity of mutation induction by ENU and iPMS may reflect differences in the pattern of DNA alkylation by these agents.

Similar to all alkylating agents, ENU and iPMS form DNA adducts by the transfer of an alkyl group to oxygen or nitrogen radicals in DNA (see 1.4.1), resulting in a wide spectrum of mutation (Favor, 1999; Justice *et al.*, 1999; van Zeeland, 1996). Despite the fact that ENU and iPMS are both monofunctional alkylating agents, their mode of action on DNA is slightly different as ENU has a higher affinity for *O*-alkyl-bases than iPMS (Sega *et al.*, 1986; van Zeeland *et al.*, 1985; Vogel *et al.*, 1996). It has been suggested that the higher ability to modify O^6 atom by ENU and other alkylating agents, resulting in their selective ability to form O^6-alkyldeoxyguanosine, could define their mutagenicity and carcinogenicity (Goth and Rajewsky, 1974; Loveless, 1969).

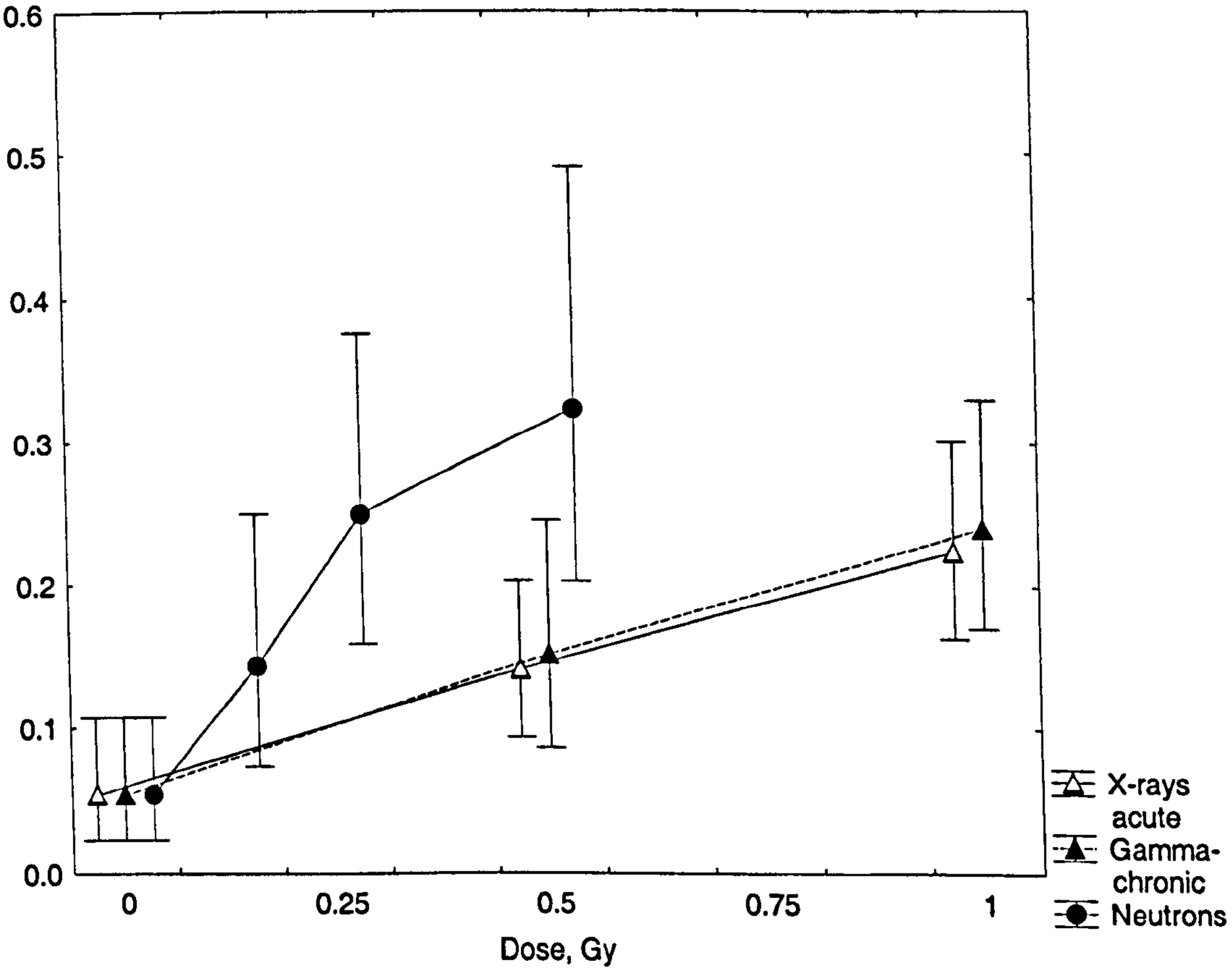
3.3.2 Experimental design

Previous studies analysing radiation-induced mutations have shown that ESTR analysis is a very powerful technique that allows the detection of germline mutations at much lower doses than traditional genetic techniques (Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a) (Figure 3-2). For this reason it was initially decided to analyse the doses of 50 mg/kg of ENU and 25 mg/kg of iPMS. These doses are in the lowest range of doses analysed with traditional systems such as the SLT (Ehling and Neuhauser-Klaus, 1988a; Favor, 1998) or the DLT (Ehling *et al.*, 1972; Ehling and Neuhauser-Klaus, 1995).

Once the results of 50 mg/kg of ENU and 25 mg/kg of iPMS had been obtained, new CBA/Ca male mice were dosed with 12.5, 25 or 75 mg/kg of ENU and 12.5 or 37.5 mg/kg of iPMS. It should be noted that the doses of 12.5 and 25 mg/kg of ENU are the lowest doses ever analysed and they are far below the proposed threshold value of 38 mg/kg (Favor, 1998), below which germline mutation induction should not occur.

Pre-meiotic exposure to 75 mg/kg of ENU or 25 mg/kg and 37.5 mg/kg of iPMS resulted in a drastic decrease in the fertility of exposed males. This was completely unexpected as no decrease in the number of litters or litter size was observed after exposure to 50 mg/kg of ENU. The results of previous studies show that male sterility can be detected at doses of ENU of 100 mg/kg or higher (Shelby and Tindall, 1997). However, a more recent study shows a very high sensitivity of CBA/CaJ mice to ENU (Weber *et al.*, 2000). The severely impaired fertility of the exposed males finally resulted in a small number of offspring available for the analysis. To overcome this problem, male mice exposed to 75 mg/kg of ENU or 25 mg/kg and 37.5 mg/kg of iPMS were additionally mated 9-10 weeks after injection. Litters conceived nine or ten weeks after treatment were derived from pre-meiotic stem cells (Searle, 1974).

Figure 3-2 Dose-response curves obtained with ionising radiation (Dubrova *et al.*, 2000a).



3.3.3 ESTR mutation rates in male mice exposed to ENU

3.3.3.1 Post-meiotic exposure

The results of the mutation induction at post-meiotic stages were obtained by mating male mice treated with 50 or 75 mg/kg of ENU three weeks after exposure (Table 3-2). From 10 males treated with 50 mg/kg of ENU, 87 offspring were obtained. The number of ESTR mutations scored are given (Table 3-3). The frequency of paternal mutation rate in this group was slightly elevated (1.85-fold, P=0.1346). These data did not allow us to distinguish whether this non-significant increase could be attributed to ENU or it was a statistical artefact. To address this issue, a dose of 75 mg/kg of ENU was studied, this dose was believed to be high enough to produce statistically significant increases in paternal mutation rate at ESTR loci. The results obtained with 75 mg/kg of ENU showed that paternal mutation rate was significantly elevated in respect to the control group. A 2.15-fold increase with a statistically significant probability of 0.0356 (Table 3-4).

Following the initial results, it was decided to study the clustering of mutations in male mice exposed to 50 and 75 mg/kg of ENU. This analysis showed that elevated ESTR mutation rate in both groups was attributed to a few instances of germline mosaicism in the exposed males and was not observed for singleton ESTR mutations (Table 3-4). Thus, ESTR mutation rate for singleton mutations in male mice exposed to 50 mg/kg of ENU was similar to that in control group and was slightly elevated in those exposed to 75 mg/kg. As post-meiotic exposure to any mutagen cannot result in mutation clustering, the elevated ESTR mutation rate in the germline of these males most probably is not attributed to the ENU exposure.

3.3.3.2 Pre-meiotic exposure

Pre-meiotic exposure to ENU was studied using the doses of 12.5, 25, 50 and 75 mg/kg six and nine weeks after exposure (Table 3-2). Different doses were used to generate a dose-response curve from which it is possible to evaluate the sensitivity of this system.

Table 3-2 Stages of mouse spermatogenesis.

	ENU and iPMS mating points	Days taken to reach ejaculate	Germ cell type	Etoposide Mating points
PRE-MEIOTIC	6, 9,10 weeks	?	Primordial cells	6 weeks
		Over 42	Type A spermatogonia	
			A_1	
			A_2	
			A_3	
			A_4	
		35-37	Intermediate spermatogonia	5 weeks
		34-36	Type B spermatogonia	
MEIOTIC			Primary spermatocytes	
		33-35	Preleptotene	
		32-33	Leptotene	
		30-32	Zygotene	4 weeks
		23-30	Pachytene	
		22-23	Diplotene	3 weeks
		21-22	Diakinesis – Metaphase I	
		21-22	Secondary spermatocytes	
POST-MEIOTIC	3 weeks	7-21	Spermatids	2 weeks
		0-7	Spermatozoa	

Table 3-3 Summary of mutation data for male mice exposed to ENU

Group	No males	No offspring	*Ms6-hm**		*Hm-2**		*Ms6-hm + Hm-2**	
			Paternal	Maternal	Paternal	Maternal	Paternal	Maternal
Control	10	121	5(5)	9 (9)	7 (7)	8 (6)	12 (12)	17 (15)
Pre-meiotic								
12.5 mg/kg	7	137	28 (22)	6 (6)	11 (9)	2 (2)	39 (31)	8 (8)
25 mg/kg	10	191	57 (49)	21 (14)	15 (15)	6 (6)	72 (64)	27 (20)
50 mg/kg	10	97	20 (15)	7 (7)	11 (7)	7 (7)	31 (22)	14 (14)
75 mg/kg	19	123	29 (25)	11 (8)	12 (12)	3 (3)	41 (37)	14 (11)
Post-meiotic								
50 mg/kg	10	87	9 (5)	4 (4)	7 (4)	8 (8)	16 (9)	12 (12)
75 mg/kg	19	123	29 (25)	11 (8)	12 (12)	3 (3)	41 (37)	14 (11)

* Numbers of singleton mutations are given in parentheses.

Table 3-4 ESTR mutation rates in male mice exposed to ENU

Group	All maternal mutations			All paternal mutations			Paternal singletons		
	Rate	Ratio*	Prob†	Rate	Ratio*	Prob†	Rate	Ratio*	Prob†
Control	0.0702	-	-	0.0496	-	-	0.0496	-	-
Pre-meiotic									
12.5 mg/kg	0.0292	0.42	**0.0491**	0.1423	2.87	**0.0006**	0.1131	2.28	**0.0130**
25 mg/kg	0.0707	1.01	1	0.1885	3.80	**3.11×10^{-7}**	0.1675	3.38	**7.19×10^{-6}**
50 mg/kg	0.0722	1.03	1	0.1598	3.22	**0.0002**	0.1134	2.29	**0.0222**
75 mg/kg	0.0569	0.87	0.8743	0.1667	3.36	**4.12×10^{-5}**	0.1504	3.03	**0.0003**
Post-meiotic									
50 mg/kg	0.069	0.98	1	0.092	1.85	0.1346	0.0517	1.04	1
75 mg/kg	0.068	0.97	1	0.1068	2.15	**0.0356**	0.0631	1.27	0.6762

*Ratio to mutation rate in control.
†Probability of difference from the control group (Fisher's exact test, two-tailed). Statistically significant values are given in bold.

In contrast to the original procedure of mating exposed males six weeks after treatment, mating of treated males nine weeks after exposure did not dramatically affect their fertility, and as a result, an extra 54 offspring from the exposed males were included in this analysis. Mutation rates in males exposed to this concentration of ENU and mated to non-exposed females six and nine weeks after exposure were similar (P=0.6032, Fisher's exact test). It has also been shown that there is no statistically significant difference between offspring conceived six and ten weeks after paternal exposure to ionising radiation (Dubrova *et al.*, 1998a). I therefore combined data from the two stages treated with 75 mg/kg of ENU to produce a single estimate of mutation rate for this group.

Litters conceived from males treated with 12.5 mg/kg of ENU showed a statistically significant 2.87-fold increase in paternal mutation rate. Similarly, exposure to 25, 50 and 75 mg/kg resulted in a statistically significant paternal mutation rate increase of 3.8, 3.22 and 3.36-fold to the control group, respectively (Table 3-4).

Mutations were occasionally shared by more than one offspring, suggesting germline mosaicism for a single mutation. Only 15.8% (29/183) of paternal mutations observed in pre-meiotic stages could have arisen as a result of germline mosaicism. The analysis of ESTR mutation induction from singletons (non-shared mutations) marginally decreased paternal mutation rates; however, compared to the control group, they remained statistically elevated (Table 3-4). These data indicate that mutation induction by ENU cannot be attributed to shifts in the level of mosaicism in germ cell populations sharing the same mutation.

These results clearly show that male mice exposed to 25, 50 and 75 mg/kg of ENU reveal a plateau of mutation induction (Figure 3-3). However, the frequency of ESTR mutation in the offspring of males exposed to 12.5 mg/kg of ENU is lower than for those exposed to higher doses. To evaluate the dose-response of mutation induction, the paternal mutation rate scored in each male (Y) and the dose, was fitted to different equations giving a quadratic regression as the best fit (Figure 3-4).

$$Y = 0.2755 + 0.0080 \times Dose - 0.0001 \times Dose^2;\ F\ (2/47) = 12.1;\ P = 0.0001$$

This analysis revealed that at doses of 25 mg/kg of ENU or below, paternal mutation rate increases linearly and reached a plateau at higher concentrations.

Figure 3-3 Mutation induction at mouse ESTR loci by pre-meiotic exposure to ENU.

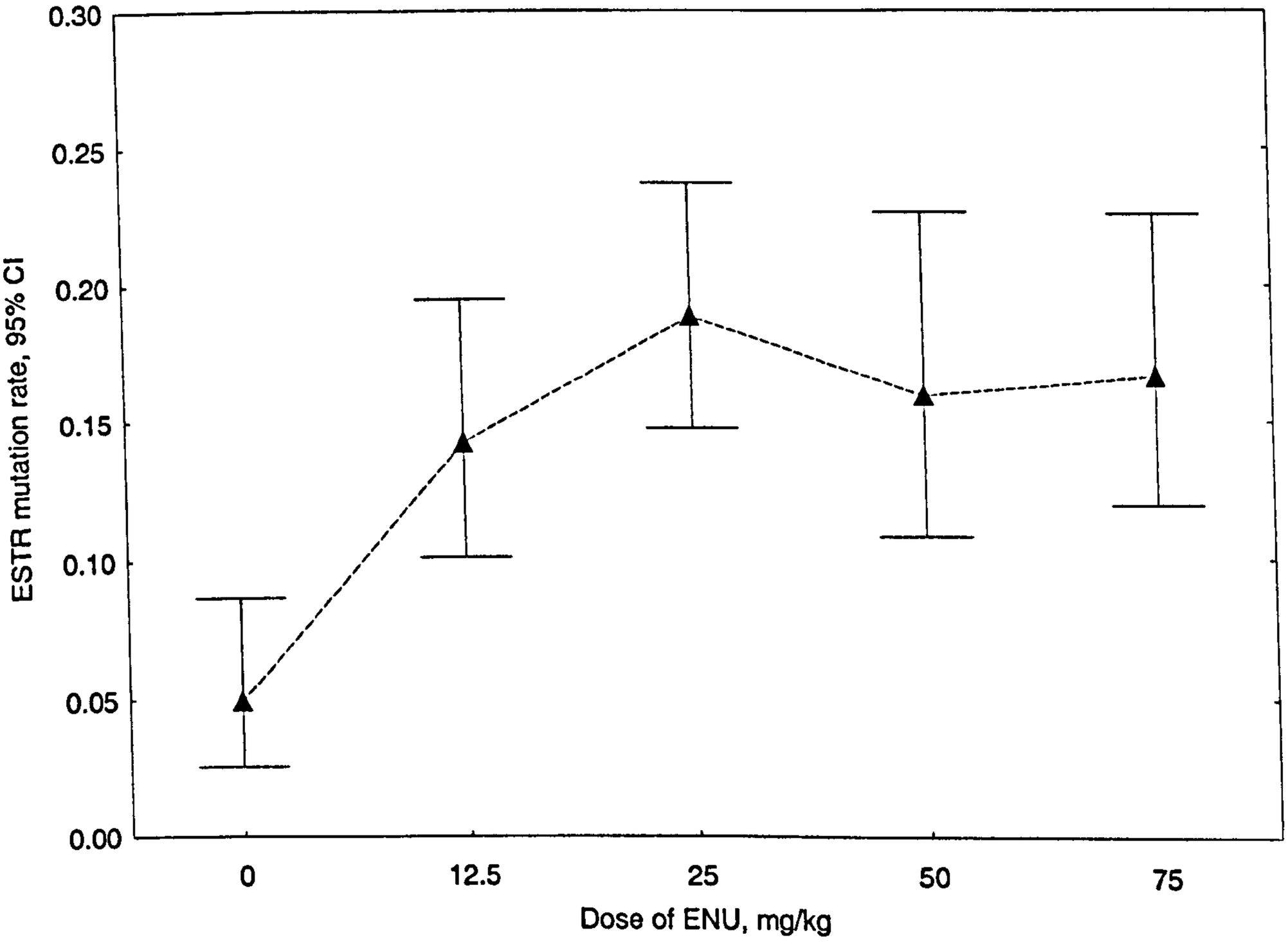
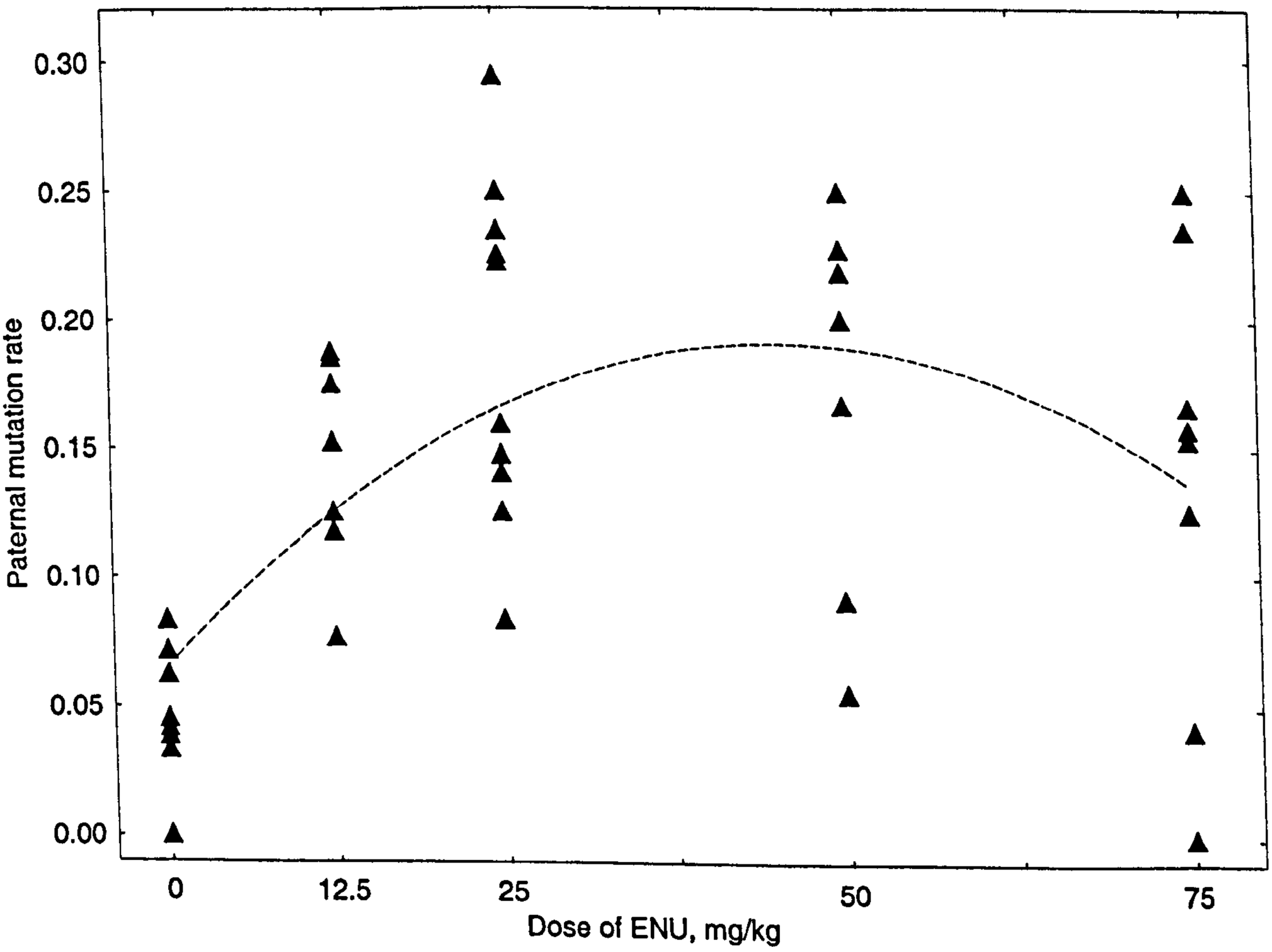


Figure 3-4 Dose-response for ESTR germline mutation induction by pre-meiotic exposure to ENU.



3.3.4 ESTR mutation rates in male mice exposed to iPMS

3.3.4.1 Post-meiotic exposure

The analysis of mutation induction at post-meiotic stages of spermatogenesis, following single exposure to iPMS, was assessed using a single dose of 25 mg/kg. This dose is in the lowest range ever used in previous studies of germline mutation induction in mice. Treated male mice were mated three weeks after exposure to the mutagenic agent (Table 3-2), generating a total of 90 offspring from 9 males (Table 3-5). The results of this study show that post-meiotic exposure to iPMS does not affect ESTR mutation rate in the germline of injected males (Table 3-6).

3.3.4.2 Pre-meiotic exposure

The study of the mutagenicity of iPMS on pre-meiotic stages of spermatogenesis was analysed using doses of 12.5, 25, and 37.5 mg/kg. Treated male mice were mated six weeks after exposure to the chemical agent. Offspring conceived at this stage represent the mutational effect of iPMS on pre-meiotic spermatogonia (Searle, 1974). As was observed in pre-meiotic stages treated with 75 mg/kg of ENU, pre-meiotic exposure to 25 or 37.5 mg/kg of iPMS produced a sharp decrease in fertility. This decrease in paternal fertility was completely unexpected, as it has been published that iPMS affects male mice fertility at doses of 75 mg/kg or over (Shelby and Tindall, 1997).

In the initial study with 25 mg/kg of iPMS, 34 offspring from 7 males were obtained. Despite the small sample size, a statistically significant 4.7-fold increase (P=4.15x10^{-5}) was found (data not shown). Nevertheless, a further experiment using the same dose and mating scheme was arranged to increase the number of offspring and therefore obtain more statistical power. In total, 104 offspring from 14 males were analysed (Table 3-5). These experiments showed that there is a statistically significant 3.88-fold increase (P=3.52x10^{-6}) in paternal mutation rate (Table 3-6).

Table 3-5 Summary of mutation data for male mice exposed to iPMS

Group	No males	No offspring	*Ms6-hm**		*Hm-2**		*Ms6-hm + Hm-2**	
			Paternal	Maternal	Paternal	Maternal	Paternal	Maternal
Control	19	121	5(5)	9 (9)	7 (7)	8 (6)	12 (12)	17 (15)
Pre-meiotic								
12.5 mg/kg	16	214	42 (34)	14 (14)	13 (13)	13 (13)	55 (47)	27 (27)
25 mg/kg	14	104	25 (19)	13 (9)	15 (7)	3 (3)	40 (26)	16 (12)
37.5 mg/kg	4	81	17 (13)	3 (3)	8 (6)	2 (2)	25 (19)	5 (5)
Post-meiotic								
25 mg/kg	9	90	10 (8)	3 (3)	4 (4)	6 (6)	14 (12)	9 (9)

* Numbers of singleton mutations are given in parentheses.

Table 3-6 ESTR mutation rates in male mice exposed to iPMS

Group	All maternal mutations			All paternal mutations			Paternal singletons		
	Rate	Ratio*	Prob†	Rate	Ratio*	Prob†	Rate	Ratio*	Prob†
Control	0.0702	-	-	0.0496	-	-	0.0496	-	-
Pre-meiotic									
12.5 mg/kg	0.0631	0.90	0.8336	0.1285	2.59	**0.0011**	0.1098	2.21	**0.0098**
25 mg/kg	0.0650	0.93	0.9600	0.1923	3.88	**3.52×10^{-6}**	0.1250	2.52	**0.0068**
37.5 mg/kg	0.0309	0.44	0.1310	0.1543	3.11	**0.0008**	0.1173	2.36	**0.0219**
Post-meiotic									
25 mg/kg	0.0500	0.71	0.5191	0.0778	1.57	0.3237	0.0667	1.34	0.5875

*Ratio to mutation rate in control.

† Probability of difference from the control group (Fisher's exact test, two-tailed). Statistically significant values are given in bold.

Once the first set of data was obtained it was decided to analyse a higher (37.5 mg/kg) and a lower dose (12.5 mg/kg) of iPMS to address the issue of dose-response. When the experiment analysing mutation induction after exposure to 37.5 mg/kg of iPMS started, a decrease in fertility was already expected, as it was previously observed in males exposed to a lower dose (25 mg/kg). To obtain enough offspring for the analysis and to overcome the low fertility period, male mice were mated six and ten weeks after exposure (Table 3-2). These two stages correspond to pre-meiotic cells (Searle, 1974) and it has been proved with ENU and radiation (Dubrova *et al.*, 1998a) that those stages generate, statistically, the same results. In total 81 offspring from 4 males were obtained (Table 3-5). From male mice treated with 12.5 mg/kg of iPMS, 214 offspring from 16 males were analysed.

Offspring derived from male mice treated with 12.5 mg/kg of iPMS showed a statistically significant 2.59-fold increase in paternal mutation rate in comparison to the control group, with a probability of 0.0011 (Table 3-6). Offspring from male mice treated with 37.5 mg/kg showed a statistically significant (P=0.0008) increase in paternal mutation rate, 3.11 times higher than that found in the control group (Table 3-6) (Figure 3-5).

Similarly to ENU, mutations were occasionally shared by more than one offspring as a result of germline mosaicism. With iPMS, 23.3% (28/120) of paternal mutations observed in pre-meiotic stages could have arisen as a result of germline mosaicism. The analysis of ESTR mutation induction from singletons marginally decreased paternal mutation rates; however, compared to the control group, they remained statistically elevated (Table 3-6). These data indicate that mutation induction by iPMS cannot be attributed to shifts in the level of mosaicism in germ cell populations sharing the same mutation.

In contrast to what was thought from the inconclusive results obtained with the SLT and the DLT (Ehling *et al.*, 1972; Ehling and Neuhauser-Klaus, 1995; Shelby and Tindall, 1997), all different doses of iPMS increase mutation frequency in pre-meiotic stages of spermatogenesis.

Figure 3-5 Mutation induction at mouse ESTR loci by pre-meiotic exposure to iPMS.

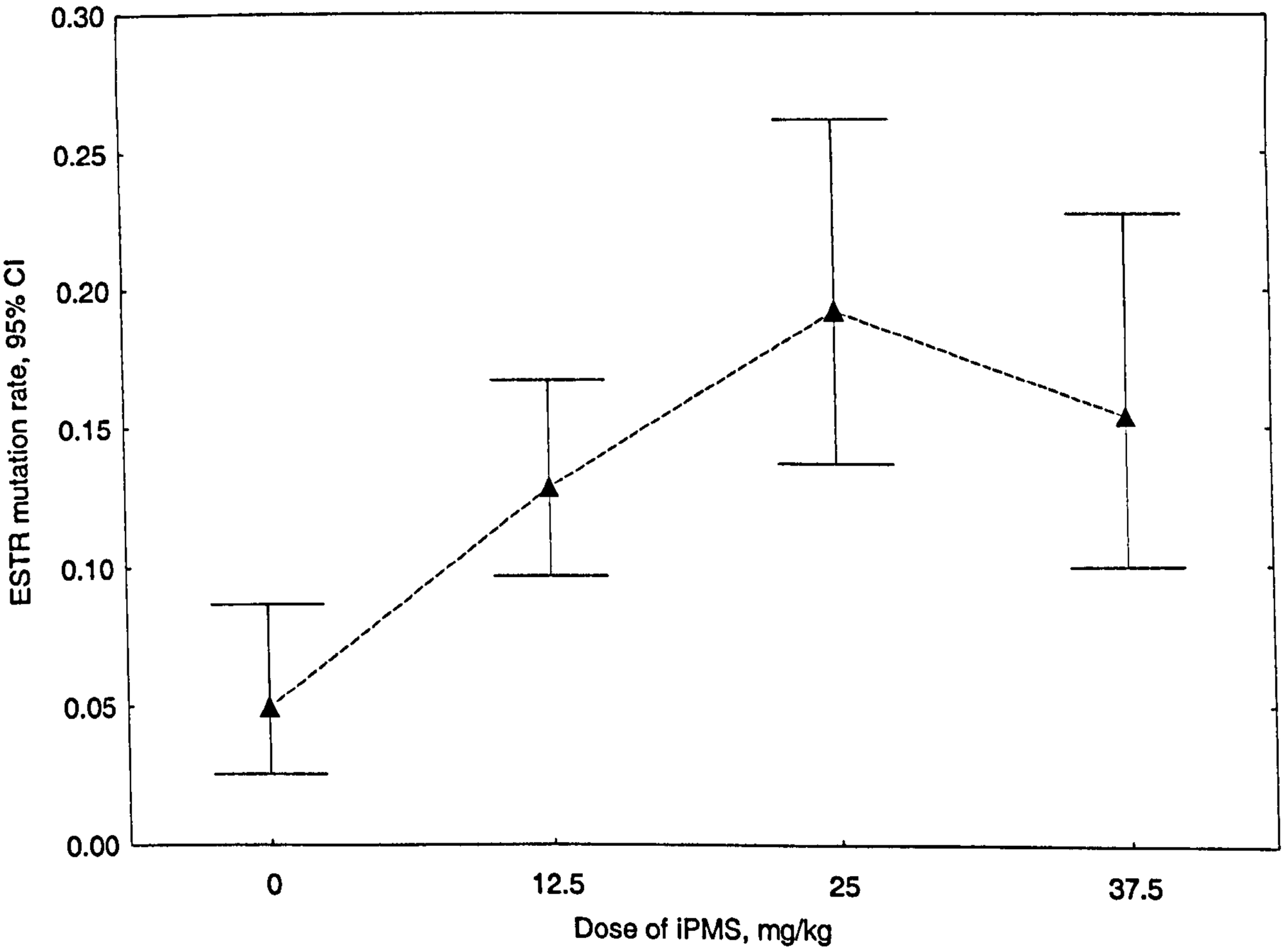
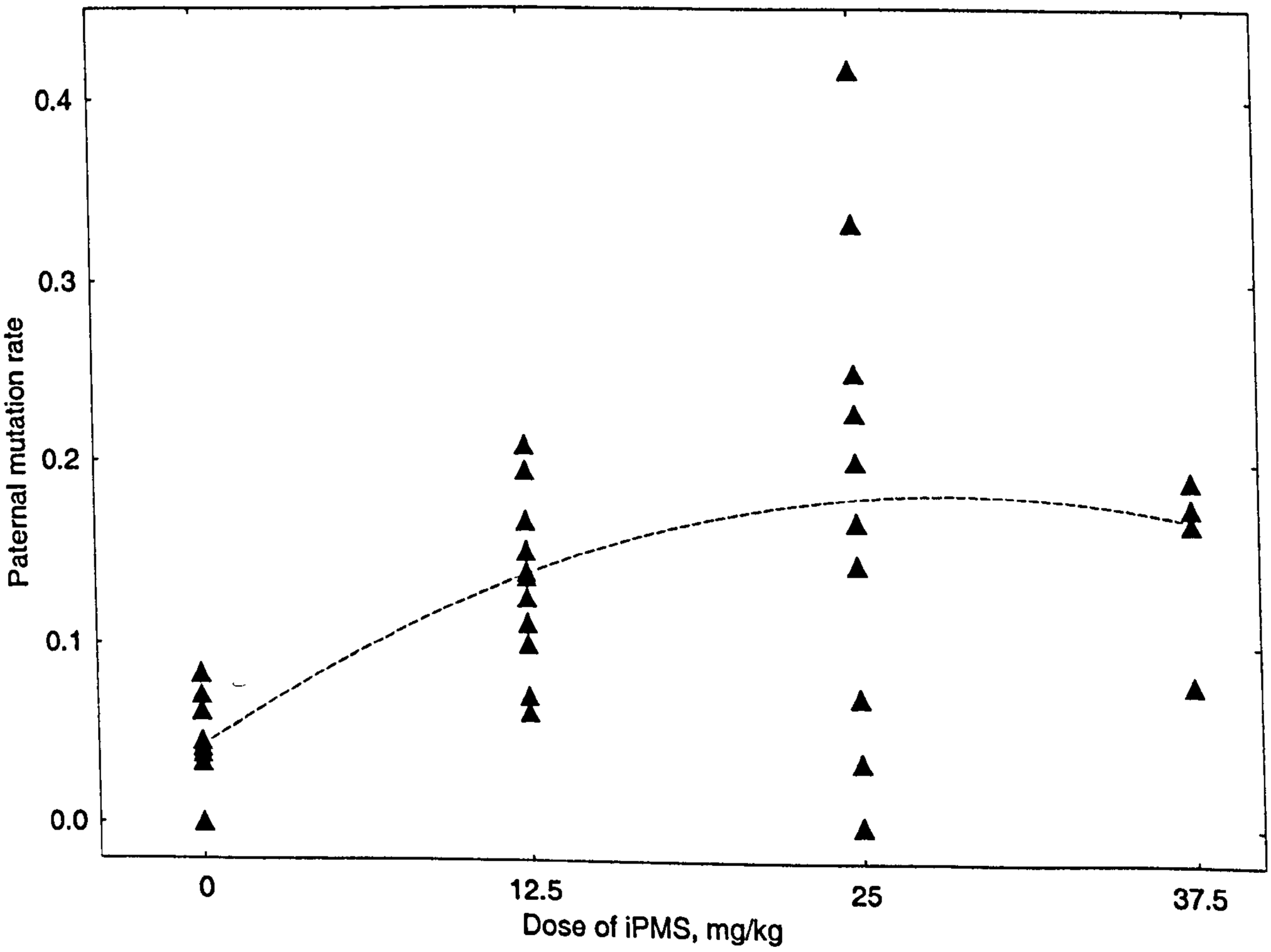


Figure 3-6 Dose-response for ESTR germline mutation induction by pre-meiotic exposure to iPMS.



To evaluate the dose-response of mutation induction, the paternal mutation rate scored in each male (*Y*) and dose, was fitted to different regressions to find the best fit. Similarly to the results obtained with ENU, the equation that best fit the results obtained with iPMS is a quadratic regression (Figure 3-6).

$$Y = 0.2415 + 0.0158 \times Dose - 0.0003 \times Dose^2;\ F\ (2/41) = 12.62;\ P = 0.0001$$

Therefore, there is a linear dose-response at doses up to 25 mg/kg followed by a saturation of the system with a plateau of mutation induction at higher doses of exposure.

3.3.5 Discussion

3.3.5.1 Stage-specificity of ESTR mutation induction by alkylating agents

The results of my study show a similar pattern of ESTR mutation induction in the germline of male mice exposed to ENU and iPMS. Thus pre-meiotic exposure to the two alkylating results in significant increases of ESTR mutation rates, but does not affect post-meiotic cells.

The stage-specificity of mutation induction in the male germline by these chemical mutagens has previously been analysed using traditional approaches for mutation scoring in mice. It was shown that ENU affects all stages of spermatogenesis when tested with the SLT (Favor, 1998; Russell *et al.*, 1979; Russell *et al.*, 1982; Russell and Hunsicker, 1984). However, the results obtained with iPMS are inconclusive (Ehling *et al.*, 1972; Ehling and Neuhauser-Klaus, 1995; Shelby and Tindall, 1997). Thus, the data on dominant lethality show that this chemical induces mutations in post-meiotic stages, with the pre-meiotic effects apparently attributed to high cytotoxicity of iPMS (Ehling and Neuhauser-Klaus, 1995). However, the results of more recent studies using transgenic *lac*Z mice show mutation induction in testicular DNA following high-dose pre-meiotic exposure to iPMS (Liegibel and Schmezer, 1997). My data therefore provide the first experimental evidence for pre-meiotic mutagenicity of low-dose exposure to iPMS in the mouse germline.

The results of this work support previous data on the stage-specificity of ESTR mutation induction by ionising radiation, showing the lack of mutation induction in post-meiotic cells (Barber *et al.*, 2000; Dubrova *et al.*, 1998a). They do not support the

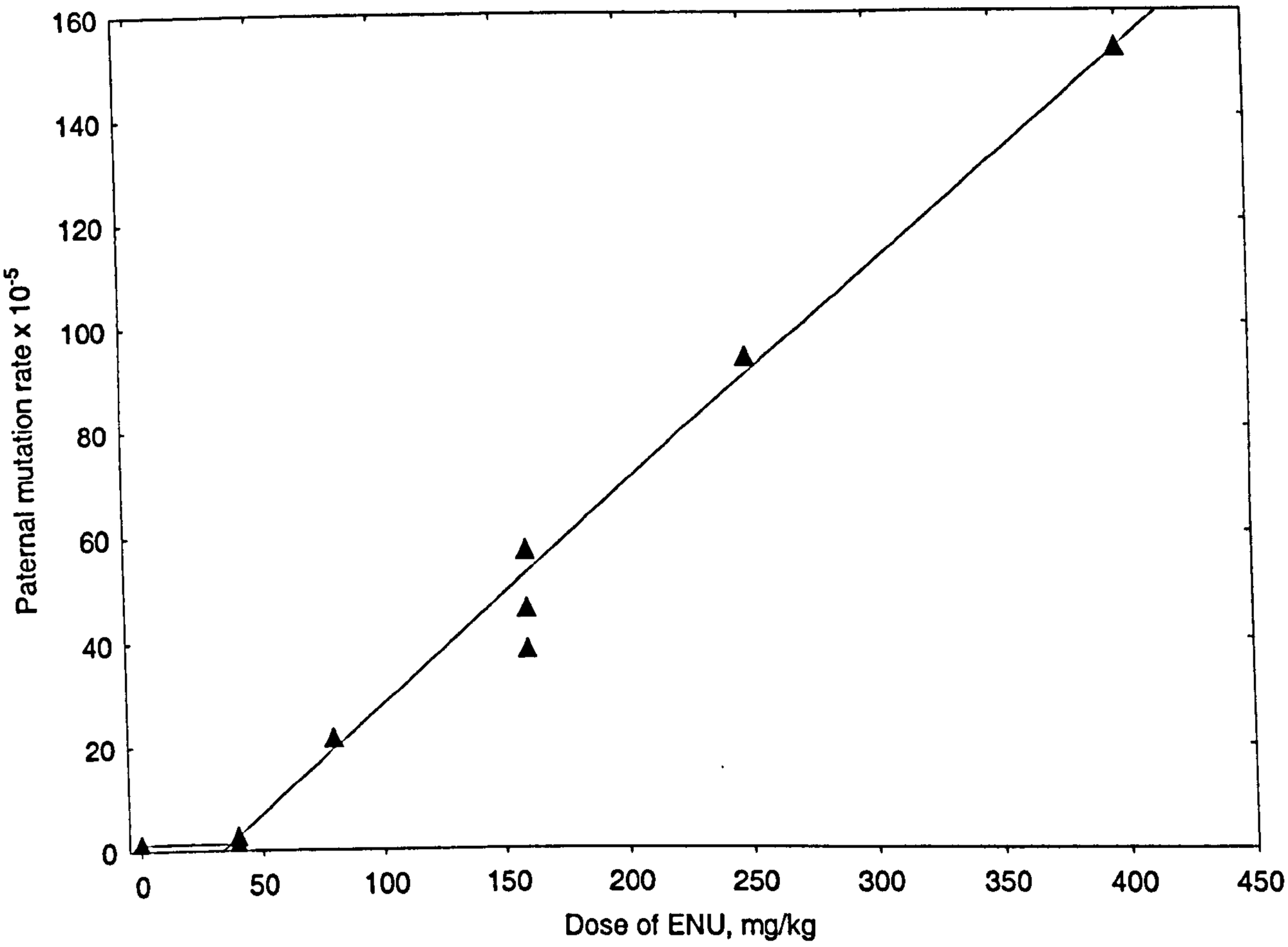
data of the Japanese group showing statistically significant increases in ESTR mutation rate in post-meiotic stages following exposure to ionising radiation (Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994).

Despite the clear differences in the initial DNA damage generated by ionising radiation and alkylating agents (Ehling *et al.*, 1985; Li *et al.*, 1990; Shelby *et al.*, 1993) (see 1.4), there is a striking similarity in the stage-specificity of mutation induction at ESTR loci by these mutagens in the mouse germline. Data obtained from radiation and chemically-induced mutations on the germline, provide strong evidence that mutation induction at ESTR loci can only occur following exposure to the diploid pre-meiotic and meiotic cells. The inability of ESTR loci to reflect induction of mutations in exposed post-meiotic male mice cells, to some extent, limits the potential application of these loci for monitoring germline mutation induction by chemicals, such as some environmental agents and anticancer drugs, which display peak mutagenic activity in post-meiotic cells (Renault *et al.*, 1997; Shelby, 1996; Witt and Bishop, 1996). These effects cannot be detected by mutation scoring at mouse ESTR loci. However, given that the spermatogonia stem cells can accumulate a substantial amount of induced mutations throughout reproductive life, the analysis of the mutagenesis of chemicals in pre-meiotic cells is crucial, as it represents a major genetic risk factor for humans. The results of this study have demonstrated that mutation induction by pre-meiotic exposure to alkylating agents can be monitored at ESTR loci; therefore, we have shown that these loci can be regarded as a useful marker for such long-term exposure to chemical mutagens.

3.3.5.2 Sensitivity of ESTR loci to pre-meiotic exposure to alkylating agents

The results of my study show that the dose-response curves obtained with ENU and iPMS are extremely similar. Exposure to both chemical mutagens results in a linear increase in mutation rate within the interval of doses from 12.5 mg/kg to 25 mg/kg with a plateau of mutation induction at higher concentrations. These results refute the hypothesis suggesting the presence of a threshold for germline mutation induction by chemical mutagens, estimated as 38 mg/kg of ENU (Favor, 1998) (Figure 3-7). My data clearly show significant increases in ESTR mutation rates in male mice exposed to doses of ENU and iPMS as low as 12.5 mg/kg.

Figure 3-7 Proposed threshold dose-response for ENU-induced mutations in mouse spermatogonia (Favor, 1998).



The establishing of dose-response curves for mutation induction by these two mutagens enabled us to evaluate the sensitivity of this system for exposure in pre-meiotic stages. Using the approach previously used for the analysis of germline mutation induction in mice by ionising radiation (Dubrova *et al.*, 1998a; Luning and Searle, 1971), the doubling doses for pre-meiotic exposure to ENU and iPMS for ESTR loci to three other mouse systems for mutation detection were compared (Table 3-7). The doubling dose for ESTR mutation induction by ENU is higher than that for the SLT; this difference is attributed to the plateau of ESTR mutation induction at doses greater than 25 mg/kg. However, within the linear part of dose-response curve for ESTR loci (4.4 mg/kg), the doubling dose is close to the mean value for the SLT. The analysis of the doubling dose for the dominant cataract system shows a very broad 95% confidence interval, which reveals the low sensitivity and reliability of this system. The Muta^{TM}Mouse assay for mutations at *LacZ* gene in testicular DNA shows very high values of doubling dose for both alkylating agents, therefore suggesting that the sensitivity of this approach is quite low.

Table 3-7 Doubling doses for acute exposure to alkylating agents in mice spermatogonia.

System	No. of mice*	Dose-Range, mg/kg†	Doubling Dose, mg/kg (95% CI)	Reference
ENU				
SLT	858,209	50-250	2.8 (1.9-3.9)	(Russell *et al.*, 1982) (Favor, 1998)
Dominant Cataract, 30 loci	38,381	160-250	4.2 (0.0-24.4)	(Ehling *et al.*, 1985)
LacZ‡	57	150	13.9 (10.4-17.4)	(Douglas *et al.*, 1997) (Liegibel and Schmezer, 1997) (Renault *et al.*, 1997) (Tinwell *et al.*, 1997)
ESTR loci	669	12.5-75	8.1 (4.0-14.5)	This thesis
iPMS				
LacZ‡	43	100-200	29.4 (21.2-37.5)	(Douglas *et al.*, 1997) (Liegibel and Schmezer, 1997) (Tinwell *et al.*, 1997)
ESTR loci	520	12.5-37.5	6.7 (3.3-12.0)	This thesis

*Including control and exposed animals.
†The range is given for the interval of doses of exposure to which resulted in a significant increase in mutation rates.
‡Data from germ cells isolated from seminiferous tubules sampled 50-91days after treatment.

Most importantly, the results of my study show that ESTR loci provide a unique opportunity for the analysis of mutation induction at very low doses of exposure to chemical mutagens. Using the SLT, doses of exposure starting from 50 mg/kg are necessary to obtain significant increase in mutation rate in paternal germ cells (Favor, 1998); the Muta^{TM}Mouse assay provided evidence for germline mutation induction at even higher doses of exposure to ENU and iPMS over 100 mg/kg (Liegibel and Schmezer, 1997; Renault *et al.*, 1997) (Table 3-7). In contrast, the data presented clearly show that the analysis of mutation induction at ESTR loci can be performed at doses as low as 12.5 mg/kg. Moreover, previous studies on mutation induction at SLT required the analysis of hundreds of thousands of mice (Favor, 1998; Russell *et al.*, 1982), whereas, as it was previously found for radiation-induced germline mutation at mouse ESTR loci (Barber *et al.*, 2000; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998a; Dubrova *et al.*, 2000a), elevated mutation rate after exposure to chemical mutagens can be detected robustly in very small numbers of offspring.

3.4 ESTR mutation induction by etoposide

3.4.1 Introduction

Following the analysis of the alkylating agents ENU and iPMS, it was decided to extend this study to another chemical mutagen, the anticancer drug etoposide. Etoposide belongs to a group of blocking agents (see 1.4.3). It is a semi-synthetic podophyllotoxin derivate widely used in cancer chemotherapy (Liu, 1989; Smith, 1990). ET does not directly affect DNA and mutation induction is attributed to the inhibition of the enzyme topoisomerase-II (topo-II). This inhibition stabilizes a ternary complex, ET/topo-II/DNA, known as cleavable complex. This complex prevents the re-joining of the DSBs generated by topo-II (Anderson and Berger, 1994; Ferguson and Baguley, 1994; Ferguson and Baguley, 1996; Smith, 1990). Blockage of the rejoining activity of topo-II by cleavable complexes, results in DNA DSBs that lead to disruption of chromosomal integrity and formation of chromosomal aberrations (Lee *et al.*, 1995; Suzuki *et al.*, 1995; Suzuki *et al.*, 1997a).

Blocking agents have not previously been studied using ESTR analysis, but it has been shown that ET is a germline mutagen with unique properties in both maternal and paternal gametes of mice. Increases in both aneuploidy and chromosomal structural aberrations have been observed in ET treated female germ cells (Mailhes and Marchetti, 1994; Mailhes *et al.*, 1994; Mailhes *et al.*, 1996). In male germ cells, using micronucleus test, ET has been shown to induce fragmentation of centromeric DNA specifically in primary spermatocytes (Kallio and Lahdetie, 1993; Kallio and Lahdetie, 1996; Lahdetie *et al.*, 1994); similarly, studies of germline mutagenicity using the SLT and the DLT, proved that ET exhibits significant mutagenicity in primary spermatocytes (Russell *et al.*, 1998; Russell *et al.*, 2000a). For its mode of action and the narrow window of mutation induction in the mouse germline detected with the SLT and the DLT (Russell *et al.*, 1998; Russell *et al.*, 2000a), the analysis of ET was thought to be the ideal model to test the capability of ESTR loci for the analysis of germline mutation induction.

As previously stated, ESTR mutations are believed to be a by-product of DNA repair activity of DSBs (see 1.6). As ET blocks the re-joining activity of topo-II enzyme generating DNA DSBs (Liu, 1989), it could, theoretically, be possible to obtain increases in paternal ESTR mutation rate in all stages of spermatogenesis.

3.4.2 Experimental design

Male mice were given a single dose of 80 mg/kg of etoposide and were subsequently mated to non-exposed females two (days 14 to 17), three (days 21 to 24), four (days 28 to 31), five (days 35 to 38), and six weeks (days 42 to 45) after treatment (Table 3-2); corresponding to the cellular stages: spermatids; secondary spermatocytes and primary spermatocytes at diakinesis, diplotene or late pachytene; primary spermatocytes at early pachytene or zygotene; primary spermatocytes at preleptotene, type B spermatogonia and intermediate spermatogonia; and type A spermatogonia respectively (Searle, 1974). This mating scheme was chosen because of the previously reported very narrow stage-specificity of germline mutation induction by ET (Kallio and Lahdetie, 1993; Kallio and Lahdetie, 1996; Lahdetie *et al.*, 1994; Russell *et al.*, 1998).

3.4.3 ESTR mutation rates in male mice exposed to ET

3.4.3.1 Post-meiotic stages (2 weeks)

The first cellular stage analysed were spermatids, those cells correspond to post-meiotic stages of spermatogenesis. One-hundred offspring derived from 9 males were analysed (Table 3-8). The analysis of offspring derived from post-meiotic spermatids did not reveal any increase in paternal mutation rate at ESTR loci (Table 3-9).

3.4.3.2 Late meiotic and early post-meiotic stages (3 weeks)

The analysis of the late stages of meiosis and the initial stages of post-meiosis correspond to the cellular stages of primary spermatocytes at late pachytene, diplotene or diakinesis, and secondary spermatocytes. Seventy-nine offspring derived from 12 treated males were profiled (Table 3-8), the analysis of these offspring showed that ET does not lead to increased mutation frequency at ESTR loci in these cellular stages (Table 3-9).

Table 3-8 Summary of mutation data for male mice exposed to ET.

Group	No. males	No. offspring	*Ms6-hm**		*Hm-2**		*Ms6-hm + Hm-2**	
			Paternal	Maternal	Paternal	Maternal	Paternal	Maternal
Control	19	121	5(5)	9 (9)	7 (7)	8 (6)	12 (12)	17 (15)
Etoposide								
2 weeks	9	100	11 (7)	9 (9)	3 (3)	7 (7)	14 (12)	16 (16)
3 weeks	12	79	7 (7)	7 (7)	2 (2)	1 (1)	9 (9)	8 (8)
4 weeks	11	82	23 (21)	8 (4)	9 (9)	4 (4)	32 (30)	12 (8)
5 weeks	10	76	20 (12)	7 (7)	6 (6)	1 (1)	26 (18)	8 (8)
6 weeks	11	95	14 (14)	4 (4)	3 (3)	5 (5)	17 (17)	9 (9)

* Numbers of singleton mutations are given in parentheses.

Table 3-9 ESTR mutation rates in male mice exposed to ET.

	All maternal mutations			All paternal mutations			Paternal singletons		
Group	**Rate**	**Ratio***	**Prob†**	**Rate**	**Ratio***	**Prob†**	**Rate**	**Ratio***	**Prob†**
Control	0.0702	-	-	0.0496	-	-	0.0496	-	-
Etoposide									
2 weeks	0.0800	1.14	0.8329	0.0700	1.41	0.4798	0.0500	1.01	1
3 weeks	0.0506	0.72	0.5679	0.0570	1.15	0.9137	0.0570	1.15	0.9137
4 weeks	0.0731	1.04	1	0.1951	3.94	**8.94×10^{-6}**	0.1829	3.69	**3.55×10^{-5}**
5 weeks	0.0526	0.74	0.6355	0.1710	3.45	**0.0002**	0.1184	2.39	**0.0224**
6 weeks	0.0473	0.67	0.4324	0.0895	1.80	0.1478	0.0895	1.80	0.1478

*Ratio to mutation rate in control.

† Probability of difference from the control group (Fisher's exact test, two-tailed). Statistically significant values are given in bold.

3.4.3.3 Meiotic stages (4 weeks)

The analysis of meiotic stages was studied using 82 offspring derived from treated primary spermatocytes at zygotene or early pachytene (Table 3-8). This analysis revealed a statistically significant 3.94-fold increase in paternal mutation rate (Table 3-9). To rule out the possibility of statistically significant increase in paternal mutation rate due to germline mosaicism, analysis of singletons were performed. This analysis resulted in a statistically significant 3.69-fold increase (P=3.55x10^{-5}).

3.4.3.4 Late pre-meiotic and early meiotic stages (5 weeks)

This mating point allowed analysis of mutation induction in intermediate spermatogonia, type B spermatogonia, and primary spermatocytes at preleptotene. Similar to meiotic stages, the analysis of 76 offspring derived from 10 treated males, showed a statistically significant increase in ESTR mutation rate. This increase was 3.45-fold higher than that found in the control group (Table 3-9). The possibility of increase due to paternal mosaicism was excluded with the analysis of singletons, which revealed a statistically significant 2.39-fold increase.

3.4.3.5 Pre-meiotic stages (6 weeks)

The analysis of pre-meiotic stages of spermatogenesis was performed in type A spermatogonia. For the analysis of mutation induction at this stage, 95 offspring derived from 11 males were profiled (Table 3-8). Similar to the data obtained in post-meiotic and late meiotic stages, the statistical analyses of pre-meiotic stages showed that the mutation frequency after exposure to ET did not statistically differ from that found in the control group (Table 3-9).

3.4.4 Discussion

Using several systems for mutation detection, germline mutation induction in male mice exposed to ET has previously been shown. The results of these studies show that ET is a very specific germline mutagen as it only affects primary spermatocytes (Kallio and Lahdetie, 1993; Kallio and Lahdetie, 1996; Lahdetie *et al.*, 1994; Russell *et al.*, 1998; Russell *et al.*, 2000a). However, the only reliable data on male mice germline mutation induction *in vivo* were generated by the DLT (Russell *et al.*, 1998) (Figure 3-8).

The analysis of ESTR mutation induction in mouse germline treated with 80 mg/kg of ET showed the same pattern of mutation induction as previously described by the DLT (Russell *et al.*, 1998) (Figure 3-9). Russell *et al.* found decreases in average litter size specifically 4 weeks after exposure followed by a gradual recovery through weeks five and six (Figure 3-8). Given that the litter size generally reflects the mortality among the offspring of exposed parents, these results were interpreted as an evidence for induction of dominant-lethal mutations in the germline of treated males. The apparent resemblance therefore shows a remarkable similarity in the stage-specificity of mutation induction between ESTR loci and loci affecting the litter size.

The very narrow stage-specificity of mutation induction by ET may be explained by the differential activity of topo-II during mouse spermatogenesis. Thus, Northern blot analysis of topo-II gene transcripts from testes has revealed that the level of topo-II transcripts steadily increases from type A and/or B spermatogonia, reaching its maximum in pachytene spermatocytes (Cobb *et al.*, 1997). It should be noted that exposure to ET results in the highest yield of dominant-lethal and ESTR mutations in males treated on this stage of mouse spermatogenesis.

The results of recent studies show that topo-II plays an important role in meiosis. For example, the analysis of the frequency of crossing-over after exposure to ET has revealed the induction aneuploidy from late pachytene to diakinesis and the reduction of crossing-over in early- and mid-pachytene stages (Russell *et al.*, 2000a). It has also been established that in *Saccharomyces cerevisiae* meiotic recombination is initiated by DNA DSBs, likely created by Spo 11 (Bergerat *et al.*, 1997; Keeney *et al.*, 1997), a type II topoisomerase homologous to a family of type-II topoisomerases known

as topoisomerase VI. Topo-II is required for homolog pairing (Loidl *et al.*, 1994; Weiner and Kleckner, 1994), as well as for axial element and synaptonemal complex (SC) formation (Dresser and Giroux, 1988; Giroux, 1988; Klein *et al.*, 1992; Moens and Earnshaw, 1989). The SC is a major chromosomal component that is unique to meiotic prophase (Moses, 1981). It is thought to be essential but not sufficient for crossing-over, and is also associated with homologous chromosome recognition, synaptic initiation, and synaptic progression. It has also been suggested that the SC serves as a regulator for meiotic chromosome segregation (Maguire, 1995). These findings correlate with the observed genotoxic effects of ET in male meiosis, which generates fragmentation of centromeres possibly due to the inhibition of the religation activity of topo-II and induction of aneuploidy as a result of failures in separation of homologous chromosomes (Kallio and Lahdetie, 1996).

The same failure in desynapsis of bivalents and malsegregation has been observed in several mice with different null mutations, including the DNA mismatch repair genes PMS2 and MLH1 (Baker *et al.*, 1995; Baker *et al.*, 1996). Similarly to topo-II, PMS2 and MLH1 are associated with the SC and are involved in meiotic synapsis and crossing-over. Null mice for those genes also showed increases in tandem repeat DNA instability. As it is widely accepted that tandem repetitive DNA expansions occur during replication (Richard and Paques, 2000), the connection between those null mice and genome instability must be through the inability to complete meiosis successfully. If the connection between inability to complete meiosis and genome instability is real, the effects of ET at meiosis could lead to genome instability causing the observed changes in ESTR loci array length.

On the other hand, the lack of mutation induction in the pre-meiotic cells could be explained by the cytotoxicity of etoposide to differentiated spermatogonia, which results in the absence of noticeable genetic effects at this stage of mouse spermatogenesis (Russell *et al.*, 1998).

Figure 3-8 Litter size in male mice exposed to etoposide (Russell *et al.*, 1998).

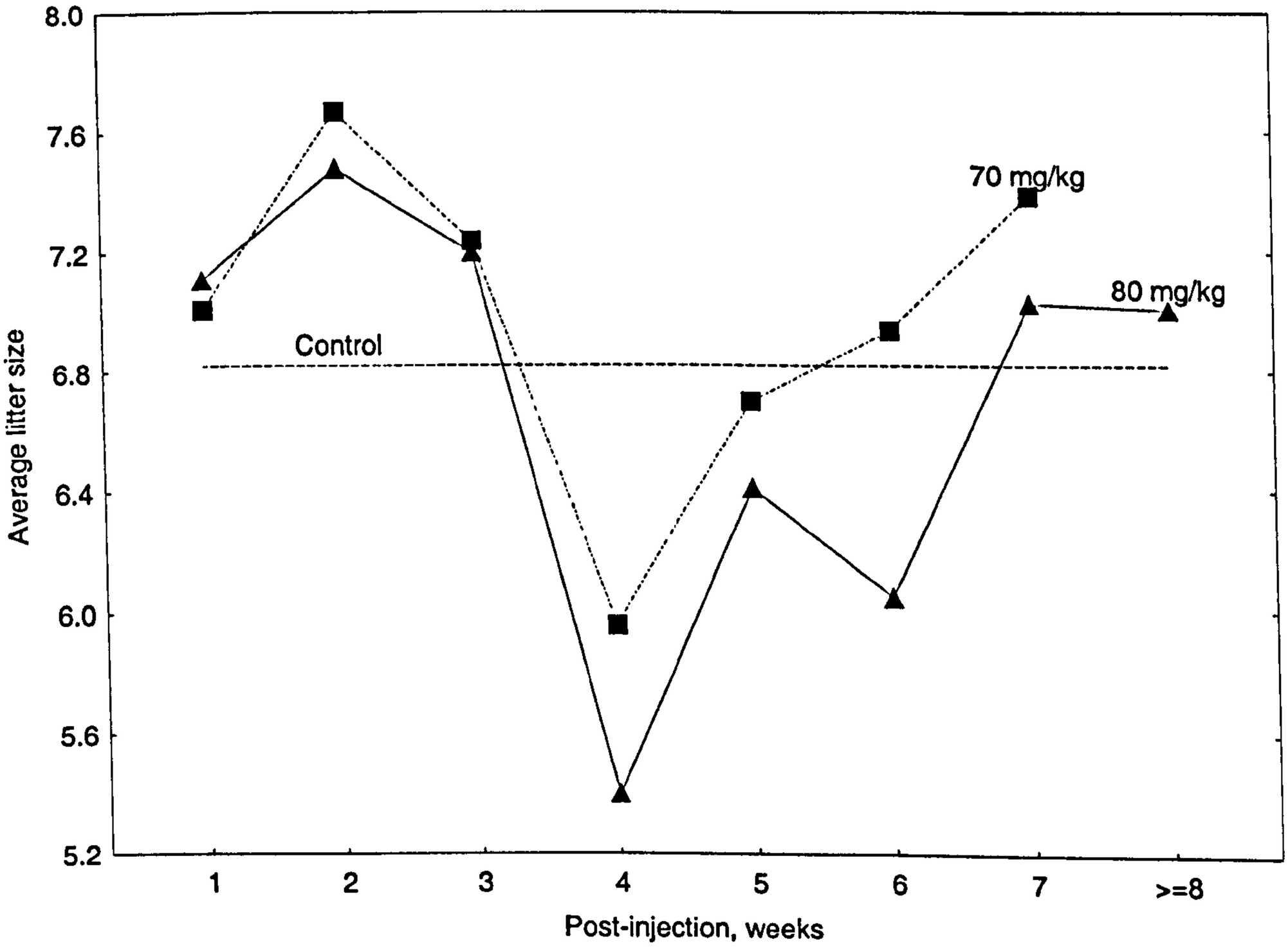
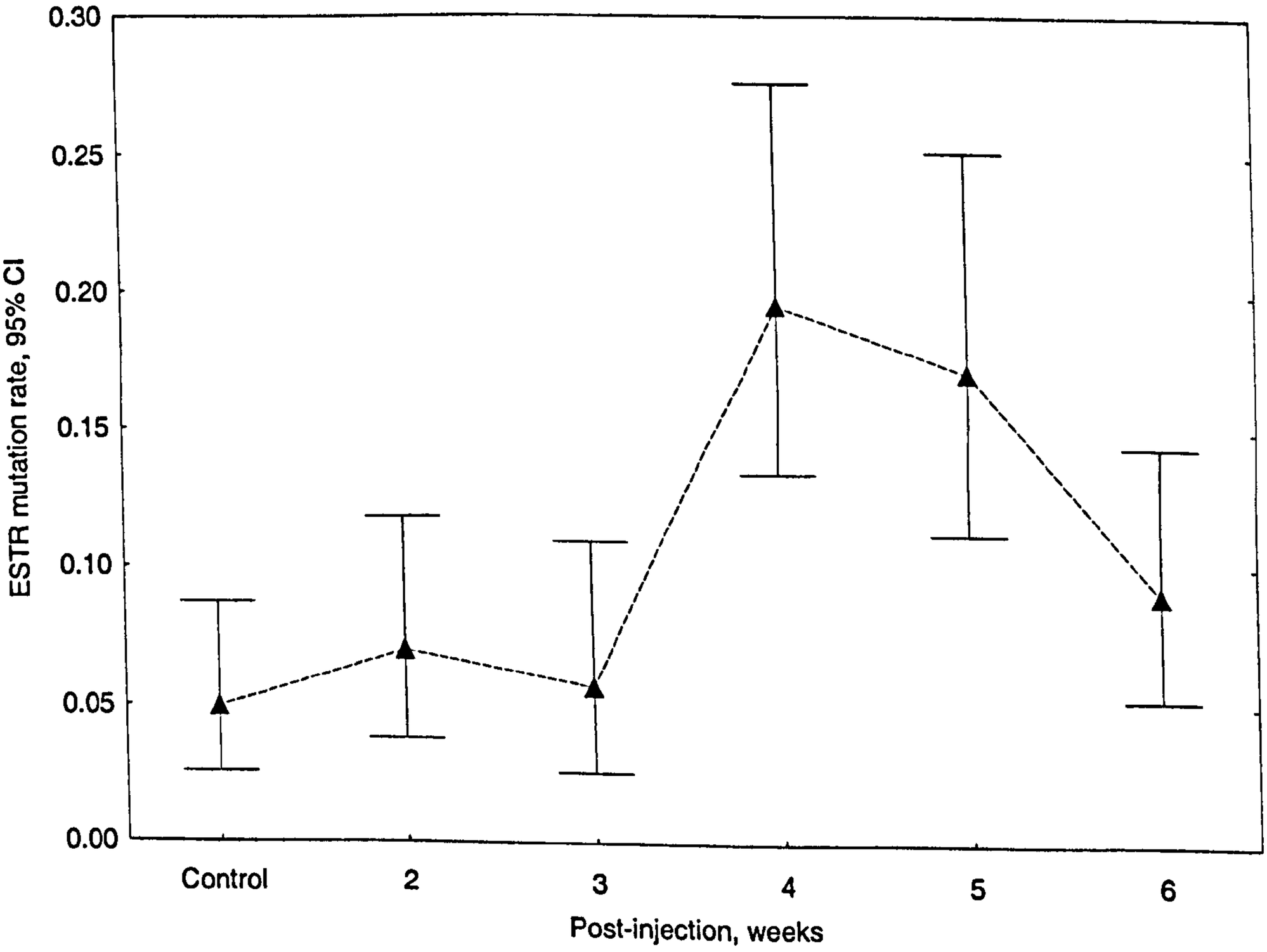


Figure 3-9 ESTR mutation rates in male mice exposed to 80 mg/kg of etoposide.



3.5 Maternal mutation rates

Statistical analysis showed that there was no increase in maternal mutation rate in the offspring derived from pre-meiotic paternal cells exposed to the three chemical mutagens (Table 3-4,-6,-9). Maternal mutation rate is used as a secondary control to ensure that any variance in mutation rate is due to the treatment and not to environmental factors. One case of decreased maternal mutation rate was observed, as this is an isolated event that has never been replicated in any of the studies of germline mutation induction at ESTR by chemicals or ionising radiation, it is considered a statistical artefact. A similar lack of maternal mutation induction in irradiated spermatids has been shown (Dubrova *et al.*, 1998a; Fan *et al.*, 1995; Sadamoto *et al.*, 1994). In contrast, small statistically significant increases in maternal mutation rate at ESTR loci have been found in irradiated spermatozoa (Fan *et al.*, 1995; Niwa and Kominami, 2001; Sadamoto *et al.*, 1994). This stage of spermatogenesis has not been included in these experiments.

3.6 Mechanisms of ESTR mutation induction by chemical mutagens

As previously described (see 1.6), mutation induction at ESTR loci by ionising radiation cannot be attributed to the direct targeting of these small genomic loci (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Sadamoto *et al.*, 1994). The main argument for non-targeted mechanisms is that an unrealistically high amount of DNA damage per genome would be required to explain the mutation induction at these loci (Dubrova *et al.*, 1998a). From these publications it also follows that the mechanism of mutation induction at ESTR loci is still unknown. That is why at the beginning of this project it was assumed that this study could generate useful data on the mechanisms of ESTR mutation induction.

The results of this study show that pre-meiotic exposure to alkylating agents dramatically increases ESTR mutation rate in the germline of treated males. Monofunctional alkylating agents, including ENU and iPMS, react with a variety of nucleophilic sites in DNA leading to different types of adducts (Li *et al.*, 1990; Shibuya *et al.*, 1982). Being mostly point mutagens, these chemicals can also induce DNA lesions, leading to elevated frequency of SCE and CA in mammalian cells (Natarajan *et al.*, 1984). It has been suggested that the ability of alkylating agents to induce CA and recombination may be attributed either to the appearance of fragile alkali-labile sites

(Friedberg *et al.*, 1995) or to the conversion of alkylation-induced DNA damage into DSBs during DNA replication (Galli and Schiestl, 1999). Therefore, although the initial damage generated by monofunctional alkylating agents is mainly represented by chemical adducts, these adducts could also lead to the induction of DSBs and mutation induction at ESTR loci.

The data presented here show that the observed increase in ESTR mutation rates in male mice exposed to ENU or iPMS appears highly unlikely to be as a result of the accumulation of alkylation-induced DNA damage within these loci. The direct measurements of the total DNA damage to the mouse testis show that exposure to 10 mg/kg of ENU results in approximately 2 alkylations per 10^6 nucleotides (Sega *et al.*, 1986). Given that the *Ms6-hm* and *Hm-2* loci together represent a ~$2x10^4$ base pairs target, such an exposure would lead to the accumulation of 0.04 damaged nucleotides within these loci ($2x10^{-6}$ x $2x10^4$ = 0.04). In fact, exposure to 12.5 mg/kg of ENU results in a 2.87-fold increase in ESTR mutation rate in the germline of exposed males (Table 3-4) and therefore cannot be attributed to such negligible amount of ENU-induced damage within the arrays. Moreover, if ESTR mutation induction were initiated by ENU-induced damage within these loci, then it should result in a linear dose-response over quite wide range of doses. On the contrary, the pattern of mutation induction by both alkylating agents shows a plateau at concentrations over 25 mg/kg (Figure 3-4,-6). Thus, ESTR mutation induction in the germline of male mice exposed to alkylating agents cannot be attributed to the direct targeting of these small genomic loci.

It has been established that ET blocks the rejoining activity of the topo-II enzyme leading to the accumulation of the DNA DSBs (Liu, 1989), indicating that they may be processed in the same way as those induced by ionising radiation. However, the results of two recent publications show that different pathways may be involved in the repair of DNA damage induced by ionising radiation and ET. It was shown that the yield of gene conversions at the MHC locus in the germline of male mice exposed to ET differs from that after exposure to ionising radiation (Hogstrand and Bohme, 1999). The authors found a linear dose-response for gene conversions in the cells exposed to ET and the lack of dose-dependent increase in the irradiated cells, which could reflect differences either in the initial DNA damage by these mutagens or the way this damage

is repaired. In addition, Froelich *et al.* have shown differences in the persistence of DNA strand breaks depending on their origin. The authors observed that the repair of strand breaks generated by various chemotherapeutic agents is either incomplete or prolonged, while radiation-induced strand breaks are repaired faster and more completely (Froelich *et al.*, 1999). The amount of initial DNA damage by topo-II poisoning remains unknown, however, it should not exceed, to any marked extent, that induced after exposure to ionising radiation. If so, mutation induction at ESTR loci by this chemical may also reflect non-targeted events.

Taken together, the results of this study and previous publications (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Sadamoto *et al.*, 1994) strongly indicate that mutation induction at ESTR loci by a wide range of mutagens results from the initial mutagen-related DNA damage elsewhere in the genome and later indirect mutation at these loci. The mechanisms of this non-targeted process remain unknown and may include DNA repair or other factors.

3.7 Conclusion

The main aim of this project was to determine whether ESTR loci can be used for monitoring chemically-induced mutations in the mouse germline. The results of this study provide the first compelling experimental evidence that ESTR mutation rate is increased following exposure to chemical mutagens and demonstrate the utility and sensitivity of ESTR mutation as a biomonitoring system for assessing meiotic and pre-meiotic mammalian germline chemical mutagenicity at very low doses and in small sample sizes. This resemblance in the pattern of mutation induction between ESTR loci to those observed with traditional systems clearly illustrates the suitability of ESTR loci to reflect DNA damage in the mouse germline after pre-meiotic and meiotic exposure to chemical mutagens. Moreover, it shows that these loci provide an efficient experimental system for detection of the genetic effects induced by chemical mutagens. Most importantly, the results of this study show that the high ESTR spontaneous mutation rate can robustly detect increases in germline mutation after very low doses of exposure to chemical agents and in very small samples sizes, capable of providing new insights into the estimation of germline effects of chemical mutagens.

4 Gene expression

4.1 Introduction

To date, it is known that changes in ESTR length are not a direct effect of the capability of ionising radiation or chemical agents to generate genetic damage. However, there is little evidence of the mechanism by which point mutations in DNA could lead to insertion/deletion events at ESTR loci. It has been hypothesised that those changes could be associated with DNA repair activity which would generate genome instability and the recombination-like events necessary to induce mutations at ESTR loci (see 1.6).

Since changes in ESTR length are only detectable in offspring derived from exposed pre-meiotic stages of spermatogenesis, it was considered likely that the recombination-like event occurring at ESTR could be linked to increases in meiotic crossing-over. However, no correlation was found between ESTR mutation rate and frequency of crossing-over in unexposed mice and those exposed to X-rays or cisplatin (Barber *et al.*, 2000)

As one approach to gain insight into the mechanisms of mutagenesis at ESTR loci, it was decided to run a pioneer study on the analysis of changes in gene expression after exposure to the chemical mutagen ENU. The results of this experiment were expected to generate the knowledge for future experimental approaches and demonstrate possible genes that might be involved in the connection between direct DNA mutagenesis by chemicals and changes in ESTR length.

The most feasible system, at the present time, for the analysis of changes in gene expression induced by chemical mutagens, is cDNA microarray analysis.

The basic mechanism of microarray analysis is based on competition for binding to a specific expressed sequence tag (EST) bound to the array, between cDNA from untreated and treated mice (Figure 4-1). Different fluorescent labelling is used for control (normally Cy3, green fluorescence) and treated (normally Cy5, red fluorescence) cDNA. If the levels of RNA of a specific gene are not affected by the exposure, the proportion of Cy3 and Cy5-labelled cDNA should be even, giving as a result yellow fluorescence at the position of the EST for that gene; however, if the

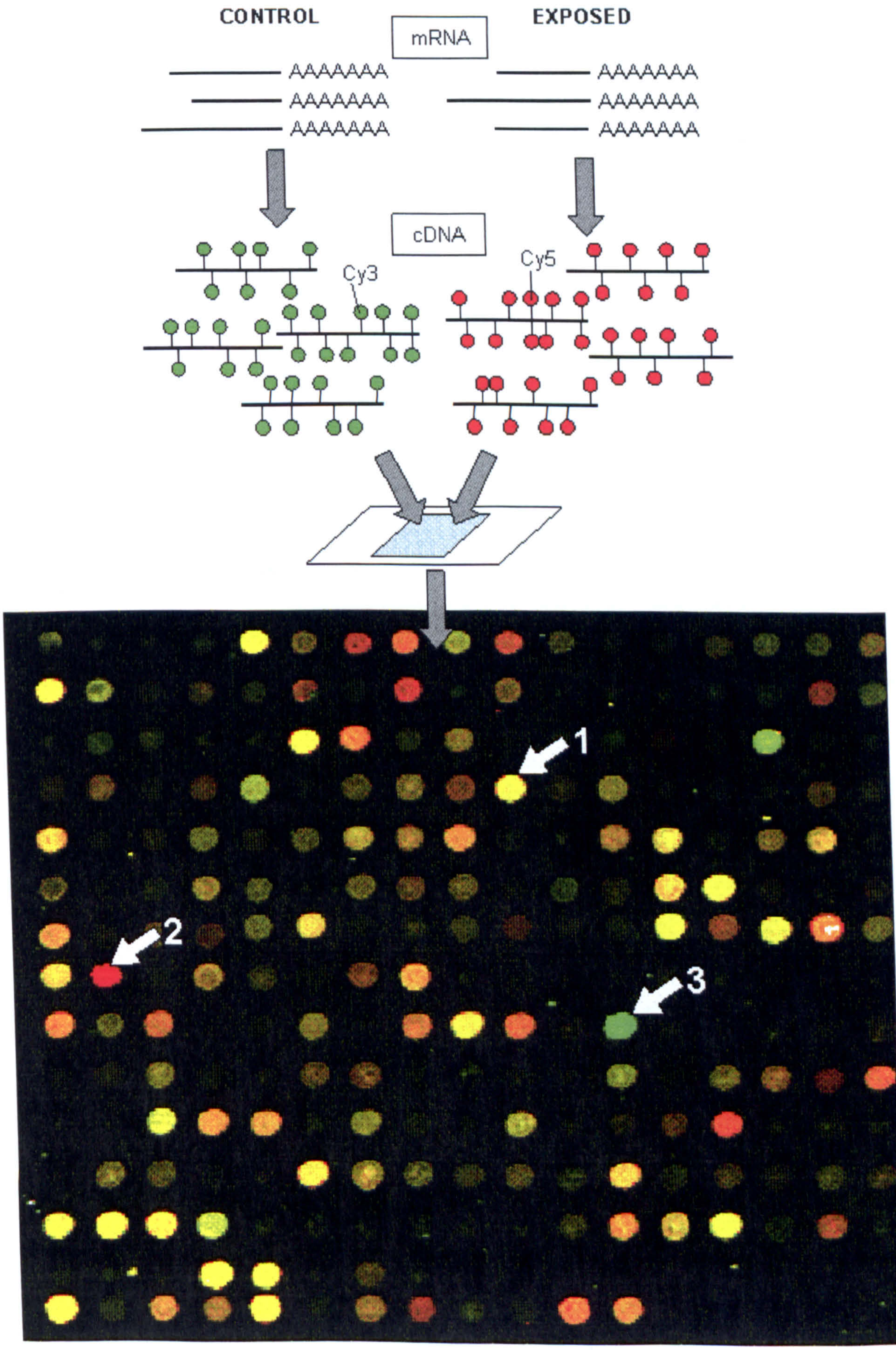


Figure 4-1 Procedure and example of a microarray. 1, gene expression not affected; 2, induction of transcription; 3, inhibition of transcription.

levels of RNA in the treated mice are elevated, the proportion of Cy5-labelled cDNA will be higher than Cy3-labelled cDNA, resulting in red fluorescence in the array. In the event of decreased levels of RNA as a result of exposure, green fluorescence should be observed. In addition, the level of gene induction/repression can be evaluated measuring the intensities of the colours, as different proportions of cDNA will generate different intensities.

4.2 Experimental design

As a pioneer study, one dose (50 mg/kg) of one chemical mutagen (ENU) was analysed. ENU was chosen, primarily, because it was previously used for the analysis of germline mutation induction at ESTR loci resulting in elevated mutation rate. In addition, ENU is one of the best characterised chemicals in terms of the genetic damage induced and the DNA repair mechanisms used to repair that damage (Friedberg *et al.*, 1995; Sega *et al.*, 1986; van Zeeland, 1996).

Similarly, the dose selected for this analysis was also used for the analysis at ESTR loci. The level of induction obtained in pre-meiotic stages of spermatogenesis with this dose, was sufficient to reach the plateau of mutation induction (see 3.3.3).

The analysis of changes in gene expression was performed using cDNA synthesised from gross testis extracted 6 or 24 hr after exposure. These two time points were believed to give an adequate time window to allow the generation of damage by ENU and the modification of the pattern of gene expression in response to the damage.

Twenty CBA/Ca male mice divided in two groups were used in this experiment. One group contained 10 mice intraperitoneally injected with a dose of 50 mg/kg of ENU. The 10 remaining mice were injected with PBS forming a control group. Five animals from each group were sacrificed 6 hours after injection and the remainder 24 hours after injection. At the time of culling testes were removed and frozen. The analysis of patterns of gene expression was performed using cDNA synthesised from testicular RNA.

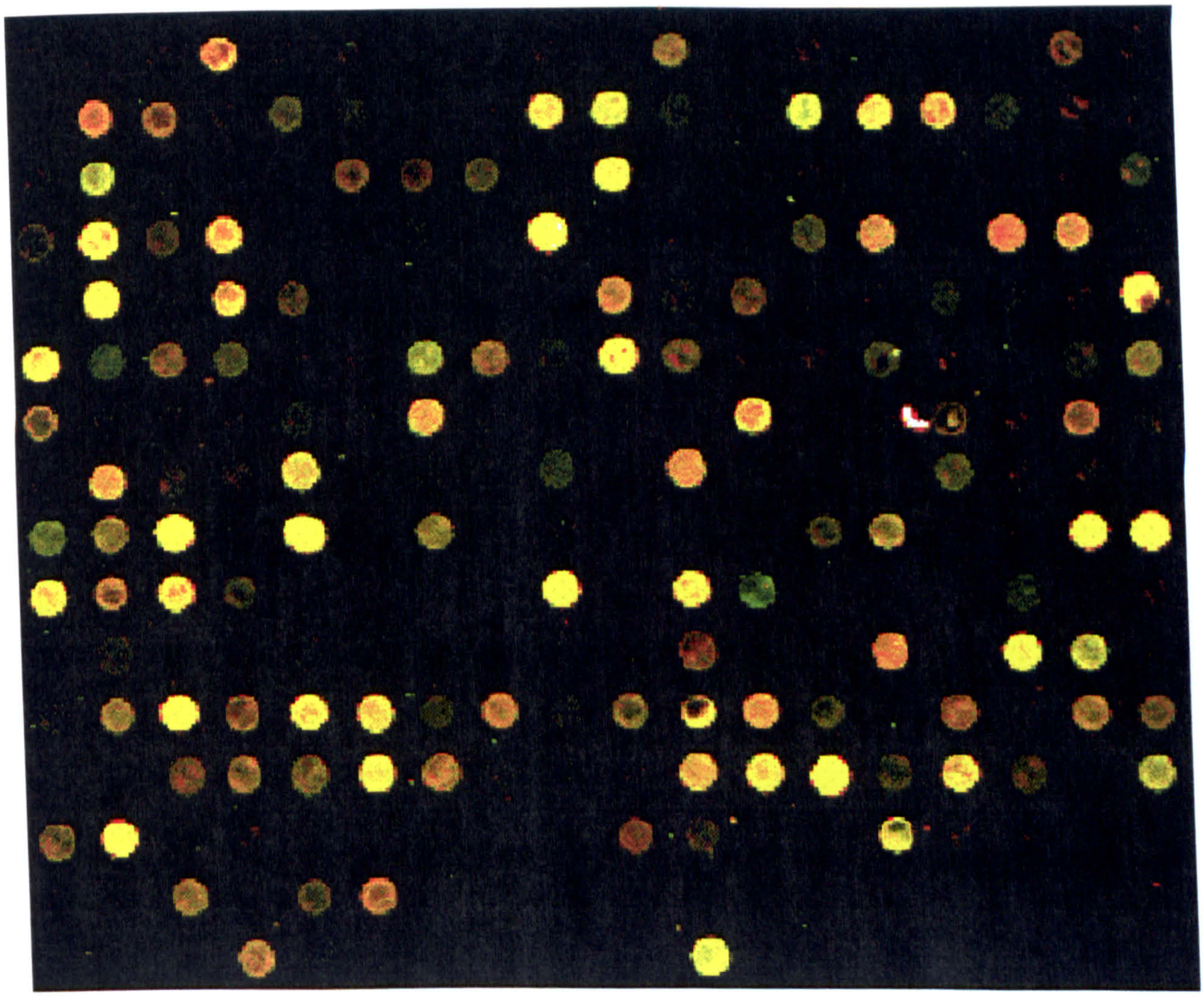


Figure 4-2 Example of microarray hybridised with cDNA from untreated mice (Cy3) and mice exposed to 50 mg/kg of ENU (Cy5).

4.3 Results

The objective of this experiment was to reveal any changes in the patterns of gene expression which might shed light on the molecular mechanism of mutation induction at ESTR loci. Since the observed changes in ESTR loci length are hypothetically associated with DNA repair and recombination-like mechanisms, the analysis of genes involved in these processes was considered a key factor. It would also seem very relevant to study genes involved in the control of the cell cycle and apoptosis as the mutational process might take place at meiosis.

Despite the expectations placed on this experiment, the visual analysis of the arrays did not allow the detection of any clear difference in the pattern of gene expression between treated and control group six or twenty-four hours after exposure, as none of the features were clearly red or green (Figure 4-2).

The statistical analysis of the cDNA ratios, from control and exposed mice, confirmed the lack of marked changes in gene expression 6 and 24 hr after exposure. Similarly, no difference was detected between the two time points analysed within the same group (data not shown). Tables 6-1 to 6-4 show a summary of the ratio of gene expression between control and treated group for genes coding for proteins involved in DNA repair, recombination, cell cycle control and apoptotic processes. Full data on the expression of genes analysed is available in the Microsoft Excel 2000 "Appendix" file in the enclosed CD.

Table 4-1 Analysis of ESTs from DNA repair genes in treated and control samples.

Name†	Average 6hr*	SD	Average 24hr*	SD	Average Total*	SD
Apex	**1.176**	0.238	**1.285**	0.287	**1.231**	0.265
Brca1	**0.720**	0.287	**1.057**	0.365	**0.846**	0.340
Cd20	**0.385**	0.035	**0.700**	-	**0.490**	0.184
Cdk7	**1.016**	0.344	**1.148**	0.064	**1.082**	0.244
Cry1	**1.186**	0.202	**1.174**	0.222	**1.180**	0.200
Csnk1a1	**1.104**	0.328	**1.000**	0.175	**1.052**	0.254
Ercc1	**0.998**	0.286	**1.515**	0.772	**1.257**	0.625
Ercc5	**0.958**	0.253	**1.270**	0.503	**1.100**	0.413
Fancc	**1.612**	0.275	**1.048**	0.277	**1.330**	0.395
G22p1	**0.873**	0.245	**1.045**	0.242	**0.954**	0.252
Gas2	**0.868**	0.292	**1.035**	0.232	**0.942**	0.266
Gtf2h1	**0.945**	0.246	**1.201**	0.227	**1.073**	0.267
Gtf2h2	**1.120**	0.338	**1.233**	0.927	**1.176**	0.649
Hells	**0.945**	0.204	**1.034**	0.141	**0.994**	0.167
Itih1	**0.580**	0.177	**0.507**	0.203	**0.540**	0.186
Itih2	**0.874**	0.262	**0.955**	0.569	**0.910**	0.397
Lig1	**0.800**	0.284	**1.620**	0.495	**1.073**	0.526
Lig3	**0.800**	0.158	**1.414**	0.516	**1.107**	0.487
Mgmt	**1.112**	0.339	**1.160**	0.066	**1.136**	0.232
Mpg	**1.057**	0.266	**1.206**	0.214	**1.126**	0.250
Msh2	**1.170**	0.193	**1.001**	0.319	**1.091**	0.266
Msh3	**0.714**	0.357	**2.300**	2.740	**1.461**	2.003
Msh6	**0.904**	0.221	**1.038**	0.174	**0.971**	0.200
Ubce7ip3	**0.620**	0.270	**1.285**	0.817	**0.916**	0.640
Nthl1	**0.880**	0.223	**0.886**	0.310	**0.883**	0.259
Ogg1	**0.901**	0.185	**1.027**	0.287	**0.959**	0.241
Pcna	**1.240**	0.197	**1.140**	0.195	**1.190**	0.200
Pcolce	**1.254**	0.243	**1.076**	0.159	**1.165**	0.215
Pcp4	**0.664**	0.153	**0.625**	0.106	**0.653**	0.133
Pms2	**0.765**	0.199	**1.437**	0.438	**1.053**	0.461
Rad1	**1.120**	0.282	**1.046**	0.181	**1.085**	0.236
Rad23b	**0.374**	0.175	**1.081**	0.745	**0.707**	0.625
Rad50	**0.750**	0.235	**0.976**	0.769	**0.863**	0.559
Rad51	**0.624**	0.266	**1.170**	0.635	**0.867**	0.519
Rad51ap1	**1.044**	0.150	**1.132**	0.112	**1.088**	0.133
Rad51l3	-	-	**0.960**	0.000	**0.960**	0.000
Rad52	**1.020**	0.335	**1.260**	-	**1.068**	0.310

Rag1	**0.710**	0.150	**0.733**	0.144	**0.720**	0.138
Rbbp4	**1.060**	0.283	**1.121**	0.271	**1.087**	0.272
Top1	**0.908**	0.212	**1.312**	0.459	**1.095**	0.399
Top2a	**0.990**	0.220	**1.120**	0.201	**1.055**	0.210
Top2b	**0.949**	0.274	**1.549**	0.911	**1.211**	0.683
Ung	**1.052**	0.199	**0.880**	0.083	**0.999**	0.187
Xpa	**0.897**	0.207	**1.116**	0.294	**1.026**	0.278
Xpc	**0.830**	0.144	**0.915**	0.593	**0.864**	0.373
Xrcc1	**0.962**	0.241	**1.295**	0.195	**1.110**	0.272
Xrcc5	**0.986**	0.398	**1.328**	0.494	**1.157**	0.470

Table 4-2 Analysis of ESTs from recombination genes in treated and control samples.

Name†	Average 6hr*	SD	Average 24hr*	SD	Average Total*	SD
Brca1	**0.720**	0.287	**1.057**	0.365	**0.846**	0.340
Clu	**1.260**	0.263	**1.142**	0.164	**1.201**	0.221
Fancc	**1.612**	0.275	**1.048**	0.277	**1.330**	0.395
Fcgr2b	**1.190**	0.265	**1.232**	0.196	**1.211**	0.221
Fen1	**0.960**	0.186	**1.570**	0.386	**1.231**	0.420
G22p1	**0.873**	0.245	**1.045**	0.242	**0.954**	0.252
Gypa	**0.460**	0.141	**1.575**	0.867	**1.203**	0.887
Hmg1	**0.936**	0.277	**1.323**	0.460	**1.115**	0.414
Lig1	**0.800**	0.284	**1.620**	0.495	**1.073**	0.526
Lig3	**0.800**	0.158	**1.414**	0.516	**1.107**	0.487
Prkdc	**0.993**	0.300	**1.164**	0.440	**1.071**	0.424
Psmc3	**1.326**	0.237	**1.174**	0.273	**1.250**	0.254
Rad50	**0.750**	0.235	**0.976**	0.769	**0.863**	0.559
Rad51	**0.624**	0.266	**1.170**	0.635	**0.867**	0.519
Rad51l3	**-**	-	**0.960**	0.000	**0.960**	0.000
Rad52	**1.020**	0.335	**1.260**	-	**1.068**	0.310
Rad54l	**0.951**	0.258	**1.086**	0.325	**1.023**	0.294
Rad9	**1.480**	0.481	**1.157**	0.402	**1.341**	0.446
Rag1	**0.710**	0.150	**0.733**	0.144	**0.720**	0.138
Rbpsuh	**0.507**	0.234	**1.335**	0.763	**0.980**	0.711
Rfp	**1.376**	0.198	**1.192**	0.196	**1.284**	0.215
Sil	**0.836**	0.255	**1.473**	0.820	**1.075**	0.581
Tsn	**0.948**	0.287	**0.957**	0.192	**0.951**	0.242

Table 4-3 Analysis of ESTs from cell cycle genes in treated and control samples.

Name†	Average 6hr*	SD	Average 24hr*	SD	Average Total*	SD
Abl1	**0.682**	0.241	**1.132**	0.178	**0.862**	0.310
Ank1	**0.965**	0.078	**1.055**	0.021	**1.010**	0.070
Bcat1	**0.640**	0.269	**1.160**	-	**0.813**	0.355
Bcl2	**0.605**	0.007	**1.005**	0.267	**0.872**	0.292
Bcl3	**1.256**	0.389	**4.125**	5.669	**2.299**	3.439
Brca1	**0.720**	0.287	**1.057**	0.365	**0.846**	0.340
Cbx1	**0.894**	0.365	**1.028**	0.423	**0.953**	0.386
Cbx5	**0.885**	0.252	**1.480**	0.862	**1.140**	0.617
Ccna2	**1.044**	0.431	**1.665**	0.787	**1.331**	0.674
Ccnb1-rs1	**0.965**	0.384	**0.664**	0.263	**0.828**	0.355
Ccnc	**0.970**	-	**2.053**	1.145	**1.836**	1.104
Ccnd1	**0.682**	0.486	**1.236**	0.457	**0.944**	0.540
Ccnd3	**1.200**	0.215	**1.330**	0.171	**1.265**	0.196
Ccne1	**0.965**	0.035	**0.965**	0.191	**0.965**	0.112
Ccnf	**0.894**	0.304	**1.774**	2.172	**1.334**	1.534
Ccng	**1.357**	0.130	**1.428**	0.257	**1.397**	0.201
Cdc20	**1.236**	0.259	**1.068**	0.171	**1.156**	0.232
Cdc25a	**0.973**	0.355	**1.012**	0.289	**0.988**	0.322
Cdc25b	**0.605**	0.474	**0.730**	-	**0.647**	0.343
Cdc25c	**1.136**	0.181	**1.241**	0.334	**1.183**	0.257
Cdc2a	**1.248**	0.302	**1.201**	0.543	**1.226**	0.418
Cdc2l2	**1.048**	0.146	**1.550**	1.149	**1.236**	0.718
Cdc42	**0.850**	0.261	**1.024**	0.190	**0.937**	0.239
Cdc6	**0.809**	0.281	**0.949**	0.441	**0.876**	0.364
Cdc7l1	**0.964**	0.279	**0.981**	0.303	**0.971**	0.284
Cdk2	**1.140**	0.129	**1.205**	0.349	**1.169**	0.235
Cdk5	**0.909**	0.215	**1.158**	0.274	**1.049**	0.274
Cdk5r	**0.786**	0.261	**0.958**	0.120	**0.862**	0.218
Cdk7	**1.016**	0.344	**1.148**	0.064	**1.082**	0.244
Cdkn1a	**0.671**	0.186	**0.968**	0.512	**0.808**	0.395
Cdkn1c	**1.424**	0.105	**1.248**	0.215	**1.336**	0.185
Cdkn2b	**1.433**	0.470	**1.413**	0.410	**1.423**	0.395
Cks1	**1.372**	0.469	**3.040**	2.588	**1.849**	1.388
Clu	**1.260**	0.263	**1.142**	0.164	**1.201**	0.221
Dnmt1	**0.924**	0.226	**0.921**	0.170	**0.923**	0.195
E2f3	**0.690**	0.071	**0.635**	0.120	**0.663**	0.087
Emd	**1.050**	0.308	**1.115**	0.318	**1.076**	0.272

Fancc	**1.612**	0.275	**1.048**	0.277	**1.330**	0.395
Farsl	**0.902**	0.289	**1.414**	0.377	**1.158**	0.416
Frat1	**1.246**	0.203	**1.340**	0.253	**1.293**	0.222
Frda	**1.042**	0.340	**1.421**	0.981	**1.232**	0.738
G22p1	**0.873**	0.245	**1.045**	0.242	**0.954**	0.252
Gas2	**0.868**	0.292	**1.035**	0.232	**0.942**	0.266
Gfi1	**0.917**	0.232	**1.057**	0.157	**0.982**	0.206
Hdac2	**0.956**	0.299	**1.196**	0.149	**1.076**	0.261
Hipk3	**1.000**	0.333	**1.288**	0.195	**1.144**	0.299
Hira	**1.034**	0.338	**1.056**	0.605	**1.048**	0.510
Hmga2	**1.014**	0.213	**1.532**	0.610	**1.273**	0.510
Hmmr	**0.814**	0.218	**0.750**	0.161	**0.782**	0.184
Itgb1	**0.890**	0.219	**1.360**	0.314	**1.099**	0.350
Lmna	**0.653**	0.213	**0.953**	0.206	**0.803**	0.252
Lmnb1	**0.792**	0.107	**1.483**	1.193	**1.051**	0.736
Lmnb2	**1.030**	0.406	**1.333**	0.788	**1.160**	0.562
Mad2l1	**0.850**	-	**0.982**	0.100	**0.960**	0.105
Mapk1	**0.712**	0.337	**1.526**	0.951	**1.150**	0.832
Mapk3	**0.626**	0.282	**1.224**	0.257	**0.925**	0.405
Mdm2	**0.809**	0.342	**1.143**	0.257	**1.002**	0.335
Mdm4	**1.077**	0.467	**1.132**	0.902	**1.111**	0.727
Meox2	**0.490**	-	**0.500**	0.127	**0.497**	0.090
Mif	**1.184**	0.375	**1.164**	0.193	**1.174**	0.290
Mki67	**1.006**	0.308	**0.986**	0.207	**0.996**	0.247
Msh2	**1.170**	0.193	**1.001**	0.319	**1.091**	0.266
Msh6	**0.904**	0.221	**1.038**	0.174	**0.971**	0.200
Myod1	**0.490**	0.096	**0.725**	0.458	**0.647**	0.386
Notch1	**-**	-	**3.230**	-	**3.230**	-
Nr4a2	**0.823**	0.075	**0.798**	0.316	**0.809**	0.228
Pcna	**1.240**	0.197	**1.140**	0.195	**1.190**	0.200
Pctk1	**1.067**	0.236	**1.227**	0.201	**1.143**	0.229
Pctk3	**0.902**	0.194	**1.428**	0.570	**1.165**	0.495
Pms2	**0.765**	0.199	**1.437**	0.438	**1.053**	0.461
Pola2	**0.520**	0.295	**1.723**	1.336	**0.971**	0.973
Ppp2ca	**1.092**	0.230	**1.042**	0.109	**1.067**	0.177
Ppp2cb	**1.228**	0.211	**1.102**	0.237	**1.165**	0.222
Rad1	**1.120**	0.282	**1.046**	0.181	**1.085**	0.236
Rad9	**1.480**	0.481	**1.157**	0.402	**1.341**	0.446
Rbl2	**0.986**	0.215	**1.300**	0.189	**1.143**	0.254
Rbms1	**1.432**	0.279	**1.304**	0.326	**1.368**	0.294
Rrm1	**1.036**	0.404	**1.122**	0.168	**1.077**	0.310

Sp100	**0.445**	0.177	**2.083**	2.311	**1.537**	1.981
Srpk2	**0.542**	0.298	**1.290**	0.533	**0.874**	0.554
Stat3	**0.658**	0.174	**1.283**	1.258	**0.970**	0.896
Stk2	**1.215**	0.714	**1.100**	1.155	**1.146**	0.894
Terf1	**1.016**	0.234	**1.176**	0.472	**1.096**	0.374
Tfdp1	**1.154**	0.182	**1.090**	0.146	**1.122**	0.159
Tff1	**0.675**	0.148	**0.833**	0.145	**0.770**	0.153
Tff3	**0.575**	0.346	**1.040**	-	**0.730**	0.363
Tgfbr1	**0.948**	0.292	**1.433**	0.924	**1.130**	0.596
Tk1	**-**	-	**0.950**	0.597	**0.950**	0.597
Tlk2	**0.900**	0.220	**1.110**	0.397	**1.012**	0.333
Top2a	**0.990**	0.220	**1.120**	0.201	**1.055**	0.210
tsg101	**1.152**	0.296	**0.968**	0.331	**1.060**	0.323
Ube2v1	**0.993**	0.312	**1.260**	0.477	**1.126**	0.419
Ung	**1.052**	0.199	**0.880**	0.083	**0.999**	0.187
Wee1	**1.018**	0.313	**1.362**	0.529	**1.190**	0.448
Xrcc5	**0.986**	0.398	**1.328**	0.494	**1.157**	0.470

Table 4-4 Analysis of ESTs from apoptotic genes in treated and control samples.

Name†	Average 6hr*	SD	Average 24hr*	SD	Average Total*	SD
Abl1	**0.682**	0.241	**1.132**	0.178	**0.862**	0.310
Akt1	**0.776**	0.281	**1.282**	0.378	**1.029**	0.412
Aplp1	**0.796**	0.226	**0.930**	0.233	**0.846**	0.223
Aplp2	**0.750**	0.177	**1.422**	0.686	**1.086**	0.590
Apoa1	**0.540**	0.232	**1.112**	0.746	**0.800**	0.582
Apoa2	**1.160**	0.167	**1.060**	0.156	**1.120**	0.152
Apoa4	**0.570**	0.190	**0.658**	0.233	**0.620**	0.203
Apobec1	**0.970**	0.235	**0.907**	0.493	**0.943**	0.332
Apobec2	**-**	-	**1.187**	0.488	**1.187**	0.488
Apoc1	**1.441**	0.089	**1.198**	0.552	**1.288**	0.451
Apoc2	**1.363**	0.212	**1.231**	0.261	**1.297**	0.241
Apod	**0.620**	0.184	**0.665**	0.318	**0.643**	0.214
Apoe	**0.988**	0.309	**1.173**	0.183	**1.076**	0.267
Apoh	**1.263**	0.254	**1.228**	0.162	**1.246**	0.208
App	**0.978**	0.230	**1.416**	0.676	**1.205**	0.550
Arnt	**0.756**	0.425	**1.134**	0.592	**0.928**	0.535
Axl	**1.090**	0.281	**1.160**	0.198	**1.125**	0.232
Bak	**0.585**	0.204	**-**	-	**0.585**	0.204

Bax	**0.822**	0.218	**1.076**	0.263	**0.949**	0.264
Bcl2	**0.605**	0.007	**1.005**	0.267	**0.872**	0.292
Bcl2l10	**1.010**	0.178	**1.230**	0.244	**1.120**	0.230
Bcl2l2	**0.872**	0.221	**0.682**	0.143	**0.785**	0.206
Bid	**0.858**	0.376	**0.887**	0.247	**0.870**	0.302
Bmp4	**0.580**	0.278	**0.600**	0.156	**0.586**	0.236
Brca1	**0.720**	0.287	**1.057**	0.365	**0.846**	0.340
Casp1	**0.762**	0.196	**0.978**	0.647	**0.875**	0.489
Casp2	**1.114**	0.322	**0.887**	0.292	**1.011**	0.323
Casp3	**0.655**	0.262	**0.637**	0.057	**0.644**	0.137
Casp6	**0.803**	0.409	**1.620**	0.085	**1.075**	0.529
Casp7	**0.430**	-	**1.940**	2.220	**1.437**	1.796
Cav2	**0.760**	0.376	**1.320**	0.453	**0.984**	0.465
Cbx4	**0.850**	0.193	**0.760**	-	**0.832**	0.172
Ccng	**1.357**	0.130	**1.428**	0.257	**1.397**	0.201
Cd36	**0.850**	-	**2.610**	-	**1.730**	1.245
Cd38	**0.640**	0.208	**1.061**	0.477	**0.874**	0.429
Cd3d	**1.149**	0.238	**1.253**	0.291	**1.192**	0.261
Cd3e	**0.850**	0.328	**1.116**	0.324	**0.983**	0.338
Cd3g	**1.442**	0.242	**1.312**	0.389	**1.377**	0.313
Cd4	**0.650**	-	**2.545**	0.601	**1.913**	1.174
Cd48	**0.787**	0.265	**2.615**	3.082	**1.831**	2.393
Cd5	**0.630**	0.113	**0.820**	0.317	**0.757**	0.269
Cd52	**1.507**	1.897	**2.298**	2.180	**2.001**	1.978
Cd59a	**1.174**	0.229	**1.590**	0.935	**1.345**	0.634
Cd6	**0.474**	0.134	**0.438**	0.185	**0.455**	0.157
Cd63	**1.042**	0.326	**1.118**	0.220	**1.076**	0.270
Cd68	**0.820**	0.195	**1.206**	0.269	**1.013**	0.301
Cd7	**0.560**	0.166	**0.613**	0.264	**0.590**	0.212
Cd83	**0.630**	0.014	**0.440**	0.283	**0.535**	0.197
Cd86	**0.480**	0.095	**1.040**	-	**0.620**	0.291
Cd8a	**0.255**	0.007	**0.740**	-	**0.417**	0.280
Cd8b	**1.257**	0.282	**1.243**	0.393	**1.250**	0.333
Cd9	**0.870**	0.120	**1.016**	0.185	**0.961**	0.171
Cdk2	**1.140**	0.129	**1.205**	0.349	**1.169**	0.235
Cdkn1a	**0.671**	0.186	**0.968**	0.512	**0.808**	0.395
Clu	**1.260**	0.263	**1.142**	0.164	**1.201**	0.221
Csf1	**0.835**	0.177	**0.480**	0.295	**0.622**	0.298
Dad1	**1.491**	0.238	**1.105**	0.132	**1.264**	0.263
Daf1	**1.290**	0.239	**1.200**	0.151	**1.245**	0.194
Dapk2	**0.620**	0.245	**0.350**	0.042	**0.543**	0.240

Daxx	**1.100**	0.290	**1.220**	0.050	**1.160**	0.206
Ddit3	**1.171**	0.224	**1.182**	0.193	**1.176**	0.206
Dffa	**0.863**	0.229	**1.250**	0.142	**1.057**	0.272
Dnase1	**1.177**	0.360	**1.147**	0.305	**1.162**	0.299
Eif2ak2	**0.375**	0.007	**0.280**	-	**0.343**	0.055
Ets2	**0.876**	0.389	-	-	**0.876**	0.389
Fadd	**0.863**	0.300	**0.970**	0.057	**0.906**	0.222
Faf1	**1.149**	0.234	**1.176**	0.297	**1.162**	0.263
Gadd45g	**0.814**	0.250	**0.710**	-	**0.797**	0.228
Gas2	**0.868**	0.292	**1.035**	0.232	**0.942**	0.266
Gzma	**0.813**	0.179	**1.186**	0.213	**1.020**	0.271
Gzmb	**0.920**	0.028	**1.245**	0.445	**1.083**	0.319
Ier3	**0.880**	0.246	**0.875**	0.049	**0.878**	0.176
Igfbp4	**0.853**	0.288	**1.200**	0.214	**1.001**	0.302
Lgals1	**1.400**	0.310	**1.116**	0.344	**1.258**	0.343
Ltbr	**0.535**	0.007	**1.140**	0.373	**0.898**	0.424
Ly64	**0.530**	0.198	**0.595**	0.361	**0.563**	0.240
Map3k1	**1.015**	0.163	**0.950**	0.234	**0.972**	0.198
Mdm2	**0.809**	0.342	**1.143**	0.257	**1.002**	0.335
Mdm4	**1.077**	0.467	**1.132**	0.902	**1.111**	0.727
Mfge8	**1.130**	0.589	**1.070**	0.225	**1.108**	0.462
Myc	**1.338**	0.468	**1.530**	0.987	**1.434**	0.756
Nfkb1	**0.828**	0.291	**1.330**	0.994	**1.079**	0.729
Nme3	**1.175**	0.156	**1.297**	0.323	**1.227**	0.226
Nrp	**0.785**	0.205	**0.870**	0.651	**0.828**	0.397
Pdcd2	**1.180**	-	**1.093**	0.198	**1.110**	0.176
Pdcd6ip	**0.860**	0.207	**0.935**	0.064	**0.890**	0.155
Pde1b	**0.933**	0.114	**1.162**	0.211	**1.076**	0.208
Pglyrp	**0.630**	0.282	**0.950**	0.212	**0.737**	0.290
Pla2g1b	**0.560**	0.170	**0.635**	0.064	**0.598**	0.113
Ptges	**1.335**	0.774	**1.235**	0.092	**1.302**	0.604
Rab6kifl	**0.906**	0.256	**1.163**	0.129	**1.018**	0.243
Req	**1.180**	0.158	**1.314**	0.380	**1.247**	0.283
Ripk1	**0.671**	0.249	**1.225**	0.504	**0.979**	0.490
Rpl13a	**1.400**	0.230	**1.134**	0.212	**1.267**	0.251
Siah1b	**0.763**	0.403	**0.718**	0.171	**0.737**	0.263
Stat3	**0.658**	0.174	**1.283**	1.258	**0.970**	0.896
Tfdp1	**1.154**	0.182	**1.090**	0.146	**1.122**	0.159
Tnfrsf1a	**0.929**	0.316	**1.272**	0.456	**1.111**	0.427
Tnfrsf5	**0.835**	0.078	**1.223**	0.551	**1.093**	0.473
Tnfrsf6	**0.595**	0.205	**0.815**	0.414	**0.742**	0.352

Traf4	**0.437**	0.265	**0.420**	-	**0.433**	0.217
Tssc3	**0.475**	0.262	**0.775**	0.290	**0.625**	0.284
Tyro3	**1.064**	0.369	**1.132**	0.265	**1.098**	0.317
Vegf	**0.652**	0.187	**1.347**	1.034	**1.031**	0.824
Ywhab	**0.908**	0.225	**0.795**	0.134	**0.876**	0.199
Ywhae	**0.945**	0.260	**1.177**	0.150	**1.061**	0.238
Ywhah	**1.097**	0.262	**1.054**	0.143	**1.078**	0.212
Ywhaq	**1.247**	0.260	**1.094**	0.200	**1.171**	0.239

† All clones containing ESTs from the same gene have been grouped under the name of the gene (for data on each clone see appendix).
* Mean ratio of Cy5 over Cy3-labelled cDNA from all animals analysed at the same time point (for individual data see appendix).

4.4 Conclusions

The analysis of changes in gene expression induced by a dose of 50 mg/kg of the chemical mutagen ENU, six and twenty-four hours after exposure, resulted in statistically the same pattern of gene expression as that of non-exposed animals. The conclusion that can be drawn from this experiment is that, at the specific time points analysed, significant changes in gene expression were not detected following the dose of ENU.

There are several hypotheses which could explain the lack of apparent induction and repression of gene transcription. Firstly, the change in the pattern of gene expression could specifically occur in certain types of spermatogenic cells. These changes in specific cell types would not be detected because of a dilution of the required cells in a mixed population of testicular cells. To avoid this possibility in further studies, the isolation of a specific type of spermatogenic cells prior to the analysis of changes in gene expression should be considered.

Secondly, it is possible that the dose of ENU used in this experiment, was not sufficient to induce the necessary DNA damage to lead to any changes in gene expression. Hence, the basal levels of transcription were adequate to repair the damage induced. In further studies it should be considered the possibility of using higher doses of ENU and it is also advisable to use more time points. However, the molecular dosimetry of ENU has revealed the presence of O^6-ethylguanine and N^7-ethylguanine in mouse testes, one hour after treatment with 10 or 100 mg/kg of ENU (Sega *et al.*, 1986).

In addition, the yield of lesions obtained with both doses is lower twenty-four hours after exposure and keeps decreasing for at least a period of six days, due to the action of DNA repair mechanisms (Sega *et al.*, 1986).

The last possibility relies on the assumption that testicular cells have an endogenous level of DNA repair proteins equal to that obtained with the maximum activation of the genes. This level of expression would not allow detection of increases in expression independently of mutagen and dose analysed. If certain, it would be reasonable to expect the observed lack of induction in the levels of transcription in genes involved in DNA repair processes. This theory is supported by two experiments. The *Saccharomyces* Genome Deletion Project (Winzeler *et al.*, 1999) has made available *S. cerevisiae* strains containing deletions of nonessential genes. These strains have allowed the confirmation of known genes and identification of novel genes involved in DNA repair damage caused by UV, ionising radiation, cisplatin and hydrogen peroxide (Bennett *et al.*, 2001; Birrell *et al.*, 2001; Birrell *et al.*, 2002).

Once the genes involved in the repair of the different type of damage were identified, the changes in pattern of expression in these genes was analysed using the cDNA microarray method. The results obtained from this analysis demonstrate that there is little, if any, correlation between the presence of the damage and the expression of genes involved in the repair of this damage (Birrell *et al.*, 2002). The authors claim that the endogenous levels of protein involved in DNA repair processes is sufficient to provide full resistance to the generated damage, making an increase in the level of transcription unnecessary.

5 Thesis summary

The aim of the project described in this thesis was to investigate the suitability of ESTR loci for the analysis of mutation induction in the germline of mice exposed to chemical mutagens. Given the growing concern over the hereditary effects of chemical mutagens for humans, the development of new sensitive approaches for evaluating the germline effects of chemical mutagens is warranted. The measurement of induced germline mutation in mice is the primary method used to evaluate genetic risk of human exposure to chemical mutagens. However, the currently available techniques are not sensitive enough to detect mutation induction in the germline of mice exposed to low and often intermediate concentrations of chemical mutagens, which represent the main concern in genetic toxicology. The results of previous publications show that ESTR loci provide a sensitive tool for the analysis of germline mutation induction in mice, capable of detecting the effects of low-dose exposure to ionising radiation (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994), therefore suggesting that these loci may also be of potential use for monitoring germline mutation by chemicals. However, at the beginning of my project, little was known about the effects of chemical mutagens on ESTR mutation rate in the mouse germline (Barber *et al.*, 2000).

The first part of my project deals with the analysis of ESTR mutation induction in the germline of male mice exposed to two alkylating agents ENU and iPMS and the topo-II inhibitor ET. The three chemicals are known to differ in their DNA-reactivity and ability to induce large-scale deletions and base substitutions, as well as in their mutagenicity in the mouse germline (Ehling *et al.*, 1972; Ehling and Neuhauser-Klaus, 1988a; Ehling and Neuhauser-Klaus, 1995; Favor, 1998; Favor, 1999; Justice *et al.*, 1999; Kallio and Lahdetie, 1993; Kallio and Lahdetie, 1996; Lahdetie *et al.*, 1994; Lee *et al.*, 1995; Mailhes and Marchetti, 1994; Mailhes *et al.*, 1994; Mailhes *et al.*, 1996; Russell *et al.*, 1998; Russell *et al.*, 2000a; Sega *et al.*, 1986; Shelby and Tindall, 1997; Suzuki *et al.*, 1995; Suzuki *et al.*, 1997a; van Zeeland *et al.*, 1985; van Zeeland, 1996; Vogel *et al.*, 1996).

The results of my study show that post-meiotic exposure to the alkylating agents ENU and iPMS does not affect ESTR mutation rate, whereas pre-meiotic exposure significantly increases mutation rate in the germline of treated males (see 3.3). These

data are consistent with previous results demonstrating a lack of ESTR mutation induction in the germline of male mice post-meiotically exposed to ionising radiation (Barber *et al.*, 2000; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a) but contradict those obtained by a Japanese group (Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994). My data also provide strong evidence that mutation induction at ESTR loci can only occur following mutagenic exposure of diploid pre-meiotic and meiotic cells.

The analysis of dose-response for ESTR germline mutation induction reveals a remarkable similarity between the pattern of pre-meiotic mutation induction by the alkylating agents ENU and iPMS (see 3.3). Exposure to these chemicals results in a linear increase in ESTR mutation rate within the interval of doses from 12.5 mg/kg to 25 mg/kg with a plateau at higher concentrations.

The comparison of the doubling doses for pre-meiotic exposure to ENU and iPMS for ESTR loci to some other mouse systems for mutation detection enables an evaluation of the sensitivity of this system to pre-meiotic mutagen exposure (see 3.3). It shows that ESTR loci are remarkably sensitive to a low-dose exposure to the alkylating agents and therefore provide a new and powerful approach for monitoring germline mutation in mice. Moreover, previous studies on mutation induction at specific loci (SLT) required the analysis of hundreds of thousands of mice, whereas, elevated ESTR mutation rate can robustly be detected in very small numbers of offspring.

The analysis of mutation rates in male mice exposed to the topo-II inhibitor ET provides important results on the stage-specificity of mutation induction at ESTR loci (see 3.4). My data showing a very narrow window of ESTR mutation induction by ET targeting meiotic spermatocytes are consistent with the results of previous studies on dominant-lethal mutations and CA (Russell *et al.*, 1998; Suzuki *et al.*, 1997a) and therefore show a remarkable similarity with other biomonitoring systems in the timing of mutation induction by ET in male mice.

The results of my study also raise the important issue of the mechanisms of mutation induction at mouse ESTR loci by chemical mutagens (see 3.6). They show that ESTR mutation induction in the germline of male mice exposed to alkylating agents or ET cannot be attributed to the direct targeting of these loci and are consistent with the

results of previous studies on radiation-induced mutation at ESTR loci (Barber *et al.*, 2000; Dubrova *et al.*, 1998a; Dubrova *et al.*, 1998b; Dubrova *et al.*, 2000a; Dubrova and Plumb, 2002; Fan *et al.*, 1995; Niwa *et al.*, 1996; Sadamoto *et al.*, 1994). They strongly indicate that mutation induction at ESTR loci by a wide range of mutagens results from the initial mutagen-related DNA damage elsewhere in the genome and later indirect mutation at these loci. The mechanisms of this non-targeted process remain unknown and may include DNA repair or other factors.

The second part of my project deals with the analysis of changes in the expression profiles in the germline of male mice exposed to ENU (see Chapter 4). The main objective of this study was to establish whether ESTR mutation induction is related to the consistent changes in the expression profiles of some genes associated with DNA repair, apoptosis, cell division/signalling and carcinogenesis. Using microarray technology, the expression profiles in testes of male mice were analysed 6 and 24 hours after exposure to ENU. The results of this study show the lack of any significant changes in the expression profiles and suggest that the repair of ENU-induced DNA damage is not accompanied by the up-regulation of transcription. Further studies are clearly needed to evaluate the expression profiles following exposure to chemical mutagens and to establish whether activation of DNA repair could affect ESTR stability.

In conclusion, the work presented in this thesis has revealed that ESTR loci provide a new and very sensitive tool for the analysis of germline mutation induction by chemical mutagens. The ability of ESTR loci to reflect pre-meiotic exposure to alkylating agents makes them a useful marker for long-term exposure, which currently represents a major genetic risk factor for humans. The results of this study also show that elevated ESTR mutation rate can robustly be detected at low doses of exposure to alkylating agents. The lack of sensitive, relatively inexpensive approaches has been a severe limitation to the study of induced germline mutagenicity to date. The work presented in this thesis demonstrates that ESTR loci provide such a system for the analysis of germline mutation induction by chemical mutagens.

5.1 Future work

5.1.1 Further analysis of ESTR mutation induction by chemical mutagens

To further verify the suitability of ESTR loci for monitoring germline mutation induction by chemical mutagens, the mutagenicity of other types of chemicals should be evaluated. Judging from the results of previous studies, the analysis of the germline effects of three chemical mutagens appears to be highly relevant:

• Procarbazine – The anticancer drug procarbazine is an alkylating agent, mainly used for the treatment of Hodgkin's lymphoma (Ehling and Neuhauser, 1979; Suzuki *et al.*, 1999). Similar to ENU, procarbazine generates DNA methylations preferentially in oxygen residues (Souliotis *et al.*, 1994) leading to increased mutations in pre- and post-meiotic stages of mouse spermatogenesis (Ehling and Neuhauser, 1979; Goldstein, 1984). Assuming that humans and mice are equally sensitive to procarbazine, the analysis of ESTR mutation induction by this chemical could provide important data on the genetic consequences of exposure to this anticancer drug in humans.

• Bleomycin – Bleomycin is well-known radiomimetic agent, exposure to which leads to accumulation of the DNA damage similar to that induced by ionising radiation (Charles and Povirk, 1998; Povirk, 1996). This apparent similarity could provide important clues on the mechanisms of germline mutation induction at ESTR loci. Using the DLT, bleomycin was initially considered a female-specific mutagen (Sudman *et al.*, 1992); however, further analyses revealed that bleomycin is also mutagenic in spermatogonia (Russell *et al.*, 2000b).

• Cyclophosphamide – Given the negative results on ESTR mutation induction by the cross-linking agent cisplatin (Barber *et al.*, 2000), further analysis of the ESTR mutagenicity by cross-linking agents are clearly needed. The cross-linking agent CP is the most widely used anticancer drug. In addition to the intentional exposure in cancer patients, exposure has also been detected in medical personnel (Sessink *et al.*, 1993; Sessink *et al.*, 1994; Sessink *et al.*, 1997). Therefore, it is important to confirm the results of germline mutagenicity of CP, obtained with the SLT and the DLT, suggesting that CP specifically affects post-meiotic cells (Ehling and Neuhauser-Klaus, 1988b; James and Smith, 1982; Jenkinson *et al.*, 1987; Sram, 1976).

5.1.2 Development of new approaches for the analysis of ESTR mutation induction by chemical mutagens

The results of a recent publication show that spontaneous and radiation-induced ESTR instability in mouse somatic and germ cells can be monitored by single molecule PCR (SM-PCR) analysis (Yauk *et al.*, 2002). SM-PCR dramatically reduces the numbers of mice needed for the measurement of germline mutation frequencies and allows detailed analysis of the spectrum of spontaneous and induced ESTR mutations. Future work should therefore establish whether this approach could also be used for the analysis of ESTR mutation induction in the germline of male mice exposed to chemical mutagens.

5.1.3 Transgenerational effects in the offspring of exposed males

The results of recent studies show that ESTR mutation rates in the germline of unexposed first- and second-generation offspring of irradiated males are remarkably elevated, demonstrating that radiation-induced instability can be transmitted to subsequent generations. (Barber *et al.*, 2002; Dubrova *et al.*, 2000b). However, to date, there have been a few publications suggesting that *in vitro* exposure to some chemical carcinogens and mutagens can also result in a delayed increase in mutation rate (Bardelli *et al.*, 2001; Li *et al.*, 2001). It therefore remains to be seen whether exposure to chemical mutagens can also affect ESTR mutation rates in the germline of first- and second-generation offspring of treated males. Judging from the results of my study, future work should address this important issue by profiling the second- and third-generation offspring of male mice exposed to the alkylating agent ENU.

5.1.4 Analysis of mechanisms of mutation at ESTR loci

The mechanisms of spontaneous and induced ESTR mutation still remain unknown. In this thesis, it was attempted to establish whether ESTR mutation induction is related to the consistent changes in the expression profiles of some genes associated with DNA repair, recombination, apoptosis, cell division/signalling and carcinogenesis. This analysis did not reveal any consistent changes in the pattern of gene expression between control and exposed males. This work should further be extended, including the analysis of corresponding changes following exposure to higher doses of chemicals and over a considerable period of time.

In addition, this work should be complemented by the analysis of ESTR mutation induction by chemical mutagens in the germline of male mice defective at genes associated with DNA repair and apoptosis. The p53 and ATM knock-out mice represent the most obvious candidates for this work. The analysis of spontaneous and induced mutation in these animals could provide important information on the mechanisms of mutation induction at ESTR loci.

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