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**The use of the transgenic mouse Muta™Mouse to study
1,8-dinitropyrene-induced mutagenesis.**

by

Katalin K.A. Bertenyi, B.Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

**Department of Biology
Carleton University
Ottawa, Canada**

1997

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The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis,

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Submitted by

Katalin K.A. Bertenyi, B.Sc. (Hons.)

in partial fulfilment of the requirements
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Chair, Department of Biology

Thesis Supervisor

Carleton University

August, 1997

Szüleimnek

Abstract

1,8-dinitropyrene (1,8-DNP) is an important environmental contaminant which is a potent *in vitro* mutagen and rodent carcinogen. The mutagenicity of 1,8-DNP in Muta™Mouse (mouse strain 40.6), which carries approximately 80 copies of the bacterial *lacZ* gene on λ gt10 vectors per somatic cell, has been studied. The studies have examined mutagenesis both in tissues which are targets for 1,8-DNP-induced tumorigenesis, and in putative nontarget tissues. Mice were treated via intraperitoneal (i.p.) injections with a total of either 0.3, 0.9 or 2.8 mg/kg 1,8-DNP (Study #1), or 5.0 mg/kg 1,8-DNP (Study #2). A statistically significant increase in mutant frequency was observed in mesentery tissue but not in bone marrow, brain, lung or liver tissues (Study #1), or bone marrow or lung tissues (Study #2). DNA sequence analysis of *lacZ* transgenes (mesentery tissue; Study #1; 2.8 mg/kg 1,8-DNP) revealed that of 12 mutations, nine were base substitutions, two were frameshift mutations and one was a tandem base substitution.

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List of Abbreviations

A	adenine
AAF	2-acetylaminofluorene
AF	2-aminofluorene
Amp	Ampicillin
APS	ammonium persulphate
bp	base pair(s)
C	cytosine
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
°C	temperature in degrees Celsius
DMF	dimethylformamide
DMN	dimethyl nitrosamine
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
1,3-DNP	1,3-dinitropyrene
1,6-DNP	1,6-dinitropyrene
1,8-DNP	1,8-dinitropyrene
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetra-acetic acid

ENU	ethylnitrosourea
EtBr	ethidium bromide
FISH	fluorescence <i>in situ</i> hybridisation
G	guanine
g	acceleration due to gravity
Glu-461	glutamate at position 461 of <i>lacZ</i>
gm	gram(s)
<i>hprt</i>	hypoxanthine phosphoribosyl transferase
IARC	International Agency for Research on Cancer
i.p.	intraperitoneal (injection)
Kan	Kanamycin
kb	kilobase(s)
kg	kilogram(s)
λ	bacteriophage Lambda
<i>lacI</i>	gene encoding the <i>lac</i> operon repressor enzyme
<i>lacZ</i>	gene encoding the enzyme β -galactosidase
LB	Luria-Bertani
m^+_k	methylation positive <i>E. coli</i> K-12 strain
M	molar (moles per litre)
<i>mcrA</i> , <i>mcrB</i>	genes encoding enzymes for methylated cytosine restriction
MFH	malignant fibrous histiocytomas

mg	milligram(s)
mL	millilitre(s)
mM	millimolar
MMC	mitomycin C
MTD	maximum tolerated dose
NAT	<i>N</i> -acetyltransferase
6-NC	6-nitrochrysene
ng	nanogram(s)
nitro-PAH	nitrated polycyclic aromatic hydrocarbon
1,8-NONP	1-nitroso-8-nitropyrene
1-NP	1-nitropyrene
nt	nucleotide
OAT	<i>O</i> -acetyltransferase
O.D.	optical density
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
P-Gal	phenyl β -D-galactoside
r	resistant

r_k	restriction deficient <i>E. coli</i> K-12 strain
RFLP/PCR	restriction fragment length polymorphism/polymerase chain reaction
rpm	revolutions per minute
RSM	restriction site mutation
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
s.c.	subcutaneous (injection)
SCE	sister chromatid exchange
SDS	sodium dodecyl sulphate
STT	short-term test
T	thymine
TAE	Tris-acetate, EDTA buffer
TBE	Tris-borate, EDTA buffer
TE	Tris-HCl, EDTA buffer
Tyr-503	tyrosine at position 503 of <i>lacZ</i>
UDS	unscheduled DNA synthesis
μg	microgram(s)
μL	microlitre(s)
UV	ultraviolet
v/v	volume/volume
V	volt(s)
w/v	weight/volume

X-Gal

5-bromo-4-chloro-3-indolyl- β -D-galactoside

%

percent

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I. Introduction and Literature Review

1.1 General toxicology testing.

There is significant human exposure to chemicals, some of which represent health concerns. As of 1993, there were approximately 60,000 synthetic chemicals being used commercially, and their number has increased by about 1,000 each year (Sullivan *et al.*, 1993). Humans also face exposure to environmental pollutants such as soot, tars and oils, which have been previously linked to occupational carcinogenesis (Dipple, 1985). Polycyclic aromatic hydrocarbons (PAHs) and their nitrated derivatives are classes of pollutants that are derived not only from specific occupational environments, but also from combustion products, originating from sources such as heat and power generation, open refuse burning, and motor vehicle emissions, and these pollutants are wide-spread in the environment, being detected in the atmosphere, soil, water and subsequently, the food chain (Dipple, 1985).

Government regulations require that all compounds intended for human use, or for release into the environment, be tested for safety and efficacy (Sullivan *et al.*, 1993). However, existing environmental contaminants, such as PAHs, are also frequently the subjects of biological testing for mutagenicity and carcinogenicity (Rosenkranz, 1982; reviewed by Rosenkranz and Mermelstein, 1983). There are over 200 short-term tests (STT) available for the detection of genotoxic agents (DeMarini *et al.*, 1989a). In contrast, carcinogens are generally determined on the basis of lifetime cancer tests in rodents (Ashby and Morrod, 1991), but these tests are more expensive than STTs and take longer to perform (Health and Welfare Canada and Environment Canada, 1986).

One possible action of chemicals is their interaction with DNA. Gene and chromosome mutations are associated with many different deleterious effects on individuals, including malignancy, birth defects, genetic diseases and aging (Gossen and Vijg, 1993a; Mirsalis *et al.*, 1995). Cancer is one biological endpoint that has been studied extensively (Weinberg, 1996; Hellman and Vokes, 1996). The initiation of malignant growth involves multiple steps, including damage by genotoxic agents to genetic targets such as oncogenes, tumour suppresser genes and DNA repair genes (Weinberg, 1996). Non-genotoxic agents can also be carcinogenic, possibly by enhancing cell turnover and increasing the number of cells with existing spontaneous mutations (Ames and Gold, 1990a; Mirsalis *et al.*, 1995), or by the stimulation of proliferative responses (Tennant *et al.*, 1993).

Genotoxic agents damage DNA in multiple ways, including base substitutions, frameshift mutations involving one or two bases, and deletions and insertions of more than two bases. Many natural and synthetic chemicals have the ability to alter DNA in these ways, though not all chemicals exert their effects identically. Much research has been carried out in both prokaryotic and eukaryotic systems in an attempt to gain a better understanding of which chemicals are genotoxic and by what mechanism(s).

1.1.1 Prokaryotic test systems.

The Ames *Salmonella typhimurium* tester strains (Ames, 1971; Ames *et al.*, 1973a) have been used extensively as a quick and relatively inexpensive

way to detect and classify mutagens. The reversion assays identify chemicals which are able to revert the *S. typhimurium his* mutants, and also provide some mechanistic information about the mutagenicity of these chemicals (Ames *et al.*, 1973a). By themselves, these reversion assays are limited to testing direct-acting mutagens. Rat or human liver homogenate, S-9, has been used in conjunction with the Ames test to allow testing of compounds that require mammalian enzymatic activation in order to be genotoxic (Ames *et al.*, 1973b). Forward mutation assays with various bacterial strains can also be used as indicators of chemical mutagens (Ames *et al.*, 1973a). Generally, these forward mutation assays detect mutations in a target gene by assaying for the loss of function in the corresponding protein, such as the repressor (*lacI* gene product) or β -galactosidase (*lacZ* gene product) of the *Escherichia coli lac* operon (Miller, 1992). These forward mutation assays are less informative than the reversion assays in the sense that generally the mutational event can be considerably less specific in the former assay and still allow phenotypic selection of mutants.

The Ames *S. typhimurium* assays are still in use today for a number of reasons. The assays are cost-efficient, quick and relatively easy to perform (Tennant *et al.*, 1987). The Ames assays also provide mechanistic information about the genotoxicity of each chemical, such as its preference for frameshift or base substitution mutagenesis (Ames *et al.*, 1973b). However, positive genotoxic responses by reverse and forward mutation assays only indicate that further testing is required, since it is rather difficult to draw parallels between prokaryotic and mammalian systems (Health and Welfare Canada and

Environment Canada, 1986). Another drawback of the Ames assay as a predictor of the carcinogenicity of compounds is that approximately 30% of the positive results have been found to be negative in long-term rodent assays, thereby over-predicting potential carcinogenicity (Tennant *et al.*, 1994). In contrast, false negative results mean that potentially harmful chemicals are considered safe and are therefore not tested in eukaryotic systems.

1.1.2 Eukaryotic test systems.

Eukaryotic test systems are able to reflect the different underlying molecular events that are involved in eliciting a genotoxic response from chemicals (Health and Welfare Canada and Environment Canada, 1986). Unlike the prokaryotic assays, the use of eukaryotic test systems facilitates the extrapolation of test results to humans with the help of animal and metabolic studies (Ames and Gold, 1990a). However, these tests still only provide a partial answer. Human exposure to chemicals usually occurs at lower than test levels and humans are simultaneously exposed to a wide variety of chemicals, some of which may act as anti-mutagens or synergistically (Health and Welfare Canada and Environment Canada, 1986). In addition, there exists an enormous difference in the metabolic efficiency in elimination, detoxification or activation of chemicals between species and even individual humans, and the DNA repair mechanisms vary between individual humans and other animals (Health and Welfare Canada and Environment Canada, 1986). Nevertheless, many variations of mammalian test systems, both *in vitro* and *in vivo*, have been

developed which allow detection of genotoxic compounds which cause gene mutations and chromosomal mutations in the hope that they will provide some indication of putative chemical hazards to humans.

1.1.2.1 Gene mutation assays.

Mammalian *in vitro* gene mutation assays detect alterations in the genes of cultured cells (Gossen and Vijg, 1993a). Cells from many different organisms and their tissues can be cultured, including Chinese hamster ovary (CHO) cells, V79 Chinese hamster lung (CHL) cells, mouse BALB/c 3T3 cells and human lymphoblasts (Shirnamé-Moré, 1995). The mutagenicity studies are carried out with or without exogenous metabolic activation, similar to the prokaryotic testing systems (Shirnamé-Moré, 1995). Genotoxicity of compounds can be determined by assaying for the presence of chemical-induced adducts on the DNA or by determining the frequency of mutations induced at a specific locus such as *hprt* (hypoxanthine phosphoribosyl transferase) (Gossen and Vijg, 1993a).

Generally, a limited number of cell types are used for *in vitro* gene mutation assays (Gossen and Vijg, 1993a; Skopek *et al.*, 1995). Although relatively easy to work with, the testing of cultured cells for chemical-induced mutations does not give information about the inherent differences in responses between tissues in a given organism (Gossen and Vijg, 1993a; Mirsalis *et al.*, 1994). Furthermore, individual loci used for *in vitro* gene mutation detection have limitations which need to be recognised before evaluating the test results. For example, in the case of the hemizygous *hprt* locus, inter-laboratory

discrepancies in results can arise from variations in scoring methods and in cloning efficiencies (Martus *et al.*, 1995). Another limitation of using hemizygous nonessential loci such as the *hprt* locus for mutation detection is the size of the damaged area. Deletions which extend into neighbouring essential genes would not be detected because they would be lethal to the cell (DeMarini *et al.*, 1989b).

Cells which undergo *in vitro* culturing often experience alterations which can affect the interpretation of mutagenicity studies. For example, several cell divisions in culture are required in order to score the 6-thioguanine-resistant mutant phenotype conferred by the *hprt* locus, and this system is therefore limited to those cell types which can grow well *in vitro* (Martus *et al.*, 1995). In contrast, Carmichael *et al.* (1996) reported that while first passage human breast cell cultures retained the enzymatic activity necessary to metabolise certain chemicals to the reactive form, which subsequently interacts with DNA to form adducts, adduct detection was greatly diminished after treatment of cells which had undergone numerous passages.

Until recently, it has been difficult to carry out *in vivo* studies of either induced or spontaneous mutations in higher organisms because of the problems associated with identifying and isolating the affected genes (Gossen and Vijg, 1993a). In addition, only a limited number of target tissues were available for testing due to technical limitations, and the procedures were expensive (Hoorn *et al.*, 1993).

An endogenous locus commonly used for *in vivo* mutagenicity assessments is the *D/b-1* locus. This locus determines the presence or absence

of the binding site for *Dolichos biflorus* agglutinin on cellular surfaces in villi in the small intestine (Winton *et al.*, 1988; Tao *et al.*, 1993; Tao and Heddle, 1994). Mutations in this locus result in the loss of the binding site for the lectin, and this can be detected by staining whole mounts of the small intestine (Tao *et al.*, 1993; Tao and Heddle, 1994). Another endogenous locus which is occasionally used for *in vivo* mutagenicity tests is the *hprt* locus. In humans, lymphocytes are isolated and used for studying mutations at the *hprt* locus (Grist *et al.*, 1992) and splenic T lymphocytes have been used for the analysis of mutations at the *hprt* locus in mice (Skopek *et al.*, 1996). However, analysis of endogenous loci such as *Dlb-1* and *hprt* is limited to a small number of tissues, and there are also culturing limitations. The transgenic rodent assay is derived from a need to have a good *in vivo* assay which allows analysis of DNA from all tissues for mutations (section 1.2).

1.1.2.2 Chromosomal mutation assays.

Genotoxic mutagens can do more than just alter the existing DNA sequence in a localized region. Chemicals can affect the entire chromosome of the organism, causing rearrangements or large scale alterations. Several *in vitro* and *in vivo* techniques exist to study these phenomena.

In vitro studies employing cultured cells can be used to observe chromosomal alterations following exposures to certain chemicals. Two of the most commonly used *in vitro* STTs to predict rodent carcinogenicity are assays which detect sister chromatid exchanges (SCE) and chromosomal aberrations

(Tennant *et al.*, 1987). SCEs have been observed in mammalian cell lines such as human lymphocytes (Barale *et al.*, 1993) and Chinese hamster V79 cells (Kerdar *et al.*, 1993) which were exposed to chemicals, and chromosomal aberrations have been detected in mammalian cell lines such as CHL following treatment with suspected clastogens (Matsuoka *et al.*, 1991). However, in the event that specific loci are used as targets to study chromosomal mutations, there are limitations. Recovery of specific locus mutants with multi-locus deletions is dependent on the presence of an autosome carrying genes which are homologous to the essential genes which flank the target gene (DeMarini *et al.*, 1989b).

In vivo tests look for chromosomal mutations following chemical treatment of a whole organism. For regulatory purposes, relevant *in vivo* genotoxicity assays are those which measure chromosomal aberrations in bone marrow and DNA repair in the liver (Blakey *et al.*, 1995), but other *in vivo* tests do exist. For example, the mouse micronucleus test is usually limited to somatic tissues such as bone marrow and peripheral blood lymphocytes (Hoom *et al.*, 1993). Occasionally, the presence of micronuclei are determined in tissues such as liver and testes, but with some technical difficulty, and with limited published data for comparison of results (Hoom *et al.*, 1993). Other *in vivo* tests used occasionally include the mouse lymphoma test, which has limited sensitivity (Gossen *et al.*, 1989), and the detection of SCEs in rodent cells following the treatment of the whole animal with the putative mutagen or clastogen (Shirnamé-Moré, 1995). As well, chromosomal aberrations have been successfully induced

in vivo in rats, and subsequently detected in lung fibroblasts (Khan and Heddle, 1991).

1.1.2.3 Long term assays.

Chronic rodent bioassays are often used to provide carcinogenicity data, with minimal regard for the occurrence of gene or chromosomal mutations.

Rodents are generally treated with sub-lethal doses of suspected human carcinogens by diverse routes of administration, including inhalation (Ishinishi *et al.*, 1986), oral dosing (Odagiri *et al.*, 1986), intraperitoneal injection (i.p.) (King *et al.*, 1986), intratracheal instillation, subcutaneous injection (s.c.) and skin applications (Kunitake *et al.*, 1986).

Of the chronic rodent bioassays done on rats and mice by 1990, 82% (350/427) of the chemicals adequately tested on these species were synthetic industrial chemicals, and about half have been classified as rodent carcinogens (Ames and Gold, 1990b). However, only 77 natural chemicals had been tested on rodents by 1990, and again about half were rodent carcinogens (Ames and Gold, 1990b).

Although informative, the chronic rodent bioassays do not depict human exposure accurately. Generally speaking, it is assumed that if a chemical is carcinogenic at the maximum tolerated dose (MTD) in rats and mice, it is also carcinogenic to humans when administered at or close to the MTD (Ames and Gold, 1990b). However, since humans often receive exposure to chemicals at levels which are hundreds or thousands of times lower than the MTD which

produced the carcinogenic effect in the rodent, it would be ideal to understand the mechanisms of mutagenesis and carcinogenesis at these levels (Ames and Gold, 1990b). Long-term bioassays are also expensive, time-consuming and require high numbers of experimental animals (Gossen *et al.*, 1994).

Unfortunately, the gene mutation and chromosomal mutation assays discussed above do not provide enough information about specific factors such as uptake of the chemical, its distribution, metabolic activation, detoxification, elimination and cell proliferation (Mirsalis *et al.*, 1994). Since each tissue in a given organism might be affected differently by a chemical (Ashby and Tinwell, 1994), it would be ideal to use *in vivo* mammalian models which allow investigation of mutations in all tissues in terms of accumulating data which would be most beneficial to humans (Hoorn *et al.*, 1993).

The development of the transgenic rodent models circumvented some of these problems by providing a mammalian model containing a recoverable target gene, allowing *in vivo* chemical treatment of a mammal, and *in vitro* characterisation of the target gene. These transgenic systems are also quick relative to the long term bioassays, with experimental times of months compared to years, respectively.

1.2 Transgenic rodent models.

Since 1989, transgenic rodent systems have received significant attention in the field of genetic toxicology. These mutagenesis models allow *in vitro* scoring of mutations in well characterised genetic targets in all cells of the

organism, somatic and germ line. While mutations to somatic cells can adversely affect the health of an individual, germ cell mutagenesis has more far-reaching consequences, with permanent genetic alterations passed on to subsequent generations. Muta™Mouse, the *lacZ*-based transgenic mouse, has been shown to be a suitable model to study germ cell mutagenesis in spermatogonial stem cells (van Delft and Baan, 1995), and several groups have reported detection of spontaneous and chemical-induced heritable mutations in the *lacZ* transgene (Douglas *et al.*, 1994; Douglas *et al.*, 1995a; Douglas *et al.*, 1997; Krebs and Favor, 1997). The transgenic systems are also useful in determining which organs are more susceptible to chemical-induced damage. Ashby and Tinwell (1994) provide several examples of organotropy in the mouse; known mouse carcinogens which are specific to certain organs induce mutations in those specific organs, and not others.

The transgenic rodent assays reduce dependence on animal experimentation relative to the long-term bioassay by being amenable to concurrent studies of several short-term genotoxicity end-points (Sullivan *et al.*, 1993). For example, following dosing with chemicals such as mitomycin C (MMC) and ethylnitrosourea (ENU), the peripheral blood from mice can be used for the micronucleus assay, and the mice can then be sacrificed for the transgenic mouse mutation assay (Suzuki *et al.*, 1993; Suzuki *et al.*, 1994). This is particularly important since genotoxic chemicals can have various endpoints (Heddle *et al.*, 1990).

1.2.1 Lambda-based transgenic mouse systems.

1.2.1.1 Muta™ Mouse

Gossen *et al.* (1989) have developed a system which allows the *in vitro* study of mutagenesis in various mouse tissues after *in vivo* treatment with a mutagenic compound by using a recoverable target gene system. The 3126 bp bacterial *lacZ* gene, used as the target gene, was cloned into the ~47 kb bacteriophage λ gt10 which was then inserted into the mouse genome (Figure 1) (Gossen *et al.*, 1989).

The production of transgenic mice was carried out by using the male pronuclei of fertilised eggs of (BALB/c X DBA/2)CD2 F₁ mice as recipients for approximately 150 copies of the λ gt10/*lacZ* vector (Hogan *et al.*, 1986; Gossen *et al.*, 1989). These eggs were then implanted into pseudo-pregnant BCBA mice, and positive offspring were identified using Southern blot analysis of tail DNA (Gossen *et al.*, 1989). Four of the positive offspring were bred to homozygosity (Myhr, 1991) and were designated as strains 20.2, 34.1, 35.5 and 40.6 (Gossen *et al.*, 1989). The transgene lacks a promoter for *in vivo* expression in eukaryotes, and the presence of λ gt10/*lacZ* does not seem to affect the normal physiological functions of the mouse cells (Myhr, 1991).

The four strains, 20.2, 34.1, 35.5 and 40.6, each contain between 3 and 80 copies of the vector per haploid genome integrated into single sites of the genome (Gossen *et al.*, 1989). This variation in location of integration was found to have an effect on the rate of the spontaneous mutation frequency (Gossen *et al.*, 1989). For example, integration into the X chromosome (strain 35.5) caused

Figure 1. A schematic representation of the ~47 kb λ gt10 shuttle vector with the 3126 bp *lacZ* insert (adapted from Gossen *et al.*, 1989). The bacterial *lacZ* gene, used as the target gene, was excised from the 3.9 kb *Dra*I fragment of the plasmid pMC1511, and was cloned into the single *Eco*RI site of the bacteriophage λ gt10 following the attachment of *Eco*RI linkers to the *lacZ* fragment (Gossen *et al.*, 1989). MutaTMMouse contains approximately 40 copies of the λ gt10/*lacZ* vector integrated in a single site in the B region of mouse chromosome 3 in a head-to-tail manner in every haploid genome (Blakey *et al.*, 1995).

chromosome integration
(80 copies/diploid genome)

a 25-100 fold increase in the spontaneous mutant frequency of the *lacZ* transgene found in liver and brain relative to the spontaneous mutant frequency of the transgene in these tissues in at least three other transgenic mouse strains (Gossen *et al.*, 1991; Gossen *et al.*, 1993). Conversely, strain 40.6 was found to have a low spontaneous mutation rate for the transgene, especially in the liver and brain (Gossen *et al.*, 1989), making it the ideal strain for mutagenicity assays. This transgenic strain was named Muta™Mouse and has been used for several spontaneous (Douglas *et al.*, 1994; Ono *et al.*, 1995) and chemical-induced (Gossen *et al.*, 1989; Myhr, 1991; Hoorn *et al.*, 1993; Douglas *et al.*, 1995a; Brooks *et al.*, 1995; Douglas *et al.*, 1996; Krebs and Favor, 1997) mutagenesis studies in both somatic and germ cells.

Muta™Mouse has approximately 40 copies of the λ gt10/*lacZ* shuttle vector (Myhr, 1991) inserted in a head-to-tail fashion into a single site in the mouse genome (Gossen *et al.*, 1989). A combination of fluorescence *in situ* hybridisation (FISH) and DAPI chromosome banding was used to localise the insertion of λ gt10/*lacZ* in Muta™Mouse to the B region in mouse chromosome 3 (Swiger *et al.*, 1994; Blakey *et al.*, 1995). The location of integration makes this strain stable in terms of deletion of the transgene, since the vector is positioned about one-third of the way between the centromere and telomere, and breaks are more likely to occur near the telomere than between the B region and the centromere (Blakey *et al.*, 1995). In addition, chromosome breakage in mice is more likely to occur in centromeric heterochromatin, and therefore the site of the

λ gt10/*lacZ* integration also confers some protection to the vector from breakage damage (Blakey *et al.*, 1995).

Recovery of the λ gt10/*lacZ* vector from Muta™Mouse involves excision of the vector from the genomic DNA and packaging the vector *in vitro* using a commercially available *in vitro* packaging extract (Gossen *et al.*, 1989). This packaging extract contains terminase enzymes which are able to recognise and cut the λ cos sites on either end of the λ gt10/*lacZ* vector, thereby packaging one vector into each empty phage head (Gossen and Vijg, 1993a).

Initially, the determination of the mutant frequency, and screening for *lacZ*⁻ mutants, was carried out by plating infected *E. coli* host cells in the presence of X-Gal (5-bromo-4-chloro-3-indolyl- β -galactoside) and observing the ratio of colourless (mutated) to blue (non-mutated) plaques (Gossen *et al.*, 1989). This method was time consuming and expensive because it required confirmation of the mutant phenotype (Gossen and Vijg, 1993a).

The Muta™Mouse system was improved greatly when Gossen *et al.* (1992) developed a positive selection method using an altered *E. coli* host (kanamycin and ampicillin resistant, galactose sensitive), *E. coli* C *galE*⁻. After allowing phage harbouring λ gt10/*lacZ* vectors to infect the *E. coli* host cells, the phage titre was determined by plating onto LB medium. The selection for phage carrying mutant *lacZ* genes was achieved by plating the infected *E. coli* cells onto medium supplemented with P-Gal (phenyl β -D-galactoside).

The biochemical basis of the positive selection method involves killing the *E. coli* host cells harbouring wild type *lacZ*⁺ phage. The λ gt10/*lacZ* infected cells

are plated onto media supplemented with P-Gal, a lactose analogue (Gossen and Vijn, 1993a). Cells carrying the wild-type phage produce active β -galactosidase which metabolises P-Gal to galactose and phenol (Figure 2) (Dean and Myhr, 1994). The galactose is then metabolised into UDP-galactose but this metabolite cannot be further converted into UDP-glucose due to a point mutation in the *galE* gene, which inactivates the UDP-epimerase (Gossen *et al.*, 1992). The build-up of UDP-galactose leads to cell lysis early in cell growth (Gossen and Vijn, 1993b). However, cells containing a mutant *lacZ* gene cannot convert the P-Gal to galactose, and consequently the cells survive (Dean and Myhr, 1994). This positive selection assay was improved by adding a multicopy plasmid which over-expresses the *galK* and *galT* genes, allowing for a rapid accumulation of UDP-galactose in cells containing *lacZ*⁺ phage, and therefore promoting swift cell death before significant viral replication and infection of neighbouring cells occurs (Mientjes *et al.*, 1994). Further experiments with this positive selection method show that it is as sensitive as the older colour (X-Gal) screening method (Dean and Myhr, 1994; Mientjes *et al.*, 1996), and compares favourably to the Big Blue™ assay (section 1.2.1.2) in terms of ease of selecting mutants (Tinwell *et al.*, 1994).

CpG islands in the vertebrate genome are often methylated (Bird, 1986), and bacteriophage lambda sequences inserted in mice experience *in vivo* methylation at all of its CCGG sites, thereby significantly decreasing the efficiency of λ vector rescue (Gossen *et al.*, 1989). Similar problems were reported by Kohler *et al.* (1990) when trying to rescue lambda vectors bearing

Figure 2. A schematic representation of the *E. coli* C *galE*⁻ positive selection assay. Following extraction of the genomic DNA from Muta™Mouse tissue, the λ gt10/*lacZ* vectors are packaged *in vitro*, and are allowed to infect *E. coli* C *galE*⁻ host cells. Selection is carried out on media supplemented with P-Gal; cells infected with *lacZ*⁺ phage will produce β -galactosidase, which uses P-Gal as a substrate to release the galactose moiety. The *galK* and *galT* genes, carried chromosomally and on plasmid pAA119, convert the galactose to UDP-galactose, but the strain is deficient in *galE*, and thereby unable to complete the pathway to form UDP-glucose. The accumulation of UDP-galactose is lethal to the host *E. coli* C *galE*⁻ cells, resulting in cell death and an absence of plaques on the plates. Conversely, if the host cells are infected with *lacZ*⁻ phage, β -galactosidase, and therefore galactose, will not be produced. The *E. coli* C *galE*⁻ cells are therefore viable on P-Gal medium, allowing viral replication and the formation of plaques amidst a lawn of bacterial cells. Phage obtained from these plaques are subsequently analysed for mutations. (Adapted from Dean and Myhr, 1994.)

lacI transgenes from mice tissue. In the case of the λ gt10/*lacZ* vectors, this high level of *in vivo* methylation can lead to host restriction during *in vitro* packaging or once in the host bacteria (Gossen *et al.*, 1989). Although initially an *E. coli* K-12 strain was used as the host for mutant selection, the *mcrA* and *mcrB* methylation-dependent host restriction systems in K-12 strains resulted in degradation of the λ shuttle vector (Gossen and Vijg, 1993a). An attempt to use a *mcrA*⁻ and *mcrB*⁻ *E. coli* K-12 host was also unsuccessful, and so finally an *E. coli* C strain which did not restrict foreign DNA and provided high rescue efficiencies was used (Gossen and Vijg, 1988).

Vectors containing mutations can subsequently be analysed using common microbiology and molecular biology techniques. Bacterial complementation assays are used to localise the *lacZ* mutation to one of three complementation groups. The *lacZ* gene is then amplified by PCR (polymerase chain reaction) and the DNA fragments are sequenced to identify the mutations.

Studies with the Muta™Mouse system (strain 40.6) have shown that the *in vivo* background mutant frequency is low, allowing the detection of low levels of mutagenesis (Gossen *et al.*, 1989). In addition, the *lacZ* insert is neutral, experiencing no selective pressures when in the mouse, whether mutated or wild-type (Cosentino and Heddle, 1996). Another feature of this system is that it allows the determination of the type of mutation, as well as the identity of the local DNA sequence content (Gossen *et al.*, 1989). Most importantly, studies have shown that the *lacZ* mutations observed using this system actually do originate in the mouse tissues and not in the *E. coli* C cells (section 4.1.3.5)

(Gossen and Vijg, 1993a). The mutational spectra of ENU-induced mutations in the *lacZ* gene has been found to be different following treatment in the mouse and treatment in *E. coli* (Gossen and Vijg, 1993a). Also, the fact that increased mutant frequencies in the *lacZ* transgene obtained from various tissues (*in vivo* treatment) often takes several days to appear, while in *E. coli* the effect is seen immediately after treatment, indicates that the mutations in the *lacZ* transgene originate in the mouse (Gossen and Vijg, 1993a).

The lambda based transgenic rodent systems have several disadvantages when compared to one of the newer emerging systems, the Xenomouse (section 1.2.2.2). Genomic DNA isolated from tissues is generally 200-300 kb in size (Gossen *et al.*, 1994). Since Muta™Mouse carries approximately 80 copies of the vector and transgene at a single genomic site, multiple double stranded breaks can be expected in this 4000 kb region (Gossen *et al.*, 1994). Though the *lacI* transgene employed in Big Blue™ is approximately one-third the size of the Muta™Mouse transgene, the same lambda bacteriophage vector is used, and the same limitations to rescue efficiency apply (Kohler *et al.*, 1991a; Short *et al.*, 1992). Damage to the *cos* sites precludes *in vitro* rescue of the lambda vectors. The lambda systems require two intact *cos* sites, and thus a minimum of one complete vector and two partially complete vectors flanking the whole vector on either side, for the packaging of one lambda monomer (Martus *et al.*, 1995; Gossen *et al.*, 1995). The terminase enzyme provided by the commercial packaging kit is then able to recognise and cut at the *cos* sites, and the bacteriophage lambda DNA is packaged into an empty

phage pre-head (Gossen *et al.*, 1989). Deletions in the vector can also interfere with successful packaging of the lambda phage. For *in vitro* packaging, the lambda vector needs to be between 42 kb and 52 kb in size (Gossen and Vijg, 1993a). As a result, deletions of more than 5 kb or insertions of more than 8 kb are not detectable by the Muta™Mouse or Big Blue™ selection systems (Gossen and Vijg, 1993a; Buettner *et al.*, 1996) and therefore clastogens could erroneously be scored as non-mutagenic in Muta™Mouse mutagenesis studies. For example, Suzuki *et al.* (1994) detected only a few induced mutations in the bone marrow of Muta™Mouse following treatment with the strong bone marrow clastogen MMC. It was suggested that this lack of increase in mutant frequency over the control was due to the fact that MMC likely causes large deletions, which are not detectable by the Muta™Mouse selection system (Suzuki *et al.*, 1994). Though few large size alterations (greater than 50 bp) have been reported in these lambda based transgenic rodent models (Gossen *et al.*, 1995), a 2072 bp deletion was recently detected in the Big Blue™ transgenic mouse system (Buettner *et al.*, 1996).

Mutagenesis of *lacZ* has also been studied in prokaryotic systems (Watanabe and Ohta, 1993; Wehner and Horneck, 1995). For example, reverse mutation assays employing the *lacZ* gene in bacteria were engineered by site-directed mutagenesis to allow the detection of base substitution and frameshift mutagens (Cupples and Miller, 1989; Cupples *et al.*, 1990a; Marwood *et al.*, 1995). Site-directed mutagenesis was also used to characterise the active site of *lacZ* (Cupples and Miller, 1988; Cupples *et al.*, 1990b; Edwards *et al.*, 1990).

Although these data provide some comparison between the mutagenesis of the *lacZ* gene in prokaryotes and eukaryotes (transgenic), mutagenesis of the prokaryotic *lacZ* gene, used as a forward mutation target, has not been studied as extensively as that of the prokaryotic *lacI* gene (section 1.2.1.2).

1.2.1.2 Big Blue™.

The Big Blue™ transgenic mouse model is also widely used to study both spontaneous (Kohler *et al.*, 1991a; Kohler *et al.*, 1991b; Nishino *et al.*, 1996) and induced (Kohler *et al.*, 1991a; Kohler *et al.*, 1991b; Mirsalis *et al.*, 1993; Shephard *et al.*, 1993; Mullin *et al.*, 1995; Skopek *et al.*, 1996) *in vivo* mutations in a *lacI* transgene. This bacteriophage lambda-based transgenic mouse model involves the use of the bacterial *lacI* gene as the genetic target. As with the Muta™Mouse system, the target gene is carried on a λ shuttle vector, along with an α *lacZ* reporter gene, and *in vitro* packaging is used to recover the target gene from the genomic DNA of various tissues, followed by plating on selective media (Kohler *et al.*, 1991a). However, instead of using the Muta™Mouse λ gt10 vector, the Big Blue™ system employs the λ LIZ vector, 40 copies of which are stably integrated in a head-to-tail fashion on chromosome 4 of mouse strain C57BL/6 (Short *et al.*, 1992). Mouse strain B6C3F1 also carries the λ LIZ vector, permitting the study of inter-strain variations in mutational spectra following exposure to various mutagens (Kohler *et al.*, 1991b). Unlike the improved positive selection assay for the Muta™Mouse system, the Big Blue™ system uses X-Gal in the selection medium, with *lacI*⁻ (mutant) phage giving rise to blue

plaques and wild-type *lacI* phage producing colourless plaques (Kohler *et al.*, 1991a).

The spontaneous mutation spectrum of the *lacI* transgene in the Big Blue™ system was found to be different from those obtained with bacterial assays (Kohler *et al.*, 1991a). This helps to support the notion that the system was detecting mutations which occurred in the transgenic animal, and not in the bacterial host (Kohler *et al.*, 1991a; Kohler *et al.*, 1991b). In addition, a preliminary study involving ENU-induced mutagenicity revealed the possibility of a fundamental difference in prokaryotic and eukaryotic mutagenesis following ENU treatment (Kohler *et al.*, 1991a).

Spontaneous and chemical-induced mutations in the *lacI* gene have been extensively characterised since the wild-type sequence was described approximately two decades ago (Farabaugh, 1978). A large amount of data has been generated on both spontaneous and chemical-induced mutations in the bacterial *lacI* gene (Miller *et al.*, 1978; Schaaper *et al.*, 1986; Halliday and Glickman, 1991; Lambert *et al.*, 1992; Jiao *et al.*, 1993; Zielenska *et al.* 1993; Bertenyi and Lambert, 1996). A computer software system was developed to aid in the management of data from *lacI* mutagenesis studies in both bacteria and transgenic animals (de Boer, 1995). In addition, an electronic data base [<http://darwin.ceh.uvic.ca/bigblue/bigblue.htm>] with information from *lacI* mutagenesis studies from different labs is continually being updated.

1.2.1.3 Comparisons between the two transgenic *lac* mouse systems.

The two transgenic *lac* mouse systems share several positive characteristics for *in vitro* analysis of *in vivo* mutagenesis in rodents. Both transgenes are neutral in the animal (Cosentino and Heddle, 1996; Short *et al.*, 1992) and have low spontaneous mutant frequencies (Gossen *et al.*, 1994), and both transgenic rodent models have relatively efficient systems for the recovery of mutant transgenes (Tinwell *et al.*, 1994). In terms of the abilities of the two systems to detect mutations following treatment with chemical, the mutation induction rates with ENU were found to be similar between the Muta™Mouse and Big Blue™ transgenic mouse systems (Kohler *et al.*, 1991a).

The *lacI* target is approximately one-third the size of the *lacZ* target, but the spontaneous mutant rates in tissues such as spleen and testis are about the same in both systems (Kohler *et al.*, 1991a). Reasons for this similarity in mutant rates include the possibility that *lacI* has increased sensitivity over *lacZ* to mutation detection, or the fact that base substitutions in the amino terminus of *lacZ* often do not alter the functioning of the β -galactosidase protein sufficiently for detection of mutations with substrates such as X-Gal and P-Gal (Kohler *et al.*, 1991a). Other advantages of the *lacI* transgenic system relative to the *lacZ* transgenic system include the fact that the *lacI* transgene was incorporated into two different mouse strains, instead of just one as for the *lacZ* system (Tinwell *et al.*, 1994), and that a large data base of more than 10,000 *lacI* mutants from both bacteria and transgenic animals is available (de Boer, 1995). Finally, while the

lacI target is only 1080 bp (Kohler *et al.*, 1991a), the *lacZ* target is 3126 bp (Gossen *et al.*, 1989), and it is therefore much easier to sequence the *lacI* gene and identify mutations.

Since the *lacZ* gene is three times larger than the *lacI* target gene, the chemical has a larger target for mutagenesis (Gossen *et al.*, 1994). Some comparative studies have found the Muta™Mouse system to be more sensitive to mutagenesis by certain chemicals, possibly due to variations in mouse strains (Tinwell *et al.*, 1995). In addition, the Muta™Mouse system allows for a more cost effective and rapid acquisition of mutagenicity data than the Big Blue™ system (Tinwell *et al.*, 1994) since the former relies on the presence of plaques on positive selection plates to indicate the presence of mutant phage (Gossen *et al.*, 1992), while the Big Blue™ system requires a subjective differentiation between blue and clear plaques to select the mutant phage (Kohler *et al.*, 1991a).

1.2.2 Other emerging transgenic rodent systems.

1.2.2.1 Rat.

The λ LIZ vector involved in the production of the Big Blue™ mouse (Kohler *et al.*, 1991a) was used to produce a transgenic rat cell line (Wyborski *et al.*, 1995) as well as a transgenic rat model (Short *et al.*, 1992). The transgenic rat model was derived from Fischer-344 rat lines (*Rattus norvegicus*) (Short *et al.*, 1992; Dycaico *et al.*, 1994). Together with the Big Blue™ mouse, it was hoped that using these two different species with the same transgene would

allow interspecies comparison of the effects of mutagens, and that this would yield an improved data set for human risk assessment based on animal models (Short *et al.*, 1992). Furthermore, the larger size of the rat relative to the mouse provides more DNA within each tissue, allowing the improved analysis of smaller tissues, or specific cell populations within certain tissues (Short *et al.*, 1992). These transgenic rats have a lower spontaneous mutation rate than the Big Blue™ mouse system (Dycaico *et al.*, 1994).

1.2.2.2 Xenomouse.

A potential successor to the Muta™Mouse is the plasmid-based transgenic mouse, Xenomouse. A C57BL/6 transgenic mouse model was constructed which carries the plasmid pUR288, with the *lacZ* reporter gene as the mutational target (Gossen *et al.*, 1995; Dollé *et al.*, 1996), integrated into two sites, one on mouse chromosome 3, the other on chromosome 4 (Huang *et al.*, 1996).

There are several advantages to this plasmid based system over the bacteriophage lambda vector systems. The first advantage of the plasmid-based system is its increased sensitivity to large size alterations (Gossen *et al.*, 1995). As long as the antibiotic resistance gene and origin of replication are intact, it should be possible to rescue plasmids with deletions that extend into the flanking regions of the host chromosomal DNA (Dollé *et al.*, 1996). Large genetic rearrangement events have also been detected with the plasmid-based mouse system in mutants that arise spontaneously or following exposure to X-rays or

ENU (Gossen *et al.*, 1995; Dollé *et al.*, 1996). The plasmid-based mouse system is also sensitive to point mutations (Martus *et al.*, 1995).

Increased rescue efficiencies over the bacteriophage λ system are another advantage of the plasmid based system (Dollé *et al.*, 1996). Rescue of the plasmid from genomic DNA is easier than rescue of the λ gt10/*lacZ* vector since the plasmid is only 5 kb, which is about a tenth the size of the λ vector (Gossen and Vijg, 1993a). The average size of genomic DNA which is extracted from tissues is 200-300 kb in length, and since rescue of the 50 kb λ vector requires three consecutive λ vectors, with two intact *cos* ends for packaging, rescue efficiencies are limited by the isolation of intact genomic DNA (Gossen and Vijg, 1993a).

Finally, more data can be obtained in a single experiment with the plasmid based system relative to the lambda based system. Large amounts of the plasmid can be purified at one time, since the Lac repressor magnetic beads have a high capacity for the plasmids (Gossen and Vijg, 1993a; Vijg and Douglas, 1996).

A disadvantage of the Xenomouse system is its spontaneous mutant rate. The spontaneous mutant frequencies of the transgenes in young *lacZ* and *lacI* mice were found to be approximately 4×10^{-5} , while the young plasmid based mice developed spontaneous mutations in their transgenes at a frequency of about 7×10^{-5} (Gossen *et al.*, 1995), implying that slight mutagenic effects might be detected more easily in the *lacZ* and *lacI* mice than in Xenomouse.

1.2.2.3 Targets other than *lacZ* or *lacI*.

There are several alternatives to using *lac* genes as targets in transgenic rodents to study mutagenesis. Burkhart *et al.* (1993) developed a transgenic mouse with the bacteriophage Φ X174 am3 as the removable mutational target. This system has two targets; the 5 kb Φ X174 and a reversion assay for a mutation at the am3 site (Burkhart *et al.*, 1993). Leach *et al.* (1996) developed the λ supF transgenic mouse containing multiple copies of a recoverable lambda phage shuttle vector which carries the *supF* reporter gene. Nohmi *et al.* (1996) developed the *gpt* delta transgenic mouse which carries lambda EG10 DNA as the transgene. This system can be used to assay for chemicals causing point mutations through selection of *gpt*⁻ mutants on 6-thioguanine plates; clastogens can be detected using the Spi selection system (Nohmi *et al.*, 1996). Finally, a bi-transgene model was developed which carries both a bacteriophage lambda transgene and a polyomavirus middle T oncogene (Jakubczak *et al.*, 1996). Forward mutations in the lambda *cII* gene were used to follow the possible mutational changes which can occur during mammary tumour progression (Jakubczak *et al.*, 1996). These are but a few examples of the many different transgenic models available for mutagenesis and carcinogenesis studies, and more are expected in the future.

1.3 Nitroarenes.

Nitropyrenes belong to a class of compounds called nitroarenes, and can be formed when PAHs react with nitrogen oxides during combustion processes

(King, 1988). Nitroarenes can be found in diesel exhaust, kerosene heater and fuel gas burner emissions (Tokiwa and Ohnishi, 1986), cigarette smoke and grilled food (Kinouchi *et al.*, 1986), and until recently, the carbon black of photocopier toners (Löfroth *et al.*, 1980; Rosenkranz *et al.*, 1980). This distribution of nitroarenes in the environment is of human health concern due to the mutagenic and carcinogenic properties of these chemicals in bacteria and mammals (reviewed by Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). Nitroarenes include many different types of chemicals, including 1-nitropyrenes (1-NP), which appear to be the most abundant of nitroarenes, and dinitropyrenes, which may be the most mutagenic of the nitroarenes in bacteria (Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). Tri- and tetranitropyrenes are also mutagenic in bacteria (Mermelstein *et al.*, 1981).

1.3.1 Genetic toxicology.

1.3.1.1 Bacterial mutagenicity.

Nitropyrenes are direct-acting mutagens in *S. typhimurium* (Mermelstein *et al.*, 1981) and have been shown to be strongly mutagenic in the Ames *S. typhimurium* tester strains (Tokiwa and Ohnishi, 1986; Watanabe *et al.*, 1989; Watanabe *et al.*, 1993). 1,8-dinitropyrene (1,8-DNP) (Figure 3) and other nitropyrenes are frameshift mutagens in *S. typhimurium* (Mermelstein *et al.*, 1981), causing predominantly -2 frameshifts (GC:CG) at alternating (GpC)_n sequences at the *hisD3052* allele (Nohmi *et al.*, 1995). The partially activated metabolite of 1,8-DNP, 1-nitroso-8-nitropyrene (1,8-NONP), has also been

Figure 3. The chemical structure of 1,8-dinitropyrene.

shown to be a powerful mutagen in *E. coli*, inducing several classes of mutations, including -1 frameshifts at contiguous G or C sequences (Lambert *et al.*, 1991; Lambert *et al.*, 1992).

Of the nitropyrenes, 1,8-DNP induced the largest number of revertants/nmole in tester strains such as *S. typhimurium* TA98 (Mermelstein *et al.*, 1981; Heflich *et al.*, 1985). In another assay, the minimum detectable mutagen concentration required to produce an effect in the *S. typhimurium* TM677 forward mutation assay was studied with various nitropyrenes (including 1,3-dinitropyrene (1,3-DNP) and 1,6-dinitropyrene (1,6-DNP)), and 1,8-DNP was found to be the most potent mutagen (0.5×10^{-3} nmole/mL) (Busby Jr. *et al.*, 1994a). Tests with other *S. typhimurium* strains, such as TA1538, have also shown 1,8-DNP to be a potent bacterial mutagen, being approximately 40-fold more mutagenic than 1-NP (Heflich *et al.*, 1985).

1.3.1.2 Eukaryotic mutagenicity/carcinogenicity.

Nitropyrenes, including 1,8-DNP, are potent eukaryotic mutagens and carcinogens. Gene mutations and chromosomal mutations have been detected in various mammalian cell lines and *Saccharomyces cerevisiae* following exposure to 1,8-DNP, and tumours have been detected in long term carcinogenicity assays upon treatment of rodents with dinitropyrenes.

Gene mutation assays testing the mutagenic potencies of nitropyrenes have employed cell lines of either human or rodent origin. For example, 1,8-DNP was mutagenic in a forward mutation assay using metabolically competent MCL-

5 human cells, as was 1,6-DNP (Busby Jr. *et al.*, 1994b). In contrast, recent work suggests that 1,8-DNP is not metabolically activated in human hepatoma HepG2 cells (Silvers *et al.*, 1994). In rodent cell lines, 1,8-DNP was found to be mutagenic at the *hprt* locus in CHO cells (Edgar and Brooker, 1985; O'Donovan, 1990) but not in hamster V79 cells, possibly due to differences in metabolic activity (O'Donovan, 1990). 1,8-DNP was also mutagenic in mouse lymphoma L5178Y cells (Cole *et al.*, 1982).

Chromosomal mutations resulting from exposure to nitropyrenes have also been well studied. 1,8-DNP has been implicated in producing chromatid and chromosomal aberrations and SCE in rodent cell lines (Edgar and Brooker, 1985; Tokiwa and Ohnishi, 1986). Nachtman and Wolff (1982) also detected significant induction of SCE in cultured CHO cells following exposure to 1,8-DNP and a rat liver S-9 mix. In contrast, the yeast *S. cerevisiae* D4 does not experience recombinogenic activity in the presence of nitropyrenes under standard aerobic conditions, perhaps due to the absence of metabolic activation (McCoy *et al.*, 1983).

The carcinogenic potential of nitropyrenes has also been well documented (Table 14). 1,8-DNP, as well as other nitropyrenes, have been found to be tumorigenic in a variety of rodent species (Tokiwa and Ohnishi, 1986). Studies have shown that 1,6-DNP and 1,8-DNP are more carcinogenic than the other nitropyrenes (King, 1988). In rats, 1,8-DNP has been found to cause mammary gland tumours, leukemias, malignant fibrous histiocytomas (MFH) and sarcomas at the site of injection (Sato *et al.*, 1986; King *et al.*, 1986;

Imaida *et al.*, 1988; Imaida *et al.*, 1995). Metastasis to the lung and liver has also been noted (King, 1988). 1,8-DNP is a proven mammary gland carcinogen in rats (King *et al.*, 1986; Imaida *et al.*, 1988; Imaida *et al.*, 1991), and has been shown to form DNA adducts in rat mammary tissue (Norman *et al.*, 1990) and human breast cells (Carmichael *et al.*, 1996). MFH were also detected in BALB/c mice following s.c. administration of 1,8-DNP (Tokiwa *et al.*, 1986; Otofujii *et al.*, 1987).

1.3.2 DNA adducts.

The formation of DNA adducts generally occurs in the presence of most potent carcinogens (Dipple, 1995) and therefore adducts can be good indicators of the mutagenicity and carcinogenicity of chemicals, both in prokaryotes and eukaryotes. Adducts were present in many rat tissues following treatment with 1,8-DNP, with adduct levels being highest in mesentery and mammary tissue, but also detectable in the liver, bladder, lung and kidney (Norman *et al.*, 1990). DNA adducts were also detected in primary rabbit tracheal epithelial cells following administration of 1,8-DNP (Norman *et al.*, 1989). The major adduct resulting from the metabolism of 1,8-DNP in rodents has been described as being 1-*N*-(2'-deoxyguanosin-8-yl)-amino-8-nitropyrene (Heflich *et al.*, 1986; Norman *et al.*, 1990).

DNA adduct formation has also been documented in *S. typhimurium* following treatment with 1,8-DNP (Andrews *et al.*, 1986) with the major adduct formed at the C8-position of deoxyguanosine (Heflich *et al.*, 1985). This major

adduct was identified as 1-*N*-(2'-deoxyguanosin-8-yl)-amino-8-nitropyrene (Andrews *et al.*, 1986).

1.3.3 Metabolism.

It is important to understand the enzymatic processes which are involved in the metabolic activation and detoxification of chemicals (Silvers *et al.*, 1992). These enzymatic processes will in fact modulate the outcome of the mutagenicity tests.

The potency of 1,6-DNP and 1,8-DNP as carcinogens in mammalian cells depends on the efficiency with which a) enzymes reduce these nitropyrenes to reactive hydroxylamine derivatives, and b) the intermediate acts as a substrate for acetyl transferase which yields an acetoxarylamine (Saito *et al.*, 1985; King, 1988). Following reductive metabolism and *O*-acetylation, nitropyrenes eventually form nitrenium ions (Figure 4), which can then go on to interact with proteins and nucleic acids (King, 1988; Kerdar *et al.*, 1993).

Several enzymes are necessary for the metabolic activation of nitropyrenes to their mutagenic forms (Bryant *et al.*, 1984; reviewed by Hein, 1988). 1,8-DNP is partially activated in both bacteria and mammals by a reductive process that is mediated by a number of enzymes that are collectively known as nitroreductases (Fifer *et al.*, 1986; Djuric *et al.*, 1986; Norman *et al.*, 1990). Nitroreduction has been shown to be an important step in the activation of dinitropyrenes, with weak mutagenic responses being observed in CHO cells

Figure 4. A schematic representation of the metabolism of 1,8-DNP.

Nitroreduction of 1,8-DNP leads to 1-nitroso-8-nitropyrene which is then further metabolised to form a hydroxylamine. O-acetylation will form an *N*-acetoxy-aryamine which can convert to an electrophilic nitrenium ion which can then go on to react with DNA to form adducts. Protonation of the hydroxylamine will also produce the nitrenium ion. (Adapted from King, 1988.)

nucleic acid adducts

following 1,8-DNP treatment, but stronger mutagenic responses observed when the cells were treated with the metabolite 1,8-NONP (Fifer *et al.*, 1986).

1,8-DNP is different from some other nitropyrenes in its requirement for an acetyltransferase enzyme for activation (Orr *et al.*, 1985). *S. typhimurium* strain TA98/1,8-DNP₆ retains the classical nitroreductase activity but has been shown to be deficient in an acetyl CoA acetyltransferase activity and this explains the observed resistance of this strain to 1,8-DNP-induced mutagenesis (Orr *et al.*, 1985). Acetyl CoA-dependent O-acetyltransferase (OAT) is involved in the metabolic activation of *N*-hydroxyarylamines formed from the oxidation of aromatic amines or reduction of nitroarenes in *S. typhimurium* (Watanabe *et al.*, 1994). Human (Land *et al.*, 1989), mouse (Mattano *et al.*, 1989; Kelly and Sim, 1994), rat (Djuric *et al.*, 1985) and rabbit tissues all possess the necessary enzymes (acetyl-CoA:arylamine *N*-acetyltransferase; NAT) to form *N*-acetoxyarylamines from hydroxylamines and acetohydroxamic acid metabolites (Norman *et al.*, 1990). The mammalian OAT and NAT are most likely the same enzymes (Watanabe *et al.*, 1994).

Variability in the rate of metabolic reactions such as *N*-acetylation can make individuals more or less vulnerable to toxic responses to chemicals (King and Glowinski, 1983). This variability in the NAT phenotype among individuals can lead to ethical questions relating to employment, insurance or credit, especially in circumstances where there is a known exposure to certain chemicals which may be more harmful to individuals who are classified as slow detoxifiers (Vineis and Schulte, 1995).

1.4 Objectives.

1.4.1 1,8-DNP has been shown to be a powerful bacterial and mammalian mutagen, and rodent carcinogen (Mermelstein *et al.*, 1981; Edgar and Brooker, 1985; Tokiwa and Ohnishi, 1986; King, 1988; Watanabe *et al.*, 1989; Imaida *et al.*, 1991; reviewed by Rosenkranz and Mermelstein, 1983). The first objective of this study was to determine whether 1,8-DNP-induced mutations are formed and can be detected in the *lacZ* transgene of the transgenic mouse Muta™Mouse.

1.4.2 1,8-DNP is a proven mammary gland carcinogen in rats (Imaida *et al.*, 1991; King, 1988), and has been shown to form DNA adducts in rat mammary tissue (Norman *et al.*, 1990) and human breast cells (Carmichael *et al.*, 1996). The second objective of this study was to determine whether Muta™Mouse would develop detectable 1,8-DNP-induced mutations in the *lacZ* transgene in mammary tissue. To facilitate this examination, retired breeder females were used in the second dosing protocol.

1.4.3 1,8-DNP is a frameshift mutagen (Mermelstein *et al.*, 1981), producing primarily -(GC:CG) frameshifts in the *S. typhimurium hisD3052* tester strain (Nohmi *et al.*, 1995) and the activated metabolite 1,8-NONP forms primarily G:C frameshifts in a forward mutation assay (Lambert *et al.*, 1991). The third objective of this study was to determine the mutational spectrum of 1,8-DNP in the *lacZ* transgene of Muta™Mouse.

II. Materials and Methods

2.1 Bacterial strains.

The bacterial strains used in this study are listed in Table 1.

2.2 Chemicals, media, enzymes and kits.

1,8-dinitropyrene (1,8-DNP) was a generous gift of Dr. Douglas Bryant, McMaster University. Stock solutions were prepared fresh as required in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corporation). Ethylnitrosourea (ENU; Sigma-Aldrich Corporation) was the generous gift of Dr. G.R. Douglas, Health Canada, and was prepared fresh as required in PBS.

Luria-Bertani (LB) broth (2% w/v LB broth base (Gibco/BRL Life Technologies Ltd., Burlington, Ontario)) with or without 1.5% Bacto agar (w/v, Difco) was used as a standard organic medium. Bottom agar and top agar were prepared by mixing LB broth base (0.5% w/v), agar (0.75% w/v) and NaCl (0.64% w/v) with distilled H₂O, and in the case of top agar, supplemented with MgSO₄ to a final concentration of 10 mM.

The media were supplemented, if required, with filter sterilised antibiotics at the following concentrations: ampicillin, 50 µg/mL; kanamycin, 20 µg/mL. Antibiotics were purchased from Sigma Chemical Co. Maltose (Fisher Scientific Co.) was used at a final concentration of 0.2% w/v and MgSO₄ (BDH Inc., Toronto, Ontario) was used at a final concentration of 10 mM. All of these supplements were prepared as stock solutions by dissolving appropriate concentrations in distilled H₂O and filter sterilising, and were stored at 4 °C. Phenyl β-D-galactoside (P-Gal; Sigma-Aldrich Corporation) was used at a final

Table 1. List of bacterial strains used.

Strain	Relevant <i>lacZ</i> genotype or phenotype	Source or reference
<i>Escherichia coli</i> K-12		
DH5 α	$\beta + \omega$ donor; <i>lacZ</i> Δ M15	Jessee, 1986
DH5 α (pUC128)	as DH5 α ; harbours pUC128 (<i>αlacZ</i>)	Keen <i>et al.</i> , 1988
W4680	$\alpha + \omega$ donor; <i>lacZ</i> 39 (deletion)	Cook and Lederberg, 1962
Hfr3000 X90	$\alpha + \beta$ donor; <i>lacZ</i> 90 (Oc)	Newton <i>et al.</i> , 1965
<i>Escherichia coli</i> C		
<i>E. coli</i> C <i>galE</i> ⁻	Δ <i>lacZ</i> , <i>galE</i> ⁻ , <i>recA</i> ⁻ , Kan ^r , pAA119 [<i>galE</i> ⁻ , <i>galK</i> ⁺ , <i>galT</i> ⁺ , Amp ^r]	Gossen <i>et al.</i> , 1992 Mientjes <i>et al.</i> , 1994
<i>E. coli</i> C <i>lacZ</i> ⁻	Δ <i>lacZ</i>	Gossen <i>et al.</i> , 1989

concentration of 0.3% w/v and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal; Research Organics Inc.) was used at a final concentration of 0.08 mg/mL. P-Gal solutions were made fresh as required and X-Gal stock solutions were stored at 4 °C.

Proteinase K, sodium dodecyl sulphate (SDS), phenol, Tris and agarose were purchased from Gibco/BRL Life Technologies Ltd., Burlington, Ontario. Chloroform was purchased from Caledon. Sucrose and NaCl were purchased from BDH Inc., Toronto Ontario. Gelatin, dimethyl formamide (DMF) and isoamyl alcohol were obtained from JT Baker Chemical Co., NJ, USA. Amberlite MB-1A Mixed Bed Exchanger was purchased from the Sigma-Aldrich Corporation, ammonium persulphate (APS), Temed from Amresco, and urea, acrylamide and bis-acrylamide were purchased from Dalton Chemical Laboratories Inc., Toronto, Ontario. Other fine chemicals were from the Sigma-Aldrich Corporation.

AmpliTaq® DNA polymerase and the related buffers and solutions were purchased from The Perkin-Elmer Corporation. Restriction enzymes were purchased from New England BioLabs Ltd., Mississauga, Ontario. Primers for PCR and sequencing were purchased from Dalton Chemical Laboratories, Inc., Toronto, Ontario (Table 2). Sequencing was carried out using an ABI Prism™ 377 DNA Sequencer and the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit was purchased from The Perkin-Elmer Corporation.

The Giga-Pack II Gold phage packaging extract was purchased from Stratagene, La Jolla, CA, USA. Promega Wizard™ Lambda Preps DNA

Table 2. List of oligonucleotide primers used for PCR amplification and/or nucleotide sequencing of *lacZ*.

Designation	Sequence	Complementation region	Base position
P1	5' - GGCGAGCGATACACCGCATCC -3'	ω	2634 - 2654
P2	5' - TGCTGTTGACTGTAGCGGCTG - 3'	ω	2915 - 2895
P3	5' - GGATGCGGTGTATCGCTCGCC - 3'	ω	2654 - 2634
P4	5' - CGCCAATCCACATCTGTG - 3'	ω	2332 - 2315
P5	5' - CGCGTTCGGTTGCACTACGC - 3'	ω	2133 - 2114
P6	5' - TGGATGCGGCGTGCGGTC - 3'	β	1863 - 1846
P7	5' - CTTTCATCCACGCGCGCGTAC - 3'	β	1543 - 1524
P8	5' - CGCCGGTAGCCAGCGCGG - 3'	β	1323 - 1306
P9	5' - AGCGTGCCGTCGGCGGTGT - 3'	β	983 - 965
P10	5' - ATGCCGCTCATCCGCCAC - 3'	α	641 - 624
P11	5' - GTGTAGATGGGCGCATCG - 3'	α	320 - 303
P12	5' - TCCTTACGCGAAATACGGGCAG -3'	ω	3133 - 3112
P13	5' - AATGTGTGGAATTGTGAG - 3'	α - β	-46 to -29
nP13	5' - TTAATGCAGCTGGCACGACAG - 3'	α - β	-170 to -150
P14	5' - TACACGCTGTGCGACCGC -3'	β - ω	1213 - 1230

Purification System and Wizard™ PCR Preps DNA Purification System for Rapid Purification of DNA Fragments were purchased from Fisher Scientific Co.

2.3 Bacterial growth conditions.

All bacterial strains were grown overnight in LB and maltose (0.2% w/v), with supplements as noted, at 37 °C and 250 rpm. Exponential cultures were prepared by 1:100 dilution of the overnight culture in LB, with supplements as noted. Exponential cells were grown, with agitation, to a cell density of approximately O.D.₆₀₀ of 1.5.

2.4 Mice.

Transgenic mice (strain 40.6), known commercially as Muta™Mouse, were donated by Ingeny BV (Leiden, The Netherlands) and were bred and tested at Health Canada, under the supervision of Dr. G.R. Douglas. The Muta™Mouse mice were used as the test animals and were dosed with varying amounts of 1,8-DNP. These mice, (BALB/c X DBA/2)CD2 F₁, carry 35-40 copies of the bacteriophage vector λ gt10/*acZ* per chromosome arranged in a head-to-tail form in every cell (Gossen *et al.*, 1989; Douglas *et al.*, 1994). Two major studies were carried out. A schematic diagram depicting these studies is displayed in Figure 5. The handling and treatment of mice were carried out under the supervision of J. Gingerich, and the necropsies were carried out with the help of laboratory members of the Mutagenesis Section, Dr. Lambert, and Dr. Bichara.

Figure 5. A schematic diagram summarising the two major studies described in this thesis. The general procedures used to obtain and analyse *lacZ*⁻ mutants from the λ gt10/*lacZ* transgene of the Muta™Mouse are also shown.

STUDY #1

- treatment with 1,8-DNP
- 4 dose levels
 - control (DMSO only)
 - low (0.3 mg/kg)
 - medium (0.9 mg/kg)
 - high (2.8 mg/kg)
- 8 weekly injections
- 29 day fixation period

STUDY #2

- treatment with 1,8-DNP and ENU
- control (DMSO only)
- 1,8-DNP (5.0 mg/kg)
- ENU (240 mg/kg) (positive control)
- 8 daily injections
- 29 day fixation period

remove tissues; store at -80 °C

extract genomic DNA from tissue

use packaging extract to remove λ gt10/*acZ* vectors,
package into empty pre-heads

propagate the phage particles in *E. coli* C *galE*⁻

plate on P-Gal for selection of *lacZ*⁻ mutants (positive selection assay)

collect plaques representing *lacZ*⁻ phage

complementation assay

PCR amplification of *lacZ* gene

DNA sequence analysis of *lacZ* gene

2.4.1 Study #1.

22 female mice of approximately 9 weeks of age were randomly assigned to one of four groups. The first two groups consisted of 5 mice each, and were assigned to either the negative control group or the low dose group. These mice received only the vehicle, DMSO (negative control), or approximately 0.04 mg/kg 1,8-DNP (low dose), respectively, per injection, for a total dose of approximately 0.3 mg/kg 1,8-DNP for the treated mice in the low dose group. The other two groups of mice contained 6 mice each, and received approximately 0.1 mg/kg 1,8-DNP (medium dose) and 0.4 mg/kg 1,8-DNP (high dose) respectively per injection. The total doses for these groups were approximately 0.9 mg/kg 1,8-DNP (medium dose) and 2.8 mg/kg 1,8-DNP (high dose). The animals were injected with their respective doses intraperitoneally once a week for 8 weeks, and their masses were recorded on a weekly basis (Figure 7). The solutions were freshly prepared each week and used immediately. The 1,8-DNP was dissolved in the DMSO with the help of a Branson 1210 Sonicator. Each treatment group of mice was housed separately in a polycarbonate cage with hardwood chips. Temperature and humidity were kept constant and food and water were available *ad libitum*.

The animals were sacrificed 29 days after the final injection. Tissues collected included brain, lungs, liver, mesentery, ovaries, bone marrow, kidneys, bladder, colon, spleen and tissue from the mammary region. Liver tissue was stored in two cryotubes, with one half of each lobe being placed into one vial. Bone marrow was collected by flushing the mouse femur with 1.0 mL of PBS

(phosphate-buffered saline without Mg^{2+} ; 8 g NaCl, 0.2 g KCl, 0.94 g Na_2PO_4 , 0.2 g KH_2PO_4 , 800 mL H_2O), and following centrifugation, the resulting pellet was stored and the buffer was discarded. The tissues were flash-frozen in liquid nitrogen immediately upon removal, and stored at $-80\text{ }^{\circ}\text{C}$.

2.4.2 Study #2.

Study #2 involved 30 female *lacZ* transgenic mice (strain 40.6; Muta™Mouse). These females had given birth to pups which were in the process of being weaned when the experiment started. Each mother was allowed to keep two of her pups to encourage lactation during the course of injections, and each family unit was housed in separate cages, which were kept in an Isotec isolator unit. The injections occurred in the same room as the housing, and temperature, humidity and air flow were kept constant, while food and water were available *ad libitum*. The cages were changed approximately every two days.

The mice were divided into 3 groups of 10 mice each; a negative control group which received DMSO only a treatment group which received a total of 5.0 mg/kg 1,8-DNP and a positive control group which received a total of 240 mg/kg ENU (in PBS), a proven Muta™Mouse mutagen (Douglas *et al.*, 1995a). The 1,8-DNP was dissolved in the DMSO with the help of a Branson 1210 Sonicator. The volume of DMSO which was administered to the negative control group and to the treatment group (as a vehicle for the 1,8-DNP) was 4 mL/kg for the first 4 days, and 3 mL/kg for the next 4 days because the animals were losing mass (Figure 8). The i.p. injections were administered daily for 8 days. The amount of

days. The amount of chemical received depended on the mass of each animal, which was recorded daily.

The pups were removed from the mothers 2 days after the final injection, and two weeks after the final treatment, the females were moved to another facility until they were sacrificed. Two animals in the negative control group did not survive the treatment and fixation periods; one died during the treatment from an unrelated uterine infection (autopsy performed by Health Canada staff) and one died during the fixation period. The necropsy of the 28 surviving animals took place 29 days after the final injection and tissues collected included brain, mesentery, mammary (two nipples from either side, lower abdominal region), lungs, liver, ovaries, bone marrow, kidneys, bladder, colon, and spleen. Liver tissue was stored in two cryotubes, with one half of each lobe being placed into one vial. Bone marrow was collected by flushing the mouse femur with 1.0 mL of PBS, and following centrifugation, the resulting pellet was stored and the buffer was discarded. The tissues were flash-frozen in liquid nitrogen immediately upon removal, and were stored at -80 °C.

The process of DNA isolation, phage packaging and mutant selection involving samples from both studies was carried out over a span of approximately 2 years. During this time, these experiments always involved samples taken from all 4 (Study #1) or all 3 (Study #2) treatment groups to avoid day-to-day biases due to natural variations in the DNA isolation, phage packaging and positive selection procedures. In addition, tissues from ENU treated animals were often obtained from J. Gingerich (Mutagenesis Section,

Health Canada), and used as positive controls for isolation procedures using samples from Study #1. Tissue samples were also provided (J. Gingerich, Health Canada) from various on-going studies for DNA isolation and packaging optimisation experiments.

2.5 DNA isolation.

Methodology for the isolation of genomic DNA prior to the packaging of the bacteriophage lambda vectors differed from tissue to tissue and optimisation was necessary for most of the tissues. The general outline of the procedures involved in extracting genomic DNA from mouse cells is as follows. Modifications for individual tissues are listed in the appropriate sections.

Phenol:chloroform:isoamyl alcohol (25:24:1) was prepared fresh daily. Approximately 25 mL of phenol and 10 mL of 1 M Tris (pH 8.0) were vigorously shaken, the phases were allowed to separate for 5 minutes, and the aqueous layer was removed. This step was repeated with 10 mL of 0.1 M Tris (pH 8.0), and the remaining organic layer was mixed with an equal volume of chloroform:isoamyl alcohol (24:1). After vigorous mixing, the solutions were centrifuged for 15 minutes at 1,600 x g in a Sorvall RC-5 Superspeed Refrigerated Centrifuge to separate the phases. The aqueous layer was then discarded, and the organic layer was ready to be used.

Samples were removed from the -80 °C storage freezer, and were placed in Falcon tubes containing 5.0 mL of lysis buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 10 mM EDTA) and proteinase K (final concentration: 0.5 mg/mL) as well

as 10% SDS (final concentration: 0.2%). After gentle inversion, the tubes were placed overnight in a water bath at 37 °C and 40 rpm. The following morning, a further 2.0 mL of lysis buffer and proteinase K (final concentration: 1.6 mg/mL) were added to each tube and the tubes were then placed into a 42 °C water bath for another 3 hours, again at 40 rpm. After this incubation, 5.0 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube. After repeatedly inverting the samples for 25 minutes and a centrifugation of 20 minutes at 1,500 x g in an IEC Centra 8 centrifuge to separate the phases, about three-fourths of the aqueous layer was removed (amount dependent on tissue type). A sufficient amount of 5 M NaCl was added to increase the final concentration to 200 mM. A volume of chloroform:isoamyl alcohol (24:1) equal to the volume of aqueous layer removed was added. Mixing by inversion then proceeded for 25 minutes, followed by a 20 minute centrifugation at 1,500 x g in an IEC Centra 8 centrifuge to separate the phases. About two-thirds of the aqueous layer was removed, and an equal volume of absolute ethanol was added. The tubes were gently inverted at room temperature for about 10 minutes, until no refractive lines remained, and the DNA had precipitated. The strands of DNA were then spooled onto Pasteur pipettes with sealed ends. The DNA was then washed in 70% ethanol, and allowed to air dry. DNA was stored at 4 °C in sterile screw-cap microtubes containing TE, pH 7.6 (10 mM Tris, pH 7.6, 1 mM EDTA). The concentration of the DNA was verified using a Beckman DU 640 Spectrophotometer at O.D.₂₆₀ and O.D.₂₈₀. An O.D.₂₆₀ of 1.0 is equal to 50 µg/mL of double-stranded DNA (Maniatis *et al.*, 1982).

2.5.1 Mesentery tissue.

The DNA extraction procedure was as described in section 2.5, with the following modifications. The mesentery tissue was prepared for proteinase K digestion by mincing it with surgical scissors. 500 μ L of lysis buffer was used to resuspend this mass, which was then transferred to an appropriately labelled Falcon tube containing 5.0 mL of lysis buffer, proteinase K (final concentration: 0.5 mg/mL) and 10% SDS (final concentration: 0.2%). Overnight incubation was as described in section 2.5.

The following day, after phenol extraction (section 2.5), 6.0 mL of the aqueous layer was removed, 60 μ L of 5 M NaCl was added and 6.0 mL of chloroform:isoamyl alcohol was added. After this extraction, 5.5 mL of the aqueous layer was transferred into a clean Falcon tube and the rest of the procedure was done as described previously in section 2.5. The resulting DNA samples were stored in 20 μ L of TE, pH 7.6.

2.5.2 Bone marrow.

The DNA extraction procedure was as described in section 2.5, with the following modifications. After the phenol extraction step on the second day of the procedure (section 2.5), 4.5 mL of the aqueous layer was removed, 45 μ L of 5 M NaCl was added and approximately 5.0 mL of chloroform:isoamyl alcohol was added. After this extraction, 3.0 mL of the aqueous layer was transferred into a clean Falcon tube and the remaining procedures are as described in section 2.5. The resulting DNA samples were stored in 20 μ L of TE, pH 7.6.

2.5.3 Brain tissue.

The DNA extraction procedure was as described in section 2.5, with the following modifications. Only the anterior portion of the brain was used, and it was kept on dry ice until ready to use. Five mL of lysis buffer and proteinase K (final concentration: 0.5 mg/mL) was added to each labelled Falcon tube. Using surgical scissors, the tissue was cut into very fine pieces, until minced. The tissue mass was resuspended in 500 μ L of lysis buffer and transferred to the Falcon tubes. After the addition of 10% SDS (final concentration: 0.2%), the tubes were gently inverted and placed overnight in a 37 °C water bath with gentle agitation.

The following day, after phenol extraction (section 2.5), 7.0 mL of the aqueous layer was removed, 70 μ L of 5 M NaCl was added and 7.0 mL of chloroform:isoamyl alcohol was added. After this extraction, 6.0 mL of the aqueous layer was transferred into a clean Falcon tube and the rest of the procedure was done as described in section 2.5. The resulting DNA samples were stored in 25 μ L of TE, pH 7.6.

2.5.4 Lung tissue.

The DNA extraction procedure was as described in section 2.5, with the following modifications. The lung tissue was prepared for digestion by mincing with surgical scissors. 500 μ L of lysis buffer was used to resuspend the minced tissue mass, which was then transferred to a Falcon tube containing 2.0 mL of PBS. The tissues were de-gassed to prevent them from floating in the solution

by placing the Falcon tubes containing the tissues into a flask and using suction to draw air out of the flask and also the tissues. The tissues were pelleted by centrifugation for 5 minutes at 760 x g in an IEC Centra 8 centrifuge and the resulting supernatants were removed by aspiration. The pellets were then resuspended in 2.0 mL PBS. The centrifugation step was repeated, and again the supernatants were aspirated. The pellets were then each resuspended in 500 μ L of lysis buffer. Following the addition of 5.0 mL of lysis buffer, proteinase K (final concentration: 0.5 mg/mL) and 10% SDS (final concentration: 0.2%), the tubes were incubated overnight as described in section 2.5.

Following overnight incubation, phenol extraction (section 2.5) was carried out, and 4.0 mL of the aqueous layer was removed and added to 40 μ L of 5 M NaCl and 4.0 mL of chloroform:isoamyl alcohol. After mixing, and separation of the phases by centrifugation, 2.5 mL of the aqueous layer was transferred into a Falcon tube and the rest of the procedure was done as described previously in section 2.5. The resulting DNA samples were stored in 40 μ L of TE, pH 7.6.

2.5.5 Liver tissue.

The DNA extraction procedure was as described in section 2.5, with the following modifications. On the first day, the liver samples were homogenised using a Duall tissue grinder (Mandell) and a Teflon pestle with 5.0 mL of cold TMST (50 mM Tris, pH 7.6, 3 mM MgSO₄, 250 mM sucrose, 0.2% Triton X-100) buffer. The samples were placed into Falcon tubes, centrifuged at 4 °C for 6

minutes at 700 x g in a Sorvall RC-5 Superspeed Refrigerated Centrifuge and the resulting supernatant was aspirated. The remaining pellets were resuspended in 5.0 mL of cold TMST buffer and the samples were spun again for 6 minutes as before. The suction and resuspension steps were repeated and a third spin for 6 minutes was carried out. After removal of the supernatant, 5.0 mL of lysis buffer and proteinase K (final concentration: 0.5 mg/mL) was added to each tube along with 10% SDS (final concentration: 0.2%), and incubation was allowed to proceed overnight as described previously in section 2.5.

The following day, after phenol extraction (section 2.5), 5.0 mL of the aqueous layer was removed, 50 μ L of 5 M NaCl was added and 5.0 mL of chloroform:isoamyl alcohol was added. After this extraction, 4.0 mL of the aqueous layer was transferred to a clean Falcon tube and the rest of the procedure was carried out as described in section 2.5. The resulting DNA samples were stored in 75 μ L of TE, pH 7.6.

2.6 Mutant isolation using the positive selection assay.

An *E. coli* C *galE*⁻ (Table 1) overnight culture was prepared as described in section 2.3, and was supplemented with 50 μ g/mL ampicillin and 20 μ g/mL kanamycin. An exponential culture was started the following day, supplemented with 0.2% w/v maltose.

Appropriate amounts (volume varies with tissue source) of extracted genomic DNA (concentration of 1-5 μ g/ μ L) which contained multiple copies of the λ gt10/*acZ* insert, were mixed with corresponding amounts of the red and

yellow phage packaging extracts (Giga-Pack II Gold, Stratagene), including empty phage heads, according to the provided protocol. Briefly, for 2.0 μL of genomic DNA, 3.0 μL of recently thawed extract from the red tube (freeze-thaw extract) was added to the DNA in an Eppendorf tube. After gently mixing, 4.5 μL of the recently thawed sonic extract from the yellow tube was added to the DNA-red tube mixture, the solutions were gently mixed, pulse-spun and incubated at room temperature. Approximately one hour after the commencement of incubation, the contents of the tubes were gently mixed and the tubes were pulse spun again. After 2.5 hours of room temperature incubation, the resultant phage were diluted in 500 μL SM buffer (100 mM NaCl, 50 mM Tris, pH 7.6, 16 mM MgSO_4 , 0.01% w/v gelatin) and were mixed for another 30 minutes by gentle inversion.

During the incubation time for phage packaging, the exponential *E. coli* C *galE*⁻ cells were grown to a cell density of approximately O.D.₆₀₀ of 1.5, and were concentrated by spinning down at 850 x g for 10 minutes at 10 °C in an IEC Centra-7R refrigerated centrifuge. The cells were resuspended in half the original volume in cold LB supplemented with 10 mM MgSO_4 .

Fifteen μL of the packaged phage was allocated to titre determination, while the remaining 485 μL of the packaged phage was allocated for mutant selection. The packaged phage particles were allowed to adsorb to the concentrated *E. coli* C *galE*⁻ cells for 25 minutes at room temperature.

Following adsorption, the mixture for titre determination was combined with 8 mL top agar (section 2.2) per plate, and plated onto bottom agar plates.

For each of the samples, four plates were used as indicators of the plaque titres. Another four plates had P-Gal, dissolved in DMF, added to the top agar (final concentration: 0.3% w/v), and these served as the indicators of phage containing non-functional *lacZ* genes (Figure 2). The plates were incubated overnight at 37 °C.

Following the overnight incubation, the number of clear plaques present on the titre and test plates (a total of eight plates) was counted and recorded. Calculations used to determine the plaque titre in the plates, the total number of phage tested and the mutant frequency are described below.

Equations:

$$1. \text{ Phage titre}/\mu\text{L} = \frac{(\text{total number of plaques on titre plates})}{(\text{volume of packaged phage tested})}$$

$$2. \text{ Total number of plaques} = (\text{volume of cells used} + \text{volume of packaged phage} - \text{volume of packaged phage removed for titre determination}) \times (\text{phage titre}/\mu\text{L})$$

$$3. \text{ Mutant frequency} = \frac{(\text{total number of mutants})}{(\text{total number of plaques})}$$

Plaques from Study #1, found on the control and high dose test plates, were removed and stored in 500 to 1000 μL SM buffer and chloroform (final concentration: 0.5% to 1% v/v). Plaques from Study #2, found on the negative

control and the 1,8-DNP-treated test plates, were collected and stored as for Study #1.

Mean mutant frequency, standard error of the mean and standard deviation calculations were carried out using the SigmaStat™ Statistical Software program (Jandel Scientific). The statistical significance of differences in the mean mutant frequency of different treatment groups relative to the mean mutant frequency of the negative control group was determined using the Mann-Whitney two sample, unpaired one-tailed statistical test (Rosner, 1986) using the InStat program (GraphPad Software Inc.).

2.7 Lambda phage amplification, phenotype verification and purification.

The titre of the phage collected previously from the positive selection assay (section 2.6) was amplified by re-infecting the *E. coli* C *galE*⁻ host and picking the resultant phage plaques. The mutation in the phage based *lacZ* gene was verified by carrying this amplification step out in top agar supplemented with X-Gal (section 2.7.1). Fresh plaques collected from the amplification experiment (section 2.7.1) were used for the infection of the *E. coli* complementation strain DH5α (section 2.8.1) and also the *E. coli* C *lacZ*⁻ strain, from which the λ phage particles in the supernatant were collected (section 2.7.2). Phage DNA was used for PCR amplification of phage *lacZ* sequences (section 2.9) which were analysed by DNA sequencing (section 2.10).

2.7.1 Lambda phage amplification and phenotype verification.

Following the positive selection assay to identify *lacZ*⁻ phage (section 2.6), the plaques of interest were stored in SM buffer and chloroform. However, due to the fact that only one plaque was stored per Eppendorf tube, and the phage were required approximately one year after the initial storage, the titre was poor. Amplification of the phage titre was carried out by infecting the *E. coli* C *galE*⁻ host with these phage stocks.

An overnight culture of *E. coli* C *galE*⁻ cells was grown, as described in section 2.6. The following day, an exponential culture was grown (section 2.3), supplemented with 10 mM MgSO₄ and 0.2% w/v maltose. 200 µL of these exponential cells was mixed with 100-300 µL of phage stock in a Falcon tube, and allowed to adsorb for 20 minutes at 37 °C. The phage stock was obtained from the P-Gal positive selection assay (section 2.6). The cell-phage mixture was then combined with 10 mL of top agar (0.08 mg/mL X-Gal) and plated onto a LB plate. *E. coli* strain DH5α (pUC128) (Table 1) was used as a positive control for β-galactosidase hydrolysis of X-Gal, and *E. coli* C *galE*⁻ cells without phage served as a negative control for phage contamination of the host cells or cell-based hydrolysis of X-Gal.

Following an overnight incubation at 37 °C, the plates were examined for growth of plaques, and their colour (blue or clear) was scored. Clear plaques indicated a *lacZ*⁻ genotype. Plugs with plaques were collected with a Pasteur pipette and stored in Eppendorf tubes in 1.0 mL SM buffer and chloroform (final concentration: 0.5%).

2.7.2 Lambda phage purification.

E. coli C *lacZ*⁻ (Table 1) was used to produce lambda phage-rich supernatant to obtain purified lambda DNA for subsequent PCR amplification (section 2.9) and sequencing (section 2.10). A culture of *E. coli* C *lacZ*⁻ was grown overnight (section 2.3). The following day, phage were allowed to adsorb to the bacteria for 15 minutes at 37 °C by mixing 100 µL of the *E. coli* C *lacZ*⁻ cells with 50 µL of the phage (section 2.7.1) in an Eppendorf tube. This phage-cell mixture was then transferred into a flask containing 10 mL LB (10 mM MgSO₄, 0.2% w/v maltose) and was incubated overnight at 37 °C with agitation.

The following day, the lambda phage in the supernatant were isolated using the Wizard™ Lambda Preps DNA Purification System (Promega). Briefly, 30 µL of chloroform was added to each flask, and agitation continued for 15 minutes. The culture was then decanted into 15 mL Falcon tubes, which were centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge at 8,200 x g for 15 minutes to remove cellular debris. The supernatants were transferred to fresh 15 mL Falcon tubes. Following the addition of 40 µL of Nuclease mix (0.25 mg/mL RNase A, 0.25 mg/mL DNase I, 150 mM NaCl, 50% glycerol) to each tube, the tubes were gently inverted and incubated at 37 °C for 15 minutes. Four mL of PEG-NaCl mix (33% polyethylene glycol [PEG-8000], 3.3M NaCl) was then added to each tube, mixed gently and the tubes were placed on ice for 30 minutes. Following the precipitation step, the tubes were spun in the Sorvall for 10 minutes at 9,900 x g. The supernatants were then discarded, leaving the pelleted phage. The tubes were drained and the residual supernatant was

allowed to air dry. The phage pellets were resuspended in 500 μ L phage buffer (150 mM NaCl, 40 mM Tris-HCl, pH 7.4, 10 mM MgSO_4) and were then transferred to 1.5 mL Eppendorf tubes. A 20 second centrifugation in an Eppendorf Centrifuge 5415C at 10,500 x g was used to pellet any remaining debris, and the supernatants were transferred to fresh 1.5 mL Eppendorf tubes. At this point, the tubes were stored overnight at 4 °C. The following day, 1.0 mL of mixed Purification Resin was added to each tube containing supernatant and mixed by pipetting. For each sample, a Minicolumn was attached to the barrel of a 5 mL disposable syringe, and the lysate/Resin mixture was transferred into the barrel of the syringe. The plunger was used to then push the mixture through, leaving the DNA in the column. The Minicolumn was then washed with 2.0 mL of 80% isopropanol which was drained completely by centrifugation in the Eppendorf Centrifuge 5415C at 10,500 x g for 20 seconds. The purified phage DNA was eluted in 50 μ L of water that had been preheated to 80 °C, by spinning it for 20 seconds at 10,500 x g. The quantity and quality of the purified DNA was verified using a Beckman DU 640 Spectrophotometer at O.D.₂₆₀ and O.D.₂₈₀, and was stored at -20 °C.

2.8 Complementation assays.

The *lacZ* gene is approximately 3.3 kb in length. Thus, sequencing the gene is a long and difficult task. In order to simplify the search for spontaneous or chemical-induced mutations, protein complementation assays were used to attempt to localise the mutation to one of three regions of the *lacZ* gene, α , β or

ω. Three *E. coli* K-12 complementation strains were used for this purpose: DH5α, W4680 and HFR3000 X90 (Table 1). Each strain lacks one of the three complementation regions and thus, when the *lacZ* gene of the phage is also present, complementation between the host and phage *lacZ* genes was determined by a blue colour in the presence of X-Gal. Specifically, a clear colour with one strain, and a blue colour with the other two strains, indicates that the mutation in the *lacZ* gene of the phage is in the region which is lacking in the first, clear strain.

2.8.1 Complementation with the *E. coli* strain DH5α.

The methylation pattern of the λ phage grown in *E. coli* C was altered prior to the complementation testing with the K-12 strains by using an *E. coli* K-12 host strain DH5α, which is restriction deficient (r^-_k, m^+_k). Phage collected following infection of this strain of DH5α are methylated in a K-12 pattern and are therefore compatible in the other two K-12 complementation strains, W4680 and HFR3000 X90.

An overnight culture of *E. coli* DH5α was set up from a single colony (section 2.3). The following day, an exponential culture supplemented with 10 mM MgSO₄ was established. 100 μL of the exponential culture was transferred to a 15 mL Falcon tube and mixed with 50 μL of the phage stock (section 2.7.1), and allowed to adsorb for 20 minutes at 37 °C. Top agar (0.08 mg/mL X-Gal) was added and the mixture was plated onto a LB plate. The plates were incubated overnight at 37 °C.

The following day, the colour of the plaques was recorded and the plates were kept for comparison to the results of the complementation with the other two strains. About 4 or 5 plaques were picked from each plate and stored in 500 μ L SM buffer supplemented with 1% chloroform at 4 °C for at least 2 hours. Phage were then used in complementation assays in strains W4680 and HFR3000 X90 (section 2.8.2).

2.8.2 Complementation with the *E. coli* strains W4680 and HFR3000 X90.

Overnight cultures were established from single colonies of the two *E. coli* K-12 strains, W4680 and HFR3000 X90, as described in section 2.3. The following day, exponential cultures supplemented with 10 mM MgSO_4 were established. 100 μ L of each exponential culture was transferred to 15 mL Falcon tubes. Fifty μ L of phage (obtained from adsorption with DH5 α ; section 2.8.1) was then added to each Falcon tube, and adsorption was allowed to proceed for 20 minutes at 37 °C. The mixture was taken up in 10 mL top agar (0.08 mg/mL X-Gal) and poured onto a LB plate. The plates were grown overnight at 37 °C.

The following day, each plate was examined for the appearance of blue plaques, indicating a functional *lacZ* gene product. The plates from each of the three complementation strains (DH5 α [section 2.8.1], W4680 and HFR3000 X90) for each mutant were compared to determine the relative intensity of the blue colour, and these results were recorded. This information was used to make an

initial assignment of the location of the mutation to one of the three complementation regions.

2.9 Amplification of the *lacZ* gene using PCR.

The *lacZ* gene is approximately 3.3 kb in length, including the promoter/operator regions (see Appendix for nucleotide sequence). The PCR amplification and sequencing strategies are depicted in Figure 6.

To simplify the DNA sequencing, separate sets of primers were used to amplify the gene using PCR. This resulted in fragments corresponding to a) the region closest to the 3' end of *lacZ* (1.7 kb or 1.9 kb), and b) the 5' end of *lacZ* (1.6 kb or 1.7 kb). There was an overlap of 331 bp in the middle section of the gene. The primers used were P14 (plus strand) and P2 or P12 (minus strand) to amplify the 1.7 kb and 1.9 kb regions, respectively (Figure 6; Table 2). This 3' section of the gene corresponds to the complementation regions of ω and some of β . The primers used to amplify the 5' end of the *lacZ* gene were P7 (minus strand) and P13 or nP13 (plus strand) to produce fragments of 1.6 kb and 1.7 kb, respectively (Figure 6; Table 2). This region corresponds to the complementation regions of α and some of β .

PCR amplification was carried out using an Amplitron[®]II Thermolyne PCR machine or an MJ Research, Inc. Peltier-Effect Cycling PCR machine. PCR reactions were done in 50 μ L volumes using the following conditions: 1x reaction buffer II, 200 μ M of each dNTP, 0.2 μ M of each primer, 2.5 units of AmpliTaq[®]

Figure 6. A schematic diagram depicting the oligonucleotide primers used in this study for PCR amplification and sequencing of the *lacZ* gene. The *lacZ* gene (0-3158 bp) is indicated by a solid line (5' to 3'), along with the promoter/operator region (-183 to 0 bp). The approximate locations of the oligonucleotides along the *lacZ* gene and their orientations are indicated. As well, the general positions of the three complementation groups, α , β and ω , are shown.

lacZ

DNA polymerase (The Perkin-Elmer Corporation), 2.5 mM MgCl₂ and 0.1-0.5 µg DNA template.

The following cycles were used. For the P14 P2 or P14 P12 amplification: 95 °C for 1 minute, 30 seconds; followed by 35 repetitions of 95 °C for 45 seconds; 55 °C for 10 seconds; and 70 °C for 1 minute, 30 seconds; an extension at 75 °C for 5 minutes after completion of the repetitions. Samples were stored at 4 °C until use. For the P13 P7 or nP13 P7 amplification: 95 °C for 1 minute, 50 seconds; followed by 35 cycles of 95 °C for 45 seconds; 45 °C for 25 seconds; 72 °C for 1 minute, 30 seconds; an extension at 75 °C for 5 minutes after completion of the repetitions. Samples were stored at 4 °C until use.

Portions of the resulting PCR products (5 µL of PCR product) were analysed on a 1% agarose gel pre-stained with ethidium bromide (EtBr; 0.5 µg/mL) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 67 V for 45 minutes. A λ *Bst*EI digest was used as a standard ladder to determine the sizes of the fragments. DNA bands were visualized under a UV light source and photographed. The PCR amplified samples were purified using the Wizard™ PCR Preps DNA Purification System for Rapid Purification of DNA Fragments (Promega) using a protocol provided by the manufacturer.

Briefly, a 1.5 mL Eppendorf tube was prepared for each PCR sample by adding 100 µL of the Direct Purification Buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.8 at 25 °C], 1.5 mM MgCl₂, 0.1% Triton® X-100). The PCR reaction was then mixed with the Direct Purification Buffer. Next, 0.8 mL of mixed Purification Resin

was added and mixed by pipetting. The mixture was placed into a 5 mL disposable syringe with an attached purification Minicolumn and pushed slowly through. DNA bound to the resin became immobilised on the column and was then washed with 3 mL of 80% isopropanol. The column was detached from the syringe and spun in the Eppendorf Centrifuge 5415C centrifuge at 10,500 x g for 20 seconds to remove residual isopropanol. DNA was eluted from the column by adding 50 μ L sterile dH₂O, leaving it at room temperature for 2 minutes, and finally spinning for 20 seconds at 4,700 x g. The quantity and quality of the purified DNA was verified using a Beckman DU 640 Spectrophotometer at O.D.₂₆₀ and O.D.₂₈₀, and was stored at -20 °C.

2.10 Sequencing of the *lacZ* gene.

DNA sequence analysis was carried out using cycle sequencing and an ABI Prism™ 377 DNA Sequencer (The Perkin-Elmer Corporation) according to the manufacturer's protocol, with some variations which are described in detail in section 2.10.1. A sequence of about 500-600 bp could be read and analysed upon sequencing with each primer. DNA sequences corresponding to the ω and β regions were sequenced first since previous experiments have suggested that this section of the gene is relatively sensitive to induced and spontaneous mutations (section 4.2.3.2). The major primers used for this section were P2, P4, P6 and P12. Other primers were used as required, especially if a mutation was found towards the end of a particular sequencing run, and confirmation was deemed necessary. In the absence of any mutations in this region, the 5' region

of the *lacZ* gene was sequenced, using primarily the primers P8 and P10. Other primers were used as necessary. A complete list of primers used for both sequencing and PCR is presented in Table 2.

2.10.1 Sequencing reactions.

The sequencing reactions were carried out in 0.2 mL thin-walled PCR tubes according to the manufacturer's protocol (The Perkin-Elmer Corporation), with some modifications. A cocktail consisting of 26 ng of the primer (4 μ L), water (8 μ L) and the Terminator Ready Reaction Mix (4 μ L) was made up. Between 0.08 μ g to 0.16 μ g of the purified PCR-amplified *lacZ* region was mixed with 16 μ L of the cocktail. An appropriate volume of water was used in the cocktail mix to produce a final volume of 20 μ L. Alternatively, some sequencing samples were prepared by using 6 μ L of the Reaction Mix, 100 ng of primer, 40-100 ng DNA template and enough water to bring the final volume to 20 μ L. Paraffin oil (40 μ L) was added to the top of each reaction mixture and the tubes were then placed in the MJ Research, Inc. Peltier-Effect Cycling PCR machine, pre-heated to 95 °C. Following a pre-dwell at 95 °C for 90 seconds, cycle sequencing was carried out using cycles consisting of 96 °C for 30 seconds, 45 °C for 20 seconds and 60 °C for 4 minutes. After 38 repetitions, an extension at 75 °C for 5 minutes occurred, followed by storage at 4 °C until use. Generally, this reaction was allowed to proceed overnight.

The cycle sequencing products were precipitated by adding 50 μL of 95% ethanol and 2.0 μL of 3M NaAcetate (pH 5.2) to 23 μL of product in a 0.5 mL Eppendorf tube. The samples were mixed and placed on ice for 10 minutes, after which time they were spun for 20 minutes at 4 °C at 16,600 x g in an Eppendorf Centrifuge 5417R centrifuge. The supernatant was then removed with a pipette, being careful not to dislodge the DNA pellet. The pellet was washed 5 times with 260 μL of 70% ethanol, and most of the liquid was then removed. The pellet was dried using a SpeedVac SVC100 for about 20 minutes.

Once the DNA samples were dry, any residual paraffin oil was removed with a pipette, and the DNA was resuspended in 5.0 μL of deionized formamide. Prior to loading, the samples were heated for 2 minutes at 90 °C and were then kept on ice until needed.

2.10.2 Sequencing gel.

The sequencing gel was pre-run for 20 minutes. The wells were rinsed with TBE (10.8 g Tris base, 5.5 g boric acid, 4 mL of 0.5 M EDTA (pH 8.3)). 2.5 μL of each denatured cycle sequencing product (section 2.10.1) was then loaded and run on a 4.25% sequencing polyacrylamide gel (acrylamide:bis-acrylamide ratio 19:1; 42% w/v urea in 1 X TBE) for 7 hours, with the data being continuously downloaded to Power Macintosh 7100/80 computer, using the ABI Prism™ 377 Collection program for data collection. Sequence analysis was carried out on a Power Macintosh 6100/66, using the Factura and Sequence Navigator programs (The Perkin-Elmer Corporation).

III. Results

3.1 Study #1.

22 female Muta™Mouse mice (strain 40.6) were obtained at approximately 9 weeks of age. These transgenic mice, carrying approximately 35–40 copies of the bacteriophage vector λ gt10/*lacZ* per haploid chromosome, were randomly assigned to one of four groups. The first two groups consisted of 5 mice each and received only the vehicle, DMSO, or a total dose of approximately 0.3 mg/kg 1,8-DNP, respectively. The other two groups contained 6 mice each, and received total doses of approximately 0.9 and 2.8 mg/kg 1,8-DNP, respectively. The mice were weighed prior to the treatments and on the day of necropsy (Figure 7). The average masses of the animals increased during the dosing period, likely due to the fact that the animals were still growing at the start of the injections, and weight gain associated with maturation might mask any loss due to DMSO toxicity, if present. All animals survived the 8 weekly injections and the 29 day fixation period following the treatments. The necropsy involved removal of the brain, lungs, liver, mesentery, ovaries, bone marrow, kidneys, bladder, colon, spleen and tissue from the mammary region. The tissues were flash-frozen in liquid nitrogen, and stored at -80 °C (section 2.4.1). No tumours were observed. The bacteriophage vectors carrying the *lacZ* transgene were isolated from the genomic DNA of mesentery, bone marrow, brain, lung and liver tissues following optimisation of the extraction procedures (section 2.5), and *lacZ* mutants were identified by the positive selection assay (section 2.6). The phage titre (phage titre/ μ L), the actual number of packaged

Figure 7. The average masses of the mice in Study #1 during the course of treatment. The masses of the mice in the negative control group (DMSO only), and the treatment groups of 0.3, 0.9 and 2.8 mg/kg 1,8-DNP were determined prior to the injections on each of the 8 weekly treatment days, and also on the day of necropsy, 29 days after the final injection. Error bars representing the standard error of the mean are shown for one treatment group per week to give an indication of the variations in the masses.

phage used for the assay (total number of plaques), and the mutant frequency for each sample were calculated as described in section 2.6.

3.1.1 Mesentery tissue.

The site of injection was chosen as the most likely site of 1,8-DNP-induced mutagenesis in the mice, since it is the region with the highest concentration of the chemical. The genomic DNA isolation technique used for other tissues (Vijg and Douglas, 1996) was modified to allow the extraction of genomic DNA from the mesentery tissue in a sufficiently intact form to allow the excision and packaging of the bacteriophage lambda particles into the phage pre-heads (section 2.5.1).

The mutant frequencies of *lacZ* transgenes obtained from mesentery tissue of mice in Study #1 were calculated following the collection of data from the positive selection assay (section 2.6). The number of packaged lambda vectors obtained from each mesentery sample ranged from 2.53×10^4 to 17.91×10^4 (mean = $[7.39 \pm 0.83] \times 10^4$) (Table 3), and was not very efficient according to the recommendations of Morrison and Ashby (1994) of a minimum of 20×10^4 phage per sample. The mean mutant frequency and standard error of the mean were determined for each treatment group.

There was a modification to the calculation of the mean mutant frequency. A mutant frequency of 0 had to be omitted for the mean mutant frequency calculation for animal #6 from the low 1,8-DNP dose group (0.3 mg/kg 1,8-DNP)

Table 3. A summary of the positive selection assay data obtained from mesentery tissue of mice treated in Study #1, with DMSO only (negative control) or 0.3, 0.9 or 2.8 mg/kg 1,8-DNP. The Mann-Whitney unpaired, one-tailed test was used to determine whether there existed any statistically significant differences between the mutant frequencies of the *lacZ* transgene from groups of Muta™Mouse mice given various doses of 1,8-DNP relative to the negative control group.

^A. Generally determined from four plates for each animal sample.

^B. Standard error of the mean.

^C. Samples with total titres of less than 1×10^4 plaques were discounted.

^D. Significant increase according to the Mann-Whitney unpaired, one-tailed test.

Mutant frequencies of the high dose group (2.8 mg/kg 1,8-DNP) and the negative control group (DMSO only) were compared for a statistically significant increase in values following treatment.

Total dose (mg/kg 1,8-DNP)	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques in the presence of P-Gal ^A	Mutant frequency (x 10 ⁻⁵)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney test (p-value)
0	1	4.01	1	2.49			
	2	3.79	2	5.27	6.50	1.52	
	3	17.91	13	7.26			
	4	5.25	3	5.71			
	5	11.91	14	11.75			
0.3	6	0.22 ^c	0 ^c	---			
	7	12.69	10	7.88	9.13	1.17	0.143
	8	6.15	4	6.51			
	9	11.38	12	10.54			
	10	7.77	9	11.56			
0.9	11	6.84	6	8.76			
	12	8.42	12	14.26	9.82	2.02	0.165
	13	5.42	2	3.69			
	14	6.51	3	4.61			
	15	4.94	6	12.15			
2.8	16	5.19	8	15.43			
	17	9.84	13	13.21			
	18	5.09	6	11.80	21.29	10.83	0.041 ^D
	19	2.70	1	3.70			
	20	2.53	19	74.96			
	21	6.23	8	12.84			
	22	10.67	12	11.25			

due to an insufficient number of plaques on the titre plates (representing a total phage titre of less than 1×10^4) (Table 3; footnote C).

There was a gradual increase in the mean mutant frequencies as the dose of 1,8-DNP was increased, from 6.50×10^{-5} for the negative control group which received only DMSO, to 21.29×10^{-5} for the highest dose group, which received a total of 2.8 mg/kg 1,8-DNP during the treatment period (Table 3). The Mann-Whitney unpaired one-tailed statistical test was used to assess whether there were any significant increases in the mutant frequencies of each treatment group relative to the negative control group (DMSO only). The InStat statistical program (GraphPad Software Inc.) was used to carry out this analysis. All one-tailed p-values greater than 0.05 indicated a lack of significant difference between the mutant frequencies of different groups. Thus, no statistically significant increases in mutant frequencies following treatment with either 0.3 or 0.9 mg/kg 1,8-DNP were observed when compared to the mutant frequencies of the negative control group using the Mann-Whitney statistical test (Table 3). However, a one-tailed p-value of 0.041 was calculated following the comparison of the mutant frequencies of the negative control group (DMSO only) and the high dose group (2.8 mg/kg 1,8-DNP), indicating that the observed increase in the mutant frequencies of the high dose group relative to the mutant frequencies of the negative control group was statistically significant according to the Mann-Whitney statistical test (Table 3; footnote D). Plaques from both the negative control group and the high dose group were collected as described in section 2.6, and the *lacZ* transgenes were analysed for DNA sequence alterations

sections 2.7-2.10). The sequencing data are presented in section 3.3, and are discussed in section 4.2.

3.1.2 Bone marrow.

The mutant frequencies of *lacZ* transgenes obtained from bone marrow were calculated following the collection of data from the positive selection assay (section 2.6). The mean mutant frequency and standard error of the mean were determined for each treatment group (Table 4). It may be noted that the mutant frequencies in bone marrow are based on the analysis of high numbers of phage (Table 4). This is borne out by the total number of phage packaged from each sample and detected on the titre plates, which ranged from 4.92×10^4 to 66.33×10^4 phage (mean = $[34.03 \pm 3.89] \times 10^4$) for Study #1 (discounting animal #4) (Table 4; footnote C). Genomic DNA is relatively easy to extract from bone marrow without damaging the DNA. This is an important characteristic for the efficient *in vitro* packaging of the transgene into the phage pre-heads.

There was a modification to the calculation of the mean mutant frequency. A mutant frequency of 0 had to be omitted for the mean mutant frequency calculation for animal #4 from the negative control group (DMSO only), due to an insufficient number of plaques on the titre plates (representing a total phage titre of less than 1×10^4) (Table 4; footnote C).

The Mann-Whitney unpaired one-tailed statistical test was used to assess whether there were any significant increases in the mutant frequencies of each treatment group relative to the negative control group (DMSO only). The InStat

Table 4. A summary of the positive selection assay data obtained from bone marrow of mice treated in Study #1, with DMSO only (negative control) or 0.3, 0.9 or 2.8 mg/kg 1,8-DNP. The Mann-Whitney unpaired, one-tailed test was used to determine whether there existed any statistically significant differences between the mutant frequencies of the *lacZ* transgene from groups of Muta™Mouse mice given various doses of 1,8-DNP relative to the negative control group.

- ^A. Generally determined from four plates for each animal sample.
- ^B. Standard error of the mean.
- ^C. Samples with total titres of less than 1×10^4 plaques were discounted.
- ^D. Marginally significant change according to the Mann-Whitney unpaired, one-tailed test. Mutant frequencies of the low dose group (0.3 mg/kg 1,8-DNP) and the high dose group (2.8 mg/kg 1,8-DNP) were compared to the negative control group (DMSO only) for a statistically significant change in values following treatment.

Total dose (mg/kg 1,8-DNP)	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques in the presence of P-Gal ^A	Mutant frequency (x 10 ⁻⁵)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney Test (p-value)
0	1	31.08	14	4.50			
	2	31.61	12	3.80	4.06	0.15	
	3	46.45	18	3.87			
	4	0.42 ^C	0 ^C	---			
	5	4.92	2	4.06			
0.3	6	16.43	7	4.26			
	7	57.78	21	3.63	3.42	0.32	0.056 ^D
	8	66.33	25	3.77			
	9	16.37	5	3.05			
	10	29.36	7	2.38			
0.9	11	12.19	3	2.46			
	12	52.48	24	4.57	6.94	3.57	0.471
	13	49.70	21	4.23			
	14	48.71	120	24.64			
	15	23.52	4	1.70			
2.8	16	22.33	9	4.03			
	17	18.49	12	6.49			
	18	62.56	41	6.55	7.59	1.72	0.057 ^D
	19	49.24	36	7.31			
	20	28.49	7	2.46			
	21	19.55	30	15.35			
	22	27.10	20	7.38			

statistical program was used to carry out this analysis. All one-tailed p-values greater than 0.05 indicated a lack of significant difference between the mutant frequencies of different groups, though p-values between 0.05 and 0.1 were considered to indicate a marginal level of significance. The Mann-Whitney test did not detect any increase in the mutant frequencies of the low (0.3 mg/kg 1,8-DNP) and medium dose (0.9 mg/kg 1,8-DNP) group relative to the mutant frequencies of the negative control group (Table 4). It should be noted however, that a marginally significant decrease in mutant frequencies was observed in the low dose group relative to the negative control group. The Mann-Whitney statistical test was used to determine that there was a marginally significant increase in the mutant frequencies of the high dose group (2.8 mg/kg 1,8-DNP) relative to the mutant frequencies of the negative control group, with a one-tailed p-value of 0.057 (Table 4; footnote D).

3.1.3 Brain tissue.

The mutant frequencies of *lacZ* transgenes obtained from brain tissue of mice in Study #1 were calculated following the collection of data from the positive selection assay (section 2.6). The anterior portion of the brain (both lobes) was tested for each animal. The packaging of the phage was not efficient as seen by the relatively low titres, with the total number of packaged phage particles ranging from 7.14×10^4 to 32.01×10^4 (mean = $[14.61 \pm 1.18] \times 10^4$; 20 of 22 samples had numbers below 20×10^4) (Table 5). The mean mutant

Table 5. A summary of the positive selection assay data obtained from brain tissue of mice treated in Study #1, with DMSO only (negative control) or 0.3, 0.9 or 2.8 mg/kg 1,8-DNP. The Mann-Whitney unpaired, one-tailed test was used to determine whether there existed any statistically significant differences between the mutant frequencies of the *lacZ* transgene from groups of Muta™Mouse mice given various doses of 1,8-DNP relative to the negative control group.

^A. Generally determined from four plates for each animal sample.

^B. Standard error of the mean.

^C. Marginally significant increase according to the Mann-Whitney unpaired, one-tailed test. Mutant frequencies of the medium dose group (0.9 mg/kg 1,8-DNP) and the negative control group (DMSO only) were compared for a statistically significant increase in values following treatment.

Total dose (mg/kg 1,8-DNP)	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques in the presence of P-Gal ^A	Mutant frequency (x 10 ⁻⁵)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney test (p-value)
0	1	11.99	7	5.84			
	2	13.58	6	4.42	5.62	0.32	
	3	14.31	9	6.29			
	4	15.97	9	5.64			
	5	15.18	9	5.93			
0.3	6	8.00	3	3.75			
	7	13.98	10	7.15	7.76	2.35	0.345
	8	15.82	7	4.42			
	9	7.14	12	16.81			
	10	11.96	8	6.69			
0.9	11	8.12	6	7.39			
	12	9.54	7	7.34	7.00	0.72	0.063 ^C
	13	19.88	14	7.04			
	14	17.76	11	6.19			
	15	20.87	9	4.31			
2.8	16	13.39	13	9.71			
	17	17.83	8	4.49			
	18	32.01	18	5.62	6.19	1.72	0.165
	19	12.57	4	3.18			
	20	19.55	9	4.60			
	21	13.05	6	4.60			
	22	8.88	13	14.64			

frequency and standard error of the mean were determined for each treatment group.

The Mann-Whitney unpaired one-tailed statistical test was used to assess whether there were any significant increases in the mutant frequencies of each treatment group relative to the negative control group (DMSO only). The InStat statistical program was used to carry out this analysis. All one-tailed p-values greater than 0.05 indicated a lack of significant difference between the mutant frequencies of different groups, though p-values between 0.05 and 0.1 were considered to indicate a marginal level of significance. The medium dose group (0.9 mg/kg 1,8-DNP) demonstrated mutant frequencies which were marginally greater than the mutant frequencies of the negative control group (DMSO only) according to the Mann-Whitney test, with a one-tailed p-value of 0.063 (Table 5; footnote C). The mutant frequencies of the low dose (0.3 mg/kg 1,8-DNP) and high dose (2.8 mg/kg 1,8-DNP) groups were not significantly different from those of the negative control group (Table 5).

3.1.4 Lung tissue.

The mutant frequencies of *lacZ* transgenes obtained from lung tissue of mice in Study #1 were calculated following the collection of data from the positive selection assay (section 2.6). Two to three lung lobes were examined for each animal. The packaging of the phage was efficient, with phage titres ranging from 17.23×10^4 to as high as 63.95×10^4 (mean = $[33.58 \pm 2.36] \times 10^4$) (Table

6). The mean mutant frequency and standard error of the mean were determined for each treatment group.

The results from lung tissue obtained from Study #1 indicate an absence of detectable *in vivo* mutagenic activity of 1,8-DNP on the transgene (Table 6). The Mann-Whitney unpaired one-tailed statistical test was used to assess whether there were any significant increases in the mutant frequencies of each treatment group relative to the negative control group (DMSO only). The InStat statistical program was used to carry out this analysis. All one-tailed p-values greater than 0.05 indicated a lack of significant difference between the mutant frequencies of the treatment groups and the negative control group. Thus, no statistically significant increases in mutant frequencies following treatment with either 0.3, 0.9 or 2.8 mg/kg 1,8-DNP were observed when compared to the mutant frequencies of the negative control group using the Mann-Whitney statistical test (Table 6).

3.1.5 Liver tissue.

The mutant frequencies of *lacZ* transgenes obtained from liver tissue of mice in Study #1 were calculated following the collection of data from the positive selection assay (section 2.6). A part of each liver lobe was generally analysed for each animal, to minimise possible inter-lobe variations in mutant frequency, which have been reported elsewhere (Tinwell *et al.*, 1995). In addition, genomic DNA extraction was facilitated by homogenisation of the liver tissue, as recommended by Tinwell *et al.* (1995). The packaging of the phage

Table 6. A summary of the positive selection assay data obtained from lung tissue of mice treated in Study #1, with DMSO only (negative control) or 0.3, 0.9 or 2.8 mg/kg 1,8-DNP. The Mann-Whitney unpaired, one-tailed test was used to determine whether there existed any statistically significant differences between the mutant frequencies of the *lacZ* transgene from groups of Muta™Mouse mice given various doses of 1,8-DNP relative to the negative control group.

^A. Generally determined from four plates for each animal sample.

^B. Standard error of the mean.

Total dose (mg/kg 1,8-DNP)	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques in the presence of P-Gal ^A	Mutant frequency (x 10 ⁻⁵)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney test (p-value)
0	1	21.40	6	2.8			
	2	27.50	28	10.2	9.16	3.17	
	3	39.16	18	4.6			
	4	25.98	54	20.8			
	5	29.62	22	7.4			
0.3	6	28.49	24	8.4			
	7	17.23	10	5.8	7.96	0.93	0.500
	8	63.95	37	5.8			
	9	34.46	35	10.2			
	10	27.04	26	9.6			
0.9	11	21.14	10	4.7			
	12	31.08	35	11.3	8.26	1.19	0.396
	13	52.42	34	6.5			
	14	46.25	45	9.7			
	15	34.06	39	11.5			
2.8	16	28.83	17	5.9			
	17	27.04	25	9.2			
	18	33.13	22	6.6	8.60	0.64	0.396
	19	43.93	36	8.2			
	20	44.60	46	10.3			
	21	27.04	19	7.0			
	22	34.39	35	10.2			

was not very efficient, with the total number of successfully packaged and detected phage particles per animal ranging from 1.39×10^4 to 35.39×10^4 plaques on the titre plates (mean = $[13.39 \pm 2.72] \times 10^4$) (Table 7). In most cases, prior to obtaining the data, the DNA extraction and packaging procedures had to be repeated multiple times, with several alterations to the established protocol. The mean mutant frequency and standard error of the mean were determined for each treatment group.

There were some modifications to the calculation of the mean mutant frequency. Results from both animal #5 and #13 were disregarded for the mean mutant frequency calculation of their respective treatment groups (DMSO only and 0.3 mg/kg 1,8-DNP) due to an insufficient number of plaques on the titre plates (representing a total phage titre of less than 1×10^4) (Table 7; footnote C). Results were also disregarded from animals #16 and #22 for the calculation of the mean mutant frequency for their respective dosage groups (0.9 and 2.8 mg/kg 1,8-DNP) due to a total lack of plaques on the P-Gal selection plates (Table 7; footnote D), which contradicts the results from the DMSO only treatment group for liver which had a mean mutant frequency of 8.13×10^{-5} (Table 7).

The Mann-Whitney unpaired one-tailed statistical test was used to assess whether there were any significant increases in the mutant frequencies of each treatment group relative to the negative control group (DMSO only). The InStat statistical program was used to carry out this analysis. All one-tailed p-values greater than 0.05 indicated a lack of significant difference between the mutant

Table 7. A summary of the positive selection assay data obtained from liver tissue of mice treated in Study #1, with DMSO only (negative control) or 0.3, 0.9 or 2.8 mg/kg 1,8-DNP. The Mann-Whitney unpaired, one-tailed test was used to determine whether there existed any statistically significant differences between the mutant frequencies of the *lacZ* transgene from groups of Muta™Mouse mice given various doses of 1,8-DNP relative to the negative control group.

^A. Generally determined from four plates for each animal sample.

^B. Standard error of the mean.

^C. Samples with total titres of less than 1×10^4 plaques were discounted.

^D. Samples contaminated; data omitted for these animals.

Total dose (mg/kg 1,8-DNP)	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques in the presence of P-Gal ^A	Mutant frequency (x 10 ⁻⁵)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney test (p-value)
0	1	14.18	11	7.76			
	2	12.92	19	14.70	8.13	2.45	
	3	6.96	2	2.87			
	4	1.39	1	7.19			
	5	0.94 ^C	0 ^C	---			
0.3	6	18.42	13	7.06			
	7	34.19	14	4.09	6.20	1.10	0.278
	8	6.11	6	9.81			
	9	20.68	13	6.29			
	10	2.68	1	3.73			
0.9	11	18.42	12	6.51			
	12	35.39	25	7.06	8.66	1.40	0.365
	13	0.61 ^C	1 ^C	16.30			
	14	5.98	5	8.36			
	15	5.52	7	12.69			
2.8	16	2.98 ^D	0 ^D	---			
	17	34.19	22	6.43			
	18	15.34	21	13.69	10.90	2.52	0.365
	19	1.77	1	5.64			
	20	2.60	5	19.22			
	21	4.19	4	9.54			
	22	1.92 ^D	0 ^D	---			

frequencies of different groups. Thus, no statistically significant increases in mutant frequencies following treatment with either 0.3, 0.9 or 2.8 mg/kg 1,8-DNP were observed when compared to the mutant frequencies of the negative control group using the Mann-Whitney statistical test (Table 7).

3.2 Study #2.

Study #2 involved 30 female *lacZ* transgenic mice (strain 40.6; Muta™Mouse). These females had given birth to pups which were being weaned when the experiment started. Each mother was allowed to keep two of her pups to encourage lactation during the course of injections.

The mice were divided into 3 groups of 10 mice each; a negative control group which received DMSO only, a treatment group which received a total of 5.0 mg/kg 1,8-DNP and a positive control group which received a total of 240 mg/kg ENU (in PBS). Twenty-eight of the 30 animals involved in Study #2 survived until necropsy. Of the 10 animals assigned to the negative control group (DMSO only), 8 survived the treatment and 29 day fixation period. However, the two deaths do not appear to be related to the injections. One animal died during the injection period, but an autopsy showed that the cause was an unrelated uterine infection. The dams had given birth to the pups only a few weeks previous to the commencement of the study. The second negative control animal died during the fixation period, but also due to natural causes. As the injections progressed on a daily basis, the animals lost weight (Figure 8). The amount of DMSO that was administered each day to the negative control group

Figure 8. The average masses of the mice in Study #2 during the course of treatment. The masses of the mice in the negative control group (DMSO only), the treatment group (5.0 mg/kg 1,8-DNP) and the positive control group (240 mg/kg ENU) were determined prior to the injections on each of the 8 days of treatment, up to 3 days post-treatment, and on the day of necropsy, 29 days after the final injection. The decline in the average masses during the first 4 days of treatment prompted the decrease in the administered dose of DMSO in both the negative control group and the treatment group (DMSO was used as the vehicle for 1,8-DNP) from 4 mL/kg to 3 mL/kg for the subsequent 4 days of treatment. Error bars representing the standard error of the mean are shown for one treatment group per day to give an indication of the variations in the masses.

and to the treatment group (as a vehicle for the 1,8-DNP) was decreased to $\frac{3}{4}$ of the original amount on the fifth day of injections, in the event that the weight loss was due to DMSO toxicity. The weights of the mice in all groups continued to decline during the treatment period, but had increased by the necropsy day (29 days after the final injection) to almost the same amount as before the treatments. It should be noted that at the commencement of the course of treatment, all but two of the pups had been weaned from the mothers, and even the remaining ones were starting to eat solid food, reducing the demand on the lactating mothers. Also, the mice in the treatment groups were fully mature, and no weight gain due to growth was expected.

Following 8 daily i.p. injections, and a 29 day fixation period, the remaining 28 animals were sacrificed. Tissues collected included brain, mesentery, mammary (two nipples from either side, lower abdominal region), lungs, liver, ovaries, bone marrow, kidneys, bladder, colon, and spleen. Immediately upon removal, the tissues were flash-frozen in liquid nitrogen and stored at -80 °C (section 2.4.2). No tumours were observed. The bacteriophage lambda vectors carrying the *lacZ* transgene were isolated from the genomic DNA of the various tissues following optimisation of the extraction procedures (section 2.5). Following multiple unsuccessful attempts at packaging the vectors from mesentery tissue, this aspect of the project was abandoned. The packaging of vectors derived strictly from mammary tissue was also abandoned because mammary tissue proved to be high in fat content, the cell type was heterogeneous and the packaging was poor despite multiple attempts.

Successfully packaged λ gt10/*lacZ* vectors bearing *lacZ* mutants were identified by the positive selection assay (section 2.6) for bone marrow and lung tissue. The phage titre (phage titre/ μ L), the actual number of packaged phage used for the assay, and the mutant frequency for each sample were calculated as described in section 2.6.

3.2.1 Bone marrow.

The mutant frequencies of *lacZ* transgenes obtained from bone marrow were calculated following the collection of data from the positive selection assay (section 2.6). The total number of phage packaged from each sample and detected on the titre plates ranged from 9.74×10^4 to 74.95×10^4 phage (mean = $[34.89 \pm 3.48] \times 10^4$) for Study #2 (discounting animal #7) (Table 8; footnote D). The mean mutant frequency and standard error of the mean were determined for each treatment group. The ease with which relatively intact genomic DNA is extracted from bone marrow is reflected in the high numbers of observed plaques and therefore a high number of packaged phage.

There were some modifications to the calculation of the mean mutant frequencies. Data for two negative control animals (DMSO only) were missing due to the premature deaths of these animals (Table 8; footnote C). Also, data for animal #7 in the negative control group was omitted because the data were compromised by bacterial contamination on the plates resulting in an excessively low number of plaques on the titre plates (representing a total phage titre of less than 1×10^4) (Table 8; footnote D).

Table 8. A summary of the positive selection assay data obtained from bone marrow of retired breeder mice treated in Study #2, with DMSO only (negative control), 5.0 mg/kg 1,8-DNP, or 240 mg/kg ENU (positive control). The Mann-Whitney unpaired, one-tailed test was used to determine whether there existed any statistically significant differences between the mutant frequencies of the *lacZ* transgene from groups of Muta™Mouse mice following the administration of 5.0 mg/kg 1,8-DNP or 240 mg/kg ENU relative to the negative control.

^A. Generally determined from four plates for each animal sample.

^B. Standard error of the mean.

^C. Two animals died.

^D. Samples with total titres of less than 1×10^4 plaques were discounted.

^E. Total dose of ENU was 240 mg/kg.

^F. Significant according to the Mann-Whitney unpaired, one-tailed test. Mutant frequencies of the positive control group (240 mg/kg ENU) and the negative control group (DMSO only) were compared for a statistically significant increase in values following treatment.

Chemical	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques (P-Gal) ^A	Mutant frequency (x 10 ⁻⁵)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney test (p-value)
DMSO ^C	1	67.39	36	5.34			
	2	50.63	28	5.53			
	3	26.84	56	20.87			
Negative Control	4	32.34	19	5.88	8.49	2.18	
	5	32.54	20	6.15			
	7	0.81 ^D	51 ^D	628.23			
	8	20.01	21	10.49			
	9	26.90	14	5.20			
1,8-DNP	11	42.01	21	5.00			
	12	63.55	50	7.87			
	13	74.95	62	8.27			
5.0 mg/kg	14	17.49	22	12.58	8.05	1.56	0.4418
	15	26.11	51	19.53			
	16	27.77	22	7.92			
	17	31.61	21	6.64			
	18	9.74	3	3.08			
	19	17.03	12	7.05			
	20	22.93	6	2.62			
	21	43.47	1012	232.80			
ENU ^E	22	55.66	1124	201.93			
	23	74.15	1176	158.59			
	24	31.34	363	115.81	171.34	11.13	0.0004 ^F
Positive Control	25	29.49	453	153.62			
	26	28.76	383	133.17			
	27	21.93	364	165.95			
	28	22.80	403	176.79			
	29	27.10	452	166.77			
	30	17.36	361	207.93			

The Mann-Whitney unpaired one-tailed statistical test was used to assess whether there were any significant increases in the mutant frequencies of the 1,8-DNP and ENU treatment groups relative to the negative control group (DMSO only). The InStat statistical program was used to carry out this analysis. All one-tailed p-values greater than 0.05 indicated a lack of significant difference between the mutant frequencies of different groups. The Mann-Whitney test did not detect any statistically significant increase in the mutant frequencies of the 1,8-DNP-treated group relative to the mutant frequencies of the negative control group, with a p-value of 0.4418 (Table 8). However, a highly significant increase in the mutant frequencies of the ENU-treated group was observed relative to the mutant frequencies of the negative control group according to the Mann-Whitney statistical test, with a one-tailed p-value of 0.0004 (Table 8; footnote F).

3.2.2 Lung tissue.

The mutant frequencies of *lacZ* transgenes obtained from lung tissue were calculated following the collection of data from the positive selection assay (section 2.6). Two to three lung lobes were examined for each animal. The mean mutant frequency and standard error of the mean was determined for each treatment group (Table 9).

Bacteriophage lambda DNA from lung tissue in Study #2 did not package efficiently (mean = $[12.82 \pm 2.34] \times 10^4$) (Table 9), and data were obtained from only 15 of the 28 animals (with total phage titres greater than 1×10^4 phage). The rest of the samples did not package, despite multiple attempts at both extracting

Table 9. A summary of the positive selection assay data obtained from lung tissue of retired breeder mice treated in Study #2, with DMSO only (negative control), 5.0 mg/kg 1,8-DNP, or 240 mg/kg ENU (positive control). Data were not obtained for some of the animals (section 4.1.3.4).

- ^A. Generally determined from four plates for each animal sample.
- ^B. Standard error of the mean.
- ^C. Two animals died.
- ^D. Total dose of ENU was 240 mg/kg.
- ^E. Insufficient data to perform the Mann-Whitney calculations.

Chemical	Animal number	Total number of plaques ^A (x 10 ⁴)	Total number of plaques in the presence of P-Gal ^A	Mutant frequency (x 10 ⁻³)	Mean (x 10 ⁻⁵)	SEM ^B (x 10 ⁻⁵)	Mann-Whitney test (p-value)
DMSO ^C Negative Control	8	19.75	23	11.65	10.72	0.92	
	9	20.41	20	9.80			
1,8-DNP 5.0 mg/kg	13	1.09	1	9.15			
	17	17.76	16	9.01	8.86	1.11	N/A ^E
	18	10.01	5	5.00			
	19	22.86	21	9.19			
	20	15.04	18	11.97			
ENU ^D Positive Control	21	1.04	4	38.33			
	22	2.55	16	62.71			
	23	2.80	19	67.86	61.38	5.95	N/A ^E
	25	1.49	5	33.53			
	27	10.07	81	80.42			
	28	19.61	127	64.75			
	29	23.99	161	67.12			
	30	23.86	182	76.29			

genomic DNA from lung tissue and packaging the bacteriophage lambda vector.

The Mann-Whitney unpaired one-tailed statistical test was not used due to insufficient sample sizes (Table 9).

3.3 Analysis of the mutant *lacZ* gene.

Following the identification of mutant *lacZ* phages using the positive selection assay (section 2.6), phage stocks were stored for several months. 1,8-DNP-induced mutants from mesentery tissue from Study #1 were selected for mutational analysis because a statistically significant increase in the mutant frequencies of the *lacZ* transgene was observed following treatment with 2.8 mg/kg 1,8-DNP (Table 3). The poor titre of the original phage stocks necessitated the re-amplification of the lambda phage, and at the same time, the phenotype was verified (section 2.7.1). This higher phage stock was the source of lambda phage for the complementation studies and was also used to infect *E. coli* C *lacZ*⁻ for lambda phage purification (section 2.7.2) and was used for subsequent PCR (section 2.9) and DNA sequence analysis (section 2.10).

3.3.1 The use of complementation assays to localise the *lacZ* mutation to one of three regions on the gene.

The *lacZ* gene is approximately 3.3 kb in length, thus making sequencing a long and difficult task. In order to simplify the search for spontaneous or chemical-induced mutations, protein complementation assays were used to attempt to localise the mutation to one of three regions of the *lacZ* gene: α , β or

ω . The limits of the α region are well characterised and are located from base positions 9-276 bp on the *lacZ* gene (Langley *et al.*, 1975), but the boundary between the β and ω regions is less clear, and is thought to occur somewhere in the vicinity of base position 1,800 (Douglas *et al.*, 1994). Three *E. coli* K-12 complementation strains are used for this purpose: DH5 α , W4680 and HFR3000 X90 (Table 1). Each strain lacks one of the three complementation regions and thus, when the *lacZ* gene of the phage is also present, complementation between the host and phage *lacZ* gene products was determined by a blue colour in the presence of X-Gal. Specifically, a clear colour with one strain, and a blue colour with the other two strains, indicates that the mutation in the *lacZ* gene of the phage is in the region which is lacking in the first, clear strain. This unique feature of *lacZ* complementation is the basis of a lot of work done with recombinant DNA (Jacobson *et al.*, 1994).

Complementation studies are possible because the removal of all large segments of the *lacZ* gene is deleterious to the proper functioning of the gene, and because *E. coli lacZ* codes for β -galactosidase, which is active in a tetrameric form, making complementation physically possible (Jacobson *et al.*, 1994).

The improved phage stock was used to infect the *E. coli* K-12 host for the complementation assays. In most cases, the mesentery (Study #1) phage stocks were revived sufficiently to allow amplification of the phage stock. A total of 68 plaques were collected from the initial positive selection assay; 29 from the negative control group (DMSO only) and 39 from the high dose group (2.8 mg/kg

1,8-DNP). Of these samples, 27 from the negative control group and 36 from the treated group were successfully amplified and used for the complementation assays. The details of the complementation studies are described in section 2.8. The predictive ability of the complementation assay to forecast the location of the mutation on the *lacZ* gene for the 26 mutants for which a mutation was identified is presented in Table 10. Of the mutations localised to one of the three complementation regions, only 45% were predicted correctly; of the mutations which were localized to two of the three regions, 64% were predicted correctly. No prediction by complementation analysis was available for 4 mutants.

3.3.2 Identification of mutations in the *lacZ* transgene.

Lambda DNA was extracted from all of the phage particles which were successfully amplified (section 2.7.1). These phage particles originated from the mesentery tissue of Muta™Mouse mice in Study #1, from both the negative control group (DMSO only) and the high dose group (2.8 mg/kg 1,8-DNP). The total number of successfully amplified phage samples was 27 from the negative control group and 36 from the treated group (2.8 mg/kg 1,8-DNP). Following purification of the lambda DNA (section 2.7.2), the lambda-based mutant *lacZ* transgene was amplified by PCR into two fragments (section 2.9). These fragments were then sequenced with the help of an ABI Prism™ 377 DNA Sequencer, an automated machine capable of reading segments of DNA up to 500-600 kb in length (section 2.10). The wild-type *lacZ* sequence (transgenic)

Table 10. A summary of the complementation assay results of *lacZ*⁻ mutants (spontaneous and 2.8 mg/kg 1,8-DNP) from mesentery tissue obtained from mice of Study # 1. DH5 α (β and ω donor), W4680 (α and ω donor) and HFR3000 X90 (α and β donor) were the complementation strains which were used to attempt to localise the *lacZ*⁻ mutation to one of the three complementation regions. The success of this predictive tool is assessed by comparing the results to the actual location of the mutation as determined by DNA sequence analysis.

Putative region of <i>lacZ</i> ⁻ mutation	Number of predicted mutations (complementation assay)	Number of observed mutations (sequencing)	Accuracy of predictions (%)
α β ω	0 7 4	 3 2	(5/11) 45 %
α, β α, ω β, ω	0 7 4	 2 4	(7/11) 64 %
unknown	4		
TOTAL	26		

appears in the Appendix. Some modification to the original sequencing protocol was required to obtain high quality sequences.

Fourteen *lacZ* transgenes originating from mesentery tissue of Muta™Mouse mice in Study #1, DMSO treatment only, were successfully sequenced (Table 11). All of the detected mutations were base substitutions. Of these, seven of the mutations were G:C → A:T transitions, three were G:C → T:A transversions, three were G:C → C:G transversions and finally, one was an A:T → C:G transversion.

DNA sequence alterations were successfully identified in 12 *lacZ* transgenes originating from mesentery tissue of Muta™Mouse mice in Study #1, high dose group (2.8 mg/kg 1,8-DNP) (Table 12). Of these mutations, nine were base substitutions, two were frameshift mutations of -2 (GC:CG) and +1 (A:T), respectively, and one tandem base substitution (GC:CG → CT:GA) was identified. Both frameshifts were found in contiguous sequences of GC and A residues, respectively (Table 12). Of the base substitutions, three were G:C → A:T transitions, four were G:C → T:A transversions, one was an A:T → G:C transition and one was an A:T → C:G transversion.

A comparison of the sequencing results from both the spontaneous and 1,8-DNP-treated *lacZ* transgenes appears in Table 13. The Monte-Carlo exact contingency table Chi-square test and the Monte-Carlo exact likelihood ratio test do not indicate a statistically significant difference between the mutational spectra of the two groups which were analysed.

Table 11. Analysis of mutations in the *lacZ* transgene of Muta™Mouse mesentery tissue, from Study #1. These animals were in the negative control group, receiving only DMSO.

^A. Sequence is 5' to 3' from the purine containing strand.

Dose	Mutant number	Site (lacZ)	Mutation	Amino acid alteration	Sequence context ^
Control (DMSO only)	1-A-1	1717	C:G -> A:T	Val -> Phe	GCTTC G TCTGG
	2-B-1	2744	C:G -> T:A	Arg -> His	CGACC G CCTTA
	3-A-1	2169	C:G -> T:A	Trp -> Stop	GCCTG G CAGCA
	3-A-2	1180	G:C -> A:T	Arg -> Cys	ACAGC G CACGG
	3-B-2	961	G:C -> C:G	Leu -> Val	GTGCA G TTCAA
	3-C-1	1732	C:G -> A:T	Asp -> Tyr	GGGTG G ATCAG
	3-C-2	2713	C:G -> T:A	Gly -> Arg	GGCTC G GATTA
	3-D-2	2713	C:G -> T:A	Gly -> Arg	GGCTC G GATTA
	5-A-1	2744	C:G -> T:A	Arg -> His	CGACC G CCTTA
	5-A-2	2714	C:G -> G:C	Gly -> Ala	GCTCG G ATTAG
	5-B-1	1370	C:G -> G:C	Pro -> Arg	CACTC G GGTGA
	5-C-1	1334	C:G -> T:A	Arg -> His	CGAAC G CGTAA
	5-C-2	511	T:A -> G:C	Ser -> Arg	AGGAC A GTCGT
	5-D-1	1083	G:C -> T:A	Asn -> Lys	TGACG G TTAAC

Table 12. Analysis of mutations in the *lacZ* transgene of Muta™Mouse mesentery tissue, from Study #1. These animals were in the high dose group, receiving a total of 2.8 mg/kg 1,8-DNP.

^A. Sequence is 5' to 3' from the purine containing strand.

Dose	Mutant number	Site (lacZ)	Mutation	Amino acid alteration	Sequence context ^A
Treated	17-B-1	2817	G:C -> T:A	Ser -> Arg	TTTTC G CTCGG
	17-C-1	1399	C:G -> A:T	Glu -> Stop	GGAAT G AATCA
	18-C-1	2708	C:G -> T:A	Trp -> Stop	AAACT G GCTCG
	20-B-1	1231	A:T -> C:G	Tyr -> Asp	GCCGT A GCGGT
	20-B-2	756	C:G -> T:A	Met -> Ile	CAGAT G TGC GG
	21-A-1	1527-1534	-(GC:CG)	Val -> Gly	ATCCA CGCGCGCG TACAT
2.8 mg/kg 1,8-DNP	21-B-3	1180	G:C -> A:T	Arg -> Cys	ACAGC G CACGG
	22-A-2	1791-1794	+(A:T)	Gly -> Trp	TCGCC AAAA TCACC
	22-B-1	1520	G:C -> T:A	Pro -> Gln	ACATC G GGCAA
	22-C-3	1406	C:G -> A:T	Gly -> Val	ATCAG G CCACG
	22-D-1	1523	A:T -> G:C	Met -> Thr	CGTAC A TCGGG
	22-D-3	1626	G:C -> C:G	Cys -> Trp	TATTC G CAAAG
		1627	C:G -> T:A	Glu -> Lys	TTTGC G AATAC

Table 13. A summary of the observed mutations in both spontaneous and 1,8-DNP-induced (2.8 mg/kg) mutations in the *lacZ* transgenes from mesentery tissues of mice in Study #1.

Type of mutation	Mutational event	Occurrence in spontaneous mutants	Occurrence in 1,8-DNP-treated mutants (2.8 mg/kg)
Base substitution			
transition	G:C -> A:T	7 (50%)	3 (25%)
transversion	G:C -> T:A	3 (21.4%)	4 (33.3%)
transversion	G:C -> C:G	3 (21.4%)	0 (0%)
transition	A:T -> G:C	0 (0%)	1 (8.3%)
transversion	A:T -> C:G	1 (7.1%)	1 (8.3%)
Frameshift			
	- (GC:CG)	0 (0%)	1 (8.3%)
	+ (A:T)	0 (0%)	1 (8.3%)
Tandem base substitution			
	GC:CG -> CT:GA	0 (0%)	1 (8.3%)
TOTAL		14	12

IV. Discussion

4.1 The mutagenicity of 1,8-DNP in Muta™Mouse.

4.1.1 Results in tissues.

The *lacZ* transgene of Muta™Mouse was examined for spontaneous and 1,8-DNP-induced mutations following doses of 0, 0.3, 0.9, 2.8 (Study #1) and 5.0 (Study #2) mg/kg 1,8-DNP. Tissues examined included mesentery, bone marrow, brain, lung, liver and mammary. Of the 1,8-DNP-treated animals, only the transgenes originating from the mesentery tissues of animals in the high dose group of Study #1 (2.8 mg/kg 1,8-DNP) demonstrated a statistically significant increase in mutant frequencies relative to the negative control (DMSO only) (Table 3). Confidence in the abilities of the Muta™Mouse system and the study design used in Study #2 to detect chemical-induced mutations is maintained by the strong mutagenic effects of the positive control ENU in Study #2 (Tables 8 and 9). This was an expected result, since ENU is a potent alkylating agent and a proven Muta™Mouse mutagen (Douglas *et al.*, 1995a; Douglas *et al.*, 1996).

Mutations are expected in the *lacZ* transgene originating in 1,8-DNP-treated mesentery tissue, not only because it is exposed to the highest concentration of 1,8-DNP, but also because in a previous study with rats, mesentery tissue was found to contain high levels of DNA adducts 12 hours following treatment with 1,8-DNP (1.17 to 1.46 mg/kg) (Norman *et al.*, 1990). Norman *et al.* (1990) speculated that mesenteric cells in rats are therefore able to activate 1,8-DNP into a reactive species which then goes on to bind to DNA. Consistent with that finding is a report that soft tissue tumours were observed in

the peritoneal cavity of CD rats following i.p. administration of 1,8-DNP (King *et al.*, 1986).

The highest packaging efficiencies, resulting in high plaque counts on the titre plates, were observed with bacteriophage vectors obtained from the bone marrow of mice from both studies presented in this thesis (Tables 4 and 8). This was not surprising in light of the fact that DNA from bone marrow is relatively easy to isolate, minimising the potential of shearing damage to DNA, which is an important factor in the efficient packaging of the lambda vector. The results from bone marrow DNA obtained from Study #1 indicate that there might be some marginally detectable *in vivo* mutagenic activity of 1,8-DNP on the transgene (Table 4). However, although almost double the dose of 1,8-DNP was employed in Study #2 relative to Study #1 (2.8 mg/kg 1,8-DNP versus 5.0 mg/kg 1,8-DNP respectively), the Mann-Whitney unpaired one-tailed statistical test did not detect any significant differences in mutant frequencies in the treated group of Study #2 relative to the negative control group of DMSO only (Table 8). This suggests that the marginally significant increase in mutant frequencies observed in the 2.8 mg/kg 1,8-DNP-treated mice in Study #1 (Table 4) could be attributed to the inherent variability of this system instead of 1,8-DNP-induced mutagenesis. The DMSO treated mice in Study #1 had mutant frequencies which ranged from 3.8×10^{-5} to 4.5×10^{-5} (Table 4) and these values compare favourably to the spontaneous data compiled by Zhang *et al.* (1995) from studies reported by various groups, which range from 2.4×10^{-5} to 4.1×10^{-5} . These studies reported by Zhang *et al.* (1995) all used 8-10 week old male

Muta™Mouse mice, while female mice were used in the studies presented in this document. The effects of gender in toxicology testing are discussed in section 4.1.3.6.

The results from brain tissue obtained from Study #1 indicate there might be some marginally detectable *in vivo* mutagenic activity of 1,8-DNP on the transgene (Table 5) at a dose of 0.9 mg/kg 1,8-DNP. However, this increase is sufficiently marginal that it is likely due to the inherent variability of the testing system rather than an actual mutagenic effect of 1,8-DNP. This is especially likely since presumably any mutagenic effect at a dose of 0.9 mg/kg 1,8-DNP would be enhanced at a dose of 2.8 mg/kg 1,8-DNP, and this effect was not observed (Table 5). In fact, the mean mutant frequency of the high dose group (2.8 mg/kg 1,8-DNP) is slightly less than for the other treatment groups. However, this decrease is too small to be attributed to cellular toxicity and no increased indication of toxicity was observed at the high dose.

A unique feature of brain tissue relative to other tissues is the blood-brain barrier which limits the accessibility of certain compounds to brain cells (Goldstein and Betz, 1986). ENU was shown to be able to pass through, providing a dose-dependent increase in mutant frequency over several days following a single treatment with ENU (Gossen *et al.*, 1989). 1,8-DNP is highly lipophilic (Carmichael *et al.*, 1996), and as the lipid solubility of a substance is an important factor in determining its ability to cross the blood-brain barrier (Goldstein and Betz, 1986), it is possible that 1,8-DNP can in fact cross this barrier. The ability of 1,8-DNP to pass through the blood-brain barrier could be

ascertained by radiolabelling the chemical, and assaying brain tissues for the presence of the chemical after treatment of the mouse. The brain is also unique as a mutational target because it is post-mitotic in adult mice, and thus an error-prone DNA-repair system and not DNA replication is most likely involved in the induction of mutations (Gossen *et al.*, 1989).

The results from lung tissue obtained from Study #1 indicate an absence of detectable *in vivo* mutagenic activity of 1,8-DNP on the transgene (Table 6). Unfortunately, bacteriophage lambda DNA from Study #2 did not package as successfully (Table 9), but although the data from Study #2 are not complete they do indicate that of the samples tested, there does not appear to be a detectable mutagenic activity by 1,8-DNP (5.0 mg/kg) relative to the negative control of DMSO only.

The results from liver tissue obtained from Study #1 indicate there is no detectable *in vivo* mutagenic activity of 1,8-DNP on the transgene (Table 7). The mean mutant frequency of the negative control group was 8.13×10^{-5} (Table 7), which is comparable to the data reported by others and compiled by Zhang *et al.* (1995) of average spontaneous mutant frequencies for liver tissue of male Muta™Mouse mice of approximately 2.1×10^{-5} to 7.0×10^{-5} . As noted before, the mice used in the study presented in this document are female, and gender differences in mutagenesis studies are addressed in section 4.1.3.6. A negative result for 1,8-DNP-induced mutagenesis in liver tissue is consistent with a DNA adduct study using the same chemical in rats. Norman *et al.* (1990) used ^{32}P -postlabelling to observe adduct formation in various tissues in rats following a

single treatment with 1,8-DNP. Although rat liver cytosol is the location where 1,8-DNP is reduced to nitroso- and amino- derivatives under aerobic conditions, relatively low levels of adducts were found as compared to levels in tissues such as mesentery and mammary (Norman *et al.*, 1990). Norman *et al.* (1990) predicted that this lack of DNA damage might reflect the ability of the hepatic enzymes to efficiently detoxify 1,8-DNP.

Mammary tissue was also considered for *in vivo* 1,8-DNP-induced mutagenesis studies. Due to the highly lipophilic nature of 1,8-DNP and its proven carcinogenicity in rodent mammary glands (King *et al.*, 1986; King, 1988; Imaida *et al.*, 1991), and because 1,8-DNP has been shown to form DNA adducts in rat mammary tissue (Norman *et al.*, 1990), it was postulated that there would be some accumulation of 1,8-DNP in Muta™Mouse mammary tissues, and that the Muta™Mouse mice would develop 1,8-DNP-induced mutations in the *lacZ* transgene in mammary tissue. To facilitate this analysis, female mice that had previously produced offspring were used, in the hope that their mammary glands would be enlarged and more defined than in young mice. To encourage lactation and active cellular turnover in the mammary glands in the dams, two pups from the three week old litter continued to be housed with each mother during the course of the injections and were removed two days after the final treatment. However, histological analysis showed that a proper analysis of mutation formation in the transgene of mammary gland tissue would not be possible since the glands were interspersed in fatty deposits, and it would not be feasible to isolate these glands for further study. In addition, repeated attempts

at the optimisation of the DNA extraction protocol used for the extraction of genomic DNA from other tissues (Vijg and Douglas, 1996) failed to provide consistent packaging of the vector. This is surprising in light of the fact that an optimised protocol, based the genomic DNA isolation technique used for certain other tissues (Vijg and Douglas, 1996) had been previously developed which allowed the successful isolation and packaging of genomic DNA from the mammary region of Muta™Mouse mice which were not involved in any study, with total phage titres ranging from approximately 8.7×10^4 to 27.7×10^4 plaques (data not shown). The spontaneous mutant frequency of these samples ranged from 5.9×10^{-5} to 9.6×10^{-5} , which is comparable to the spontaneous values calculated for other tissues. The failure of the successful DNA isolation and packaging of vectors from animals in Study #2 could be due to a number of reasons, including damage to the DNA, either by shearing forces, or by nucleases or other agents which destroy the DNA or interfere with the cutting of the vectors at the *cos* sites of the packaging reaction. The initial interest in the mammary region would be more justified in an animal such as the rat, which is 8-10 times the size of the mouse (Dycaico *et al.*, 1994) and would therefore simplify the isolation of the tissues.

4.1.2 Factors related to 1,8-DNP.

4.1.2.1 The dose relative to other rodent studies.

Sub-chronic levels of 1,8-DNP were used in the two studies presented in

this thesis, for a total of 0.3, 0.9, 2.8 mg/kg 1,8-DNP (Study #1) or 5.0 mg/kg 1,8-DNP (Study #2). With the exception of the transgenes originating from mesentery tissue in study #1 (2.8 mg/kg 1,8-DNP), no significant increase in mutant frequency was observed in the various tissues and 1,8-DNP treatment groups relative to the negative control group. These results are somewhat surprising in light of the fact that both DNA adducts and carcinogenesis were observed in rodents treated with both less and more 1,8-DNP than was used in the studies presented in this document (Table 14). Sarcomas were observed at the site of injection (s.c.) in rats with as little as approximately 0.31 mg/kg 1,8-DNP (Sato *et al.*, 1986), and adducts were observed in several tissues following i.p. injection of 1.17 to 1.46 mg/kg 1,8-DNP into rats (Norman *et al.*, 1990). At higher doses, 1,8-DNP was shown to be a very potent carcinogen, with 100% acquisition of MFH or leukemias in several studies (Table 14). Tumours were not observed in any of the animals employed in this study at the time of necropsy.

There exists a debate as to whether transgenic mice should be dosed according to the subchronic MTD, which can be related to the chronic cancer bioassays, or whether the acute MTD should be used (Ashby and Tinwell, 1995). Ashby and Tinwell (1995) support the use of the one time acute dose because the results can then be compared to those obtained previously using the bone marrow micronucleus assay or the liver UDS (unscheduled DNA synthesis) assay, which is an indicator of DNA repair (Norman *et al.*, 1989). Tao and Heddle (1994) suggest treating with multiple doses until the highest possible dose is achieved and allowing a fixation time which is equivalent to the tissue

Table 14. The *in vivo* effects of 1,8-DNP in rodents. A summary of the various biological end-points observed in different studies which involved the treatment of rodents with 1,8-DNP.

- A. Injections were either s.c. (subcutaneous) or i.p. (intraperitoneal).
- B. At site of injection unless otherwise noted.
- C. Actual value given was in mg 1,8-DNP per mouse (total of 1 mg per mouse).
Assume 25 gm mass per mouse.
- D. Malignant fibrous histiocytoma (MFH).
- E. Actual value given was in mg 1,8-DNP per rat (total of (i) 4 mg, (ii) 0.4 mg, (iii) 0.04 mg per rat). Assume 130 gm mass per rat.
- F. Actual value given was in mg 1,8-DNP per mouse (total of 1 mg per mouse).
Assume 25 gm mass per mouse.
- G. Actual value given was in μmol 1,8-DNP per rat (total of 6.3 μmol per rat).
Assume 50 gm mass per rat.

Animal type	Strain and age	Treatment with 1,8-DNP (total) ^A	Biological end-point ^B	Source
Mouse	BALB/c; 6 weeks old; male	~ 40 mg/kg ^C ; s.c.	30% developed MFH ^D	Tokiwa <i>et al.</i> , 1986
Rat	F344; 6 weeks old; male	(i) ~ 31 mg/kg ^E ; s.c. (ii) ~ 3.1 mg/kg ^E ; s.c. (iii) ~ 0.31 mg/kg ^E ; s.c.	100% developed sarcomas 100% developed sarcomas 90% developed sarcomas	Sato <i>et al.</i> , 1986
Rat	CD; weanling; female	35 mg/kg; i.p.	91% developed MFH ^D ; 50% developed mammary tumours	King <i>et al.</i> , 1986
Mouse	CD; newborn; female BALB/c; 6 week old; male	35 mg/kg; s.c. ~40 mg/kg ^F ; s.c.	100% developed MFH ^D 40% developed MFH ^D	Otofuji <i>et al.</i> , 1987
Rat	CD; weanling; female CD; newborn; female	35 mg/kg; i.p. 18 mg/kg; s.c.	88% developed MFH ^D ; 50% developed mammary tumours 100% developed MFH ^D ; leukemia observed	Imaida <i>et al.</i> , 1988
Rat	Spague-Dawley; retired breeder; female	1.17 to 1.46 mg/kg; i.p.	highest DNA adduct levels in mesentery and mammary tissues; also detected in bladder, lung, kidney and liver tissues	Norman <i>et al.</i> , 1990
Rat	CD; newborn female	~36 mg/kg ^G ; s.c.	100% developed MFH ^D ; 22% developed leukemia	Imaida <i>et al.</i> , 1995

with the longest fixation period before testing the tissues of interest (section 4.1.3.3). However there is some concern that these assays do not provide an accurate reflection of the genotoxic potential of a chemical in a regulatory sense, since tissue toxicity invoked by the chemical can lead to an increased number of cell divisions (Ames and Gold, 1990b). Cell division reduces the amount of time available for the repair of DNA adducts, caused either by endogenous events such as oxidative DNA damage, or exogenously administered chemicals, and this may then lead to the fixation of promutagenic DNA adducts, and possibly carcinogenesis (Ames and Gold, 1990a; Ames and Gold, 1990b; Swenberg, 1995). Indeed, about half of the chemicals tested, both natural and synthetic, are rodent carcinogens when tested at the MTD (Ames *et al.*, 1990a; Ames and Gold, 1990b). In this case, because the relevant human exposure would be to lower, non-toxic doses of the chemical, the risk of the chemical as a potential human carcinogen would be exaggerated (Ames and Gold, 1990b; Ashby and Morrod, 1991; Swenberg, 1995). In contrast, a study using known nongenotoxic carcinogens and the Big Blue™ *lacI* transgenic mouse did not demonstrate any link between the use of the chemicals at dose levels which elicited positive responses in the 2-year bioassays and increased levels of mutations in the *lacI* gene (Gunz *et al.*, 1993). Explanations for this include the possibility that mitogenesis by nongenotoxic carcinogens is not a sustained effect and the natural variability between strains of mice in their responses to chemicals (Gunz *et al.*, 1993).

4.1.2.2 Adducts.

The formation of DNA adducts generally occurs in the presence of most potent carcinogens and can therefore be a good indicator of the mutagenicity of a chemical, both in prokaryotes and eukaryotes, and also of its carcinogenic risk (La and Froines, 1993; Dipple, 1995).

Numerous studies of adduct formation following exposure of bacteria, mammalian cells in cultures and rodents to nitropyrenes. The observation of *in vitro* (calf thymus DNA) and *in vivo* (*S. typhimurium* strain TA98NR) adduct formation following treatment with 1,8-DNP revealed that the same predominant DNA adduct, 1-*N*-(2'-deoxyguanosin-8-yl)-amino-8-nitropyrene, was formed in both the calf thymus DNA and in *Salmonella* (Andrews *et al.*, 1986). The same DNA adduct was observed in cultured rabbit tracheal epithelial cells (Norman *et al.*, 1989) and in female CD rats (Norman *et al.*, 1990) following treatment with 1,8-DNP.

4.1.2.3 Accessibility of the chemical.

Nitropyrenes have low solubility in solutions, including DMSO (Imaida *et al.*, 1995), and in this study, 1,8-DNP was dissolved in DMSO with the help of a Branson 1210 Sonicator. This low solubility characteristic limited the amount of chemical that could be injected in the mice at any given time, and it also presented possible limitations in the amount of chemical which might be distributed to the tissues of the mice following i.p. administration of the 1,8-

DNP/DMSO mixture. However, precipitation of 1,8-DNP was rarely observed at the site of injection at the time of necropsy.

DMSO is generally recommended as a good alternative to water as a solvent and vehicle for the test chemical, but care must be taken to ensure that there are no interactions between the test chemical and its solvent which could produce artifacts (Nestmann *et al.*, 1985). For example, DMSO has been shown to enhance the mutagenic effect of hexachloroacetone in *Salmonella* tester strains (Nestmann *et al.*, 1985). Norman *et al.* (1990) have previously reported the presence of DNA adducts following 1,8-DNP treatment of rats, with DMSO as the solvent; no adducts were detected in the animals treated with DMSO only.

The portal circulatory system is mostly responsible for dealing with chemicals that are administered i.p., and these chemicals are delivered to the liver before passing on to other organs (Lukas *et al.*, 1971). Compounds administered s.c. are expected to be distributed systemically without first experiencing hepatic metabolism (Imaida *et al.*, 1995). Studies employing both the i.p. and s.c. routes of mutagen administration have shown that at least for the mammary gland, mutagenic and carcinogenic responses are independent of the route of administration (Imaida *et al.*, 1995). Another study showed that 1,8-DNP is absorbed from the peritoneal cavity of rats relatively quickly (Norman *et al.*, 1990).

It would be expected that the largest amount of chemical exposure, and possibly genotoxic effects, would be at the site of injection (mesentery) and the liver due to the portal circulatory system (Lukas *et al.*, 1971). A mutagenic effect

was observed in the *lacZ* transgene of Muta™Mouse mesentery following the administration of 2.8 mg/kg 1,8-DNP to animals in Study #1 (Table 3), though the mean mutant frequency was low (21.29×10^{-5}) compared to those observed following treatment with agents such as ENU (Table 8) (70.7×10^{-5} for liver, 285.6×10^{-5} for bone marrow (Douglas *et al.*, 1996); 61.4×10^{-5} for lung (this thesis, Study #2)). It should be noted that the study presented in this document used 240 mg/kg ENU while Douglas *et al.* (1996) used 250 mg/kg ENU. No 1,8-DNP-induced mutagenesis of the *lacZ* transgene was observed in liver samples (Table 7), but these results are in accordance with a study using the same nitropyrene in rats. Norman *et al.* (1990) used ^{32}P -postlabelling to observe adduct formation in various tissues in rats following a single treatment with 1,8-DNP and detected the highest adduct levels in mesentery tissue and lower levels in liver tissue. However, adducts were also detected in mammary, bladder, lung and kidney tissues (Norman *et al.*, 1990), suggesting that the chemical does get distributed to most, if not all, tissues.

4.1.2.4 Metabolism.

1,8-DNP is partially activated in both bacteria and mammals by nitroreduction (Fifer *et al.*, 1986; Heflich *et al.*, 1986; King *et al.*, 1986; Norman *et al.*, 1990), with subsequent acetylation being necessary for the formation of the DNA reactive species (Norman *et al.*, 1990). Reductive metabolism yields intermediates such as electrophilic aryl nitrenium ions which are the ultimate reactive species with specific sites on DNA bases (Kerdar *et al.*, 1993). It is

believed that one of the critical steps in mutagenesis is the reaction of the electrophilic species with the nucleophilic centres of DNA (Kerdar *et al.*, 1993).

One possibility for the lack of observed 1,8-DNP-induced mutagenesis in the transgenic mice of the studies presented in this document is the absence of activation of the chemical to a reactive electrophilic aryl nitrenium ions. Reasons for this may include the low activity of enzymes capable of catalysing this reaction. Nitrenium ion formation requires NAT or OAT action for the metabolic activation of the *N*-hydroxyarylamines (Watanabe *et al.*, 1994). Liver cytosols of mice and humans have been shown to be capable of *N*-acetylating carcinogenic arylamines, and in certain inbred mice, other tissues have also shown this ability (King and Glowinski, 1983). The Muta™Mouse strain originates from a parental cross between DBA/2 and BALB/c (Gossen *et al.*, 1989). A survey of 20 inbred mouse strains with respect to the NAT activities in liver and blood determined that both the BALB/cJ and DBA/J strains are fast acetylators (Glowinski and Weber, 1982; King and Glowinski, 1983), indicating that Muta™Mouse possesses some of the enzymes required for the metabolic activation of 1,8-DNP to its mutagenic form. In fact, three NAT-encoding genes have been identified in BALB/c mice (Kelly and Sim, 1994). It is therefore likely that the rate limiting step for activation of 1,8-DNP to the DNA reactive species is the initial nitroreduction part (Figure 4). This has been shown to be case with *in vitro* tests using CHO, with strong mutagenic responses being observed when the cells were treated with the metabolite 1,8-NONP, and weaker mutagenic responses

being observed when treatment was with the parent compound, 1,8-DNP (Fifer *et al.*, 1986).

Limited metabolic activation of 1,8-DNP would also minimise the extent of its mutagenic action on DNA. In a study of adduct formation in rat tissues following a single dose of 1,8-DNP, low levels of adducts were found in liver relative to other tissues (Norman *et al.*, 1990). Norman *et al.* (1990) speculated that this lack of DNA damage might reflect the ability of the hepatic enzymes to efficiently detoxify 1,8-DNP. No significant increase in mutant frequency was observed in the transgenes of liver tissues of mice from Study #1 following 1,8-DNP treatment (Table 7).

4.1.3 Factors related to the transgenic mouse model.

4.1.3.1 Treatment and selection.

The design of the tests done with the different transgenic mice have varied widely, making comparison between the results difficult. A survey of several studies has concluded that the accepted number of animals (mice or rats) in each group should be at least 5 or 6 (Morrison and Ashby, 1994). These goals were met in both studies presented in this document; Study #1 (section 2.4.1) consisted of mice in groups of 5 or 6, while Study #2 (section 2.4.2) consisted of mice in groups of 10. Although it is recommended that about 20×10^4 plaques should be assessed per tissue per animal (Morrison and Ashby, 1994), this was not always achieved in the two studies presented in this thesis for various reasons, including difficulties in consistently packaging sufficiently

high numbers of the bacteriophage lambda. Others have also reported difficulties in achieving a sufficiently high packaging efficiency to produce 20×10^4 plaques with only one packaging reaction (Douglas *et al.*, 1994). There was also a laboratory-wide problem with bacterial contamination over the course of several months, making it difficult to assess the correct phage titres for the compromised samples.

When performing the positive selection assay (section 2.6), samples from both control groups and the treatment group(s) were assayed in parallel. This served as an internal control for the many different steps of the positive selection assay, and it also provided a baseline for identifying the significance of variations in bacteriophage lambda packaging efficiency. This concept of blocked experiments is suggested as a way to reduce variations within and between laboratories (Tinwell *et al.*, 1995; Piegorsch *et al.*, 1995). A positive control group did not exist in Study #1 and thus samples were provided by J. Gingerich (Health Canada), while a known Muta™Mouse mutagen, ENU (Douglas *et al.*, 1995a; Douglas *et al.*, 1996), was used as a positive control for chemical induction of mutations in the *lacZ* transgene in Study #2. Samples from other Muta™Mouse studies (J. Gingerich, Health Canada) were also used successfully as internal controls for DNA extraction procedures and packaging efficiency on occasions where there was considerable difficulty in packaging samples from either study presented in this document, or when protocols were being optimised. Growth of plaques on the titre and P-Gal selection plates can be affected by many factors, including bacterial and viral contamination of

consumables such as maltose and MgSO_4 , and variability in the growth pattern of the *E. coli* C *galE*⁻ cells (Tinwell *et al.*, 1996).

4.1.3.2 Sources of variation in the assay.

Potential sources of variability in the data obtained from transgenic animals have been described using both the *lacI* mouse, Big Blue™ (Piegorsch *et al.*, 1994), and the *lacZ* mouse, Muta™Mouse (Piegorsch *et al.*, 1997). It was determined that variability between the plates following a given packaging reaction using either animal model was not significant (Piegorsch *et al.*, 1994; Piegorsch *et al.*, 1997). There was occasionally a slight difference in mutant frequency in a given animal following different packaging reactions using both the Big Blue™ model and the Muta™Mouse model (Piegorsch *et al.*, 1994; Piegorsch *et al.*, 1997). Reasons for this observed variation might include the alteration of stored genomic DNA due to factors such as cytosine deamination and depurination and nuclease contamination and residual organic material left over from the extraction procedures, causing single- or double-stranded nicks in DNA, DNA strand breaks and modified bases (Piegorsch *et al.*, 1994). Both mutant frequency and packaging efficiency can be affected by factors such as pH, temperature and storage time of greater than 80 days (Piegorsch *et al.*, 1994). Generally speaking, the positive selection assays presented in this document were carried out within days of the DNA extraction procedure. In addition, care was taken to carry out the DNA extraction and positive selection assay in blocked experiments to ensure that factors affecting mutant frequency

on a given day would affect the numbers from all treatment groups. Certain tissues (mesentery, lung, mammary; Study #2) did not package adequately, despite multiple attempts at DNA extraction, as well as several attempts at the positive selection assay. It is possible that a contaminated solution would affect the integrity of the genomic DNA, thus diminishing the packaging efficiency which depends on the length of intact DNA (section 1.2.1.1). Finally, Piegorsch *et al.* (1994, 1997) found some animal-to-animal variability in the results presented for both Big Blue™ and Muta™Mouse mice in some of the studies surveyed. Variability between animals in each treatment group was observed in both studies presented in this document (sections 3.1 and 3.2), but data was only discarded if the packaging was poor (with a titre of less than 1×10^4 phage), if the number of plaques on the test plates (P-Gal) totalled 0 (liver; Study #1), or the mutant frequency was greater than three standard deviations away from the mean.

Variations in the spontaneous mutant frequency between various tissues in a given mouse can be due to several factors, such as the rate of cell turnover in the different tissues, variable exposure to endogenous mutagens and differences in DNA repair depending on the tissue type (Gossen and Vijg, 1993a). The spontaneous mutation rate of the *lacZ* or *lacI* transgene in the Muta™Mouse or Big Blue™ transgenic mouse models can be affected by a number of experimentally determined factors, which in decreasing order of importance include the site of integration of the transgene, the age of the animal,

the tissue examined, the strain of mouse and inter-laboratory variations (Zhang *et al.*, 1995; Ono *et al.*, 1995).

4.1.3.3 Dosing regime and mutation fixation.

Previous studies with Muta™Mouse and ENU as the mutagen have shown that in order to effectively compare either the effects of a chemical on different tissues, or the effects of various chemicals on one tissue, it is important to be consistent in both the dose delivery and time course of the treatments with the chemical(s) (Myhr, 1991). The two studies presented in this thesis involved different doses of 1,8-DNP, but in both cases, i.p. was used as the route of administration, and the fixation time was kept at 29 days. Although both i.p. and s.c. are often used to administer nitropyrenes (Table 14), i.p. was used as the route of administration in Studies #1 and #2 because it has been used successfully in other studies in which 1,8-DNP-induced DNA adducts or tumours have been reported (Table 14), and because a study has shown that 1,8-DNP is absorbed from the peritoneal cavity relatively quickly (Norman *et al.*, 1990).

Many studies, including those presented in this thesis, use multiple treatments to deliver the total chemical dose to the animals (Tokiwa *et al.*, 1986; Douglas *et al.*, 1996). It has been shown that a higher mutant frequency is obtained after multiple treatments than following a single acute dose (Tao and Heddle, 1994). This has been demonstrated using the *Dlb-1* locus, but other loci with neutral mutations, such as those involving the *lacZ* transgenic mutation assays, are expected to behave in the same way (Tao and Heddle, 1994). Since

both studies presented in this document involved multiple doses of 1,8-DNP and ENU (section 2.4), these chronic protocols were optimal for the detection of a strong positive induced mutant frequency, assuming 1,8-DNP- and ENU-induced mutations in Muta™Mouse were detectable.

The fixation time following treatment of a rodent with a chemical has an effect on the observed mutant frequency and appearance of chromosomal aberrations (Khan and Heddle, 1992; Brooks *et al.*, 1995; Douglas *et al.*, 1996). Tissue-specific variation in the timing of the expression of the optimal mutant frequency has also been observed (Heddle *et al.*, 1995; Douglas *et al.*, 1996). Brooks *et al.* (1995) have determined that following treatment of Muta™Mouse mice with 2-acetylaminofluorene (AAF; 100 mg/kg), a mutagenic response in the liver was detected from 28 days to 112 days after a single exposure to the chemical, and the mutant frequency remained relatively the same during this time. Douglas *et al.* (1996) reported that following the treatment of Muta™Mouse mice with ENU, the mutant frequency in bone marrow reached its peak after 5 days, and only began to decline 45 days after the last treatment; in liver, the peak mutant frequency was achieved 35 days post-treatment. At least for these two tissues, the fixation time of 29 days used in the two studies presented in this thesis are reasonable for the detection of mutations.

4.1.3.4 Packaging.

Deletions of more than 5 kb or insertions of more than 8 kb are not detectable by the Muta™Mouse because for *in vitro* packaging the lambda vector

needs to be between 42 kb and 52 kb in size (section 1.2.1.1) (Gossen and Vijg, 1993a; Buettner *et al.*, 1996). In *E. coli* and *S. typhimurium*, 1,8-DNP induces primarily frameshift mutations involving the deletion of 1 or 2 bases or the addition of 1 base (Lambert *et al.*, 1992; Nohmi *et al.*, 1995). Base substitutions and large deletions have also been detected following treatment (Lambert *et al.*, 1992). On the basis of the mutational specificity of 1,8-DNP in prokaryotes it is unlikely that the inability of the Muta™Mouse system to detect large deletions interfered with the selection of 1,8-DNP-induced *lacZ* mutants and the subsequent analysis of the resulting mutational spectrum.

As mentioned in section 1.2.1.1, the size of the genomic DNA isolated from tissues needs to be large enough to contain a minimum of two intact *cos* sites for the *in vitro* rescue of one λ gt10/*lacZ* vector. This limitation is a serious consideration when carrying out the DNA isolation protocol (section 2.5). Isolation of genomic DNA from tissues involves a significant amount of manual manipulation, and strand breakage is a strong possibility. In fact, some of the observed packaging difficulties in tissues such as mesentery, lung, liver and mammary, resulting in lower than expected plaque forming units (pfu), can possibly be attributed to DNA strand breakage. An attempt was made to mitigate this problem by being very gentle during the genomic DNA extraction procedure, especially when precipitating and spooling the genomic DNA.

4.1.3.5 *In vivo* versus *ex vivo* mutations.

Mutations detected with the Muta™Mouse system are ultimately scored in the bacterial host, and therefore there is some question as to whether the lesions on the DNA are fixed into mutations in the mouse tissues or in the bacterial hosts. At least three studies have addressed this question. The first involved comparing the spectrum of mutations in the *lacZ* gene arising from ENU treatment in *E. coli* cells and the *lacZ* genes isolated from ENU treated mouse brain tissue; the pattern of mutations in these two cases differed from each other (Gossen and Vijg, 1993a). The other involved the analysis of the DNA from post-mitotic cells such as brain tissue at various time points after ENU treatment (Gossen *et al.*, 1989). An increase in mutant frequency over the background was observed seven days after the treatment but not after one day (Gossen *et al.*, 1989). This might indicate that the mutations did not originate in the *E. coli* cells since the increase in mutant frequency would have then been expected immediately after the treatment (Gossen *et al.*, 1989), assuming the adducts were formed in the mouse tissue DNA immediately after treatment. Finally, the positive selection assay used with the Muta™Mouse system selects against any mutations which would occur *in vitro*, thereby avoiding the confusion of sectorized or mosaic plaques seen with the non-selecting Big™Blue assay (Paashuis-Lew and Heddle, 1997; Paashuis-Lew *et al.*, 1997). These studies have shown that the majority, if not all, of the *lacZ* mutations observed using this system actually do originate in the mouse tissues and not in the *E. coli* C cells (Gossen and Vijg, 1993a). This finding indicates that a portion of the DNA sequence alterations in

the *lacZ* transgene from mesentery tissue from Study #1 are induced by 1,8-DNP (2.8 mg/kg).

4.1.3.6 Strain, gender and age.

Factors such the strain of rodents, the gender and the age can all affect the observed mutant frequency in an endogenous or transgenic gene.

Inbred mouse strains have been shown to vary in their genetic susceptibility to the carcinogenic effects of various chemicals (Festing, 1995) and also in their acetylation phenotypes, with strains such as C57BL/6J exhibiting rapid acetylator activity in the blood, and A/J exhibiting slow acetylator activity in the blood (Glowinski and Weber, 1982). Studies indicate that Muta™Mouse mice also possess the fast acetylator phenotype (Glowinski and Weber, 1982; King and Glowinski, 1983) which is necessary for the activation of 1,8-DNP to its mutagenic form (Figure 4). It follows that some other feature is behind the apparent lack of 1,8-DNP-induced mutagenicity in the *lacZ* transgene observed in these studies presented in this document (section 4.1.2.4). The dangers of relying on one species of rodent to classify chemicals as mutagenic is illustrated by a study involving two strains of mice and the chemical DMN (dimethyl nitrosamine). A stronger positive UDS response was observed in Muta™Mouse mice following a single dose with DMN than in the Big™ Blue mice under identical conditions (Tinwell *et al.*, 1994). This suggests a difference in the activities of the liver enzyme involved in the metabolism of DMN into an alkylating species between the two strains of mice, rather than a mutagen

sensitivity between the two constructs (Tinwell *et al.*, 1994). It has therefore been suggested that, for example, four rat and mouse strains, including those already used in transgenic rodent lines, such as the mice strains DBA/2 and BALB/c and the rat strain F-344, could be used concurrently in mutagenicity testing (Festing, 1995). Of the various strains of *lacZ* transgenic mouse produced, studies showed that the Muta™Mouse system (strain 40.6) had a relatively low level of *in vivo* background mutant frequency compared to the other strains, allowing the detection of low levels of mutagenesis (Gossen *et al.*, 1989).

The studies presented in this thesis employed female transgenic mice, since the study of mammary tissue was one of the objectives. However, the gender of mice employed for mutagenicity studies can have an effect on the observed mutagenesis. A study with C57BL/6J mouse kidneys which were exposed to AF (2-aminofluorene) indicated that in males, there is a 2-5 fold higher AF-NAT activity than in females, and similar sex differences were observed in mouse kidney microsomes during the activation of AAF (King and Glowinski, 1983). Concurrent studies using newborn male and female mice treated with various nitrochrysene isomers have shown that under identical treatment conditions, tumour formation can vary between the sexes, with the administration of 6-NC (6-nitrochrysene) resulting in the induction of liver tumours in male newborn mice only (El-Bayoumy *et al.*, 1992). As a comparison, ENU was used as a positive control in Study #2, while others have published data involving ENU treatment of male Muta™Mouse mice. The mice in study #2

were female, approximately 4 months old, and were treated with 240 mg/kg ENU with a fixation time of 29 days, while Douglas *et al.* (1996) treated 9-10 week old male mice with 250 mg/kg ENU followed by a fixation time of 25 days. The mutant frequency observed in bone marrow of the animals in Study #2 ranged from 115.8×10^{-5} to 232.8×10^{-5} with a mean of $(171.3 \pm 11.1) \times 10^{-5}$, while the data presented by Douglas *et al.* (1996) range from 245×10^{-5} to 284×10^{-5} with a mean of $(264.3 \pm 23.7) \times 10^{-5}$. The mutant frequencies observed in Study #2 are lower than those of Douglas *et al.* (1996), but considering that slightly less ENU was used in Study #2, the observed differences cannot be attributed only to gender or age related variations.

The age of the rodents used in mutagenicity testing can affect the outcome of the study. The newborn state in rats confers more sensitivity than the weanling stage to carcinogenic compounds (Imaida *et al.*, 1995). Also, as organisms age, more spontaneous mutations are accumulated (Grist *et al.*, 1992; Ono *et al.*, 1995), though in a non-linear manner, with two-thirds of the spontaneous mutations in mice arising before adulthood (Paashuis-Lew and Heddle, 1997). The mice in study #1 were approximately 9 weeks of age at the start of the study and 21 weeks at the time of necropsy. In contrast, retired breeders were used in Study #2, with an approximate age of 4 months. Using the data from bone marrow, for example, the negative control mice in the first study had a mean mutant frequency of $(4.06 \pm 0.15) \times 10^{-5}$ (Table 4) while the spontaneous mutant frequency of the mice in the second study was approximately twice that value, at $(8.49 \pm 2.18) \times 10^{-5}$ (Table 8).

4.2 Molecular analysis of mutations.

4.2.1 Complementation studies.

1,8-DNP-induced mutants from mesentery tissue from Study #1 were selected for mutational analysis, because a statistically significant increase in the mutant frequencies of the *lacZ* transgene were observed following treatment with 2.8 mg/kg 1,8-DNP (Table 3). Initially, complementation studies were performed to localise the mutations on the *lacZ* transgene to one of three complementation groups. Subsequent sequence analysis of the mutants indicated that the ability of the complementation studies to identify the location of the mutations on the *lacZ* gene was not every efficient. The greatest amount of information can be obtained from those results which can narrow down the region of mutation to one of α , β or ω . However, only 42% of the analysed mutants yielded such a clear response with the complementation studies, and of these mutants, only 45% were found to contain mutations in the assigned regions following sequence analysis (Table 10). In contrast, a study by Douglas *et al.* (1996) reported an 85% success rate in the correlation between the location of the mutation reported by the complementation study, and what was subsequently identified by sequence analysis. A larger region is involved when the location of the mutation can only be narrowed down to two of the three complementation regions; 42% of the mutations analysed in this study were predicted to be in either the α or ω region, or the β or ω region, but in fact only 64% of the mutants identified by sequence analysis were in these regions (Table 10). Douglas *et al.* (1996) reported a 100% predictive ability of the

complementation assays for these regions. Four of the mutants identified by sequence analysis did not yield data from the complementation studies.

The complementation studies appear to be negligible in their usefulness. Their predictive ability was low enough that direct sequencing would save time. Reasons for the failure of the complementation studies include the fact that the complementation regions are not clearly defined, and therefore the process of complementation is not always a precise event. In addition, some mutations do not completely eliminate the function of the β -galactosidase protein, resulting in a light blue colour with all three complementation strains, and protein complementation is not able to easily restore all mutations (Vijg and Douglas, 1996). Finally, the determination of the colour of the plaques following the exposure of the *lacZ* gene product to X-Gal is subjective, and the resulting colour can be influenced by factors such as length of incubation.

4.2.2 PCR amplification and sequencing.

Prior to sequencing, PCR was used to generate a large number of copies of the mutant transgene to facilitate the sequencing process. The possibility that some of the mutations that were identified by sequencing actually occurred due to mis-incorporation during the PCR cycles is one which needs to be addressed. It is estimated that for 1 μ g of mammalian genomic DNA which contains approximately 10^5 copies of a single copy gene, the errors induced by *Taq* polymerase would be less than 1 in 10^5 (Myers and Parry, 1994). Since all observed mutant frequencies were greater than what would be expected from

mis-incorporation during PCR, it is likely that this source of error, if present, was insignificant relative to the *in vivo* derived mutations. In addition, the most frequently reported *Taq* polymerase errors are A:T → G:C transitions (Sandy *et al.*, 1992), but this was not observed in any of the negative control (DMSO only) mutants, and in only one of the 1,8-DNP-treated mutants, accounting for 8.3% of the characterised mutants from the high dose group (Table 13). Also, due to the nature of the PCR technique, no more than 50% of the newly created DNA strands should contain a base alteration which was due to a mis-incorporation error. Since only obvious mutants are scored as such when analysing the DNA sequence results, it is highly unlikely that a mutation due to a PCR mis-incorporation was scored as a mutation originating in the mouse.

Spontaneous mutations can occur by various routes, and may be mediated by oxidative damage and methylation. Methylation is known to play an important role in the formation of spontaneous mutations. Under normal circumstances in bacteria, the spontaneous deamination of cytosine forms uracil, which is then excised from the DNA by uracil-DNA glycosylase (Duncan and Miller, 1980). However, methylation of cytosine leads to 5-methylcytosine residues which form thymine upon deamination, and this error cannot be repaired by uracil-DNA glycosylase, leading to spontaneous mutations (Duncan and Miller, 1980). In vertebrates, 60% to 90% of the CpG sites are methylated at the 5 position on the cytosine ring, and the DNA repair mechanisms are unable to recognise the deamination of 5-methylcytosine to form thymine (Bird, 1986).

Fourteen *lacZ* transgenes originating from mesentery tissue of Muta™Mouse mice in Study #1, DMSO treatment only, were successfully sequenced (Table 11). All of the detected mutations were base substitutions. Of these, seven of the mutations were G:C → A:T transitions (likely due in part to the methylation of the genome), three were G:C → T:A transversions, three were G:C → C:G transversions and finally, one was an A:T → C:G transversion (Table 13). The spontaneous mutation spectrum in the transgene from mesentery has not been reported elsewhere. Douglas *et al.* (1994) examined the spontaneous sequence spectrum in the transgene of Muta™Mouse in liver and bone marrow. In both tissues the majority of spontaneous mutations which were detected were G:C → A:T transitions (44.4% and 71.4%, respectively), and of the observed transversions, G:C → T:A were predominant (3.7% and 14.3%, respectively) (Douglas *et al.*, 1994). This compares favourably to the spontaneous mutation spectrum reported in this thesis of 50% G:C → A:T transitions and 21.4% G:C → T:A transversions. Of the observed G:C → A:T transitions in this study, 86% were at 5'-CpG sites, while Douglas *et al.* (1994) reported an incidence of 83% for liver and 93% for bone marrow. The location of the mutations at 5'-CpG sites suggests that deamination of methylated cytosine residues, leading to the formation of thymine, was responsible for these spontaneous mutations (Bird, 1986). A:T → G:C transitions were observed in 25.9% (liver) and 4.8% (bone marrow) of the samples reported by Douglas *et al.* (1994), but none were detected in the analysis of spontaneous mutations in mesentery tissues of Muta™Mouse (Table 13).

DNA sequence alterations were successfully identified in 12 *lacZ* transgenes originating from mesentery tissue of Muta™Mouse mice in Study #1, high dose group (2.8 mg/kg 1,8-DNP) (Table 12). Although the number of analysed mutants is low (section 3.3.2), certain patterns emerge. Unlike in the collection of spontaneous mutants in which 50% of the mutations were G:C → A:T transitions, only 25% (3 of 12) of the mutations from the treated samples were this type of mutation (Table 13). The predominant types of mutations (4 of 12) observed in the treated samples were G:C → T:A transversions (33%). This class of transversion mutation was also the predominant base substitution observed in the *lacI* gene of *E. coli* following treatment with 1,8-NONP (Lambert *et al.*, 1992). No G:C → C:G transversion base substitutions were observed in the treated samples, while an A:T → G:C transition was observed in the treated samples but not in the spontaneous group. An A:T → C:G transversion base substitution was observed in each of the spontaneous and treated groups (Table 13). In addition to base substitutions, frameshift mutations and one tandem base substitution were also discovered in the treated samples (Table 13).

Nitropyrenes are direct-acting frameshift mutagens in *S. typhimurium* (Mermelstein *et al.*, 1981). One -(GC:CG) frameshift (Table 12) was observed in the *lacZ* transgene originating in mesentery tissue of 1,8-DNP-treated mice (2.8 mg/kg 1,8-DNP), in a region of alternating GC residues. Minus 2 frameshifts involving the loss of GC:CG residues were also observed in regions of alternating GC sequences in the *lacI* gene of *E. coli* following treatment with 1,8-NONP, a metabolite of 1,8-DNP (Lambert *et al.*, 1992). Lambert *et al.* (1992)

postulated that a slippage-incorporation mechanism involving slippage of the GC repeat could be responsible for this event. Others propose that a saturation or inactivation of the mismatch repair system is responsible for frameshift activity (Cupples *et al.*, 1990a). A survey of the literature did not reveal any reported -(GC:CG) frameshifts in the *lacZ* transgene, suggesting that the occurrence of this mutation is relatively rare, and therefore likely a result of the mutagenic action of a metabolite of 1,8-DNP. The other detected frameshift in the *lacZ* transgene following 1,8-DNP was a +(A:T), in the same treatment group, and was located in a contiguous sequence of A residues (Table 12). Similar frameshift mutations were observed in the *lacI* gene of *E. coli* following 1,8-NONP treatment, and were possibly due to a strand slippage mechanism (Lambert *et al.*, 1992).

Two amino acids have been shown to be important for the catalytic activity of β -galactosidase: glutamate at position 461 (Glu-461) and tyrosine at position 503 (Tyr-503) (Cupples and Miller, 1988). Of these, Glu-461 was found to be altered to a nonsense mutation in sample 17-C-1, mesentery tissue, 2.8 mg/kg 1,8-DNP (Study #1) (Table 12). Mutations at Tyr-503 were not detected in this study.

Mutation sites were not shared by any of the sequenced mutants, and therefore no hot spots were identified. As well, this lack of duplicate mutations suggests that there were no mutations in stem cells, which would give rise to a clonal expansion of mutations (Douglas *et al.*, 1995a).

4.2.3 The nature of the transgene.

4.2.3.1 The transgene as a mutational target.

Muta™Mouse (strain 40.6) has been found to be relatively stable in terms of homozygosity and the total copy number of the transgene, due at least in part to the location of integration of the transgene (Blakey *et al.*, 1995).

There are several reasons why a given chemical might give different results when mutagenicity is measured in a transgene or an endogenous locus. The bacterial *lacZ* inserts are prokaryotic, heavily methylated, are not expressed, and there are multiple copies of them positioned in a head-to-tail fashion (Tao *et al.*, 1993). A difference in sequence context or chromosomal conformation between the transgene and endogenous gene may affect the reactivity of a mutagen with the DNA, and there might exist a difference in repair between a transcribed locus and a non-expressed transgene (Skopek *et al.*, 1995). Finally, there might be a difference in the number of mutations detected when different selection methods are used for transgenes and endogenous genes (Skopek *et al.*, 1995). A study with the *lacI* transgene of the Big Blue™ transgenic mouse and the endogenous locus *Dlb-1* determined that the transgenes and host genes respond in a similar fashion to acute doses of the mutagen ENU (Tao *et al.*, 1993). In contrast, chronic doses of ENU over a 4 month period yielded an increase in the somatic mutation rate at the host locus but not the transgene (Shaver-Walker *et al.*, 1995). Both studies presented in this document involved a maximum of 8 doses of the test agent, and therefore it

is likely that any observed increase in mutation rate is representative of damage at endogenous loci.

4.2.3.2 Sequence biases.

This α region of the *lacZ* gene product is involved in the tetramer formation of β -galactosidase (Cupples and Miller, 1988). The *lacZ* gene has been shown to tolerate base substitutions in the amino terminus, resulting in the lack of detection of some mutations (Cupples and Miller, 1988; Kohler *et al.*, 1991a). As a result, those *lacZ* mutants which were detected by the positive selection assay probably contained mutations in a region other than the amino terminus. For this reason, the detection of mutations in the *lacZ* gene generally involved sequential sequence analyses commencing at the 3' end of the gene, with the P2 primer, P4 primer and so on until a mutation was detected (Figure 6). In addition, at least 81 residues throughout the *lacZ* gene can tolerate base substitution changes with minimal disruption of enzyme activity (Cupples and Miller, 1988).

4.3 The future of Muta™Mouse.

Transgenic mouse systems, including the Muta™Mouse system, can provide more detail about the mutagenic effects of substances than either bacterial or *in vitro* techniques (section 1.2). However, these transgenic systems also have significant disadvantages.

Despite the lack of convincing evidence for the mutagenicity of 1,8-DNP in the transgenic mouse (sections 3.1 and 3.2), other data suggests that 1,8-DNP, or its metabolite, interacts with rodent DNA or is carcinogenic (Table 14), and therefore is a potential human health hazard. There is therefore the potential for false-negative conclusions if other STTs are not used in conjunction with this test system. A more general disadvantage of this transgenic mouse system is that the utility of Muta™Mouse as a screening tool for harmful chemicals is limited by the need to screen for multiple end-points (genotoxic and non-genotoxic); not all chemicals listed as carcinogenic by IARC (International Agency for Research on Cancer) can be detected in transgenic mice which screen for genotoxic chemicals (Heddle *et al.*, 1991). Other short comings of using rodent models to predict human carcinogenicity are emerging. For example, there has recently been some debate over the appropriateness of using rats as models for human lung cancer resulting from inhalation of chemicals such as PAHs and nitro-PAHs, since a variety of conflicting results have been published, and the testing of rats is rather different from the human situations for exposure to nitro-PAHs (Rosenkranz, 1996). Other considerations in using animals for experimentation include the differences between the human and non-human responses to chemicals, the inability of animals to communicate subjective discomfort and the inability to detect subtle changes to the higher nervous system function (Sullivan *et al.*, 1993).

Alternatives to the Muta™Mouse transgenic system for detecting *in vivo* mutations include the restriction site mutation (RSM) assay and the restriction

fragment length polymorphism/polymerase chain reaction (RFLP/PCR). RSM detects mutations *in vivo*, but does not require the selection of a mutated phenotype, and therefore the analysis is not limited to a few genes (Myers and Parry, 1994). RSM works on the basis that endogenous or transgenic genes containing restriction enzyme recognition sites which are resistant to digestion due to mutations are isolated, amplified using PCR, and are visualised by electrophoresis (Myers and Parry, 1994). The constraints are that there needs to be adequate DNA sequence information to construct PCR amplification primers and to determine the suitable restriction enzyme recognition sequences for the amplified region (Myers and Parry, 1994). Another approach to detecting point mutations in endogenous genes is the RFLP/PCR method, which determines mutations in restriction enzyme recognition sites (Sandy *et al.*, 1992). Endonucleases are used to cleave the wild-type recognition sites, such as *TaqI* of exon 2 of the human c-H-*ras1* gene, and the remaining mutated recognition sequences are amplified (Sandy *et al.*, 1992). However, because this technique is limited to base substitutions certain classes of mutagens and clastogens go undetected.

V. Conclusions

The focus of this work was to determine the suitability of the *lacZ* transgenic Muta™Mouse model to study and detect the *in vivo* tissue specific genotoxicity of 1,8-DNP.

The first objective of this study was to determine whether 1,8-DNP-induced mutations are formed and can be detected in the *lacZ* transgene of the transgenic mouse Muta™Mouse. Statistically significant mutant frequencies were only observed in the transgenes obtained from mesentery tissue of mice from Study #1 (2.8 mg/kg 1,8-DNP), and also in the transgenes of the positive control animals (240 mg/kg ENU). The lack of observed 1,8-DNP-induced mutagenicity in the transgenes of other tissues can be due to multiple factors, including metabolic deficiencies in the Muta™Mouse strain which would result in a dearth of DNA-reactive metabolites, and difficulties in the isolation and packaging of the transgenes from various tissues. As 1,8-DNP is known to cause DNA adducts and tumours in rodents (Mermelstein *et al.*, 1981; Edgar and Brooker, 1985; Tokiwa and Ohnishi, 1986; King, 1988; Watanabe *et al.*, 1989; Norman *et al.*, 1990; Imaida *et al.*, 1991; reviewed by Rosenkranz and Mermelstein, 1983), the results of this study indicate that caution should be exercised when using the Muta™Mouse system to interpret the genotoxic potentials of chemicals. The utility of the Muta™Mouse system as an indicator of genotoxic chemicals, and by extension a predictor of carcinogens, should be further evaluated.

The second objective of this study was to determine whether Muta™Mouse would develop detectable 1,8-DNP-induced mutations in the *lacZ*

transgene in mammary tissue. To facilitate this examination, retired breeder females were used in the second dosing protocol (Study #2). The packaging of vectors derived strictly from mammary tissue was abandoned because mammary tissue proved to be high in fat content, the cell type was heterogeneous and the packaging was poor despite multiple attempts. As DNA adducts were reported in rats following treatment with 1,8-DNP (Norman *et al.*, 1990), it would be interesting to use a transgenic rat (section 1.2.2.1) to determine the mutant frequency and spectrum of 1,8-DNP in the transgene.

The third objective of this study was to determine the mutational spectrum of 1,8-DNP in the *lacZ* transgene of Muta™Mouse. 14 spontaneous and 12 1,8-DNP-treated mutant *lacZ* transgenes from mesentery tissue (2.8 mg/kg 1,8-DNP) were sequenced and the mutations identified (Table 12). Of the induced mutations, base substitutions (nine), a tandem base substitution (one) and frameshifts (-2 and +1; two) were identified. A -(GC:CG) frameshift was detected, which is characteristic of 1,8-DNP, and has been reported in a *S. typhimurium* reversion assay (Nohmi *et al.*, 1995) and in an *E. coli* forward mutation assay (Lambert *et al.*, 1991) following 1,8-DNP or 1,8-NONP treatment, respectively.

VI. Appendix

The complete nucleotide sequence (5' -> 3') of the *lacZ* transgene (0 to 3158 nt) and the promoter and operator regions (-183 to 0 nt) which are inserted into the Muta™Mouse genome on λ gt10 shuttle vectors (section 1.2.1.1).

```

-183  GAATTCCGAT TCATTAATGC AGCTGGCACG ACAGGTTTCC CGACTGGAAA
-133  GCGGGCAGTG AGCGCAACGC AATTAATGTG AGTTAGCTCA CTCATTAGGC
-83   ACCCCAGGCT TTACACATTT ATGCTTCCGG CTCGTATAAT GTGTGGAATT
-33   GTGAGCGGAT AACAAATTTCA CACAGGAAAC AGCT
0     ATGACC ATGATTACGG ATTCACTGGC ATTCCTGGGG ATGCCCGTCG
47    TTTTACAACG TCGTGACTGG GAAAACCCTG GCGTTACCCA ACTTAATCGC
97    CTTGCAGCAC ATCCCCCTTT CGCCAGCTGG CGTAATAGCG AAGAGGCCCCG
147   CACCGATCGC CCTTCCCAAC AGTTGCGCAG CCTGAATGGC GAATGGCGCT
197   TTGCCTGGTT TCCGGCACCA GAAGCGGTGC CGGAAAGCTG GCTGGAGTGC
247   GATCTTCCTG AGGCCGATAC TGTCGTCGTC CCCTCAAAC TGGCAGATGCA
297   CGGTACGAT GCGCCCATCT ACACCAACGT GACCTATCCC ATTACGGTCA
347   ATCCGCCGTT TGTTCCACAG GAGAATCCGA CGGGTTGTTA CTCGCTCACA
397   TTTAATGTTG ATGAAAGCTG GCTACAGGAA GGCCAGACGC GAATTATTTT
447   TGATGGCGTT AACTCGGCGT TTCATCTGTG GTGCAACGGG CGCTGGGTCTG
497   GTTACGGCCA GGACAGTCGT TTGCCGTCTG AATTTGACCT GAGCGCATTT
547   TTACGCGCCG GAGAAAACCG CCTCGCGGTG ATGGTGCTGC GCTGGAGTGA
597   CGGCAGTTAT CTGGAAGATC AGGATATGTG GCGGATGAGC GGCATTTTCC
647   GTGACGTCTC GTTGCTGCAT AAACCGACTA CACAAATCAG CGATTTCCAT
697   GTTGCCACTC GCTTTAATGA TGATTTCAGC CGCGCTGTAC TGGAGGCTGA
747   AGTTCAGATG TGCGGCGAGT TGCGTGAATA CCTACGGGTA ACAGTTTCTT

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797 TATGGCAGGG TGAAACGCAG GTCGCCAGCG GCACCGCGCC TTTCGGCGGT
 847 GAAATTATCG ATGAGCGTGG TGGTTATGCC GATCGCGTCA CACTACGTCT
 897 GAACGTCGAA AACCCGAAAC TGTGGAGCGC CGAAATCCCG AATCTCTATC
 947 GTGCGGTGGT TGAAGTGCAC ACCGCCGACG GCACGCTGAT TGAAGCAGAA
 997 GCCTGCGATG TCGGTTTCCG CGAGGTGCGG ATTGAAAATG GTCTGCTGCT
 1047 GCTGAACGGC AAGCCGTTGC TGATTCGAGG CGTTAACCGT CACGAGCATC
 1097 ATCCTCTGCA TGGTCAGGTC ATGGATGAGC AGACGATGGT GCAGGATATC
 1147 CTGCTGATGA AGCAGAACAA CTTTAACGCC GTGCGCTGTT CGCATTATCC
 1197 GAACCATCCG CTGTGGTACA CGCTGTGCGA CCGCTACGGC CTGTATGTGG
 1247 TGGATGAAGC CAATATTGAA ACCCACGGCA TGGTGCCAAT GAATCGTCTG
 1297 ACCGATGATC CGCGCTGGCT ACCGGCGATG AGCGAACGCG TAACGCGAAT
 1347 GGTGCAGCGC GATCGTAATC ACCCGAGTGT GATCATCTGG TCGCTGGGGA
 1397 ATGAATCAGG CCACGGCGCT AATCACGACG CGCTGTATCG CTGGATCAAA
 1447 TCTGTCGATC CTTCCCGCCC GGTGCAGTAT GAAGGCGGCG GAGCCGACAC
 1497 CACGGCCACC GATATTATTT GCCCGATGTA CGCGCGCGTG GATGAAGACC
 1547 AGCCCTTCCC GGCTGTGCCG AAATGGTCCA TCAAAAAATG GCTTTCGCTA
 1597 CCTGGAGAGA CGCGCCCGCT GATCCTTTGC GAATACGCCC ACGCGATGGG
 1647 TAACAGTCTT GGCGGTTTCG CTAAATACTG GCAGGCGTTT CGTCAGTATC
 1697 CCCGTTTACA GGGCGGCTTC GTCTGGGACT GGGTGGATCA GTCGCTGATT
 1747 AAATATGATG AAAACGGCAA CCCGTGGTCG GCTTACGGCG GTGATTTTGG
 1797 CGATACGCCG AACGATCGCC AGTTCTGTAT GAACGGTCTG GTCTTTGCCG
 1847 ACCGCACGCC GCATCCAGCG CTGACGGAAG CAAAACACCA GCAGCAGTTT
 1897 TTCCAGTTCC GTTTATCCGG GCAAACCATC GAAGTGACCA GCGAATACCT
 1947 GTTCCGTCAT AGCGATAACG AGCTCCTGCA CTGGATGGTG GCGCTGGATG
 1997 GTAAGCCGCT GGCAAGCGGT GAAGTGCCTC TGGATGTCGC TCCACAAGGT
 2047 AAACAGTTGA TTGAACTGCC TGAACTACCG CAGCCGGAGA GCGCCGGGCA
 2097 ACTCTGGCTC ACAGTACGCG TAGTGCAACC GAACGCGACC GCATGGTCAG

2147 AAGCCGGGCA CATCAGCGCC TGGCAGCAGT GGCGTCTGGC GGAAAACCTC
2197 AGTGTGACGC TCCCCGCCGC GTCCCACGCC ATCCCGCATC TGACCACCAG
2247 CGAAATGGAT TTTTGCATCG AGCTGGGTAA TAAGCGTTGG CAATTTAACC
2297 GCCAGTCAGG CTTTCTTTCA CAGATGTGGA TTGGCGATAA AAAACAACCTG
2347 CTGACGCCGC TGC GCGATCA GTTCACCCGT GCACCGCTGG ATAACGACAT
2397 TGGCGTAAGT GAAGCGACCC GCATTGACCC TAACGCCTGG GTCGAACGCT
2447 GGAAGGCGGC GGGCCATTAC CAGGCCGAAG CAGCGTTGTT GCAGTGCACG
2497 GCAGATACAC TTGCTGATGC GGTGCTGATT ACGACCGCTC ACGCGTGGCA
2547 GCATCAGGGG AAAACCTTAT TTATCAGCCG GAAAACCTAC CGGATTGATG
2597 GTAGTGGTCA AATGGCGATT ACCGTTGATG TTGAAGTGGC GAGCGATACA
2647 CCGCATCCGG CGCGGATTGG CCTGAACTGC CAGCTGGCGC AGGTAGCAGA
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2747 TTACTGCCGC CTGTTTTGAC CGCTGGGATC TGCCATTGTC AGACATGTAT
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2847 ATTGAATTAT GGCCACACC AGTGGCGCGG CGACTTCCAG TTCAACATCA
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2997 TGGTGGCGAC GACTCCTGGA GCCCGTCAGT ATCGGCGGAA TTACAGCTGA
3047 GCGCCGGTCG CTACCATTAC CAGTTGGTCT GGTGTCAAAA ATAATAATAA
3097 CCGGGCAGGC CATGTCTGCC CGTATTTTCGC GTAAGGAAAT CCATTATGTA
3147 CTATTTGAAT TC

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