Transgenic Rodent Gene Mutation Assays:

Performance Characteristics and Exploration of the Effects of Critical Variables Affecting the Development of a Standardized Experimental Protocol

By

Timothy Michael Singer

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Department of Biology

Carleton University

Ottawa, ON Canada

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Abstract

The development of transgenic rodents (TGR) containing multiple copies of chromosomally integrated bacterial reporter genes has provided an *in vivo* model that may allow for more thorough assessments of genotoxic hazards. This work aims to provide further clarification of two critical issues that will determine if TGR assays can be used in genotoxicity hazard identification. First, the performance of TGR assays as genotoxicity tests and as predictors of carcinogenicity has been determined using a database containing the results of all published TGR mutation experiments. Widespread acceptance of TGR assays also depends on better definition of the effects of critical variables affecting the experimental protocol – the treatment duration and sampling time. This work aims to characterize the effects of these variables on mutant frequencies and to determine if the recommended treatment duration of 28 days and sampling time of 3 days would be suitable for general testing purposes.

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Chapter 1

Introduction

Review of Tests Used to Identify Chemicals Causing Gene Mutations or Chromosomal Aberrations

1.1 Introduction

The benefits of chemicals to human society have been tremendous; chemicals with pharmacological activity are used to prevent and treat disease, agricultural chemicals have increased the production and quality of crops, and industrial chemicals are constituents of many important consumer products. However, along with naturally occurring chemicals, those of anthropogenic origin have become ubiquitous in the environment upon which humans and other life depend. For this reason, tests to identify the potential toxic effects of these chemicals must be conducted during compound development. Critical among these tests are those that identify genotoxicity, changes in the structure of DNA that may ultimately cause cell death, transmission of heritable diseases, and neoplasia.

Typically, genotoxic effects can occur through two mechanisms: via gene mutations and/or via chromosomal mutations. For the purposes of this chapter, gene mutations are considered to be permanent single mutations involving only one gene, while chromosomal mutations are considered to be changes in the structure of the chromosome (structural aberration), or a change in chromosome number (numerical aberration). Generally, chromosomal mutations affect more than one gene. Genotoxicity can be manifested through one or both of these mechanisms.

Since experience with genetic toxicology testing over the past several decades has demonstrated that no single assay is capable of detecting the full spectrum of genotoxic effects, the potential for a chemical to cause genotoxicity is typically determined through a battery of short and long term tests, involving both *in vitro* and *in vivo* model systems. *In vitro* assays offer advantages in that they are relatively inexpensive and easy to

conduct and do not directly involve the use of animals. However, *in vitro* tests usually require supplementation with exogenous metabolic activation enzymes in order to simulate mammalian metabolism; they also generally do not allow for the consideration of factors including pharmacokinetics and DNA-repair that are relevant when considering potential effects to humans. Nevertheless, *in vitro* assays are typically used to provide an initial indication of the genotoxicity of a chemical, and the results often inform the choice of appropriate subsequent *in vivo* studies.

The testing of chemicals for the purposes of determining safety requires reproducibility between laboratories and standardization in test methods. For this reason, testing protocols have been developed under the auspices of international organizations, such as the Organization for Economic Cooperation and Development (OECD), to promote the use of reliable and reproducible methods in testing laboratories. These protocols recommend conditions under which the tests should be conducted, based upon an extensive understanding of the validity of the test for the particular endpoint of interest.

In this chapter, the existing genetic toxicology tests are reviewed and their benefits and limitations are described (see also Table 1-1). For the purposes of this discussion, assays are grouped into four categories: *in vitro* genotoxicity assays (briefly reviewed), *in vivo* somatic cell gene mutation assays, *in vivo* somatic cell chromosomal aberration assays and *in vivo* indicator assays. A new test system is described, which is based upon transgenic rodent (TGR) models containing one of several possible chromosomally integrated transgenes that are derived from prokaryotic sources. These TGR assays have the potential to fill a gap in the existing battery of tests and contribute

to a more complete understanding of the genotoxic properties of chemicals. The objectives of this thesis relate to the determination of the role of TGR assays in genotoxicity testing and the proper conduct of the TGR assay.

1.2 *In vitro* Genotoxicity Assays

1.2.1 *In vitro* Chromosomal Aberration Assays

Chemicals causing chromosomal aberrations may be identified with an *in vitro* cytogenetics assay [1]. Mammalian cell cultures, such as those derived from the Chinese hamster (i.e. CHO or V79 lines) are treated with the test chemical; after an appropriate exposure period, mitosis in the cultures is arrested in metaphase with an inhibitor, such as colchicine. The stained metaphase spreads are examined by light microscopy to detect chromosome or chromatid aberrations. An increase in the frequency of cells with structural aberrations compared with that of the concurrent control group indicates the chemical exhibits clastogenic activity. A limitation of this assay, in comparison with some of the other *in vitro* assays, is the subjectivity and cost of having the metaphase spreads scored by an observer.

Recently, a harmonized protocol for the *in vitro* micronucleus test has been developed by an International Workshops on Genotoxicity Testing (IWGT) working group [2; 3]. A proposal for an OECD test guideline is under development and the European Centre for the Validation of Alternative Methods is undertaking a retrospective validation exercise. However, the transition of this assay from the development stage to routine use has not yet occurred.

Table 1-1: Comparison of existing tests used to examine somatic cell genotoxicity *in vivo*

Study	Rodent erythrocyte micronucleus	Bone marrow chromosomal aberration	Mouse spot test	RetinoBlast *	*Hprt* *
Species	Rodent: rat or mouse	Rodent: rat or mouse	Mouse	Mouse	Rat or mouse
Number & sex	Minimum 5/dose/sex	Minimum 5/dose/sex	Sufficient number of females (~50) to produce about 300 F_1 mice/dose	A minimum of 10 females/dose should produce a sufficient number of F_1 mice	5/sex/dose should be sufficient
Dose levels	At least 3 plus controls; maximum should be MTD; limit test acceptable where no toxicity observed at 2000 mg/kg	At least 3 plus controls; maximum should be MTD; limit test acceptable where no toxicity observed at 2000 mg/kg	At least 2 doses (plus controls), one of which should induce mild toxicity or reduced litter size	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity
Route of administration	Oral or i.p.; others where justified	Oral or i.p.; others where justified	Oral or i.p.; others where justified	Route relevant to human exposure (oral, i.p. or inhalation)	The route considered most relevant to human exposure
Treatment schedule	Single administration; multiple dosing where justified	Single administration	Single administration on the 8^{th}, 9^{th} or 10^{th} day of gestation	Single administration on the 10^{th} day of gestation	Single or multiple administration
Tissue analyzed	Bone marrow or peripheral blood erythrocytes	Bone marrow	Fur	Retinal epithelial cells	Splenic t-lymphocytes (primarily)
Sampling (examination) times	24-48 hours for bone marrow; 48-72 hours for peripheral blood	1.5x normal cell cycle length (12-18 hours) and 24 hours after first sampling	After birth of F_1 generation at about 4 weeks	After birth of F_1 generation at about 20 days	Mutant frequency determined after a selection period of several days in 6-TG
Analysis	At least 2000 PCE/animal scored by microscopy or flow cytometry	Microscopy: at least 1000 cells/animal for mitotic index; at least 100 cells/animal for chromosomal aberrations	Presence of colour spots on the coat of F_1 animals	Presence of colour spots within the retinal epithelium of F_1 animals	Calculation of induced mutant frequency
Approximate duration	About 1 week	About 2 weeks	About 6 weeks	About 2-3 months	About 2 months

Study	*Aprt* *	*TK* $^{+/-}$ *	*Dlb-1* *	Sister chromatid exchange *	Unscheduled DNA synthesis
Species	*Aprt*$^{+/-}$ or *Aprt*$^{-/-}$ mouse	*Tk* heterozygous mouse	*Dlb-1* heterozygous mouse	Rat or mouse	Rat; also mouse
Number & sex	5/sex/dose should be sufficient	5/sex/dose should be sufficient	5/sex/dose should be sufficient	5/sex/group should be sufficient	At least 3/group; usually males are sufficient
Dose levels	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least 2, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity
Route of administration	The route considered most relevant to human exposure	The route considered most relevant to human exposure	Oral administration	Gavage or intraperitoneal injection are most common	Oral (i.p. injection is not recommended)
Treatment schedule	Single or multiple administration	Single or multiple administration	Single or multiple administration	Single administration	Single administration
Tissue analyzed	Splenic t-lymphocytes or skin fibroblasts (primarily)	Splenic t-lymphocytes (primarily)	Intestinal epithelial cells	Bone marrow (other cell types may be used)	Liver
Sampling times	Mutant frequency determined after a selection period of several days in 8-AA or DAP	Mutant frequency determined after a selection period of 10-12 days in BrdU		Sacrifice 24 hours after administration; animals receive BrdU at 2-3 hours and colchicine at 21 hours	2-4 and 12-16 hours after administration; liver cells are prepared and incubated for 3-8 hours in ^{3}H-TdR
Analysis	Calculation of induced mutant frequency	Calculation of induced mutant frequency	Calculation of induced mutant frequency	Scoring SCE by examination using light microscopy	Determination of net nuclear grain count by autoradiography in at least 100 cells per animal
Approximate duration	About 2 months	About 2 months	About 2 months	About 1 week	About 1 week

* non-guideline study

1.2.2 *In vitro* Gene Mutation Assays

The *in vitro* assays commonly used in genetic toxicity testing include the bacterial reverse mutation assay (Ames assay), which detects chemicals that cause point mutations or frameshift mutations in histidine auxotrophic strains of *Salmonella typhimurium* (e.g. strains TA100, TA98, TA102), and a reverse mutation assay using a tryptophan auxotrophic strain of *Escherichia coli* (e.g., WP2*uvr*A) [4]. Revertant cells grow on minimal agar containing trace amounts of histidine or tryptophan, while wild-type cells rapidly deplete the limiting amino acid and stop growing. If there is an increase in the number of revertant colonies compared with the concurrent negative control (typically considered a two-fold or greater increase), the chemical is concluded to be mutagenic.

Mammalian cell forward mutation assays, such as the thymidine kinase (*Tk*) assay or the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) assay, detect mutations at the heterozygous *Tk* or hemizygous *Hprt* gene [5]. Cells such as L5178Y mouse lymphoma cells (*Tk* locus), several Chinese hamster cell lines (*Hprt* locus), and human lymphoblastoid cells (*Tk* locus) are most commonly used. Mutations are selected by incubation of the cell cultures with the selective agents trifluorothymidine (*Tk* assay) or 6-thioguanine (*Hprt* assay). Cells having forward mutations at the *TK* or *Hprt* genes survive in the presence of the selective agent, while wild-type cells accumulate a toxic metabolite and do not proliferate. Comparison of the mutant frequency of the treatment groups with the concurrent negative control group allows the identification of a mutagenic chemical.

1.3 General Factors Associated With *In Vivo* Tests

In vivo genotoxicity assays offer advantages in that they are affected by mammalian DNA repair processes and pharmacokinetic factors that are difficult to reproduce in most *in vitro* systems; consequently, the degree of uncertainty in extrapolating to humans is lower than for *in vitro* tests. Despite this advantage, the *in vivo* assays are considerably more time consuming to conduct because of the need to administer the test compound to animals; this period of time can in some cases be days or weeks in length. The complexity and logistics associated with *in vivo* studies is also greater than for *in vitro* assays, and this is reflected in their increased cost.

1.4 *In vivo* Assays for Somatic Cell Chromosomal Aberrations

In vivo chromosomal aberration assays assess the potential of a chemical to cause DNA damage that may affect chromosome structure, or interfere with the mitotic apparatus causing changes in chromosome number. There are several short-term assays that detect somatic cell chromosomal aberrations; these include the rodent erythrocyte micronucleus assay and the bone marrow chromosomal aberration assay.

1.4.1 Rodent Erythrocyte Micronucleus Assay

Because of its relative simplicity and its sensitivity to clastogens, the rodent erythrocyte micronucleus assay has now become the most commonly conducted *in vivo* assay. It has achieved widespread use and a recommended test method has been described in OECD Test Guideline 474 [6].

1.4.1.1 Principles

The micronucleus assay detects chromosome damage and whole chromosome loss in polychromatic erythrocytes, and eventually in normochromatic erythrocytes in peripheral blood as the red cells mature. A micronucleus is a small structure (1/5 to 1/20 the size of the nucleus) containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. Micronuclei can be found in cells of any tissue, but only form in dividing cells.

There are four generally accepted mechanisms through which micronuclei can form: a) the mitotic loss of acentric chromosome fragments (forming structural aberrations), b) mechanical consequences of chromosomal breakage and exchange, such as from lagging chromosomes, an inactive centromere or tangled chromosomes (forming structural aberrations), c) mitotic loss of whole chromosomes (forming numerical aberrations) and, d) apoptosis [7]. However, nuclear fragments resulting from apoptosis are usually easy to identify because they are much more numerous or pyknotic than those induced by clastogenic or aneugenic mechanisms. Structural aberrations are believed to result from direct or indirect interaction of the test chemical with DNA, while numerical aberrations are often a result of interference with the mitotic apparatus preventing normal nuclear division.

Bone marrow is the major haematopoietic tissue in the adult rodent. Administration of a chemical during proliferation of haematopoietic cells may cause chromosome damage or inhibition of the mitotic apparatus. These chromosome fragments or whole chromosomes may lag behind during cell division and form

micronuclei. The erythrocyte is particularly well suited to analysis for micronuclei because during maturation of the erythroblast to the polychromatic erythrocyte (a period of about 6 hours following the final mitosis), the nucleus is extruded, making detection of micronuclei easier [8]. In addition, the polychromatic erythrocyte (PCE) still contains RNA, and so it stains blue-grey with Giemsa or reddish with acridine orange. This allows differentiation from mature, haemoglobin-containing erythrocytes, which stain orange with Giemsa or are unstained by acridine orange, and facilitates identification of the cells where micronuclei induced by the test substance may be present [9]. Sampling of PCEs from the bone marrow or peripheral blood prior to their differentiation to mature erythrocytes is critical; once a PCE has matured, associating the presence of micronuclei in these cells with acute chemical exposure is not possible. Mature erythrocytes persist in peripheral circulation for about 1 month [8].

The micronucleus assay is conducted using the bone marrow or peripheral blood of rodents, typically mice, as the target tissue; the peripheral blood of species other than the mouse can be used if micronucleated erythrocytes are not rapidly removed by the spleen [6]. The usual routes of administration are via gavage or intraperitoneal injection, and generally several doses must be administered so that the dose range spans from the maximum tolerable dose to a dose without appreciable toxicity. The length of time between treatment and sacrifice is a critical parameter, which is dependent on the cell cycle time. This delay between treatment and sampling of PCEs is necessary to allow sufficient time for the number of micronucleated PCEs to rise to a peak, and corresponds to the time necessary for absorption and metabolism of the chemical, the completion of

the erythroblast cell cycle, including any test chemical-induced cell-cycle delay, and for extrusion of the erythroblast nucleus [8].

The incidence of micronucleated PCEs is low in untreated animals. To allow for appropriate statistical power, a large number of PCEs (usually at least 2000 PCEs per animal) must be scored for the incidence of micronuclei; the proportion of PCE among total erythrocytes is also determined as a measure of cytotoxicity [6].

Because micronuclei are relatively rare, manual enumeration by light microscopy is time consuming. For that reason, newer flow cytometric or image analysis methods have been adapted for the rapid processing of slides. These methods offer the potential to improve the sensitivity and the efficiency of the assay.

Any test chemical that induces an increase in the frequency of micronucleated PCEs has induced chromosomal aberrations *in vivo*, but further mechanistic information useful to distinguish micronuclei induced by clastogenic or aneugenic chemicals can also be obtained. Micronuclei of aneugenic origin will contain centromeres, the presence of which can be verified using molecular cytogenetic methods: immunofluorescent CREST-staining or fluorescence *in situ* hybridization (FISH) with pancentromeric DNA probes (see [7; 9]).

1.4.1.2 Benefits & Limitations

Based on the mechanism for micronucleus formation, the micronucleus assay, in principle, is able to detect both clastogens and some aneugens. There is a low spontaneous micronucleus frequency in erythrocytes (typically <3 micronucleated PCE/1000 PCE), which provides fairly high sensitivity to small test chemical-induced increases in micronucleus frequency [10]. In addition, there is a very large population of

cells from which to sample, making scoring easier and increasing the power of the test. Since the assay has been used in genetic toxicology testing for many years, numerous laboratories have developed considerable expertise with the assay and a large database exists to allow for comparisons.

However, as only PCEs are scored for the presence of micronuclei, the effect of a chemical on germ cells and other somatic cells is not determined. Despite evidence suggesting the bone marrow micronucleus assay can detect most germ cell clastogens [11], if the test chemical under investigation is suspected to target germ cells, separate investigations should be performed; these studies are particularly resource intensive. The identification of N-hydroxymethylacrylamide as a mutagen that induces dominant lethal mutations in germ cells but not micronucleated PCE, highlights a potential drawback of mutation assays exclusively involving somatic cells [12]. Although the assay can detect both clastogenic and aneugenic effects, it cannot distinguish between the two mechanisms unless further work is conducted using CREST-staining or FISH techniques; this is rarely done. In addition, the micronucleus assay, in principle, does not identify gene mutations *in vivo*, therefore, the use of the *in vivo* micronucleus assay to confirm a positive result obtained in an *in vitro* gene mutation assay is not mechanistically justified.

1.4.2 Mammalian Bone Marrow Chromosomal Aberration Assay

The mammalian bone marrow chromosomal aberration assay can detect clastogenic effects of a test agent. However, in the chromosomal aberration assay, these effects are observed directly by examination of metaphase chromosome spreads. A protocol is described in OECD Test Guideline 475 [13].

1.4.2.1 Principles

The assay is based on the ability of a test agent to induce chromosome structural or numerical alterations that can be visualized microscopically. The target tissue for the chromosomal aberration assay is the bone marrow because it is a rapidly dividing, well-vascularized tissue. Groups of mice, rats or Chinese hamsters are administered the test chemical, preferably only once, by a relevant route of exposure, typically by gavage or intraperitoneal injection. Doses are selected that span a range from the maximum tolerable to that which does not induce appreciable toxicity. The maximum tolerable dose by definition produces mild toxicity that at higher doses would be expected to lead to mortality, or causes bone marrow cytotoxicity (i.e., >50% reduction of the mitotic index).

In order to accumulate metaphase cells, cell division is arrested by administration of a mitotic inhibitor, such as colchicine, 3-5 hours prior to sacrifice. After a time period equivalent to 1.5x the normal cell cycle length (usually 12-18 hours for most rodent species), animals are euthanized and bone marrow cells from the femur in their first metaphase after administration are examined. Because of the potential for some chemicals to induce mitotic delay, a second sampling is conducted with a parallel group of animals 24 hours after the first sampling time. Using light microscopy, a minimum of 1000 cells/animal are scored to determine mitotic index and a minimum of 100 metaphase cells/animal are scored for chromosomal aberrations. A chemical that induces an increase in the frequency of structural aberrations, including chromosome-type and chromatid-type aberrations is considered to be clastogenic under the test conditions.

1.4.2.2 Benefits and Limitations

The chromosomal aberration assay detects clastogenic effects of a test chemical by direct examination of metaphase cells; this often is more informative because the types of aberrations can be described and classified, which allows the assay to provide mechanistic information more readily than the micronucleus assay. Although bone marrow cells are the usual target, the assay can be adapted (for example, by altering sampling times) to allow examination of other cell types, including hepatocytes and spermatogonia. This allows the assay to be used to evaluate the potential for germ cell chromosome aberrations that could lead to heritable genetic effects.

However, conducting a chromosomal aberration assay is much more time consuming than the micronucleus assay. It requires skilled personnel to correctly identify aberrations and is not adaptable for automated scoring. As a result, it is necessarily subjective. Because of cytotoxicity, poorly spread metaphases or chromosome loss during slide preparation, the number of scoreable metaphases may be low; this could make it difficult to find a sufficient number of intact metaphases/animal. Furthermore, like the micronucleus assay, the chromosomal aberration assay does not, by design, provide information regarding whether the chemical induced gene mutations in the target cells or other tissues besides the bone marrow within the animal.

1.5 *In vivo* Assays for Somatic Cell Gene Mutation in Endogenous Genes

In vivo gene mutation assays in endogenous genes are rarely used for testing purposes because of the lack of effective methods. The tests that currently exist are cumbersome and generally not suitable for routine use. The mouse spot test is the only

test for which an OECD test guideline exists, but some promising mutation tests using endogenous genes have also been developed. These endogenous gene mutation assays include RetinoBlast, *Hprt*, *Aprt*, $Tk^{+/-}$, and *Dlb*-1.

1.5.1 Mouse Spot Test

The mouse spot test was developed as a rapid screening test to detect gene mutations and recombinations in somatic cells of mice. A protocol is described in OECD Test Guideline 484 [14].

1.5.1.1 Principles

Although coat colour spots were induced experimentally by X-irradiation in 1957 [15], the ability of chemicals to induce these genetic changes was not recognized until 1975, when colour spots were induced by treatment of mice with N-ethyl-N-nitrosourea (ENU) [16]. The mouse spot test is based on the observation that chemical mutagens can induce colour spots on the fur of mice exposed *in utero*. The colour spots arise when mouse melanoblasts heterozygous for several recessive coat colour mutations lose a dominant allele through a gene mutation, chromosomal aberration or reciprocal recombination, allowing the recessive gene to be expressed [17]. Melanoblasts migrate from the neural crest to the midline of the abdomen during days 8-12 of embryonic development, while continuing to divide to produce melanocytes [18]. Those melanocytes that carry a coat colour mutation will result in differing pigmentation of the fur in a band stretching from the back to the abdomen.

Mice of the T-strain are mated with those of the HT or C57/B1 strain to produce embryos with the desired genetic characteristics. In general, treatment of about 50 dams with the test chemical would be sufficient to produce the desired ~300 F_1 animals per

group. Dams are treated on days 8, 9 or 10 of gestation by gavage or intraperitoneal injection. Three or four weeks after birth, the mice are examined for coat spots. There are three classes of spots: white ventral spots that are presumed to be the result of chromosomal aberrations leading to cell death; yellow, agouti-like spots that are likely to be a result of misdifferentiations; and pigmented black, grey, brown or near white spots randomly distributed over the whole coat, which are the result of somatic mutations. However, only the last class of spot has genetic relevance. A chemical that induces a biologically significant increase in the number of genetically relevant (somatic mutation) spots is considered to be mutagenic in this test system [14].

By examining fur from the spot using fluorescence microscopy, it is possible to distinguish the classes of spots from each other and to distinguish, from somatic mutation spots, the gene loci affected [19]. It is also possible to distinguish different types of genetic events. Identifiable gene mutations are caused by a mutation at the *c* locus that produces cells with the *c* (albino) and c^{ch} (chinchilla) alleles, which causes light brown spots. Reciprocal recombinations result from a crossing-over involving the linked *p* (pink eyed dilution) and *c* (albino) loci; the resulting recombinants are homozygous for either the wild type (visible as black spots) or mutant alleles (visible as white spots) [18].

1.5.1.2 Benefits and Limitations

The mouse spot test is capable of detecting both gene mutations and some types of chromosomal aberrations. It is relatively easy to conduct, does not require specialized expertise, and the endpoint of interest (coat spots) can be directly identified by visual examination. Further information regarding the causes of different types of mutagenic events and the gene loci involved can be inferred by examining fur from the spots

microscopically. Basic information regarding the reproductive toxicity or teratogenicity of the test chemical can also be obtained by looking for obvious malformations or reduced numbers of pups in each litter. However, mutagenic activity is only detected within the small melanocyte population very early in development and not all possible phenotypes have genetic relevance. Furthermore, the test requires a large number of animals, making it very costly to conduct. The high cost and the trend towards reduction of animals used in toxicological testing has greatly limited the use of this assay.

1.5.2 RetinoBlast (eye-spot) Assay

The retinoblast (eye-spot) assay is a variant of the mouse spot test that identifies deletion mutations by scoring colour spots in the retinal pigment epithelium instead of the coat. This assay is not commonly used, other than for basic research applications.

1.5.2.1 Principles

The eye-spot assay is similar in principle to the mouse spot test. It uses the C57BL/6J p^{un}/p^{un} strain of mouse, which carries the pink eyed unstable mutation (p^{un}), a 70-kb tandem duplication at the pink-eyed dilution locus [20]. The p^{un} mutation carried by the test strain is an autosomal recessive mutation that produces a light grey coat colour and pink eyes. Loss of one copy of the p^{un} tandem duplication causes reversion of the p^{un} mutation to the wildtype p in a retinal pigment epithelial (RPE) precursor cell and leads to the production of a RPE cell with black pigmentation, which is visible against the remaining non-pigmented RPE cells [21]. With this assay, the frequency of mutations (deletions) affecting one copy of the tandem duplication at the p^{un} locus can be measured.

The assay is most commonly conducted by treating dams from the C57BL/6J p^{un}/p^{un} strain with the test chemical by a relevant route of exposure (gavage, intraperitoneal

injection or inhalation) at approximately day 10 of gestation. Offspring are sacrificed at the age of 20 days, the eyes are removed, and the retina is placed on a slide and examined by light microscopy. The number of spots, which are observed as a single pigmented cell or groups of pigmented cells separated from each other by no more than one unpigmented cell, are counted. Each eye-spot corresponds to one p^{un} mutation. A test chemical that induces a significant increase in the frequency of eye spots compared with the negative control is mutagenic under the conditions of this assay [22].

Reversion of the p^{un} mutation arises from intrachromosomal recombination that results in the deletion of one of the tandem fragments at the p^{un} loci. The deletion can occur by several mechanisms, such as intrachromosomal crossing-over, single strand annealing, unequal sister chromatid exchange, and sister chromatid conversion [23].

1.5.2.2 Benefits and Limitations

A number of carcinogens have been found to induce intrachromosomal recombinations [23-26], so the endpoint scored by this assay has some relevance to the assessment of carcinogenicity. The assay assesses a type of genetic effect (deletion mutation) that is not identified by many other assays. The eye-spot assay requires fewer animals than the mouse spot test to achieve a high sensitivity [26] and it allows for direct examination at the single cell level. However the target cells in this assay are RPE precursor cells, which proliferate only during the period starting at about embryonic day 9 until shortly after birth [27]. Because the assay is used to determine the frequency of deletions occurring in embryos, it may not necessarily provide the most useful model if the intent is to determine if deletions occur in adult animals.

1.5.3 Gene Mutation Assays Using Endogenous Genes with Selectable Phenotypes

1.5.3.1 *Hprt*

The *Hprt* assay uses one of the few genes that is suitable for mutation analysis in wild-type animals *in vivo*. It has been widely used in basic research applications, but has yet to be used for routine testing.

1.5.3.1.1 Principles

The *Hprt* gene is located on the X-chromosome and spans 32 kb and 46 kb in human and rodent cells, respectively. Both male and female cells carry only one active copy of the *Hprt* gene; in female cells one copy of the X-chromosome is inactivated. The *Hprt* gene codes for hypoxanthine-guanine phosphoribosyltransferase (HPRT), which plays a key role in the purine salvage pathway. HPRT catalyses the transformation of purines (hypoxanthine, guanine, or 6-mercaptopurine) to the corresponding monophosphate, which is cytotoxic to normal cells in culture. The assay is based on the observation that cells with mutations in the *Hprt* gene have lost the HPRT enzyme and survive treatment with purine analogues.

Mice or rats are treated with the test chemical by an appropriate route of exposure. After a fixation period of several weeks, the spleens are removed from sacrificed animals and cultures of splenic T-lymphocytes are established. T-lymphocytes are particularly useful because they circulate throughout many tissues, which affords them a greater probability of contacting an administered mutagen than cells that are permanently resident in a single tissue. T-lymphocytes are also long-lived in circulation and they continue to undergo cell division, which makes the identification of mutant cells

possible. In addition to T-lymphocytes, mutant frequency has also been determined in other cells, including those from the kidney, thymus and lymph nodes.

Mutant selection has been described by Tates *et al.* [28]. Cells are incubated in microwell culture plates with the selective agent 6-thioguanine, a purine analogue that is a substrate for HPRT and is toxic to non-mutant cells. After 8-9 days, cloning efficiency plates are scored, while mutant frequency plates are scored after a 10-12 day expression period. The mutant frequency is calculated as the ratio between the cloning efficiencies in selective media versus cloning media. A significant increase in mutant frequency in treatment cultures compared with controls indicates the test chemical has induced mutation at the *Hprt* locus. The average spontaneous mutant frequency at the *Hprt* locus is in the range of 10^{-6} [29]. Using standard techniques, further molecular analysis of *Hprt* mutations can be performed, if desired.

1.5.3.1.2 Benefits and Limitations

The *Hprt* assay detects point mutations, frameshifts, small insertions and small deletions. As an endogenous gene it is transcriptionally active and thus is subject to transcription-coupled DNA repair [30]. However, because *Hprt* is an X-linked gene and is therefore functionally hemizygous, it is not particularly efficient at detecting large deletions, chromosomal recombination and nondisjunction events that may disrupt essential flanking genes, and which are more effectively identified with assays using endogenous autosomal genes [31]. Deletions extending into adjacent essential genes in hemizygous regions are usually lethal to the cell because there is no homologous region to compensate for the loss of essential gene function. Because *Hprt* is not a neutral gene, there is selection pressure against *Hprt* deficient lymphocytes, particularly in young

animals [32]. In addition, dilution of mutant T-lymphocytes in circulation occurs as peripheral lymphocyte populations are renewed; this is also affected by the age of the animal. The time from exposure to maximum average mutant frequency was found to be 2 weeks in the spleen of ENU exposed pre-weanling mice and 8 weeks in adult mice [33]. As a result, sampling in the spleen must be carefully timed to detect the maximum mutant frequency based on these factors. Although *Hprt* mutant frequency can be determined from any tissue that can be subcultured, it is typically only assessed in T-lymphocytes, which prevents identification of mutagenic effects that may arise preferentially in other target tissues.

1.5.3.2 *Aprt*

The *Aprt* assay uses a constructed *Aprt* heterozygous mouse model. Like the *Hprt* model, *Aprt* is widely used in research, but is not yet used in routine genetic toxicology testing.

1.5.3.2.1 Principles

Aprt is the gene coding for an enzyme (adenine phosphoribosyltransferase) that catalyzes the conversion of adenine to AMP in the purine salvage pathway; it is expressed in all tissues. The mouse *Aprt* gene is located on chromosome 8 [34], while the human gene is located on chromosome 16 [35]. In the mouse, because of its location near the telomere, the *Aprt* gene is a large target for chromosomal events such as translocation and mitotic recombination [36].

Several *Aprt* knockout models have been created. A heterozygous *Aprt* $^{+/-}$ mouse has been developed by disrupting the *Aprt* gene in embryonic stem cells using a conventional gene targeting approach [29]. This model can be used to investigate

induced forward mutations leading to the loss of the autosomal dominant locus in T-lymphocytes and skin fibroblasts, as well as mesenchymal cells from the ear [37] and epithelial cells from the kidney [38]. Using methods similar to those used for the *Hprt* model, *Aprt* heterozygous mice are treated with the test chemical. After a fixation period of several weeks, the animals are sacrificed and typically splenic T-lymphocytes or skin fibroblasts are isolated and cultured. *Aprt* deficient mutants are selected using purine analogues such as 8-azaadenine (8-AA) or 2,6-diaminopurine (DAP), which are toxic to *Aprt* proficient cells. After a 6-8 day expression period, the cloning frequency and mutant frequency are determined using methods similar to those used for the *Hprt* model [28]. The spontaneous mutant frequency at the *Aprt* locus in heterozygous mice is approximately 8.7×10^{-6} in T-lymphocytes [29] and 1.7×10^{-4} in skin fibroblasts [39]. Using standard techniques, further molecular analysis of *Aprt* mutations can also be performed, if desired.

An *Aprt* $^{-/-}$ homozygous knockout mouse has also been developed, which is capable of detecting chemicals that cause point mutations [39]. These mice have *Aprt* alleles inactivated by reversible point mutations. Mice are administered the test chemical and held for a fixation period of several weeks, after which they are injected with ^{14}C-adenine. Cells that have reverted to *Aprt* $^{+}$ have a functional adenine phosphoribosyltransferase enzyme and can sequester radiolabelled adenine by conversion to AMP, which is subsequently incorporated into nucleic acids. Using autoradiography or scintillation counting, the frequency of revertant cells can be determined. The *in situ* method also enables identification of the cell types that are most susceptible to mutation [39].

1.5.3.2.2 Benefits and limitations

Because it is an autosomal heterozygous locus, *Aprt* can detect, in addition to the point mutations, frameshifts and small deletions detectable by *Hprt*, events that may lead to loss of heterozygosity, such as large deletions, mitotic non-disjunctions, mitotic recombinations and gene conversions, if the function of the deleted essential gene is provided by the homologous chromosome. The ability to detect the full spectrum of autosomal mutations allows *Aprt* to be a much more versatile biomarker than *Hprt*. However, the use of *Aprt* as a mutational target is limited to only a few tissues due to the detection method, which relies on culturing techniques. Like *Hprt*, *Aprt* is not a neutral gene and there may be negative selection pressures on *Aprt* mutant cells, as well as influences on mutant frequency arising from dilution as the T-lymphocyte pool is renewed.

1.5.3.3 *Tk*$^{+/-}$

A *Tk* heterozygous mouse model has been constructed [40] and used in a number of basic research investigations. It is in an early stage of development and has not yet been used for routine testing.

1.5.3.3.1 Principles

The heterozygous *Tk*$^{+/-}$ mouse was created by using a mouse embryonic stem cell line with one allele of the *Tk* gene inactivated through targeted homologous recombination [40; 41]. *Tk* is generally expressed only in dividing cells and encodes thymidine kinase, which is involved in pyrimidine salvage, catalyzing phosphorylation of thymine deoxyriboside to form thymidylate. The *Tk* gene is located on the distal portion of mouse chromosome 11 [42]. Cells that have lost the second *Tk* allele through mutation

to become $Tk^{-/-}$ are easily selected because they survive when cultured in the presence of a pyrimidine analogue such as 5-bromo-2'-deoxyuridine (BrdU), while thymidine kinase-competent cells ($Tk^{+/+}$ or $Tk^{+/-}$) do not.

C57BL/6 $Tk^{+/-}$ mice receive the test chemical by a relevant route of exposure either once or in multiple administrations. Approximately 4-5 weeks after administration, mice are sacrificed and splenic lymphocytes are isolated and cultured. Mutant selection is performed as described by Dobrovolsky *et al.* [40]. Cells are incubated in microwell culture plates with the selective agent BrdU, a pyrimidine analogue that is toxic to non-mutant cells. After incubation for 10-12 days, cloning efficiency in treatment and control plates is scored. The mutant frequency is calculated by dividing the cloning efficiency of cells cultured in the presence of the selecting agent by the cloning efficiency of cells cultured in the absence of selection. A significant increase in mutant frequency in treatment cultures compared with controls indicates the test chemical is mutagenic in this assay. Again, using standard techniques, further molecular analysis of *Tk* mutations can be performed, if desired. The spontaneous mutant frequency is approximately 2×10^{-5} [40].

1.5.3.3.2 Benefits and limitations

The $Tk^{+/-}$ mouse is the analogous *in vivo* model to the commonly used *in vitro* mouse lymphoma assay. As such, it is a useful model to investigate the *in vivo* responses of chemicals found to be mutagenic in the *in vitro* assay. The *Tk* model detects intragenic mutations (point mutations, frameshifts and small deletions) as well as larger effects, such as chromosome recombination, nondisjunction and large deletions that often led to loss of heterozygosity, for which it is particularly sensitive [40; 43; 44]. However, it too

is limited to examining tissues where cells can be easily cultured. In addition, the use of BrdU, which is itself a mutagen, as a selective agent can introduce the possibility that some mutants would be produced by exposure to the selective agent. Dobrovolsky *et al.* [40] suggest this potential can be minimized by keeping cultures under tight selection pressure during the culture phase, since studies with $Tk^{+/-}$ mouse lymphoma cells have indicated several cell divisions in the absence of the selective agent are required in order to fix mutations [45].

1.5.3.4 *Dlb-1*

The *Dlb-1* specific locus test measures mutations occurring in the small intestine of treated *Dlb-1* heterozygotes. The assay has been in existence for about 15 years [46; 47], but it remains in the development stage and has not been widely used in the research community.

1.5.3.4.1 Principles

The *Dlb-1* specific locus test identifies mutations occurring in the small intestine of mice heterozygous at the *Dlb-1* (beta-1,4-N-acetyl-galactosaminyl transferase 2) locus. *Dlb-1* is a polymorphic genetic locus that exists on chromosome 11 in the mouse [48] and has 2 alleles. $Dlb\text{-}1^{b}$ is an autosomal dominant gene that determines the expression of binding sites for the lectin *Dolichos biflorus* agglutinin (DBA) in intestinal epithelium, while $Dlb\text{-}1^{a}$ determines DBA receptor expression in vascular endothelium [46]. Mice heterozygous at the *Dlb-1* locus ($Dlb\text{-}1^{a}/Dlb\text{-}1^{b}$) that develop a mutation of the $Dlb\text{-}1^{b}$ allele in an intestinal stem cell can be detected by staining an intestinal epithelial cell preparation with a peroxidase conjugate of DBA and scoring non-staining cell ribbons on the villus; these cell ribbons are cells derived from a stem cell carrying a *Dlb-1* mutation

[47]. The spontaneous mutation frequency of the *Dlb-1* gene has been reported to be approximately 1.6 x 10^{-5} mutants/villus per animal per week [49].

1.5.3.4.2 Benefits and limitations

The *Dlb-1* assay can be used for studies of animals *in utero*, as well as adult animals. The number of animals used is consistent with most assays using an endogenous reporter gene, which is substantially fewer than are required for the mouse spot test. However, the assay is restricted to analysis of mutations in the intestinal epithelium following oral or intraperitoneal administration of the test chemical. Mutagens acting preferentially at another target tissue may not be detected. In addition, the *Dlb-1* gene has not yet been cloned, so the molecular nature of any observed mutations cannot be determined. The assay is still in the development stage and has not been used, other than for research purposes in a small number of laboratories.

1.6 Indicator Tests

Indicator tests are those that do not directly measure consequences of DNA interaction (i.e. mutation), but rely on other markers that suggest some type of interaction occurred. The two most commonly conducted tests are the sister-chromatid exchange assay and the unscheduled DNA synthesis assay.

1.6.1 Sister-Chromatid Exchange Assay

The sister chromatid exchange (SCE) assay is a widely used method for assessing chromosome breakage and repair, though it is much more commonly conducted as an *in vitro* test. A protocol for the *in vitro* assay is described in OECD Test Guideline 479 [50], but there is no guideline for the *in vivo* assay.

1.6.1.1 Principles

Sister chromatid exchanges (SCE) are reciprocal exchanges of DNA segments between sister chromatids of a chromosome that are produced during S-phase. Although the molecular mechanism of these exchanges remains unknown, it is presumed to require chromosome breakage, exchange of DNA at homologous loci and repair. Work with the model genotoxicant, ENU, has provided direct evidence that suggests the replication fork is the site of SCE production [51]. However, SCE may not necessarily be caused by direct DNA interaction in all cases. A chemical that does not damage DNA but instead creates intracellular conditions that favour inhibition of DNA replication could, in itself, create SCE. It is also noteworthy that several strong clastogens, such as ionizing radiation and bleomycin, have failed to produce an increase in SCE [52]. Because SCE induction does not, itself, indicate a chemical is mutagenic, the interpretation of the toxicological relevance of SCE is often difficult.

Rodents are most commonly used for SCE assays. Bone marrow cells are usually sampled because there is always a pool of dividing cells and they are easy to prepare for scoring. Generally, groups of animals are administered the test chemical once by gavage or by intraperitoneal injection. At 2-3 hours following administration, the animals are administered bromodeoxyuridine, followed at twenty-one hours by an injection of the mitotic inhibitor colchicine or colcemid. Three hours later, all animals are sacrificed and slides of bone marrow cells are prepared and scored by light microscopy. Other cell types, including spermatogonial cells, may also be used; the treatment and sampling times for other cells will depend on the cell cycle time of the target cells and what is known of the toxicokinetic factors specific to the chemical of interest.

1.6.1.2 Benefits and limitations

The SCE assay offers a rapid and relatively inexpensive assessment of potential test chemical-induced DNA damage; any tissue from which a cell suspension can be made can be analyzed. A significant number of SCE assays for a wide variety of chemicals have previously been conducted, facilitating the comparison of the relative potencies of test chemicals. However, the major drawback remains the unknown molecular basis of SCE induction. Because factors other than direct DNA interaction can cause SCE, an increase in SCEs does not necessarily indicate mutagenicity, making interpretation in the context of genetic toxicity testing difficult. As a result, this assay has fallen out of favour and is now rarely conducted.

1.6.2 Unscheduled DNA Synthesis Assay

The unscheduled DNA synthesis (UDS) assay is a commonly used method of assessing test chemical-induced DNA excision repair. The induction of repair mechanisms is presumed to have been preceded by DNA damage. Measuring the extent to which DNA synthesis occurred offers indirect evidence of the DNA damaging ability of a chemical. A protocol is described in OECD Test Guideline 486 [53].

1.6.2.1 Principles

The UDS assay measures DNA synthesis induced for the purposes of repairing an excised segment of DNA containing a region damaged by a test chemical. DNA synthesis is measured by detecting tritium-labelled thymidine (^{3}H-TdR) incorporation into DNA, preferably using autoradiography. The liver is generally used for analysis because, under normal circumstances, there are a low proportion of primary hepatocytes in S-phase of the cell cycle; therefore, an increase in DNA synthesis can be more easily

attributed to repair of induced DNA damage, rather than DNA synthesis supporting normal cell division. The liver is also the site of first-pass metabolism for chemicals administered orally or by intraperitoneal injection.

A larger number of nucleotides excised and repaired will cause a greater amount of detectable ^{3}H-TdR becoming incorporated into DNA. For that reason, the UDS assay is more sensitive in detecting DNA damage that is repaired through nucleotide excision repair (removal of up to 100 nucleotides) as compared to base excision repair (removal of 1-3 nucleotides) [53]. Test chemicals more prone to inducing nucleotide excision repair, such as those that form bulky DNA adducts, have a greater potential to cause detectable UDS. However, the UDS assay does not, in itself, indicate if a test chemical is mutagenic because it provides no information regarding the fidelity of DNA repair, and it does not identify DNA lesions repaired by mechanisms other than excision repair.

The UDS assay is usually conducted using rats, though other species may be used. Dose levels are selected with the highest dose being the MTD. Animals are administered the test chemical once by gavage; intraperitoneal injection is not recommended because it could potentially expose the liver directly to the chemical. A group of animals is sacrificed at 2-4 hours and another at 12-16 hours after treatment. Cultures of hepatocytes are prepared and incubated for 3-8 hours in ^{3}H-TdR. Slides are prepared and processed for autoradiography using standard techniques. At least 100 cells per animal are examined and both nuclear and cytoplasmic grains are counted to determine the net nuclear grain count (cytoplasmic grains subtracted from nuclear grains). Chemicals inducing a significant increase in net nuclear grain count for at least one treatment group have induced UDS [53; 54].

1.6.2.2 Benefits and limitations

Theoretically, any tissue with a low proportion of cells in S-phase can be used for analysis. Though only liver is routinely used, a UDS assay using spermatocytes has been developed [55-57], allowing the measurement of DNA interactions that may be germ cell specific. Because UDS is measured in the whole genome, it is potentially much more sensitive than assays examining only specific loci. However, the extent of UDS gives no indication of the fidelity of the repair process. For that reason, UDS does not provide specific information on the mutagenic potential of a test chemical, but only information suggesting it does or does not induce excision repair.

1.7 Transgenic Rodent Assays

The absence of a practical *in vivo* gene mutation assay prompted the development of a new test involving transgenic rodents within which multiple copies of a bacterial reporter gene had been stably integrated. A variety of transgenic rodent (TGR) assays have since been developed, of which the *lacZ* transgenic mouse (Muta™Mouse) and the *lacI* transgenic (Big Blue®) mouse and rat are commercially available and, consequently, have become the most commonly used models. Other more recently developed models include the *lacZ* plasmid mouse and the *gpt* delta mouse and rat. TGR assays are unique in that mutations occurring in the animal are detected *in vitro* following the rescue of reporter gene vectors from the rodent genomic DNA by *in vitro* packaging into lambda shuttle vectors and infection of a bacterial host, or following the excision and religation of integrated plasmids and electroporation into the host cell [58-60]. TGR gene mutation assays are the subject of the present work.

Transgenic rodent assays are gene mutation tests that detect point mutations and small deletions and they have limited sensitivity to detect clastogenic effects. Because the transgenes are of bacterial origin and are heavily methylated [61-63], they are unlikely to be expressed in the rodent, and thus are not subject to transcription-coupled repair.

Recommendations for the experimental conduct of the TGR assays have recently been made by an expert panel [64; 65]. The treatment phase of the experiment is similar to that of other *in vivo* genotoxicity assays. Transgenic rodents (groups of 5-7 animals per dose) are administered the test compound by a relevant route of administration for a period of time (the treatment duration) during which mutagenic lesions in the bacterial reporter gene may be induced. Doses are selected to span a relatively wide range, with a group of animals receiving the MTD and the remaining two groups receiving either two-thirds or one-third of the MTD. At the end of the treatment period, the animals are maintained without treatment for a further period prior to sacrifice (the sampling time), during which induced mutagenic lesions may become fixed as mutations. A positive result is one in which the data for one or more tissues show a statistically significant dose-response relationship and/or a statistically significant increase in any dose group compared to the concurrent negative controls using an appropriate statistical model [64].

1.7.1 Muta™Mouse

LacZ transgenic mice were first produced by microinjecting the λgt10*lacZ* vector, carrying the bacterial *lacZ* gene, into fertilized CD2F1 mouse oocytes [58]. This vector is approximately 47 kb in length and the *lacZ* transgene is approximately 3100 bp. Strain 40.6, which carries 40 copies of the transgenes in a head to tail manner at a single site on

chromosome 3 [66], is commercially available from Covance Laboratories Limited (UK). More recent research indicates the actual copy number may be lower than originally determined (P. Shwed, personal communication). The Muta™Mouse model has been used in approximately 36% of the publications involving TGR models.

After treatment of the transgenic rodent, the mutant frequency in any tissue of interest can be determined by sacrificing the animal and extracting high molecular weight genomic DNA from the tissue, packaging the lambda shuttle vector *in vitro* into empty lambda phage pre-heads and infecting the *Escherichia coli C* (*lacZ*) host cells, which permit the efficient rescuing of methylated genomic DNA [67]. Cells that receive an intact *lacZ* gene (i.e. without inactivating point mutations or small deletions) will be $lacZ^{+}/lacZ^{-}$ and capable of producing functional β-galactosidase, while cells that receive a non-functional *lacZ* gene lack this capability. Wildtype cells with functional β-galactosidase cleave the non-inducing chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) to produce galactose and 5-bromo-4-chloroindigo (a blue metabolite), while cells producing only non-functional enzyme do not cleave X-gal. Thus, blue plaques containing a wild-type *lacZ* gene can visually be distinguished from white plaques containing a mutant *lacZ* gene when the *E. coli* cells are plated on media containing X-gal [68]. More recently, a simpler and faster selective system was developed in which *E. coli C* ($galE^{-}lacZ^{-}$) was used as the host and mutant selection was carried out using medium supplemented with phenyl-D-galactopyranoside (P-gal) [60]. Metabolism of P-gal by a $galE^{-}lacZ^{+/-}$ *E. coli* C cell produces galactose. This forms a toxic UDP-galactose intermediate through the enzymatic activity of galactose kinase (*galK*) and transferase (*galT*), which the cell is unable to convert to non-

toxic UDP-glucose in the absence of the epimerase enzyme [69]. Only *lacZ*$^{-/-}$ cells fail to metabolize P-gal and survive to form plaques. The *lacZ* mutant frequency is determined by dividing the total number of plaques containing *lacZ* mutations (survivors on P-gal medium) by the total number of plaque forming units, which is estimated on titre plates under non-selective conditions. Spontaneous mutant frequencies for the bone marrow, liver and small intestine are approximately 5.0×10^{-5}, 5.9×10^{-5}, and 8.7×10^{-5}, respectively [70].

1.7.2 Big Blue® mouse and rat

Big Blue® mouse and rat transgenic models exploit the bacterial *lacI* gene. The λLIZα shuttle vector, carrying the bacterial *lacI* gene (1080 bp) as a mutational target, the *lacO* operator sequences and the *lacZ* gene, was injected into a fertilized C57BL/6 mouse oocyte to produce transgenic progeny [59; 71; 72]. This transgenic mouse line was also crossed with an animal of the C3H line to produce a transgenic B6C3F1 mouse with the same genetic background as the National Toxicology Program bioassay test strain. The 45.6 kb construct is present in approximately 40 copies per chromosome [58], with integration occurring at a single locus on chromosome 4 in a head-to-tail arrangement [73]. A *lacI* transgenic rat was also produced in a Fisher 344 background in the same manner [73]. The λLIZα vector occurs as a single integration of 15-20 copies on rat chromosome 4 (Stratagene, unpublished data). Both the C57BL/6 and B6C3F1 transgenic mouse strains and the F344 rat strain are commercially available from Stratagene. The Big Blue® models have been used in approximately 50% of the publications involving TGR models.

Mutations arising in the rodent are scored in *E. coli* SCS-8 cells (*lacZ*ΔM15) following *in vitro* packaging of the λLIZα vector. Phage infecting SCS-8 host cells containing a wildtype (functional) *lacI* gene lead to the production of a functional lac repressor, which prevents transcription of *αlacZ* and the formation of β-galactosidase when the cells are plated on X-gal containing medium; these plaques will be colourless. Mutations in *lacI* produce a lac repressor that is unable to bind to the operator; consequently, *αlacZ* transcription will be de-repressed and a functional β-galactosidase will be formed. Cleavage of X-gal by β-galactosidase forms 5-bromo-4-chloroindigo, which produces a blue coloured plaque. The proportion of blue plaques among all plaques provides a measure of mutant frequency. To date, there has not been an effective positive selection method developed for the *lacI* based mouse or rat systems. Spontaneous mutant frequencies for the bone marrow, liver and small intestine are approximately 2.7×10^{-5}, 5.5×10^{-5}, and 9.5×10^{-5}, respectively for the mouse and 0.95×10^{-5}, 3.5×10^{-5}, and 5.6×10^{-5}, respectively for the rat [70].

1.7.3 Other Transgenic Rodent Models

In addition to the *lacZ* and *lacI* based models, there are several other TGR models. Two of the most well developed of these, the *lacZ* plasmid mouse and the *gpt* delta mouse and rat, have been used on a much more limited basis than the *lacZ* and *lacI* based models. However, these models circumvent some of the limitations of the other models in that they may also detect deletion mutations of limited size.

The *lacZ* plasmid mouse contains approximately 20 copies per haploid genome of the pUR288 plasmid containing the *lacZ* gene. The plasmid has been inserted into both chromosomes 3 and 4 of the C57BL/6 mouse [74]. Genomic DNA isolated from the

mice is digested with *Hin*dIII, releasing copies of the linearized plasmid, which are then purified by adsorption onto magnetic beads coated with Lac repressor [75]. The plasmid DNA is then recircularized by T4 DNA ligase and electroporated into *E. coli* C (*galE*$^-$ *lacZ*$^-$). Mutant frequency is determined using the same P-gal positive selection method described for the *lacZ* transgenic mouse. The *lacZ* plasmid mouse model has been used in approximately 4% of the publications involving TGR models.

The *gpt* delta mouse was created by microinjection of λEG10 DNA into fertilized eggs of the C57BL/6J mouse. The transgene was incorporated at a single site of chromosome 17 at approximately 80 copies per haploid genome [76]. A *gpt* delta rat has also been developed from the Sprague-Dawley strain containing approximately 10 copies on chromosome 4 [77]. Infection of *E. coli* YG6020 (*gpt*$^-$ *cre*$^+$) with λEG10 rescued from the rodent leads to excision and propagation of the plasmid region, containing the *gpt* and chloramphenicol acetyltransferase (CAT) genes, from the phage DNA by Cre-*lox* recombination. When plated on media containing 6-thioguanine (6-TG), only mutant *E. coli* cells survive, since mutation of the *gpt* gene prevents the formation of functional guanine phosphoribosyltransferase and the incorporation of toxic 6-TG into DNA. Deletion mutations can be detected by *spi*$^-$ selection. The *gpt* delta rat and mouse has been used in approximately 10% of the publications involving TGR models.

1.7.4 Benefits and Limitations of the Transgenic Rodent Models

The advantages of the TGR models include the ability to investigate gene mutations occurring in any tissue using substantially fewer animals than the conventional approaches. Mechanistic information can be derived from the use of sequence analysis. Flexibility in the route of administration allows the route selected to correspond with the

most relevant for human exposure, and permits investigation of a wide variety of compounds, including those chemicals believed to act only at the site of contact. Unlike the *in vivo* cytogenetic and UDS assays, transgenic rodent assays are not dependent on the chemical reaching the bone marrow or liver if these tissues are not the primary target of the compound.

However, the transgenic loci are comprised of heavily methylated exogenous (prokaryotic) DNA sequences. Because of spontaneous deamination of 5-methylcytosine occurring at CpG sites, TGR assays have a high spontaneous mutant frequency in most tissues, which reduces the sensitivity of the assays to detect small increases in mutant frequency. Unlike endogenous genes, such as *Hprt*, *Aprt* or *Tk*, the transgenes are not transcribed in mammals and are not subject to the same mechanisms of DNA repair (i.e. transcription-coupled repair). The assays are also comparatively very expensive to conduct and the Muta™Mouse and Big Blue® models, in particular, have very limited sensitivity to most clastogens, since deletions disrupting the cohesive ends of the lambda vector will prevent packaging.

1.7.4 Transgenic Rodent Gene Mutation Assays in the Context of Existing Genotoxicity Testing Batteries

The existing International Conference on Harmonization S2B standard genotoxicity test battery for pharmaceuticals [78], and similar test batteries required by regulatory agencies for pesticides or industrial chemicals, is comprised of an *in vitro* bacterial gene mutation assay, an *in vitro* cytogenetic assay for chromosomal aberrations in mammalian cells or a mouse lymphoma $tk^{+/-}$ assay, and an *in vivo* test for chromosomal aberrations in rodent hematopoietic cells (*in vivo* cytogenetics or micronucleus assay).

However, an *in vivo* gene mutation assay is not a component of the core battery. As described in sections 1.5.1 and 1.5.2, the existing *in vivo* gene mutation assays are extremely resource intensive and inconsistent with the principles of reduction, refinement and replacement that now define the types of animal tests that are ethically justifiable to institutional animal care committees. The development of TGR assays has provided a model that would allow further investigation of chemicals previously found to cause gene mutations *in vitro*, using substantially fewer animals than would be required using the mouse spot test. Since gene mutations and chromosomal aberrations are mechanistically distinct molecular events, the *in vivo* cytogenetic and micronucleus assays do not provide any information to indicate if a chemical mutagenic to bacteria causes gene mutations *in vivo*. Such information has significant value in the refinement of genotoxicity conclusions for suspected mutagens.

1.7.5 Factors Limiting the Use of Transgenic Rodent Mutation Assays in Genotoxicity Testing

Acceptance of a new toxicological test method is contingent upon determination of appropriate performance characteristics that demonstrate the assay identifies the endpoint of interest reliably and reproducibly. Since they are relatively new tests, the TGR assays have not yet undergone a detailed assessment of their performance characteristics as genotoxicity tests or as predictors of genotoxic carcinogenicity. Such a performance assessment would establish if TGR assays can identify mutagenic compounds as well or better than the existing tests described in the previous sections. Since many *in vivo* genotoxicity assays have exhibited poor predictive ability for rodent carcinogenicity, it is also essential that the predictive ability of TGR assays be evaluated

in a comparative context. This performance assessment would provide the necessary information to determine if the TGR assays have value in genotoxic hazard identification.

The use of TGR assays is also limited by the absence of an internationally accepted test guideline. The development of an OECD Test Guideline, which would facilitate more widespread acceptance and use of TGR mutation assays, is dependent on the creation of a standard test protocol. The current lack of understanding of the effects of some key elements of the experimental procedure hinders protocol development. In particular, the treatment duration and sampling time are the two factors with perhaps the largest influence on the assay results. A recent opinion paper by an International Workshops on Genotoxicity Testing expert panel on TGR assays recommended a treatment duration of 28 days and a sampling time of 3 days as a sufficient protocol for general testing purposes; however, the majority of TGR experiments conducted to date have used combined treatment and sampling times considerably shorter than this [70], making it difficult to evaluate the recommendation using the available experimental data. In addition, the majority of chemicals that have been used for testing in TGR assays have been highly potent mutagens, which are often mutagenic even after very short treatment periods. However, the majority of environmental mutagens are considerably less potent than the typical 'positive control' compounds, and the suitability of the recommended protocol for these weaker mutagens has not been determined.

1.8 Conclusions and Objectives

In vivo assays are necessary components of any thorough genetic toxicity testing scheme. Unlike *in vitro* assays, *in vivo* assays are capable of accounting for toxicokinetic

factors and DNA repair processes that may, in some cases, modulate genotoxicity within the whole animal. However, existing assays are seriously limited by a range of different factors, including cost of the assay, the number of tissues in which genotoxicity may be measured, the state of understanding of the endpoint, and the nature of the chemicals that will be detected. Transgenic rodents may offer some advantages in that they allow for the assessment of mutagenicity in any tissue of interest, require substantially fewer animals than the existing gene mutation assays, and through sequence analysis, may permit further mechanistic insights. However, the assays are costly, are generally limited to point mutations and small deletions, and do not detect chromosomal aberrations.

This thesis aims to provide further clarification of two critical issues that will determine if TGR assays can be used in genotoxicity hazard identification.

1) The performance of TGR assays as genotoxicity tests, and as predictors of carcinogenicity, must be characterized. The questions of performance and predictivity are addressed in Chapter 2.

2) Widespread acceptance of TGR assays depends on better definition of the effects of critical variables affecting the experimental protocol – the treatment duration and sampling time. In the context of the protocol recommendations made by the IWGT working group [65], the experiments presented in Chapter 3 aim to better characterize the effects of these variables on mutant frequencies using the weak mutagen, urethane.

Chapter 2

The predictive ability of transgenic rodent mutation assays

2.1 Introduction

DNA damage caused by chemical agents has a high degree of relevance to human health, as it may be the initiating event in the multi-step process of carcinogenesis or in the production of germ cells that may transmit a heritable genetic disease to the subsequent generation. It is for this reason that a variety of tests capable of identifying compounds that are genotoxic have been developed over the past thirty years and are now routine in the pre-clinical or pre-manufacturing phase of compound development, as required by regulatory agencies worldwide. Typically, most short-term genotoxicity tests may be classified into two broad categories – those that detect gene mutations and those that detect chromosomal mutations – and are usually combined into test batteries to facilitate the detection of a broad spectrum of genotoxic effects. Although both *in vitro* and *in vivo* tests are components of standard test batteries, the results of *in vivo* assays are believed to have greater relevance to humans because of the interactions of metabolic and other pharmacokinetic factors that are not easily or reliably reproduced *in vitro*. For this reason, the results of the *in vivo* tests are usually considered definitive when they contradict an *in vitro* test result addressing the same endpoint. While a typical test battery could include a *Salmonella* reverse mutation assay (gene mutations), an *in vitro* mouse lymphoma assay (gene mutations and, by inference, chromosomal mutations), an *in vitro* mammalian cell cytogenetic assay (chromosomal aberrations) and an *in vivo* cytogenetic assay such as the rodent erythrocyte micronucleus assay (chromosomal mutations), for many years there was no practical or reliable *in vivo* assay for gene mutations. The development of transgenic rodent gene mutation (TGR) assays has provided an *in vivo* follow-up test to the *in vitro* gene mutation assays (*Salmonella*, *in*

vitro mouse lymphoma, or other mammalian cell gene mutation assays) that could provide significant refinements in genotoxicity testing.

Because of the association between genotoxicity and carcinogenicity, the results of short-term genetic toxicity studies are commonly used as predictors of the potential carcinogenic activity of a chemical; identification of genotoxic activity of a new product in pre-clinical/pre-manufacture testing often leads either to the compound being dropped from further development or regulatory action by a government authority, since it is often assumed that, in the absence of additional information, a genotoxic compound is also carcinogenic. Because of the consequences of falsely identifying a chemical as genotoxic, particularly for new potentially useful therapeutic agents, it is essential that any new genotoxicity tests be thoroughly characterized for their accuracy in identifying genotoxicants. In light of the widespread use of these tests as indicators of carcinogenic potential it is also useful to determine the predictive ability of a new genotoxicity assay for carcinogenicity. It should be noted, however, that genotoxicity and carcinogenicity are only associated and there are numerous examples of carcinogens that are active by non-DNA reactive (non-genotoxic) mechanisms; as such, the assessment of the benefits or utility of any genotoxicity test solely on the basis of its carcinogen predictive ability is without merit.

This chapter presents the results of an analysis of the operational characteristics of the TGR assays. The first intent is to address the extent of the association between results in TGR assays and other short-term genotoxicity tests and the accuracy of the TGR assays in identifying gene mutations. The second aims to address the comparative carcinogenic predictive ability of TGR assays versus other tests, and the predictive ability

of various test batteries. This analysis was facilitated by the use of the Transgenic Rodent Assay Information Database (TRAID) which contains records for all published (and some unpublished) transgenic rodent mutagenesis experiments that were identified in a comprehensive search of the literature. In addition to the results of TGR assays, the database contains results from other common short-term assays, including the *Salmonella* reverse mutation assay, the *in vitro* chromosomal aberration (CA) assay, the mouse lymphoma $TK^{+/-}$ assay (MLA), the *in vivo* CA assay, the *in vivo* unscheduled DNA synthesis assay (UDS) and the *in vivo* rodent erythrocyte micronucleus (MN) assay. These short-term study results, where available, were obtained from the Gene-Tox and Chemical Carcinogenesis Research Information System databases (http://toxnet.nlm.nih.gov), the Genetic Activity Profiles database [79] and the published scientific literature. Rodent (both rat and mouse) carcinogenicity data were obtained from the Carcinogenic Potency Database, published literature, International Agency for Research on Cancer (IARC) monographs and National Toxicology Program (NTP) technical reports. Overall, a chemical was concluded to be positive in a short-term assay or in the rodent carcinogenicity bioassay if a single positive result (as defined by the original study author or database curator) was reported in any of these sources, irrespective of the number of negative results also reported.

Although this analysis is comprehensive, it is important to note the inherent limitations that arise from the use of this dataset. Among the 217 agents that have been examined using TGR assays, there are records for 136 agents whose carcinogenicity to the mouse or rat has been determined. Table 2-1 provides a summary of the genotoxicity test results for the 114 rodent carcinogens and 22 rodent non-carcinogens contained in the

database. Within this subset of agents, very few have been evaluated with a full set of short-term genotoxicity tests; among those tests that have been conducted, a majority of agents have returned positive (mutagenic) results in one or more short-term tests. These include 107/151 (71%) for *Salmonella*, 78/95 (82%) for *in vitro* CA, 50/62 (81%) for *in vitro* MLA, 35/50 (70%) for *in vivo* CA, 60/87 (69%) for *in vivo* MN, 29/44 (66%) for *in vivo* UDS, and 159/217 (73%) for the TGR assay. Because many of the published TGR studies were intended to investigate specific mechanistic questions, the database is comprised disproportionately by model mutagenic carcinogens. As a result, conclusions regarding the performance of TGR assays made from the analysis of these data may not be broadly generalizable because the prevalence of mutagens/carcinogens in the database is substantially greater than expected universally.

2.2 Questions for investigation

Using data contained in the TRAID for a number of chemicals tested using a variety of commonly used short-term assays, TGR assays, and rodent carcinogenicity, questions within the following two general areas could be investigated.

2.2.1 Performance of TGR assays in identifying genotoxic agents

TGR assays are genotoxicity tests and the principal benchmark of any such assay's performance should be its ability to correctly identify genotoxicants. The following questions are addressed specifically:

1. What is the degree of agreement between results of the various short-term assays?

2. From an analysis of the studies in which sequence analysis was completed, what conclusions can be drawn regarding the ability of TGR assays to identify genotoxicants?

2.2.2 Predictivity of TGR assays for rodent carcinogenicity

Since TGR assays, like all short-term genotoxicity tests, will likely be used as a predictor of carcinogenicity, it is important to address the following additional questions:

3. What are the comparative performance characteristics of each of the short-term assays in predicting rodent carcinogenicity?
4. When combined into a test battery, what is the predictive value of the various short term assays for rodent carcinogenicity?

2.3 Approach to the questions

2.3.1 Characterization of the agreement between short-term assay results

The lack of an accepted definitive genotoxicity test hinders the evaluation of any new test because there is no assay against which the new test can be measured. Instead, new genotoxicity tests must be compared against the existing assays that are known to detect mutations, despite differences in the mechanisms that lead to mutations identified using the various assays. In the analyses conducted in this chapter, ***concordance*** is used as a measure of the proportion of agreements (positive or negative) between any two assays. The ***kappa coefficient*** (K) is a more quantitative measure of agreement between tests with nominal outcomes ([80]). Kappa can range from -1 to 1 and has a magnitude that reflects the strength of agreement, where a coefficient of 1 represents complete agreement of the tests. When the observed agreement is significantly better than

Table 2-1: Summary of the chemical records in the Transgenic Rodent Assay Information Database.

Chemical	*In vitro* assays			*In vivo* assays						
							TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
1,10-Diazachrysene	+	nd	nd	nd	nd	nd	1	11	+	nd
1,2:3,4-Diepoxybutane	+	+	+	nd	+	+	9	0	-	+
1,2-Dibromo-3-chloropropane	+	+	+	+	+	+	1	1	+	+
1,2-Dibromoethane	+	+	+	-	-	+	7	1	+	+
1,2-Dichloroethane	+	nd	nd	nd	-	nd	11	0	-	+
1,2-Epoxy-3-butene	+	-	nd	+	+	nd	7	2	+	nd
1,3-Butadiene	+	nd	-	+	+	-	4	15	+	+
1,6-Dinitropyrene	+	+	nd	nd	nd	+	2	1	+	+
1,7-Phenanthroline	+	nd	nd	nd	nd	nd	15	5	+	nd
1,8-Dinitropyrene	+	+	+	nd	nd	nd	15	2	+	+
1.5 GHz electromagnetic near field	nd	nd	nd	nd	nd	nd	4	0	-	nd
10-Azabenzo[a]pyrene	+	nd	nd	nd	nd	nd	13	3	+	nd
114mIn internal radiation	nd	nd	nd	nd	nd	nd	5	1	+	nd
17b-Estradiol	-	-	-	nd	-		2	0	-	+
1-Chloromethylpyrene	+	nd	nd	nd	nd	nd	6	6	+	nd
1-Methylphenanthrene	+	nd	nd	nd	nd	nd	24	0	-	nd
1-Nitronaphthalene	+	+	nd	nd	nd	nd	3	0	-	-
2,3,7,8-Tetrachlorodibenzo-p-dioxin	-	-	+	-	-	nd	2	0	-	+
2,4-Diaminotoluene	+	+	+	nd	nd	+	5	6	+	+
2,6-Diaminotoluene	+	nd	nd	nd	+	+	7	0	-	-
2.45 GHz radiofrequency	-	-	-	nd	+	nd	4	0	-	-
2-Acetylaminofluorene	+	+	+	+	+	+	12	16	+	+
2-Nitronaphthalene	+	nd	nd	nd	nd	nd	1	2	+	nd
2-Nitro-p-phenylenediamine	+	+	+	-	-	inc	1	1	+	+
3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)	+	+	nd	+	nd	nd	3	0	-	+

Chemical	*In vitro* assays			*In vivo* assays			TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
3-Chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	+	+	+	nd	+	+	17	0	-	+
3-Fluoroquinoline	-	nd	nd	nd	nd	-	3	0	-	-
3-Methylcholanthrene	+	+	+	nd	inc	inc	0	4	+	+
3-Nitrobenzanthrone	+	nd	nd	nd	+	nd	4	2	+	+
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	+	nd	nd	nd	nd	nd	1	17	+	+
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) + 8-methoxypsoralen	nd	nd	nd	nd	nd	nd	2	0	-	-
4,10-Diazachrysene	+	nd	nd	nd	nd	nd	0	12	+	nd
4-Acetylaminofluorene	+	-	+	-	inc	-	0	1	+	-
4-Aminobiphenyl	+	+	+	nd	+	+	5	14	+	+
4-Chloro-o-phenylenediamine	+	+	nd	nd	+	nd	3	5	+	+
4-NQO	+	+	+	nd	+	nd	15	25	+	+
4-NQO + p-XSC	nd	nd	nd	nd	nd	nd	0	3	+	nd
5-(2-Chloroethyl)-2'-deoxyuridine (CEDU)	+	nd	nd	nd	-	nd	0	3	+	nd
5-(p-Dimethylaminophenylazo)benzo thiazole	+	nd	nd	nd	nd	+	0	2	+	-
5,9-Dimethyldibenzo[c,g]carbazole (DMDBC)	nd	nd	nd	nd	nd	nd	10	20	+	+
5,9-Dimethyldibenzo[c,g]carbazole + carbon tetrachloride	nd	nd	nd	nd	nd	nd	0	1	+	nd
5,9-Dimethyldibenzo[c,g]carbazole + phenobarbital	nd	nd	nd	nd	nd	nd	0	1	+	nd
5-Bromo-2'-deoxyuridine	-	nd	nd	nd	+	nd	2	0	-	+

Chemical	*In vitro* assays			*In vivo* assays						
							TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
5-Fluoroquinoline	+	nd	nd	nd	nd	nd	2	1	+	+
6-(p-Dimethylaminophenylazo)benzo thiazole	nd	nd	nd	nd	nd	+	0	5	+	+
6,11-Dimethylbenzo[b]naphtho(2,3-d)thiophene	nd	nd	nd	nd	nd	nd	2	1	+	inc
6-Nitrochrysene	+	nd	nd	nd	nd	nd	0	2	+	+
7,12-Dimethylbenzanthracene	+	+	+	nd	+	+	23	60	+	+
7,12-Dimethylbenzanthracene + 17b-estradiol	nd	nd	nd	nd	nd	nd	0	3	+	nd
7,12-Dimethylbenzanthracene + daidzein	nd	nd	nd	nd	nd	nd	0	4	+	nd
7,12-Dimethylbenzanthracene + daidzein + genistein	nd	nd	nd	nd	nd	nd	0	2	+	nd
7,12-Dimethylbenzanthracene + genistein	nd	nd	nd	nd	nd	nd	0	7	+	nd
7,12-Dimethylbenzanthracene + high fat diet	nd	nd	nd	nd	nd	nd	0	2	+	nd
7,12-Dimethylbenzanthracene + low fat diet	nd	nd	nd	nd	nd	nd	0	2	+	nd
7H-Dibenzo[c,g]carbazole (DBC)	+	nd	nd	nd	nd	nd	0	6	+	+
7-Methoxy-2-nitronaphtho[2,1-b]furan (R7000)	+	nd	nd	nd	nd	nd	8	18	+	nd
87-966	nd	nd	nd	nd	nd	nd	1	2	+	nd
89Sr-internal radiation	nd	nd	nd	nd	nd	nd	5	1	+	nd
A-alpha-C	+	+	nd	nd	nd	nd	2	4	+	+
Acetaminophen	-	+	nd	+	-	nd	1	0	-	+
Acetic Acid	-	nd	nd	nd	nd	nd	2	1	+	-
Acetone	-	-	nd	nd	-	nd	2	0	-	-

Chemical	*In vitro* assays			*In vivo* assays						
							TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
Acrylamide	-	+	+	+	+	+	19	7	+	+
Acrylonitrile	+	+	+	nd	-	-	15	0	-	+
Adozelesin	nd	nd	nd	nd	nd	nd	1	2	+	nd
Aflatoxin B1	+	+	nd	+	+	+	2	7	+	+
Aflatoxin B1 + Phorone	nd	nd	nd	nd	nd	nd	1	2	+	nd
Aflatoxin B1 + TCDD	nd	nd	nd	nd	nd	nd	1	1	+	nd
Agaritine	+	-	-	nd	nd	nd	10	2	+	+
all-trans-Retinol	+	nd	nd	nd	nd	nd	2	0	-	-
alpha-Chaconine	-	nd	nd	nd	nd	nd	0	2	+	nd
alpha-Hydroxytamoxifen	nd	nd	nd	nd	nd	nd	1	4	+	nd
alpha-Solanine	-	nd	nd	nd	nd	nd	0	2	+	nd
Aminophenylnorharman	+	nd	nd	nd	nd	nd	0	6	+	+
Amosite asbestos	-	+	nd	+	nd	nd	11	4	+	+
Amosite asbestos + B[a]P	nd	nd	nd	nd	nd	nd	4	5	+	+
AMP397	+	nd	-	nd	-	nd	6	0	-	nd
Aristolochic acid	+	+	+	nd	+	nd	8	15	+	+
Arsenite Trioxide	nd	nd	+	nd	+	nd	5	0	-	+
Azathioprine	+	+	nd	+	+	nd	6	3	+	+
Benzene	-	+	+	+	+	-	8	4	+	+
Benzo(a)pyrene	+	+	+	nd	+	+	33	118	+	+
Benzo(a)pyrene + Eugenol	nd	nd	nd	nd	nd	nd	0	1	+	nd
Benzo(a)pyrene + Green tea	nd	nd	nd	nd	nd	nd	0	1	+	nd
Benzo(a)pyrene + Lycopene	nd	nd	nd	nd	nd	nd	1	5	+	nd
Benzo(a)pyrene-diolepoxide	nd	nd	nd	nd	nd	nd	3	0	-	nd
Benzo(a)pyrene-diolepoxide + TPA	nd	nd	nd	nd	nd	nd	0	1	+	nd
Benzo(f)quinoline	+	+	nd	nd	nd	nd	19	1	+	-
Benzo(h)quinoline	+	+	nd	nd	nd	nd	9	1	+	-
beta-Propiolactone	+	+	+	+	+	nd	1	9	+	+

Chemical	*In vitro* assays			*In vivo* assays						Carc
							TGR			
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
Bitumen fumes	nd	nd	nd	nd	nd	nd	3	0	-	nd
Carbon tetrachloride	-	+	nd	-	-	-	5	0	-	+
CC-1065	-	nd	nd	nd	+	nd	0	1	+	+
Chlorambucil	+	+	nd	+	+	nd	18	22	+	+
Chloroform	+	-	+	+	+	-	5	0	-	+
Chrysene	+	nd	nd	-	nd	nd	4	8	+	+
Cisplatin	+	+	nd	+	+	nd	0	2	+	+
Clofibrate	-	+	nd	-	nd	-	2	0	-	+
CM 44 glass fibers	nd	nd	nd	nd	nd	nd	8	0	-	nd
Coal Tar	+	nd	nd	nd	nd	nd	1	1	+	+
Comfrey	-	nd	nd	nd	nd	nd	0	1	+	+
Conjugated linoleic acid (CLA)	nd	nd	nd	nd	nd	nd	3	0	-	nd
Conjugated linoleic acid (CLA) + PhIP	nd	nd	nd	nd	nd	nd	0	1	+	nd
Crocodolite Asbestos	-	nd	nd	nd	nd	nd	4	2	+	+
Cyclophosphamide	+	+	+	+	+	+	18	24	+	+
Cyproterone acetate	-	-	nd	nd	+	+	15	31	+	+
Di(2-ethylhexyl)phthalate	-	-	-	-	-	-	8	2	+	+
Dichloroacetic acid	+	+	+	nd	inc	nd	4	2	+	+
Diesel Exhaust	+	+	nd	nd	-	nd	23	1	+	+
Diethylnitrosamine (DEN)	+	+	+	nd	-	+	22	23	+	+
Diethylnitrosamine (DEN) + phenobarbital	nd	nd	nd	nd	nd	nd	0	7	+	nd
Dimethylarsinic acid	-	nd	nd	+	-	nd	5	0	-	+
Dimethylnitrosamine (DMN)	+	+	+	nd	+	+	45	71	+	+
Dinitropyrenes	nd	nd	nd	nd	nd	nd	6	10	+	nd
Dipropylnitrosamine (DPN)	+	+	nd	nd	-	+	7	9	+	+
d-Limonene	-	-	-	nd	nd	nd	2	0	-	+
Ellagic acid	-	+	-	nd	nd	nd	1	0	-	-

Chemical	*In vitro* assays			*In vivo* assays			TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
Ethylene oxide	+	+	+	+	+	inc	27	7	+	+
Ethylmethanesulfonate	+	+	+	+	+	nd	12	5	+	+
Etoposide	+	+	+	+	+	nd	13	0	-	nd
Eugenol	-	+	+	nd	+	-	1	0	-	-
Fasciola hepatica	nd	nd	nd	nd	nd	nd	0	1	+	nd
Flumequine	-	nd	nd	-	nd	nd	4	0	-	+
Folic acid	-	nd	nd	nd	nd	nd	8	0	-	-
Gamma rays	+	+	nd	+	+	+	10	10	+	+
Genistein	+	+	+	nd	-	nd	4	0	-	+
Glass wool fibres	-	nd	nd	nd	nd	nd	9	0	-	-
Glass wool fibres + B[a]P	nd	nd	nd	nd	nd	nd	0	3	+	nd
Glycidamide	+	nd	nd	nd	+	+	2	2	+	nd
Green tea	+	nd	nd	nd	nd	nd	1	0	-	-
Heavy-ion	nd	nd	nd	nd	nd	nd	2	3	+	nd
Heptachlor	-	+	+	nd	nd	nd	2	0	-	+
Hexachlorobutadiene	+	-	nd	nd	nd	nd	22	2	+	+
Hexavalent chromium	+	+	+	+	+	nd	4	5	+	+
High energy charged particle (Fe)	nd	nd	nd	nd	nd	nd	8	4	+	nd
High fat diet	nd	nd	nd	nd	nd	nd	12	0	-	+
Hydrazine sulfate	+	+	+	inc	inc	nd	24	0	-	+
Hydroxyurea	+	+	+	nd	+	nd	1	2	+	nd
Hydroxyurea + X-ray	nd	nd	nd	nd	nd	nd	1	2	+	nd
Hyperglycemia	nd	nd	nd	nd	nd	nd	0	1	+	nd
IQ	+	+	nd	+	-	nd	8	14	+	+
IQ + sucrose	nd	nd	nd	nd	nd	nd	8	4	+	nd
Isopropylmethanesulfonate	+	nd	nd	+	+	nd	6	9	+	+
Jervine	nd	nd	nd	nd	nd	nd	0	2	+	nd
Kojic acid	+	+	nd	nd	+	-	2	0	-	+

Chemical	*In vitro* assays			*In vivo* assays			TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
Leucomalachite green	-	nd	nd	nd	+	nd	8	1	+	-
Levofloxacin	-	nd	nd	nd	nd	nd	14	0	-	nd
Malachite green	+	-	nd	nd	-	nd	1	0	-	equivocal
MeIQ	+	+	nd	nd	nd	nd	7	10	+	+
MeIQx	+	-	nd	+	-	nd	21	30	+	+
Methyl bromide	+	+	+	-	+	-	2	0	-	+
Methyl clofenapate	-	nd	nd	nd	-	-	2	0	-	+
Methyl clofenapate + Dimethylnitrosamine (DMN)	nd	nd	nd	nd	nd	nd	0	2	+	nd
Methylmethanesulfonate	+	+	+	+	+	+	47	10	+	+
Metronidazole	+	+	nd	nd	-	nd	4	0	-	+
Mitomycin-C	+	+	+	+	+	nd	20	11	+	+
MMS + 4-AAF	nd	nd	nd	nd	nd	nd	0	1	+	nd
MMS + DMN	nd	nd	nd	nd	nd	nd	0	2	+	nd
N7-Methyldibenzo[c,g]carbazole (NMDBC)	+	nd	nd	nd	nd	nd	3	7	+	+
N-Ethyl-N-nitrosourea	+	+	nd	+	+	nd	74	326	+	+
N-Ethyl-N-nitrosourea + 8-methoxypsoralen	nd	nd	nd	nd	nd	nd	0	1	+	nd
N-hydroxy-2-AAF	+	+	nd	nd	nd	nd	0	4	+	+
Nickel subsulfide	-	+	nd	nd	+	nd	4	0	-	+
N-Methyl-N'-nitro-N-nitrosoguanidine	+	+	+	+	+	+	7	24	+	+
N-Methyl-N-nitrosourea	+	+	+	+	+	+	11	43	+	+
N-Nitrosodibenzylamine (NDBzA)	+	nd	nd	nd	nd	nd	20	2	+	nd
N-Nitrosomethylbenzylamine	+	nd	nd	nd	nd	nd	0	3	+	+
N-Nitrosomethylbenzylamine + ellagic acid	nd	nd	nd	nd	nd	nd	0	1	+	nd

Chemical	*In vitro* assays			*In vivo* assays						
							TGR			
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	Carc
N-Nitrosomethylbenzylamine + green tea	nd	nd	nd	nd	nd	nd	0	1	+	nd
N-Nitrosonornicotine (NNN)	+	nd	nd	nd	+	nd	1	8	+	+
N-Nitrosopyrrolidine	+	-	+	nd	+	nd	0	1	+	+
NNK + Green Tea	nd	nd	nd	nd	nd	nd	2	3	+	nd
N-Propyl-N-nitrosourea	+	+	nd	nd	nd	nd	12	19	+	+
o-Aminoazotoluene	+	nd	+	nd	nd	+	6	8	+	+
o-Anisidine	+	+	+	nd	-	-	10	2	+	+
Oxazepam	-	-	-	nd	-	nd	4	2	+	+
p-Cresidine	+	+	nd	nd	-	nd	0	4	+	+
Peroxyacetyl nitrate (PAN)	+	nd	nd	nd	nd	nd	0	1	+	nd
Phenobarbital	+	+	+	-	+	nd	14	1	+	+
PhIP	+	+	nd	-	nd	nd	51	70	+	+
PhIP + 1,2-dithiole-3-thione (DTT)	nd	nd	nd	nd	nd	nd	0	3	+	nd
PhIP + conjugated linoleic acid (CLA)	nd	nd	nd	nd	nd	nd	0	6	+	nd
PhIP + high fat diet	nd	nd	nd	nd	nd	nd	1	1	+	nd
PhIP + low fat diet	nd	nd	nd	nd	nd	nd	1	1	+	nd
Phorbol-12-myristate-13-acetate (TPA)	-	+	nd	nd	nd	nd	1	0	-	+
Polyphenon E	-	nd	+	nd	-	nd	12	0	-	nd
Potassium bromate	+	+	+	+	+	nd	13	3	+	+
Procarbazine HCl	-	nd	+	+	+	+	15	14	+	+
Proton radiation	nd	+	nd	nd	+	nd	19	11	+	nd
Quinoline	+	+	+	-	+	inc	5	4	+	+
Riddelliine	+	+	nd	nd	+	+	1	4	+	+
Rock wool fibres	nd	nd	nd	nd	nd	nd	6	3	+	-
Rock wool fibres + B[a]P	nd	nd	nd	nd	nd	nd	0	3	+	nd
Sodium saccharin	-	+	-	nd	nd	nd	2	0	-	+

Chemical	*In vitro* assays			*In vivo* assays						
							TGR			Carc
	Sal	CA	MLA	CA	MN	UDS	# negative	# positive	Overall result	
Solanidine	-	nd	nd	nd	nd	nd	0	2	+	nd
Solasodine	nd	nd	nd	nd	nd	nd	1	1	+	nd
Streptozotocin	+	nd	nd	nd	nd	nd	2	2	+	+
Sucrose	-	nd	-	nd	inc	nd	9	5	+	-
Tamoxifen	-	nd	nd	nd	nd	nd	2	11	+	+
Tamoxifen + Phenobarbital	nd	nd	nd	nd	nd	nd	0	3	+	nd
Thiotepa	+	+	nd	+	+	nd	0	1	+	+
Toremifene citrate	nd	nd	nd	nd	nd	nd	1	0	-	-
Trans-4-hydroxy-2-nonenal	-	+	nd	nd	-	nd	4	0	-	-
Trichloroethylene	+	-	+	-	+	-	33	0	-	+
Tris-(2,3-dibromopropyl)phosphate	+	+	+	-	+	nd	26	7	+	+
Uracil	-	nd	nd	nd	nd	nd	3	1	+	+
Urethane	+	+	-	+	+	nd	4	15	+	+
UVB	+	nd	nd	nd	nd	nd	1	29	+	+
Vitamin E	-	nd	nd	nd	nd	nd	5	0	-	nd
Wyeth 14,643	-	nd	nd	nd	nd	nd	2	3	+	+
X-rays	+	+	nd	+	+	+	9	54	+	+

Sal – *Salmonella* nd – not determined
Carc – carcinogenicity inc – inconclusive

expected by chance, kappa is positive and has a 95% confidence interval that does not include 0. Kappa is negative when the observed agreement is less than expected by chance.

2.3.2 Ability of TGR assays to identify genotoxicants

Because the various transgenes can be sequenced and specific mutations identified, where sequence analysis was conducted, the results of TGR assays can be confirmed against an indicator that identifies whether mutations actually occurred. When mutations are confirmed by sequence analysis as having occurred with greater frequency and with a differing mutation spectrum in treatment groups compared with the negative control, the test compound is conclusively mutagenic in the test system.

2.3.3 Characterization of the carcinogen predictivity of the short-term assays

The performance of each of the identified test methods in predicting rodent carcinogenicity was assessed using Bayesian inference, with methodology similar to that employed in previous studies [81-84]. For the purposes of this analysis, a short-term assay prediction was considered 'correct' if it agreed with the result of the rodent carcinogenicity bioassay.

Along with concordance (Section 2.3.1), which measured the proportion of all agents whose carcinogenic activity was correctly classified by each short-term assay, sensitivity, specificity, positive predictive value and negative predictive value could also be calculated. In this context, ***sensitivity*** is the proportion of carcinogens determined to be positive (genotoxic), and ***specificity*** is the proportion of non-carcinogens that were determined to be negative (non-genotoxic) in the short-term assay. These give measures of how well the assay classified chemicals belonging to each class (carcinogenic or non-

carcinogenic). However, in practice, the short-term assay result is typically all that is known; accordingly, the probability that a short term assay result is correctly indicative of the carcinogenic activity of an agent is indicated by the predictive value of the assay. ***Positive predictive value (PPV)*** is the probability a chemical found to be genotoxic in the short-term test is a rodent carcinogen, while ***negative predictive value (NPV)*** is the probability a chemical found to be negative in the short-term test is a rodent non-carcinogen. However, predictive values are so greatly influenced by ***prevalence***, the proportion of chemicals that are rodent carcinogens, that the ability to make generalizations is limited usually to those datasets with similar prevalence.

In order to provide a baseline against which some of the indices previously described can be compared (particularly in this case where prevalence is high), it is useful to examine the expected values of concordance, sensitivity and specificity in a hypothetical situation where the short-term assay results are completely unrelated to carcinogenic activity. In this case, the proportion of carcinogens and non-carcinogens that would be correctly identified if the assay was able to discriminate no better than chance is the ***concordance expected by chance*** (see [85]), which is directly related to the proportion of carcinogens in the database (prevalence). The sensitivity expected by chance is equivalent to the proportion of compounds positive in the test, while the specificity expected by chance is the proportion of compounds negative in the test. Similarly, the PPV and NPV expected simply by chance (PPVrand and NPVrand) are given by prevalence and 1-prevalence, respectively.

2.3.4 Characterization of the performance of TGR in a test battery

The performance characteristics of 8 possible 2-assay test battery combinations were examined where the TGR assay was paired with another short-term test. For each paired combination of assays, a decision regarding how to interpret the combination of tests in the context of predicting carcinogenicity could in theory be made in one of two ways: (a) the chemical could be predicted to be carcinogenic if the outcome of *either* short-term assay was positive (hereafter called the '*or*' scenario), or (b) if the outcomes of *both* short-term assays were positive (the '*and*' scenario). In all other cases, the chemical would be classified non-carcinogenic. In Figure 2-1, the decision rules for classifying a test battery outcome are outlined, taking the example of a test battery of TGR combined with *Salmonella*.

2.3.4.1 Performance of TGR versus *in vivo* MN in a test battery with *in vitro* assays

Currently, the most commonly conducted short-term *in vivo* assay is *in vivo* MN. Thus, it is important to consider if the performance of TGR is better than the performance of *in vivo* MN, when either assay is interpreted in conjunction with the most commonly conducted *in vitro* tests.

Salmonella and *in vitro* CA were used to form a two *in vitro* test battery, where a positive result in either assay resulted in an overall *in vitro* test battery conclusion of positive. This *in vitro* test battery was combined with either TGR or *in vivo* MN in a manner described by Figure 2-2. The performance characteristics of each test battery could be examined.

Figure 2-1: Decision rules for determining the outcome of a 2 assay battery, taking the example of *Salmonella* paired with TGR. The shaded area contains the possible assay outcomes that would lead to a battery prediction of carcinogenic. For scenario (a), the chemical is classified as carcinogenic if the result of either *Salmonella* or TGR is positive, otherwise the chemical is classified as non-carcinogenic. For scenario (b), the chemical is classified as carcinogenic if the results of both *Salmonella* and TGR are positive, otherwise the chemical is classified as non-carcinogenic.

(a) scenario: 'TGR *or Salmonella*'

Salmonella result	TGR result	Battery conclusion
+	+	
+	-	+
-	+	
-	-	-

(b) scenario: 'TGR *and Salmonella*'

Salmonella result	TGR result	Battery conclusion
+	+	+
+	-	
-	+	-
-	-	

Figure 2-2: Decision rules for determining the outcome of the 3 assay battery. *Salmonella* and *in vitro* CA formed the *in vitro* battery. The results of the *in vitro* battery and either TGR or *in vivo* MN determined the overall test battery conclusion. This is the same 'or' scenario described in Figure 2-1(a).

In vitro battery result	*In vivo* result	Overall battery conclusion
+	+	
+	-	+
-	+	
-	-	-

2.4 Results of the analysis

2.4.1 Characterization of the agreement between short-term assay results

Table 2-2 shows the pair-wise concordance between each of the assays using the data available for the agents shown in Table 2-1. The inter-assay concordance was relatively high, not unexpected considering these systems are used to detect genetic toxicity and the dataset was heavily weighted by genotoxic carcinogens. The kappa coefficient indicated the observed pair-wise agreement was significantly better than expected by chance for 11 short-term assay combinations, but the magnitude of kappa (0.25-0.60) suggested the strength of the associations was not substantial. As all the assays are genotoxicity tests and known genotoxicants are overrepresented within the database, one would expect a high degree of association between the tests; this was not always observed. Interestingly, *in vivo* UDS, an indicator assay that is not directly indicative of mutagenicity, seemed to have stronger agreement with TGR, *in vivo* CA, *Salmonella* and *in vitro* CA than did many of the other assays.

2.4.2 Ability of TGR assays to identify genotoxicants

The studies where sequence analysis was conducted are identified in the TRAID. Of the 2977 experimental records, sequencing was conducted for 440. Sequence analysis definitively identified 303 mutagens and 137 non-mutagens, demonstrating the ability of the assays to identify compounds causing gene mutations. There were 3 experimental records (leucomalachite green, phenobarbital) where sequencing indicated the assay conclusion should be changed from mutagenic to non-mutagenic [86-88] and 2 experimental records (oxazepam) where the assay conclusion should be changed from non-mutagenic to mutagenic [88; 89].

Table 2-2: Agreement between short-term assays – concordance and kappa coefficient (K).

	Salmonella					
In vitro **CA**	72/94 (77%) K = 0.28 *	***In vitro*** **CA**				
In vitro **MLA**	50/61 (82%) K = 0.48 *	47/55 (85%) K = 0.51 *	***In vitro*** **MLA**			
In vivo **CA**	34/50 (68%) K = 0.18	32/44 (73%) K = 0.24	18/29 (62%) K = -0.01	***In vivo*** **CA**		
In vivo **MN**	59/85 (69%) K = 0.25 *	50/70 (71%) K = 0.24	38/49 (78%) K = 0.29	34/43 (79%) K = 0.39 *	***In vivo*** **MN**	
In vivo **UDS**	32/43 (74%) K = 0.39 *	26/35 (74%) K = 0.34 *	20/28 (71%) K = 0.24	17/21 (81%) K = 0.60 *	26/36 (72%) K = 0.32	***In vivo*** **UDS**
TGR	112/151 (74%) K = 0.38 *	65/95 (68%) K = 0.16	42/62 (68%) K = 0.18	36/50 (72%) K = 0.28	62/87 (71%) K = 0.34 *	36/44 (82%) K = 0.58 *

* observed agreement is significantly better than expected by chance

2.4.3 Performance characteristics of the short-term assays for predicting carcinogenicity

Table 2-3 shows the indices of each assay's performance in identifying rodent carcinogens and non-carcinogens.

2.4.3.1 Performance of the existing short-term assays for predicting carcinogenicity

The concordance values for six of the most common short-term assays presented in Table 2-3 represent the proportion of agreements between the short-term genotoxicity assay and rodent carcinogenicity. Concordance ranged from a low of 67-68% for *in vivo* MN and *in vivo* UDS to a high of 83% for *in vitro* MLA. When these values were compared with the concordance expected by chance (the expected number of correct predictions based on prevalence), it was apparent that none of the assays predicted carcinogenicity appreciably better than chance, which is further evident as in all cases the expected concordance fell within the 95% confidence intervals of the observed concordance.

Sensitivity is the proportion of carcinogens that had positive results in the short-term assay. For these six assays, sensitivity ranged from a low of 68% (*in vivo* UDS) to 85% (*in vitro* CA and *in vitro* MLA). This suggests that most of the carcinogens were mutagenic in these short-term test systems; however, sensitivity was likely also affected by chance agreements due to the high prevalence. By contrast, specificity, which is the proportion of non-carcinogens with negative results in the short-term assay, was low. Specificity ranged from 33% (*in vitro* CA, *in vivo* MN) to 58% (*Salmonella*).

Table 2-3: Performance of the short-term assays in predicting rodent carcinogenicity.

	Sal	*In vitro* CA	*In vitro* MLA	*In vivo* CA	*In vivo* MN	*In vivo* UDS	TGR
Concordance (%)	73	80	83	71	68	67	74
Concordance expected by chance (%)	64	77	76	68	67	62	63
Sensitivity (%)	75	85	85	70	71	68	75
Specificity (%)	58	33	60	100	33	60	64
PPV (%)	91	92	96	100	93	93	91
NPV (%)	29	20	27	7	9	20	33
Prevalence (%)	85	90	91	98	92	88	84
Proportion positive in test (%)	70	83	81	69	71	65	69
Number of chemicals	128	90	58	48	78	43	136

Sal – Salmonella

For *in vivo* CA, which had an apparent specificity of 100%, only a single non-carcinogen was tested that was correctly identified. Because of this, specificity for this assay is almost certainly greatly overstated.

PPV is the probability that a positive short-term assay result is indicative of carcinogenicity. PPV for all assays was extremely high, with a range from 91% (*Salmonella*) to 96% (*in vitro* MLA). Again, for *in vivo* CA only a single chemical was tested, so the PPV of 100% for this assay is unlikely to be accurate. The differential between PPV and PPV expected by chance for the assays was 6% (*Salmonella*), 2% (*in vitro* CA), 4% (*in vitro* MLA), 2% (*in vivo* CA), 0% (*in vivo* MN) and 4% (*in vivo* UDS). The PPV expected by chance fell within the 95% confidence intervals of PPV for all assays, suggesting no assay was able to predict carcinogenicity significantly better than chance. In contrast, NPV is the probability that a negative short-term assay result is indicative of non-carcinogenicity. The NPV was very low, with a range from 7% (*in vivo* CA) to 29% (*Salmonella*). The differential between NPV and NPV expected by chance for the assays was 14% (*Salmonella*), 10% (*in vitro* CA), 19% (*in vitro* MLA), 5% (*in vivo* CA), 1% (*in vivo* MN) and 8% (*in vivo* UDS). The NPV expected by chance fell within the 95% confidence intervals of NPV for all assays except *Salmonella*, suggesting this was the only assay able to predict non-carcinogenicity significantly better than chance. Considering both PPV and NPV together, it can be concluded that the probability was no better than chance that chemicals genotoxic in the existing short-term assay were carcinogenic; only chemicals with negative results in *Salmonella* had a better than chance probability of being non-carcinogenic. None of the other existing short-term assays were appreciably predictive.

However, PPV and NPV are highly affected by prevalence, particularly in situations where specificity is low. Since prevalence was extremely high in the database used for this analysis, the actual PPV of each of these assays would have been much lower and the actual NPV would have been much higher than that observed, if a dataset that was representative of the proportion of carcinogens within the known chemical universe had been available (see Table 2-4).

A high PPV is a good characteristic for a test used in error-intolerant applications, such as in the screening of novel pharmaceuticals for genotoxic properties prior to the initiation of phase I clinical trials with healthy human volunteers. In an ideal situation, however, a short-term assay would also have high NPV, together indicating there is a high probability that both positive and negative test results are correct. Specificity and NPV are most important when an assay is used to screen sets of chemicals where the expected number of carcinogens is small [90]. Approximately 52% of chemicals tested by the NTP were carcinogenic in at least one organ in one of the four sex/species groups [91], which is much fewer than the proportion of carcinogens in the database and much higher than that generally expected within the known universe of chemicals.

Table 2-4: Impact of differing prevalence of carcinogens on PPV and NPV of the various assays, provided sensitivity and specificity of the assays is maintained

Assay	Prevalence							
	10%		25%		50%		90%	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
Salmonella	0.17	0.95	0.37	0.87	0.64	0.70	0.94	0.20
In vitro CA	0.12	0.95	0.30	0.87	0.56	0.69	0.92	0.20
In vitro MLA	0.19	0.97	0.41	0.92	0.68	0.80	0.95	0.31
In vivo CA	n/a *	0.97	n/a *	0.91	n/a *	0.77	n/a *	0.27
In vivo MN	0.11	0.91	0.26	0.77	0.51	0.53	0.91	0.11
In vivo UDS	0.16	0.94	0.36	0.85	0.63	0.65	0.94	0.17
TGR	0.19	0.96	0.41	0.88	0.68	0.72	0.95	0.22

* limited number of carcinogens, none with positive genotoxicity test results (specificity 100%)

2.4.3.2 Performance of TGR assays

The concordance of TGR assays with rodent carcinogenicity was 74% and the expected concordance due to chance was 63%; the difference (11%) between the observed and expected concordance was greater than all other assays and was comparable only to *Salmonella*. Unlike all the other assays, the expected concordance also fell outside the 95% confidence interval of the observed concordance, suggesting the proportion of agreements was significantly better than expected by chance. The sensitivity value suggests that most of the carcinogens were mutagenic; however, chance agreements may account for a large proportion of the observed sensitivity because of the high prevalence. TGR had the highest specificity (64%) of any of the short-term assays examined. PPV was comparable to the other assays; however, the differential between PPV and the expected PPV due to chance (6%) was slightly higher than the other assays. Unlike any of the other assays, the PPV expected by chance did not fall within the 95% confidence interval of PPV, suggesting the probability an agent mutagenic to TGR was carcinogenic was significantly better than chance. The NPV was the highest of all the assays examined and the differential between the NPV and the expected NPV due to chance (17%) was higher than the other assays. The NPV expected by chance did not fall within the 95% confidence interval of NPV, suggesting the probability an agent non-mutagenic to TGR was non-carcinogenic was also significantly better than chance. Overall, this suggests that there was a better than chance probability a positive result in TGR was predictive of carcinogenicity and a negative TGR result was predictive of non-carcinogenicity. Re-examination of the performance of TGR assays with a larger and more representative database will help to determine whether TGR truly has a better PPV

and NPV than those previously reported for many of the other short-term assays [81; 82; 84; 92]. It appears that the operational characteristics of TGR are at least comparable, and perhaps marginally better than *Salmonella*.

2.4.4 Performance of TGR assays in a test battery

Since the available information would suggest, at least on a preliminary basis, that TGR assays have some predictive value, the question of whether the inclusion of TGR would improve the predictivity of the standard genotoxicity test battery could be addressed. Table 2-5 shows the 32 possible test battery outcomes for 4 short-term tests plus TGR and the number of carcinogenic and non-carcinogenic test chemicals corresponding to each outcome.

Consistent with the observation that most chemicals in the dataset are genotoxic carcinogens, the majority of rodent carcinogens with results from all 5 of the short-term test systems shown in Table 2-5 were mutagenic in each of the 5 tests. None of the 22 non-carcinogens had a complete set of test battery results.

2.4.4.1 Performance of potential test battery combinations

Table 2-6 shows the performance characteristics of 8 potential test battery combinations involving TGR. Combining TGR with one of the other short-term assays shown in Table 2-6 in the 'or' test battery scenario resulted in improvements in the proportion of agreements (concordance) compared with each of the assays alone.

Table 2-5: Contingency table of short-term test battery results (n = 37).

In vitro assays		*In vivo* assays				
Salmonella	CA	CA	MN	TGR	Carcinogenicity	
					+	-
+	+	+	+	+	21	0
+	+	+	+	-	0	0
-	+	+	+	-	0	0
-	+	+	+	+	2	0
+	+	-	+	+	3	0
+	+	-	+	-	1	0
-	+	-	+	-	0	0
-	+	-	+	+	0	0
+	-	+	-	+	1	0
+	-	+	-	-	0	0
-	-	+	-	-	0	0
-	-	+	-	+	0	0
+	-	-	-	+	0	0
+	-	-	-	-	0	0
-	-	-	-	-	1	0
-	-	-	-	+	1	0
+	+	+	-	+	1	0
+	+	+	-	-	0	0
-	+	+	-	-	1	0
-	+	+	-	+	0	0
+	+	-	-	+	2	0
+	+	-	-	-	0	0
-	+	-	-	-	1	0
-	+	-	-	+	0	0
+	-	+	+	+	0	0
+	-	+	+	-	1	0
-	-	+	+	-	0	0
-	-	+	+	+	0	0
+	-	-	+	+	0	0
+	-	-	+	-	1	0
-	-	-	+	-	0	0
-	-	-	+	+	0	0

Table 2-6: Performance characteristics of 8 possible test battery combinations.

	TGR or *Salmonella*	TGR and *Salmonella*	TGR or *in vitro* CA	TGR and *in vitro* CA	TGR or *in vivo* CA	TGR and *in vivo* CA	TGR or *in vivo* MN	TGR and *in vivo* MN
Concordance	0.80	0.66	0.87	0.67	0.85	0.63	0.82	0.60
Concordance expected by chance	0.73	0.55	0.84	0.59	0.86	0.60	0.79	0.54
Sensitivity	0.87	0.64	0.94	0.65	0.87	0.62	0.86	0.58
Specificity	0.42	0.79	0.22	0.78	0.00	1.00	0.33	0.83
PPV	0.90	0.95	0.92	0.96	0.98	1.00	0.94	0.98
NPV	0.36	0.28	0.29	0.20	0.00	0.05	0.17	0.14
Proportion positive in test battery	0.83	0.58	0.92	0.61	0.88	0.60	0.85	0.55
Prevalence	0.85	0.85	0.90	0.90	0.98	0.98	0.92	0.92

In addition, sensitivity of the battery also showed marked improvement compared with the assays alone. Although specificity of the batteries in this scenario was slightly decreased compared with the component tests, PPV remained essentially unchanged and NPV generally showed slight improvement. When compared with the predictive values expected by chance, the greatest differential in PPV was provided by 'TGR or *Salmonella*' (4%), but no test battery had a PPV significantly better than expected by chance. Only the 'TGR or *Salmonella*' battery had a differential between NPV and expected NPV (13%) that was significantly better than expected by chance. The specificity and NPV of '*in vivo* CA or TGR' was 0% because the single carcinogen tested in both assays was incorrectly classified.

Overall, it appeared that pairing TGR with each of the existing short-term assays in the 'or' scenario slightly improved predictivity beyond that observed for the single tests. None of the batteries appeared to improve PPV beyond that expected simply by chance and only 'TGR or *Salmonella*' improved NPV beyond the expected level. However, if the test battery maximizing both PPV and NPV was considered the optimum for carcinogen identification, the 'TGR or *Salmonella*' test battery appeared to be superior to the others.

In the 'and' scenario, both concordance and sensitivity were decreased compared with the single tests; however, specificity was substantially increased. Nevertheless, PPV and NPV remained essentially unchanged from the assays alone, suggesting the probability that positive or negative battery results were correctly indicative of carcinogenic activity was not any better than for the single tests. When predictive values were compared with those expected due to chance, only the 'TGR and *Salmonella*'

battery had better PPV (10% better) and NPV (13% better) than expected. The specificity and PPV of 100% for 'TGR and *in vivo* CA' should be interpreted with caution since the only non-carcinogen in these scenarios was correctly classified.

Overall, the 'TGR and *Salmonella*' and 'TGR and *in vitro* CA' scenarios appeared to be superior to the others in the 'and' scenario because specificity was highest, sensitivity did not decline substantially and the PPV and NPV remained similar to the component assays alone. However, none of the batteries except 'TGR and *Salmonella*' were able to improve the predictive values beyond what would be expected by chance.

Considering all the test batteries and single assays examined, the 'TGR or *Salmonella*' battery, TGR alone and *Salmonella* alone had the best predictive values for this high prevalence dataset. Because the 'or' scenario serves to minimize false negatives, this battery interpretation may have value in situations where sensitivity and PPV are important, such as when the battery outcome is used as a means to facilitate priority setting for further testing or for safety assessment; however, the trade-off is that false positives are increased. In contrast, battery interpretations using the 'and' scenario may be appropriate in situations where minimizing false positives is important.

2.4.4.2 Complementarity – tests used to identify genotoxicants

Most genotoxic compounds will induce both chromosomal mutations and gene mutations, although the extent to which each of these two endpoints are induced by any given genotoxic agent may differ significantly. As a result, the corresponding sensitivity of tests specific to these endpoints will differ. Short-term assays are complementary when they offer greater predictivity for the detection of mutagens when combined than

when alone. Since no single assay is likely to detect all genotoxic effects, a battery comprised of tests that assay mechanistically distinct events (i.e. primarily gene mutations or chromosomal aberrations) may offer the greatest chance of detecting genotoxicity. In the ideal situation where there are no false positives, the degree of complementarity between assays is indicated by the lack of association between their conclusions regarding genotoxicity. Perfect complementarity occurs in the situation where each assay alone identifies none of the mutagens detected by the other, but identify all mutagens when combined. Such perfect complementarity between any two assays, however, is unlikely ever to occur because of the very narrow spectrum of genotoxic mechanisms detected by most assays and the number of genotoxic compounds that cause both gene mutations and chromosomal aberrations. As shown in Table 2-2, there is agreement better than expected by chance for many of the assay pairs, consistent with the belief that genotoxic compounds often induce both chromosomal aberrations and gene mutations, while non-genotoxic compounds should induce neither. In the case of the TGR assay, the lack of significant association with *in vitro* CA and *in vivo* CA would suggest that tests assessing mechanistically different endpoints would exhibit stronger complementarity; however, this conclusion is not supported by the poor complementarity exhibited by TGR paired with *in vivo* MN. Pairing of the TGR assays with *Salmonella* or *in vivo* UDS also appeared to lack strong complementarity. However, because known potent mutagens were overrepresented among the chemicals in the database, it is difficult to accurately determine complementary relationships, since strong mutagens are likely to efficiently induce both gene mutations and chromosomal mutations.

2.4.4.3 Complementarity – tests used to identify carcinogens

When the short-term assays are used as a means to predict carcinogenicity, the most complementary assays are those that, when combined, minimize the false negative rate and maximize the predictive values. Examining Table 2-6, it is apparent that the predictive values of the two assay batteries shown are not substantially greater than those of the component assays alone. However, in the cases where the overall battery is interpreted as positive when either assay is positive (the *or* scenario described in 2.3.3), the false negative rate of these test batteries is lower (and sometimes markedly so) than either assay alone. For situations where minimizing the number of false negatives is important, such as when the battery is used as a screening test prior to the initiation of clinical trials, it is apparent that the use of TGR assays in a test battery may have clear advantages, despite the lack of substantial improvement of the batteries over the component tests in terms of predictive values.

2.4.4.4 Performance of TGR versus *in vivo* MN in a test battery with *in vitro* assays

The performance characteristics of TGR when combined in a test battery with *Salmonella* and *in vitro* CA (described in Figure 2-2) are presented in Table 2-7. The performance characteristics of *in vivo* MN in this same battery are shown in Table 2-8.

It appears that TGR and *in vivo* MN performed similarly when combined in a battery with both *Salmonella* and *in vitro* CA, with the exception that TGR had higher NPV. However, these results do not suggest a clear advantage of one assay over the other on the basis of the current data.

Table 2-7: Performance of the *in vitro* test battery with TGR.

Salmonella	*In vitro* CA			Carcinogenicity +	Carcinogenicity -	Total
+	+	TGR	+	50	2	52
			-	9	1	10
		Total		59	3	62
	-	TGR	+	4	1	5
			-	2	0	2
		Total		6	1	7
-	+	TGR	+	3	0	3
			-	7	3	10
		Total		10	3	13
	-	TGR	+	3	0	3
			-	3	2	5
		Total		6	2	8

		95% C.I.
Concordance	0.89	0.81 - 0.95
Expected concordance	0.86	0.77 - 0.92
Sensitivity	0.96	0.90 - 0.99
Specificity	0.22	0.03 - 0.60
PPV	0.92	0.84 - 0.97
NPV	0.40	0.05 - 0.85
Prevalence	0.90	0.82 - 0.95

Table 2-8: Performance of the *in vitro* test battery with *in vivo* MN.

Salmonella	*In vitro* CA			Carcinogenicity +	Carcinogenicity -	Total
+	+	*In vivo*	+	36	0	36
		MN	-	11	0	11
		Total		47	0	47
	-	*In vivo*	+	3	0	3
		MN	-	1	0	1
		Total		4	0	4
-	+	*In vivo*	+	3	1	4
		MN	-	2	1	3
		Total		5	2	7
	-	*In vivo*	+	1	1	2
		MN	-	4	1	5
		Total		5	2	7

		95% C.I.
Concordance	0.89	0.79 - 0.96
Expected concordance	0.87	0.77 - 0.93
Sensitivity	0.93	0.84 - 0.98
Specificity	0.25	0.01 - 0.81
PPV	0.95	0.86 - 0.99
NPV	0.20	0.01 - 0.72
Prevalence	0.94	0.85 - 0.98

2.4.5 Conclusions

Any interpretation of results from this analysis must recognize the extent to which the predictive values are influenced by prevalence (see Table 2-4), which was high within the available database. Consequently, perhaps the more useful indicators of assay and battery performance are provided by the extent of the differences between the observed agreement or predictive values and those expected simply by chance (prevalence and 1-prevalence for PPV and NPV, respectively). The following conclusions regarding the performance of the TGR assay arise from the analysis carried out in this chapter:

1. Sequencing has indicated that TGR assays are capable of identifying compounds causing gene mutations.
2. TGR assays exhibited agreement significantly better than expected by chance with several short-term tests assessing both gene mutations and chromosomal aberrations; however, known genotoxicants are overrepresented within the existing database and, consequently, it is difficult to assess complementarity.
3. The TGR assays exhibited high sensitivity and PPV, meaning that most carcinogens had positive TGR assay results and there was a high probability that a chemical mutagenic to TGR was a carcinogen. If the result of TGR assays was used as an indicator of carcinogenic potential when screening a database with a low prevalence of carcinogens, the PPV would be substantially lower. Of the seven genotoxicity assays examined, only TGR assays provided PPV significantly better than expected simply by chance.
4. The TGR assays exhibited low specificity and NPV, meaning relatively few non-carcinogens were non-mutagenic in the TGR assay and there was a low

probability that a chemical with a negative TGR result was a non-carcinogen; however, it was no worse than *Salmonella* in this regard. If the result of TGR assays was used as an indicator of carcinogenic potential when screening a database with a low prevalence of carcinogens, the NPV would be substantially higher. Of the genotoxicity assays examined, only TGR assays and *Salmonella* provided NPV significantly better than expected simply by chance.

5. Considering all the test batteries and single assays examined using the current dataset, the 'TGR or *Salmonella*' battery, TGR alone and *Salmonella* alone had the best PPV and NPV, which were generally also significantly better than expected by chance. Despite the lack of substantial increases in predictive values of the test batteries compared with the component assays alone, the '*or*' test batteries had higher false positive rates and lower false negative rates than the single tests.
6. Both TGR assays and *in vivo* MN performed similarly when combined in a battery with *Salmonella* and *in vitro* CA, suggesting there was no clear advantage of one assay over the other.

2.5 Further discussion

TGR assays offer the capability to identify chemicals that induce gene mutations in any tissue, since the animal models contain multiple copies of a chromosomally integrated, genetically neutral transgene within every somatic and germ cell. Current test battery approaches do not routinely employ an *in vivo* test for gene mutations as an adjunct to *Salmonella* because the existing *in vivo* gene mutation assays are difficult to

conduct, expensive, not well validated and are generally not accepted by regulatory agencies. TGR assays could potentially fill this void.

TGR assays are gene mutation tests and their ability to detect gene mutations has been demonstrated repeatedly by sequencing. In addition to assessing genotoxicity, many short-term genetic toxicity tests are also used as indicators of carcinogenic potential despite the inherent problems associated with this approach. In the analysis presented in this chapter, the ability of TGR to identify rodent carcinogens did not differ greatly from that of *Salmonella*. Nevertheless, TGR did demonstrate an ability to identify carcinogens when used as a stand-alone test and when combined in a battery with other short-term genotoxicity tests.

Previous work has shown that *Salmonella* is the short term assay most predictive of carcinogenicity, but that it is not necessarily highly predictive of non-carcinogenicity. Zeiger *et al* (1990) [83] augmented, with an additional 41 chemicals, the work of Tennant *et al* (1987) [84] who analyzed the results of rodent carcinogenicity and short-term genotoxicity studies (*Salmonella*, *in vitro* CA, sister chromatid exchange (SCE), and MLA) of 73 chemicals tested by the NTP. Logistic regression analysis indicated there were no significant differences between the 41 and 73 chemical datasets, so they were combined for further analysis. The resulting dataset was composed of 59% carcinogens, 26% non-carcinogens, and 15% equivocals. *Salmonella* was clearly superior to *in vitro* CA, SCE, and MLA for discriminating carcinogens from non-carcinogens. Concordance of the short-term assays with carcinogenicity studies ranged from 59% (SCE & MLA) to 66% (*Salmonella*), PPV ranged from 63% (MLA) to 89% (*Salmonella*) and NPV ranged from 50% (SCE & MLA) to 55% (*Salmonella*). There were no combinations of short-

term assays that improved the concordance or predictivity of *Salmonella* alone; however, there were 24 carcinogens that were non-mutagenic to *Salmonella* but mutagenic in another short-term assay [83].

In additional work, Zeiger (1998) examined the NTP database (182 carcinogens, 106 non-carcinogens, 42 equivocals) to determine the performance of several widely used short-term assays (*Salmonella*, *in vitro* CA, MLA, *in vivo* MN) and to determine if any complementarity existed. Like the previous analysis, *Salmonella* was found to be the only assay predictive of carcinogenicity, but not non-carcinogenicity. The assays did not complement each other and additional tests added in a battery with *Salmonella* did not increase the predictivity over that of *Salmonella* alone [82].

Kim and Margolin (1999) investigated the performance of *Salmonella* and the *in vivo* CA and MN assays and determined if combining these 3 assays would improve the predictivity for rodent carcinogenicity. In their dataset of 82 chemicals compiled from NTP databases and from the published literature, *Salmonella* outperformed *in vivo* CA and *in vivo* MN for the prediction of carcinogenicity. *Salmonella* was the only assay of the three that had a significant positive association with carcinogenicity. When combined in 4 potential 2-assay battery combinations, no combination improved the predictivity over that of *Salmonella* alone [81].

Kirkland *et al* compiled a large database of *in vitro* genotoxicity test results (*Salmonella*, *in vitro* CA, *in vitro* MLA, *in vitro* MN) and used the data to conduct perhaps the most comprehensive investigation of the carcinogen predictive abilities of various short-term genotoxicity tests and batteries composed of one or more of these tests [92]. The dataset contained 717 compounds that had been tested in at least one *in vitro*

test system (541 carcinogens and 176 non-carcinogens). Salmonella had the lowest sensitivity (59%) and highest specificity (74%) of the four assays. PPV was 87% (Salmonella) to 74% (*in vitro* MLA) and NPV was 30% (*in vitro* MN) to 37% (Salmonella), generally consistent with the results of the TGR assay evaluation presented herein. Combining the assays into two-test batteries that were considered indicative of genotoxicity when one of the tests was positive increased sensitivity at the expense of specificity; however, the impact on PPV and NPV was more variable, with PPV decreasing slightly and NVP increasing slightly (*Salmonella* + *in vitro* MLA, *in vitro* MLA + *in vitro* CA), decreasing (*Salmonella* + *in vitro* MN, *in vitro* MLA + *in vitro* MN) or remaining essentially unchanged (*Salmonella* + *in vitro* CA). These results are inconsistent with the results of the TGR analysis presented herein, most likely because prevalence differed between the two datasets.

Despite its comprehensiveness, the major limitation of the analysis presented in this chapter is the significant overrepresentation of genotoxic carcinogens within the database currently available. Consequently, the results presented in this analysis must be interpreted with care. It is a useful reminder to consider the first comparisons between *Salmonella* mutagenicity and carcinogenicity [93; 94]. The chemicals in the datasets used for these initial analyses were primarily genotoxic carcinogens, such as direct alkylating agents, polycyclic aromatic hydrocarbons and nitrosamines that were nearly always mutagenic to *Salmonella*; consequently, the performance of *Salmonella* was greatly overestimated (PPV 89-95%). Once larger, more representative datasets (such as the NTP database) became available for analysis, more accurate assessments of PPV (70-90%) could be made [83; 84]. It is also important to consider that the tissue specificity

for mutagenicity and carcinogenicity of many of the tested mutagens was previously known; this was a significant advantage that would not necessarily be available when TGR assays are used in a true predictive capacity in the absence of prior knowledge. Accordingly, this analysis of the predictivity of TGR assays should be revisited when a more representative dataset is available.

2.5.1 Potential uses of TGR in genetic hazard identification

There are potentially two uses for TGR assays in a genotoxicity testing strategy. A primary use of the assay could be for confirming or refuting *Salmonella* gene mutation results using an *in vivo* test system. Secondarily, TGR assays could be used as the first *in vivo* assay in a test battery. In cases where the results of *in vitro* testing indicate a greater potential for gene mutations than chromosomal aberrations (i.e. mutagenic to *Salmonella*, non-mutagenic to *in vitro* CA), a TGR assay could be substituted for *in vivo* MN to identify whether the chemical causes gene mutations *in vivo*.

The application of the confirmatory approach is demonstrated in the following example. There were 11 cases identified in the TRAID where chemicals had positive results in *Salmonella* and *in vitro* CA, but negative *in vivo* MN results (Table 2-9). Greater weight is placed on *in vivo* tests because extrapolation to humans carries less uncertainty than the *in vitro* models. Since it is inappropriate to use the *in vivo* MN assay to discount *Salmonella* results because these tests assess mechanistically distinct endpoints, a final decision regarding the genotoxicity of the chemical could not be made. The addition of TGR to the test battery for the confirmation of *Salmonella* results correctly predicted carcinogenicity in 8/11 cases. Notably, the *in vivo* MN assay

provided no predictive value in these cases, either because of a false negative genotoxicity result or because these compounds were in fact not clastogenic *in vivo*.

2.5.2 Incidences of discordant *Salmonella* and TGR results

There were 32 chemicals where the results of *Salmonella* and TGR did not agree. These chemicals are listed in Table 2-10. All of them were carcinogenic, with the exception of acetic acid, leucomalachite green, sucrose, 1-nitronaphthalene, 2,6-diaminotoluene, all-trans-retinol and green tea. A review of the quality of the TGR data indicated all studies were well conducted, having adequate administration and sampling times and a sufficient number of animals per group, with the exception of all-trans-retinol, acetic acid and possibly uracil, where the experimental protocol may not have been sufficiently robust. The *Salmonella* mutagenicity data were also questionable for all-trans-retinol.

Among chemicals mutagenic to TGR, there were a number of carcinogens where evidence suggests they act at least partly by indirect mechanisms, such as crocidolite asbestos, oxazepam and Wyeth 14,643, in which an increase in oxidative DNA damage is thought to contribute to carcinogenesis. These indirect mechanisms do not contribute to mutagenicity in *Salmonella*, so a positive *Salmonella* mutagenicity assay was not expected.

Table 2-9: Chemicals for which the standard Salmonella + *in vitro* CA, *in vivo* MN test battery did not provide a clear conclusion.

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
1,2-Dibromoethane	+	+	-	?	+	+
2-Nitro-p-phenylenediamine	+	+	-	?	+	+
Acrylonitrile	+	+	-	?	-	+
Diesel Exhaust	+	+	-	?	+	+
Diethylnitrosamine (DEN)	+	+	-	?	+	+
Dipropylnitrosamine (DPN)	+	+	-	?	+	+
Genistein	+	+	-	?	-	+
IQ	+	+	-	?	+	+
Metronidazole	+	+	-	?	-	+
o-Anisidine	+	+	-	?	+	+
p-Cresidine	+	+	-	?	+	+

Table 2-10: Cases of discordant *Salmonella* and TGR results.

Chemical	*Salmonella*	TGR	Carcinogenicity
Acetic Acid	-	+	-
Acrylamide	-	+	+
Amosite asbestos	-	+	+
Benzene	-	+	+
CC-1065	-	+	+
Comfrey	-	+	+
Crocodolite Asbestos	-	+	+
Cyproterone acetate	-	+	+
Di(2-ethylhexyl)phthalate	-	+	+
Leucomalachite green	-	+	-
Oxazepam	-	+	+
Procarbazine HCl	-	+	+
Sucrose	-	+	-
Tamoxifen	-	+	+
Uracil	-	+	+
Wyeth 14,643	-	+	+
1,2:3,4-Diepoxybutane	+	-	+
1,2-Dichloroethane	+	-	+
1-Nitronaphthalene	+	-	-
2,6-Diaminotoluene	+	-	-
3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)	+	-	+
3-Chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	+	-	+
Acrylonitrile	+	-	+
all-trans-Retinol	+	-	-
Chloroform	+	-	+
Genistein	+	-	+
Green tea	+	-	-
Hydrazine sulfate	+	-	+
Kojic acid	+	-	+
Methyl bromide	+	-	+
Metronidazole	+	-	+
Trichloroethylene	+	-	+

Oxazepam, phenobarbital and Wyeth 14,643 were mutagenic in TGR only after very long treatment times (i.e. 180 days). Sequencing of the mutants observed in colorimetric or positive selection methods identifies those mutants that arose by expansion of a single mutant clone, representing a single mutational event. Eliminating the mutants arising from a single event (clonal correction) yields the mutation frequency, which represents the proportion of independent mutations. Phenobarbital was not mutagenic after clonal correction, while clonal correction confirmed the mutagenic response for oxazepam and Wyeth 14,643. These examples illustrate the importance of sequencing and clonal correction for chemicals that are administered over extended periods.

2.6 Case Studies

There were two carcinogenic compounds where an interpretation of the *Salmonella, in vitro* CA and *in vivo* MN test battery would have lead to the conclusion the compounds were non-genotoxic – a conclusion that was also supported by the TGR assay (Table 2-11). Other instances occurred where carcinogenic compounds were concluded to be genotoxic based on an interpretation of the results of the *Salmonella, in vitro* CA and *in vivo* MN test battery, but were not mutagenic to TGR (Table 2-12). Two carcinogens were mutagenic only to TGR (Table 2-13). These examples are reviewed as case studies in the following sections. These case studies highlight the failure of TGR assays, like other genotoxicity tests, to identify non-genotoxic carcinogens, as well as the importance of robust test protocols and the selection of the correct target tissues for analysis.

2.6.1 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

Kociba *et al* (1978) investigated the carcinogenicity of TCDD administered to Sprague-Dawley rats via the diet. The female rat liver was the primary target, where an increased incidence of hepatic neoplastic nodules was observed. An increase in squamous cell carcinoma of the lung was also noted in females. In addition, an increased incidence of squamous cell carcinoma of the hard palate or nasal turbinates was observed in both sexes [95]. The carcinogenicity of TCDD administered by gavage was also investigated by the National Toxicology Program. TCDD was carcinogenic to Osborne-Mendel rats, inducing follicular-cell thyroid adenomas in males and hepatocellular carcinomas and adrenal cortical adenomas/carcinomas in females. TCDD was also carcinogenic to B6C3F1 mice, inducing hepatocellular carcinomas in males and females and follicular-cell thyroid adenomas in females [96]. Male and female rodents appeared to be affected differently by TCDD. The primary target for TCDD carcinogenicity in female rats was the liver, while male rats were more likely to develop tumours of the thyroid. Other target tissues included the lung (female rat), hard palate and nasal turbinates (male and female rat), thyroid (female mouse), adrenal cortex (female rat), and skin (female mouse).

Although there is clear evidence of the carcinogenicity of TCDD, there is little evidence of genotoxicity on the basis of a large number of studies (for a detailed review, see [97]). TCDD is generally accepted to produce tumours via a non-genotoxic mechanism. The evidence for this conclusion includes the absence of DNA adduct formation and negative results in the majority of genotoxicity tests, tumour formation commonly found in association with increased cell proliferation, and evidence of tumour promotion activity [97; 98].

Carcinogens acting by a non-genotoxic mechanism usually are not detectable with TGR assays [99]. Since most evidence consistently suggests TCDD induces carcinogenesis by a non-DNA reactive (non-genotoxic) mechanism, it is not surprising that TCDD failed to induce gene mutations in *lacI* transgenic rats.

2.6.2 Acetaminophen

Acetaminophen induced hepatocellular carcinomas among male mice administered a diet containing 0.5% or 1.0% (250 or 500 mg/kg/day) for up to 18 months [100]. Administration of 0.5% or 1% (300 or 600 mg/kg/day) in the diet to Leeds strain rats also induced the incidence of hepatocellular neoplastic nodules and bladder tumours among males and females [101]. However, a NTP carcinogenicity study indicated acetaminophen administered continuously in the diet of F344/N rats and B6C3F1 mice for up to 104 weeks at concentrations of 0, 600, 3,000, or 6,000 ppm produced only equivocal evidence of carcinogenicity to female rats (based on increased incidences of mononuclear cell leukemia) and no evidence of carcinogenicity to male rats and male and female mice [102]. Other studies involving mice and rats have suggested acetaminophen lacks carcinogenic initiating activity, but may act as a tumour promoter at hepatotoxic doses (reviewed in [103]).

Table 2-11: Carcinogens that would have been concluded to be non-carcinogenic based on the results of the short-term test battery.

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
2,3,7,8-Tetrachlorodibenzo-p-dioxin	-	-	-	-	-	+
Acetaminophen	-	+	-	-	-	+
Carbon tetrachloride	-	+	-	-	-	+

Table 2-12: Carcinogens that were concluded to be genotoxic based on the standard 3-assay test battery, but were non-mutagenic to TGR.

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
1,2:3,4-Diepoxybutane	+	+	+	+	-	+
3-Chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	+	+	+	+	-	+
Chloroform	+	-	+	+	-	+
Kojic acid	+	+	+	+	-	+
Methyl bromide	+	+	+	+	-	+
Nickel subsulfide	-	+	+	+	-	+
Trichloroethylene	+	-	+	+	-	+

Table 2-13: Carcinogens that were non-genotoxic in all short-term assays, except TGR.

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
Di(2-ethylhexyl)phthalate	-	-	-	-	+	+
Oxazepam	-	-	-	-	+	+

In numerous studies, acetaminophen has not induced gene mutations in bacteria or mammalian cells *in vitro*; however, chromosomal aberrations were induced *in vitro* in CHO cells, V79 cells and human lymphocytes, although more effectively in the absence of S9 and at cytotoxic concentrations (reviewed in [103]). An increase in mutation frequency was also observed in a mouse lymphoma fluctuation test, but the relative contribution of gene mutations or chromosomal aberrations was not determined by colony sizing [104]. *In vivo* chromosomal aberration and micronucleus studies have not provided any consistent evidence of clastogenic activity; however, at high doses a general association of clastogenicity and toxicity was observed, which is consistent with saturation of the low capacity sulfation metabolic pathway and the subsequent cytochrome P450 mediated formation of reactive (genotoxic) intermediates (reviewed in [103]).

Five female *gpt* delta transgenic rats received acetaminophen in the diet at a dose of 525 mg/kg/day for 13 weeks. There was no increase in *gpt* mutation frequency in the liver compared with the negative control. The *gpt* mutation spectra indicated the most common mutations were G:C to A:T transitions (39%), followed by A:T to T:A transversions (34.8%) and G:C to T:A transversions (21.7%). The induction of a greater proportion of G:C to T:A transversions in acetaminophen treated animals compared with controls, suggests that acetaminophen may cause some oxidative DNA damage [105].

Evidence of the carcinogenicity of acetaminophen is not strong, especially at non-hepatotoxic doses. Generally, acetaminophen lacked initiating activity, which was supported by the absence of mutagenic activity in bacterial and mammalian cell models and the lack of clear evidence of clastogenic activity both *in vitro* and *in vivo*.

Acetaminophen was negative in a TGR assay, consistent with the results of most other short-term genotoxicity studies that indicated an absence of genotoxic activity.

2.6.3 Carbon tetrachloride (CCl_4)

A number of carcinogenicity studies of CCl_4 have been conducted. Oral administration of CCl_4 consistently induced hepatomas, as well as hepatocellular adenomas, hepatocellular carcinomas and pheochromocytomas in both sexes of mice (reviewed in [106; 107]). In rats, CCl_4 induced increases in the incidence of neoplastic nodules and hepatocellular carcinomas after oral administration [107]. Increased incidences of hepatocellular carcinomas and hyperplastic nodules accompanied by severe cirrhosis were also observed after subcutaneous administration [108], while hepatocellular adenomas and hepatocellular carcinomas were observed after whole body inhalation exposure (reviewed in [106]). These studies suggest CCl_4 is primarily a liver carcinogen in both male and female mice and rats.

The genotoxicity of CCl_4 has been reviewed extensively [109; 110] and there has been little evidence of genotoxicity. No evidence of carbon tetrachloride mutagenicity to *Salmonella* has been observed in a large number of studies. No evidence of DNA damage, unscheduled DNA synthesis, sister-chromatid exchanges or chromosomal aberrations was apparent *in vitro*, except a weak clastogenic effect in an *in vitro* micronucleus assay with human lymphocytes [111]. In several *in vivo* studies, carbon tetrachloride did not induce unscheduled DNA synthesis, micronuclei, chromosomal aberrations, or aneuploidy. However, binding of carbon tetrachloride to liver cell DNA has been observed in rats, mice and Syrian hamsters *in vivo* (reviewed in [109]). While it appears that the mechanism for carbon tetrachloride induced carcinogenesis could likely

have a non-genotoxic component, there appears to be insufficient evidence to make any definite conclusion.

Tombolan *et al.* [112] assessed the effect of regenerative cell proliferation induced by carbon tetrachloride on the mutagenicity of 5,9-dimethyldibenzo[*c,g*]carbazole in *lacZ* transgenic mice. As a component of this study, five male mice were administered a single 80 mg/kg dose of CCl_4 in corn oil by gavage and were sacrificed 14 days after administration. No significant increase in mutant frequency was observed. Hachiya and Motohashi [113] investigated the liver mutagenicity of carbon tetrachloride in *lacZ* transgenic mice. CCl_4 was administered by gavage to groups of 2 or 3 animals/dose/sampling time at doses of 700 or 1400 mg/kg, and the animals were sacrificed 7 days (1400 mg/kg), 14 days (700 and 1400 mg/kg) or 28 days (1400 mg/kg) after administration. In this case, a statistically significant increase in mutant frequency was observed (a maximum of approximately 2.3x or 56%), but because of high variability in the data, the authors concluded the results lacked biological significance.

The Tombolan study was not specifically designed to assess the mutagenicity of CCl_4; rather, CCl_4 was used as a means to induce cell proliferation in the liver, where CCl_4 mutagenicity was also determined. Animals were treated only once with a small dose. The Hachiya study employed a much higher dose and longer duration prior to sampling. It remains unclear, however, whether the methodological differences alone are the source of the different conclusions reached by these two laboratories.

From this information, it is difficult to conclusively determine if the negative TGR results are a result of the study methodology or are a result of an inability of CCl_4 to

induce gene mutations in general. It is also difficult to assess the performance of TGR in the absence of more concrete mechanistic data. However, it should be noted that the protocols used for both TGR studies did not conform to the subsequently proposed IWGT protocol [65], meaning the experimental conditions may not have been sufficient to observe a response. An additional TGR assay conducted according to the IWGT recommendations would provide more clarity to this situation.

2.6.4 1,2:3,4-Diepoxybutane

Female B6C3F1 mice and Sprague-Dawley rats were exposed to 1,2:3,4-diepoxybutane (DEB) by inhalation to 0, 2.5, or 5.0 ppm, 6 hours/day, 5 days/week for 6 weeks. Following exposure, animals were maintained for 18 months for observation of tumour development. Among exposed rats, a dose-dependent increase in neoplasms of the nasal mucosa was observed in the treatment groups; however, there was no evidence of neoplasia of the nasal mucosa in mice [114].

1,2:3,4-Diepoxybutane is a potent bifunctional alkylating agent (see [115] for review). DEB is a direct acting mutagen to *Salmonella* [116; 117]. Gene mutations at the *hprt* locus were induced by DEB in human TK6 cells exposed *in vitro* [118] and in the splenic lymphocytes of B6C3F1 mice and F344 rats exposed by inhalation to 4 ppm for 6 hours/day, 5 days/week for 4 weeks [33]. DEB induced micronuclei in cultured human lymphocytes [119; 120]and increased the frequency of chromosomal aberrations in human bone marrow cultures [121]. Single i.p. injections of 9-30 mg/kg DEB also increased the frequency of micronuclei in splenocytes and bone marrow of mice and rats in a number of studies (reviewed in [115]). DEB also caused chromosomal aberrations in

the bone marrow cells of mice and Chinese hamsters exposed by inhalation or i.p. injection to doses in the range of 22-34 mg/kg [122].

In contrast to the potent mutagenicity observed in other experimental models, no increase in mutant frequency in the bone marrow was observed two days after female *lacZ* transgenic mice (7-8/group) were administered DEB by i.p. injection at doses of 6, 12 or 24 mg/kg/day, each day for 5 consecutive days (G.R. Douglas, unpublished data). The discrepancy between these results and the potent mutagenic activity observed in *in vitro* assays and in the bone marrow of rodents *in vivo* (at similar doses) cannot easily be reconciled.

2.6.5 3-Chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)

Wistar rats received MX in the drinking water for 104 weeks at concentrations providing average daily doses of 0.4 mg/kg, 1.3 mg/kg or 5.0 mg/kg for males and 0.6 mg/kg, 1.9 mg/kg or 6.6 mg/kg for females. Increases in tumour incidence were observed at all doses, including those which caused no appreciable evidence of toxicity. In particular, MX caused an increased incidence of follicular adenoma and carcinoma in the thyroid glands and cholangioma in the liver. In addition, cortical adenomas of the adrenal glands (both sexes), alveolar and bronchiolar adenomas of the lungs and Langerhans' cell adenomas of the pancreas (males) and lymphomas, leukemias, and adenocarcinomas and fibroadenomas of the mammary glands (females) were increased. These data suggest MX is a potent multi-site rat carcinogen [123].

MX has been studied extensively and there are a large number of publications reporting the genetic toxicology of this compound; it is a strong electrophile and is a relatively potent bacterial mutagen. MX has induced gene mutations in numerous strains

of *Salmonella typhimurium* and *Escherichia coli*, forward mutations at the thymidine kinase locus of mouse L5178Y lymphoma cells and at the *hprt* locus of Chinese hamster ovary and Chinese hamster V79 cells, chromosomal aberrations in Chinese hamster ovary cells, and chromosomal aberrations and micronuclei in mouse L5178Y lymphoma cells and rat peripheral blood lymphocytes; MX has also induced unscheduled DNA synthesis in mouse and rat hepatocytes *in vitro* (reviewed in [124]). However, among *in vivo* models, MX has not demonstrated the same degree of mutagenic activity. Nuclear anomalies were induced in the forestomach and duodenum of mice administered a single dose of MX by gavage at doses of 0.28, 0.37 or 0.46 mmol/kg [125]. Micronuclei were also induced in peripheral blood lymphocytes of rats treated by gavage on three consecutive days with doses of 25-150 mg/kg [126]. However, in other *in vivo* studies MX did not induce micronuclei in polychromatic erythrocytes, bone marrow or the liver of mice receiving single or repeated exposures to doses ranging from 4.4 to 144 mg/kg, or in polychromatic erythrocytes of rats exposed via drinking water for 104 weeks at concentrations from 5.9-70 mg/L (reviewed in [124]). It is apparent from these results that MX has consistently failed to induce genetic damage to the bone marrow; it is also possible that toxicokinetic factors and the rates of mutation fixation and DNA repair also contribute to the discrepancies between tissues.

Groups of five male and female 7-week-old *gpt* delta C57BL/6J transgenic mice were given MX at doses of 0, 10, 30, or 100 ppm in their drinking water for 12 weeks. Immediately thereafter, the mutant frequency in the liver and lungs was assessed using 6-thioguanine and *Spi*$^-$ selection. Further groups of 5 male and 5 female *gpt* delta mice were given 0 or 100 ppm MX for 78 weeks in order to detect neoplastic lesions

histopathologically. There were no increases in mutant frequency in either tissue and there also was no evidence of cell proliferative activity using immunohistochemistry for proliferating cell nuclear antigen or any increase in neoplastic lesions [127]. These results are consistent with those of other *in vivo* models, in which MX has exhibited inconsistent results. MX may not have *in vivo* genotoxic activity of comparable potency to its *in vitro* mutagenicity because of metabolic and other toxicokinetic factors; however, any species-specific differences influencing the mutagenic activity in TGR assays could be ruled out by further studies using transgenic rat models.

2.6.6 Chloroform

Chloroform was administered by gavage to Osborne-Mendel rats and $B6C3F_1$ mice (50/sex/group). A significantly increased incidence of kidney epithelial tumours was found in male rats. The incidence of hepatocellular carcinoma was significantly increased in both sexes of mice and was accompanied by nodular hyperplasia of the liver in many of the male mice that had not developed hepatocellular carcinomas [128]. Lifetime administration of chloroform in drinking water also increased the yield of renal tubular adenomas and adenocarcinomas in male Osborne-Mendel rats in a dose-related manner, but failed to increase the incidence of hepatocellular carcinomas in female $B6C3F_1$ mice [129] and, in addition, significantly increased incidence of hepatic neoplastic nodules in female Wistar rats [130]. Exposure by inhalation also lead to an increase in renal cell adenomas and carcinomas in male BDF_1 mice at the highest exposure level, but it was noted that the inhaled dose likely exceeded the MTD (reviewed in [131]).

Ten female *lacI* transgenic B6C3F$_1$ mice per group were exposed to chloroform within an inhalation chamber at concentrations of 0, 10, 30 and 90 ppm for 10, 30, 90 or 180 consecutive days, so that mice would be exposed to non-hepatotoxic, borderline hepatotoxic or substantially hepatotoxic concentrations leading to regenerative cell proliferation. Long term exposures were included in the event that mutations secondary to cytotoxicity required longer exposure periods to become manifest. Following the final exposure, animals remained untreated for 10 days to allow for fixation of mutations and to allow clearance of remaining chloroform from the tissues prior to isolation of genomic DNA. No significant chloroform-induced increases in the mutant frequencies were observed in any exposure group at any time point, suggesting chloroform lacks mutagenic activity in the liver of female B6C3F$_1$ *lacI* transgenic mouse [132].

Chloroform has induced gene mutations in *Salmonella*, Chinese hamster V-79 cells, as well as mouse lymphoma L5178Y cells. It also induced sister chromatid exchanges in cultured human lymphocytes and in mice *in vivo*. An increased frequency of micronucleated kidney cells was observed in rats orally administered chloroform at 4 mmol/kg [133]. In addition, chloroform induced chromosomal aberrations in rat bone marrow cells *in vivo* after repeated 5 day oral administration or a single intraperitoneal injection at a dose of 1 mmol/kg [134]. While not providing evidence of genotoxicity, chloroform also induced a significant increase S-phase synthesis in mouse liver following *in vivo* treatment, an indirect indicator of hepatocellular proliferation [135].

Although chloroform has induced gene mutations and chromosomal aberrations in several test systems, it did not induce mutations of the *lacI* gene in the liver of transgenic mouse in a well conducted study. This underscores the importance of the test

battery in short-term genotoxicity testing, since one test system may not detect all genotoxicants.

2.6.7 Kojic acid

Male and female B6C3F1 mice were administered 0, 1.5 or 3.0% kojic acid (KA) in the diet beginning at 6 weeks of age for 20 months. Thyroid weights were significantly increased in both sexes and were accompanied by a markedly increased incidence of diffuse hyperplasia and follicular adenomas. The serum free triiodothyronine (T3) levels in both sexes receiving 3.0% after 6 months was significantly lower than the controls, while the serum thyroid-stimulating hormone (TSH) levels were transiently higher. Dietary administration of KA induces thyroid adenomas in male and female B6C3F1 mice, which was speculated to occur via a non-genotoxic mechanism involving alteration in serum free T3 and TSH levels [136].

KA was a weak mutagen to *Salmonella*, exhibiting mutagenic activity only at concentrations greater or equal to 1 mg/plate [137-140]. KA failed to induce biologically relevant, dose responsive and reproducible increases of the mutant frequency at the *hprt* locus of L5178Y mouse lymphoma cells [137]. Chromosomal aberrations were induced by KA in Chinese hamster ovary cells at concentrations of 3000 μg/mL and above in the presence or absence of S9 [138], but were not induced subsequently in Chinese hamster V79 cells at lower concentrations, except for a very slight increase at 1000 μg/mL in cultures treated continuously for 18 or 28 hours [137]. Micronuclei were not induced by KA in human keratinocytes, even where marked toxicity was induced; however, an increased frequency of micronuclei was induced in human hepatoma cells, associated with severe cytotoxicity at concentrations above 6000 μg/mL [137]. *In vivo*, KA did not

induce a significant increase in the frequency of micronucleated hepatocytes but did increase the frequency of micronucleated reticulocytes isolated from groups of male rats administered 1000 or 2000 mg/kg [141]. Other investigators have failed to induce an increased frequency of micronucleated polychromatic erythrocytes upon i.p. administration of KA to NMRI mice at doses up to 750 mg/kg [137]. KA also did not cause an increase in unscheduled DNA synthesis in the liver of male rats administered single oral doses of KA at 1500 mg/kg (2 and 16 h evaluation) or 150 mg/kg (16 h evaluation) [137].

KA was administered orally to groups of 10 male *lacZ* transgenic mice at doses of 0 (corn oil), 800 or 1600 mg/kg/day (the experimentally determined MTD) for 28 days. Seven days after the final dose, the animals were euthanized and the livers were removed for analysis. No increase in mutant frequency was observed in the liver at either dose compared with the negative control [137].

Overall, KA has induced an inconsistent pattern of results among short-term genotoxicity tests. Furthermore, there is some evidence to suggest the carcinogenicity of KA is at least partly mediated by perturbation of thyroid hormone homeostasis. The relative contribution of non-genotoxic and genotoxic mechanisms to the observed carcinogenicity of KA has not been clearly demonstrated; any such investigations should include further studies in TGR models using other tissues, including thyroid and bone marrow, to determine if the absence of mutagenic activity observed in the liver is indicative of a failure of the assay or a failure to select the appropriate target tissue.

2.6.8 Methyl bromide

Oral administration of methyl bromide to rats for 90 days produced squamous cell carcinomas of the forestomach and a marked diffuse hyperplasia of the epithelium of the forestomach [142]. However, subsequent examination by an NTP panel concluded that the forestomach lesions represented inflammation and hyperplasia rather than malignant lesions [143]. Additional studies found inflammation, acanthosis, fibrosis, and a high incidence of pseudoepitheliomatous hyperplasia in treated animals that regressed upon cessation of treatment [144], which suggests any carcinogenic activity to the rodent forestomach may be mediated through an irritant mechanism. No evidence of carcinogenicity was observed following administration of methyl bromide fumigated diets to rats for up to 2 years [145].

Inhalation is the most relevant route of human exposure to methyl bromide, as it is extensively used as a fumigant for disinfestation of food products. A NTP study of mice exposed by inhalation to methyl bromide for up to 103 weeks found no evidence of carcinogenicity [146]. There was also no evidence of carcinogenicity when rats were exposed by inhalation for 29 months [147]. This evidence suggests considerably uncertainty remains regarding the carcinogenic activity of methyl bromide. The International Agency for Research on Cancer has recognized this uncertainty, classifying methyl bromide as having limited evidence of carcinogenicity in experimental animals and inadequate evidence in humans [148].

Groups of 6 male *lacZ* transgenic mice were treated by gavage with single doses of 5 or 12.5 mg/kg methyl bromide in corn oil, or with 25 mg/kg every 24 hours for 10 days. Following the final exposure, animals used for DNA adduct analysis were

sacrificed after 4-6 hours, while those used for mutant frequency analysis were held without treatment for 14 days to allow a period for fixation of mutations. Animals receiving a single dose of 5 mg/kg did not exhibit any O^6-meG adducts, though animals receiving a single dose of 12.5 mg/kg exhibited O^6-meG adducts in the liver, spleen and lung. Following multiple doses of 25 mg/kg, animals exhibited adducts in liver, spleen lung, forestomach, glandular stomach, bone marrow and blood leukocytes. No increases in the *lacZ* transgene mutant frequency in the liver, spleen, lung and bone marrow were apparent after the single 12.5 mg/kg dose, or in the liver and glandular stomach following 10 consecutive daily doses of 25 mg/kg. Despite forming O^6-meG adducts in a wide range of tissues, methyl bromide did not induce mutations in the *lacZ* transgene in the liver or glandular stomach of transgenic male mice [149].

Methyl bromide has demonstrated conclusive genotoxic activity. It has produced gene mutations in *Salmonella* and in the mouse lymphoma assay, induced micronuclei in polychromatic erythrocytes of both mouse and rat, and produced sister-chromatid exchanges in cultured human lymphocytes and in the bone marrow cells of exposed mice (reviewed in [143]). Methyl bromide has also been found to cause systemic DNA methylation [150].

The absence of evidence of mutagenicity in TGR, despite clear evidence of genotoxicity in other assays is not easily reconciled. Slightly fewer animals than is considered ideal were used in the TGR assay for the determination of mutant frequency in the glandular stomach and a sampling time of 14 days may be slightly short for determining mutant frequency in the liver. Despite these minor issues, the absence of

mutations of the *lacZ* gene in liver and glandular stomach is quite conclusive. Further investigation is likely warranted.

2.6.9 Nickel subsulfide

The carcinogenicity of nickel subsulfide was investigated by the NTP. Rats and mice were exposed to nickel subsulfide by inhalation for 6 hours per day, 5 days per week for 2 years. Clear evidence of carcinogenicity was noted in rats, based on increased incidences of alveolar/bronchiolar adenoma or carcinoma in both sexes and on increased incidences of benign (males and females) or malignant (males) pheochromocytoma of the adrenal medulla. There was no evidence of carcinogenicity in male or female mice [151]. Nickel subsulfide was also demonstrated to be carcinogenic in other tissues resulting from routes of administration other than inhalation (reviewed in [152])

Mayer [153] investigated the mutagenic potential of nickel subsulfide. Groups of 6-8 *lacZ* transgenic mice and groups of 4-5 *lacI* transgenic rats were treated by a single nose only inhalation exposure (10 mg/kg for mice, 6 mg/kg for rats) for 2 hours. Following an expression period of 14 days, the mutant frequency in lung and nasal mucosa was determined. No evidence of an increased mutant frequency was apparent, suggesting nickel subsulfide was not mutagenic in the lung and nasal mucosa of *lacZ* transgenic mouse and *lacI* transgenic rat.

Although nickel subsulfide was not mutagenic to *Salmonella*, it did induce a significant increase in the frequency of chromosomal aberrations in human peripheral blood lymphocytes *in vitro* as well as an increased frequency of micronuclei in mouse polychromatic erythrocytes *in vivo* [154].

Oxidative DNA damage has been proposed as a potential mechanism for nickel subsulfide carcinogenicity. Two mechanisms for nickel subsulfide-induced oxidative damage have been proposed: nickel subsulfide could react with endogenous or nickel subsulfide-produced hydrogen peroxide to produce reactive oxygen species that directly damage DNA, or the release of reactive oxygen species from phagocytes in response to nickel subsulfide-induced inflammation could cause indirect DNA damage [155]. Further mechanistic work by Lee *et al* (1995) demonstrated a transgenic Chinese hamster cell line was susceptible to inactivation of the *gpt* target gene by nickel subsulfide; inactivation did not appear to occur through gene mutation, but through mechanisms such as DNA methylation that silenced *gpt* expression [156]. These studies, and the results of *in vitro* and *in vivo* chromosomal aberration studies, suggest the carcinogenic activity of nickel subsulfide could be mediated by both genotoxic and non-genotoxic mechanisms. However, since oxidative DNA damage is mutagenic, and appears to be detectable using a TGR assay [87; 89], it is reasonable to question whether there was sufficient exposure to nickel subsulfide to produce DNA damage. Although the nickel distribution in the respiratory tract of treated rats was about 400 times greater than in control animals, the ability of nickel subsulfide to produce DNA damage at the target site may have been low *in vivo* because of increased clearance and various protective mechanisms within the lungs [153]. Thus, TGR-detectable gene mutations *in vivo* may play a less important role in the carcinogenicity of nickel subsulfide than one would be lead to believe from the results of *in vitro* studies.

2.6.10 Trichloroethylene (TCE)

A number of carcinogenicity studies of TCE have been conducted. An initial National Cancer Institute study found an increased incidence of hepatocellular carcinomas in male and female mice but no evidence of an increased incidence of neoplastic lesions in male or female rats following gavage administration for 78 weeks [157]. Further study of TCE administered by gavage found inadequate evidence to evaluate carcinogenicity in male rats because of reduced survival and no evidence of carcinogenicity to female rats after 2 years of administration. Trichloroethylene, however, was again carcinogenic to mice, causing increased incidences of hepatocellular carcinomas in males and females and hepatocellular adenomas in females [158].
The ability of trichloroethylene to induce gene mutations and small deletions was examined using male and female *lacZ* transgenic mice. Animals were exposed to TCE in a whole body inhalation chamber at concentrations of 0, 203, 1153 or 3141 ppm, 6 hours/day for 12 days. Following the exposure period, animals were held without treatment for a 14 or 60 day fixation period. The *lacZ* mutant frequency was determined in bone marrow, kidney, spleen, liver, lung and testicular germ cells. No increased mutant frequency was observed in the lungs of animals 14 days after exposure, nor were increases in the mutant frequency observed in any of the examined tissues sampled 60 days after exposure. Inhalation exposure to TCE did not induce gene mutations or small deletions in *lacZ* transgenic mice [159].

TCE has exhibited evidence of genotoxic activity in several test systems. Covalent binding to DNA has occurred in the presence of liver microsomes *in vitro* but little DNA binding occurred *in vivo*. TCE is a weak inducer of sister chromatid

exchanges in mammalian cell culture and can produce DNA single strand breaks *in vivo*. Several studies have shown some weak mutagenic activity in *Salmonella*, but the evidence overall suggests it is not a potent mutagen. TCE was also weakly mutagenic in the mouse lymphoma assay and produced sister chromatid exchanges in the presence of metabolic activation, as well as aneuploidy at high concentrations *in vitro*. It did not induce structural chromosomal aberrations *in vitro* or *in vivo*, but caused an increase in micronuclei in mammalian cell culture and in bone marrow cells of rats *in vivo* (reviewed in [160]).

From the results in *Salmonella*, it seems that TCE is unlikely to cause significant induction of gene mutations. However, evidence of sister chromatid exchanges and aneuploidy *in vitro* and micronuclei in bone marrow cells *in vivo* would suggest that recombination and aneuploidy could play roles in the carcinogenicity of TCE, as has been speculated by several authors [159-161]. Since genotoxic effects other than gene mutations and small deletions are not detectable with TGR, the results of the study with *lacZ* transgenic mice can be taken as further evidence that TCE is not primarily a gene mutagen.

2.6.11 Di(2-ethylhexyl)phthalate (DEHP)

Male and female F344 rats and B6C3F$_1$ mice (50/group/sex) were administered DEHP in the diet for 103 weeks. A significantly higher incidence of hepatocellular carcinomas in female rats and male and female mice was observed. Male rats had significantly increased incidence of hepatocellular carcinomas or neoplastic nodules [162]. The liver carcinogenic activity of DEHP to rats was also demonstrated [163; 164],

however no carcinogenic activity was observed following intraperitoneal or inhalation exposure of Syrian golden hamsters [165].

The mutagenicity of DEHP administered in the diet was investigated using female *lacI* transgenic mice. Groups of three mice received either 3000 or 6000 mg/kg feed for 120 days. No significant increase in mutant frequency was observed in either group and no evidence of an increased rate of cell division was observed based on the labelling index for incorporation of BrdU into DNA. This would indicate that gene mutations were not produced at dose levels below that inducing hepatocyte cell division. However, the authors suggest, based on Melnick [166], that any mitogenic effect may not have been sustained throughout the treatment period [99].

In numerous short-term studies, DEHP has not demonstrated clear genotoxic activity. No evidence of DEHP-induced gene mutations in *Salmonella* or in mammalian assays was apparent. There was also no consistent evidence of DNA binding, induction of unscheduled DNA synthesis or single strand breaks, chromosomal aberrations, micronuclei or sister-chromatid exchanges in a variety of assays, both *in vitro* and *in vivo* (reviewed in [167; 168]).

As there is no clear evidence of genotoxicity, DEHP does not appear to act as an initiator of carcinogenesis. There is broad agreement that DEHP is, instead, a promoter whose rodent carcinogenic activity is mediated primarily (but not necessarily exclusively) via activation of the hepatic peroxisome proliferator activated receptor (PPAR). PPAR activation leads to enhanced cell proliferation (a mitogenic, rather than regenerative response) and also to increased oxidative stress, since peroxisomes are the primary site for hydrogen peroxide generation within hepatocytes. The absence of evidence of

DEHP-induced gene mutations in numerous studies would suggest that oxidative stress contributes proportionately less to the carcinogenicity of DEHP than does increased cell proliferation.

2.6.12 Oxazepam

Swiss-Webster mice were administered oxazepam in the diet for 57 weeks. The incidence of hepatocellular adenomas and carcinomas was increased in exposed mice. The incidence of eosinophilic foci was also increased and there was evidence of increased centrilobular hepatocyte hypertrophy. $B6C3F_1$ mice were administered oxazepam in the diet for 2 years. Clear evidence of carcinogenicity was evident, based on an increase in hepatoblastoma, hepatocellular adenomas and carcinomas in males and females. This was accompanied in both sexes by moderate hypertrophy of the centrilobular hepatocytes and an increase in the incidence of follicular cell hyperplasia of the thyroid gland, as well as thyroid gland follicular cell adenoma in females. The liver was the primary target of oxazepam carcinogenesis for mice [169].

An additional study investigated the carcinogenicity to F344 rats of oxazepam administered in the diet for 2 years. Only male rats showed a significant increase in the incidences of renal tubule adenoma and hyperplasia. It was concluded there was equivocal evidence of oxazepam carcinogenicity to male rats and no evidence in female rats [170].

Eight *lacI* transgenic mice per group were administered 0 or 2500 ppm oxazepam daily for 180 days in the feed. At the termination of the exposure period, all mice were immediately sacrificed for the determination of mutant frequency. Oxazepam induced a significant increase in mutant frequency in the liver, which remained significant after

sequencing for clonal correction. It was speculated that the significant increase in the percentage of G:C to T:A and G:C to C:G mutations may have resulted from oxidative damage, since these mutations have been known to arise from oxidative conversion of guanine to 8-oxoguanine. This study demonstrated that the *lacI* transgenic mouse assay may have the potential to detect mutations that are not detected by other assays, and that dietary administration can be an appropriate route of administration. The authors suggest that longer term administration may be necessary for carcinogens that have weak or no mutagenic effects *in vitro* [89]

Additional work examined the mutagenicity of oxazepam at the *cII* locus in transgenic mouse liver using similar experimental methods. Oxazepam was administered in the diet at concentrations of 0 or 2500 ppm for 180 days. Again, there was a significant increase in mutant frequency in the liver, which remained significant after sequencing for clonal correction. No significant differences were observed between the mutation spectrum of the treatment group and the control group, though a large increase was noted in the percentage of G:C to A:T transitions at CpG sites [88].

Oxazepam was not mutagenic in *Salmonella* or in the mouse lymphoma assay. However, a dose dependent increase in micronuclei was found in Syrian hamster embryo fibroblast, human amniotic fluid fibroblast-like and mouse L5178Y cell lines. Whole chromosomes or centric fragments as well as acentric fragments were observed in the micronuclei, suggesting both a clastogenic and aneugenic effect could have been the cause (reviewed in [171]). In NTP genetic toxicity studies, oxazepam was not mutagenic to *Salmonella*, did not induce sister chromatid exchanges or chromosomal aberrations in

cultured Chinese hamster ovary cells and did not cause an increase in the frequency of micronucleated peripheral blood erythrocytes in $B6C3F_1$ mice treated for 14 weeks [169].

It appears that chronic administration was required to detect a mutagenic response in *lacI* transgenic mouse. If the primary mechanism involved in oxazepam mutagenicity is oxidative damage, then it is not surprising that an administration time long enough to induce this damage would be necessary. Long term administration is not part of a typical study design, and it is likely no mutagenic effect would have been detected in a study conducted in accordance with the IWGT recommended protocol [65]. For chemicals that are weak mutagens based on *in vitro* testing, or for those that are thought to be indirectly mutagenic by mechanisms such as oxidative DNA damage, consideration of longer administration times, as proposed by Shane [89], may have some merit. Further investigation will be required.

2.6.13 Case Study Conclusions

The cases described in the previous sections lead to several conclusions regarding the performance of the TGR assays. TGR assays are gene mutation tests that identify compounds causing genotoxicity; thus, compounds that are carcinogenic primarily by non-genotoxic mechanisms will typically not be identified using TGR assays. In addition, test protocols that do not employ a sufficiently long treatment period may fail to induce enough mutations to provide sufficient sensitivity. Likewise, a sampling time that is too short for the particular tissue of interest may also fail to provide sufficient sensitivity. Although TGR assays allow the selection of any tissue for analysis, determining whether a compound is mutagenic also requires the analysis of the correct target tissue.

Chapter 3

Characterization of Treatment and Sampling Time Effects in a Transgenic Rodent Gene Mutation Model in Relation to the Development of a Standardized Experimental Protocol

3.1 Introduction

The involvement of gene mutations in the development of cancer, genetic disease and developmental abnormalities is widely accepted. Historically, the lack of practical and efficient *in vivo* gene mutation assays has hindered investigations aiming to identify those compounds expected to cause mutation and the mechanisms by which these mutations are induced. Although the currently used *in vitro* gene mutation models can provide reliable and valuable information, they lack the ability to mimic the complex biological processes and interactions of *in vivo* systems. These factors include absorption, metabolism, tissue distribution, rates of excretion and interactions with biological macromolecules, among others. The development of transgenic rodent (TGR) gene mutation assays has provided a potential solution to this missing component in genotoxicity testing and mechanistic research. TGR mutation assays offer significant advantages to existing *in vivo* gene mutation tests, including the ability to determine mutant frequencies and mutation spectra within any tissue of interest, such as those known to be cancer target tissues, using a relatively small number of animals.

The large volume of published TGR assay data was recently reviewed by Lambert *et al* who concluded the assays have the appropriate characteristics for use in regulatory applications [70]. However, one of the major hurdles remaining before widespread acceptance can be achieved is the development of an internationally accepted harmonized test guideline. Test guidelines provide a recommended experimental protocol that is known to be sufficient to characterize the endpoint of interest in a reliable and reproducible manner by testing laboratories. To facilitate the formation of a guideline, the impact of key experimental parameters on the observed responses of the assay to

mutagenic compounds must be more thoroughly understood. Of these parameters, the administration time and sampling time are expected to have the most impact on the assay results. Based on the genetic neutrality of mutations occurring at transgenic loci [172], the effects of multiple treatments are believed to be additive. As a result, the length of the treatment period (and by extension, the total dose administered) is critical to allow the induced mutant frequency to increase to the point at which the mutant frequency nears a maximum, or at least reaches a detectable level. After exposure, there are a number of events that may occur to influence the rate and extent to which mutations are induced, including toxicokinetic factors, DNA repair, fixation of mutations and cell division. Sampling time, the interval between the end of the treatment period and sacrifice, is the period during which these events may occur. Evidence suggests that the sampling time necessary to allow the manifestation of the maximum mutant frequency varies between tissues in relation to the turnover time of the cell population [173; 174]. Sampling time is, therefore, a critical experimental variable if the assay is to exhibit acceptable sensitivity when analyzing multiple tissues collected at the same time.

There have been several studies characterizing the relationships between treatment duration, sampling time and mutant frequency, but very few that have examined the effects of longer administration times combined with various sampling times [70]. Such experiments are critical to determine the extent of any additive effect, differences between tissues and the long term stability of induced mutant frequencies during the post-treatment period. However, most studies have only examined the effect of sampling time on the mutant frequency following a single administration of a strong mutagen [70]. If the goal of a particular study is simply to allow differentiation of strong

mutagens from non-mutagens, there appears to be little advantage of repeated dosing, since many of these strong mutagens induce an obvious increase in mutant frequency after a single administration and short sampling times. It has been hypothesized, however, that for weak mutagens, repeated dosing will be required in order to allow mutations in the genetically neutral transgene to accumulate to a detectable level [64; 65]. Experimental protocols that involve repeated dosing inherently incorporate varying sampling times for each dose interval, as daily administration for a period of *n* days in effect constitutes a series of *n* single doses, each with a sampling time equal to the remaining days until sacrifice. This may permit the development of a generalized experimental protocol where repeated-dosing for a period equivalent to the optimal sampling time for slowly proliferating tissues may allow an accumulation of mutations and provide the sensitivity necessary to detect mutations induced by weak environmental mutagens.

The subject of generalized protocols for TGR assays was discussed at two meetings of the International Workshop on Genotoxicity Testing (IWGT) held in 1999 and 2002. The discussions at these meetings lead to the recommendation, based on both theoretical considerations and limited experimental data, that a generalized protocol intended for genotoxicity evaluation should optimally involve an administration time of 28 days and a sampling time of 3 days [65].

The purpose of the following work is to experimentally challenge this IWGT protocol recommendation. The effects of repeated dosing over various administration times, and the effects of shorter or longer sampling times, on the mutant frequency induced by the weak environmental mutagen urethane (ethyl carbamate) has been

characterized in tissues with differing rates of proliferation. Based on the recommendation that one rapidly dividing tissue, one more slowly dividing tissue, and one tissue with an intermediate rate of proliferation should be sampled [70], this experiment focused on bone marrow, liver and small intestine. The resulting data are used to draw inferences regarding the general applicability of the IWGT recommendation for the testing of compounds with mutagenic potency markedly lower that the strong mutagens which have provided much of the data on which the protocol recommendations are based.

3.2 Materials and Methods

3.2.1 Chemicals

Urethane (CAS# 51-79-6; purity >99%), N-ethyl-N-nitrosourea (CAS# 759-73-9; purity 67.8%, stabilized with acetic acid) and phenyl beta-D-galactopyranoside (CAS# 2818-58-8; purity >98%) were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Urethane was dissolved in sterile Milli-Q® filtered water (Milli-Q Synthesis System; Millipore, Mississauga, ON, Canada), while N-ethyl-N-nitrosourea (stored at -30°C) was dissolved in 1/15 M phosphate buffer (pH 6) immediately before use. Proteinase K (CAS# 39450-01-6; $\geq$ 20 U/mg) was obtained from Invitrogen Corporation (Burlington, ON, Canada) and stored at 4°C until use.

3.2.2 Animals

Male *lacZ* transgenic mice (Muta™Mouse, strain 40.6 [58]) were obtained from a breeding colony maintained by the Animal Resources Division, Health Canada (Ottawa, ON, Canada). During the in-life phase of the study, animals were singly housed in

polypropylene cages within flexible-film isolators (Harlan Isotec, Blackthorn, Bicester, UK) maintained in a level 2 barrier animal facility. The room was temperature controlled and the lighting was automatically set to provide a 12 hour light/dark cycle. Animals were provided with wood shavings for bedding, nesting material, and were allowed free access to food and tap water at all times. Animals were maintained, treated and euthanized under conditions approved by the Health Canada Animal Care Committee (Protocol # 2004-027) and in accordance with the Canadian Council on Animal Care guidelines.

3.2.3 Preliminary Dose Range-Finding Study

Twenty mice (age 21 weeks) were randomly allocated across four dose groups (5 mice per group). Animals were administered urethane by gavage at doses of 0 (sterile Milli-Q water), 100, 200 or 450 mg/kg/day for 28 consecutive days, with the dose volume for each animal determined based on its bodyweight on the day of dosing (dose volume 5 mL/kg). The maximum tolerated dose (MTD) was defined as the highest dose that did not cause treatment-related mortality or a decrease in bodyweight exceeding 10% relative to the control group.

3.2.4 Experimental Design and Treatment

Ninety-seven mice (age 12 weeks) were obtained and allocated across groups by random number generation, with minor adjustment to ensure the mean bodyweight difference between groups was within 10% and to ensure that siblings were separated, to the extent possible. The study was designed in a hierarchical manner with five major groups each consisting of 2-3 dose subgroups plus negative and positive controls. Each major group corresponded to a different treatment or sampling time, as follows: (a) 7 day

treatment period, 3 day sampling time; (b) 7 day treatment, 28 day sampling time; (c) 28 day treatment, 3 day sampling time; (d) 28 day treatment, 28 day sampling time; (e) 56 day treatment, 3 day sampling time. Dose subgroups were selected to ensure that 5-7 mice in each group were administered urethane by gavage at the MTD or at 2-3 lower doses selected to ensure there was overlap between groups with respect to the total dose administered.

Urethane was administered daily for the duration of the treatment period in sterile Milli-Q water at a dose volume of 5 mL/kg, based on the bodyweight of each animal on the day of administration. Vehicle control group animals were administered sterile Milli-Q water daily at a dose volume of 5 mL/kg. The positive control, N-ethyl-N-nitrosourea (ENU) dissolved in 1/15 M phosphate buffer (pH 6) was administered once to a single animal per group by intraperitoneal injection seven days before euthanasia (a sampling time of 7 days). The experimental design is detailed in Table 3-1.

At the conclusion of the treatment and sampling periods, the mice were euthanized by cervical dislocation. The liver was removed, cut into two roughly equal sections and flash frozen in liquid nitrogen. Bone marrow was flushed from both femurs into a microcentrifuge tube using a 1 mL syringe filled with phosphate buffered saline (PBS); the suspension was pelleted and resuspended in 1 mL PBS, split into two samples and frozen in liquid nitrogen. The small intestine was removed, flushed with approximately 3 mL PBS, cut longitudinally and frozen in liquid nitrogen. All tissue samples were stored at -80°C until DNA isolation.

Table 3-1: Outline of experimental design

Group	Treatment Duration (days)	Sampling Time (days)	Dose (mg/kg/day)	Number of Animals	Total Dose (mg/kg)
A	7	3	0 (vehicle)	5	0
	7	3	25	5	175
	7	3	100	6	700
	1	7	100 ENU	1	100 ENU
B	7	28	0 (vehicle)	5	0
	7	28	25	5	175
	7	28	100	5	700
	1	7	100 ENU	1	100 ENU
C	28	3	0 (vehicle)	6	0
	28	3	25	5	700
	28	3	100	5	2800
	1	7	100 ENU	1	100 ENU
D	28	28	0 (vehicle)	6	0
	28	28	25	7	700
	28	28	100	6	2800
	1	7	100 ENU	1	100 ENU
E	56	3	0 (vehicle)	6	0
	56	3	12.5	6	700
	56	3	50	7	2800
	56	3	100	7	5600
	1	7	100 ENU	1	100 ENU

3.2.5 DNA Isolation

High molecular weight genomic DNA was isolated from bone marrow, liver, and small intestine using a modified phenol/chloroform isolation method, adapted as described. Tissue samples were handled in a block, whereby the bone marrow, liver or small intestine samples from all the animals in each group (A-E) were processed on the same day.

3.2.5.1 Bone Marrow

Frozen bone marrow from each animal was thawed in 4.5 mL lysis buffer (10 mM Tris, pH 7.6; 10 mM EDTA; and 100 mM sodium chloride) with proteinase K (1 mg/mL final volume) and 0.5 mL 10% sodium dodecyl sulfate (SDS) and allowed to digest overnight (12-18 hours) at 37°C.

Genomic DNA was isolated by phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by chloroform/isoamyl alcohol (24:1) extraction; the DNA was precipitated by addition of 2 volumes of absolute ethanol, and then spooled out on the end of a sealed glass Pasteur pipette. After washing in 70% ethanol, the DNA was dissolved in TE buffer (10 mM Tris, pH 7.6; 0.1 mM EDTA). The DNA concentration of each sample was estimated by determining the optical density at 260 nm (A_{260}) and adjusted where necessary to approximately 1 μg/μL.

3.2.5.2 Liver

Approximately one-half of a partially thawed liver was transferred to a conical glass tube containing approximately 2 mL of cold (4°C) TMST buffer (50 mM Tris, pH 7.6; 3 mM magnesium acetate; 250 mM sucrose; 0.2% Triton X-100) and homogenized using a tissue homogenizer equipped with a Teflon pestle (approximately 6-12 rotations)

in order to release intact nuclei. The samples were centrifuged (600 x g, 6 minutes at 4°C), and the pellet was washed twice with cold TMST buffer. The nuclei pellet was resuspended in lysis buffer and 1 mg/mL proteinase K plus 0.5 mL 10% SDS was added immediately. The contents were allowed to digest overnight (12-18 hours) at 37°C. Phenol/chloroform DNA isolation was conducted in an identical manner as for the bone marrow.

3.2.5.3 Small Intestine

7/3, 7/28 and 28/3 Groups

The whole small intestine from each animal was placed in 1 mL 75 mM KCl/20 mM EDTA solution on a plate until completely thawed (1-2 minutes), and then was drawn rapidly in and out of a 1 mL syringe (with the needle removed) in order to strip off the epithelial cell layer (a procedure similar to that described by Tao, *et al.* [49]. The tissue remaining after stripping was discarded and the epithelial cell suspension was allowed to digest at 37°C overnight in approximately 4.5 mL lysis buffer, 1 mg/mL proteinase K and 0.5 mL 10% SDS. Phenol/chloroform DNA isolation was conducted in an identical manner as for the bone marrow.

28/28 and 56/3 Groups

Because of the poor packaging efficiency observed with DNA samples isolated from Groups A-C, the tissue preparation and DNA isolation methods were altered in an attempt to reduce the activity of any endogenous nucleases and the potential for mechanical stress, which may have sheared the DNA into fragments too small to allow the lambda shuttle vector to be rescued. The frozen intestine was placed in 1 mL hypotonic 75 mM KCl/20 mM EDTA solution on a small plastic plate and gently

manipulated with forceps to dislodge the epithelial cell layer. Cell suspensions were incubated overnight at 37°C in 5 mL of lysis buffer containing 2 mg/mL proteinase K and 2% SDS. DNA was isolated by one phenol/chloroform/isoamyl alcohol (25:24:1) extraction, followed by two chloroform/isoamyl alcohol extractions and precipitation with 2 volumes of absolute ethanol in a -30°C freezer for ~30 minutes. The DNA was re-dissolved in TE buffer (10 mM Tris, pH 7.6; 0.1 mM EDTA).

3.2.6 Transgene Recovery and Identification of Mutants

Single copies of the λgt10 shuttle vector were recovered from the genomic DNA by *in vitro* packaging into lambda phage heads using commercially available packaging extracts (Transpack®, Stratagene, La Jolla, CA, USA). Packaging reactions were conducted as recommended by the manufacturer, except that 1-4 μL DNA was incubated with 4.8 μL packaging extract.

An overnight culture of *E. coli* C (Δ*lacZ*, *galE*⁻, *recA*-, pAA119) was established in LB medium containing 0.2% maltose, 25 μL ampicillin and 20 μL kanamycin. A subculture, containing a 1/100 dilution of the overnight culture in LB, was prepared the next morning and was incubated at 37°C in a rotary bath. After 4 hours, the cells were pelleted and resuspended in ½ volume LB containing 10 mM $MgSO_4$. Each packaged phage suspension was allowed to adsorb to 2 mL of the bacterial culture at room temperature for 30 minutes.

To estimate the total number of plaque-forming units (pfu) per sample, 15 μL of the transformant cells was removed and added to 2 mL bacteria without adsorbed phage; 32 mL top agar (0.5% LB, 0.75% agar, 0.64% NaCl, 10 mM $MgSO_4$) was then added and the suspension was divided on four 9 cm diameter plates (8 mL/plate) containing 8 mL

bottom agar (0.5% LB, 0.75% agar, 0.64% NaCl). To select for mutants, 32 mL top agar containing 0.3% phenyl beta-D-galactopyranoside (p-gal) was added to the remaining transformant cells and the contents were distributed (8 mL/plate) onto plates containing 8 mL bottom agar. The plates were incubated overnight at 37°C and scored manually the following day. The mutant frequency is the number of plaques observed on selective media divided by the number of plaque-forming units estimated on non-selective plates.

3.2.7 Statistical Analysis

3.2.7.1 Dose Range-Finding Study

The One-Sample Kolmogorov-Smirnov test procedure was used to compare the observed cumulative distribution function for animal terminal bodyweight with a theoretical normal distribution in order to indicate whether this variable is normally distributed. Homogeneity of variance was confirmed with Levene's statistic. One-way ANOVA was then used to analyze terminal bodyweights. Where a significant difference was observed ($p<0.05$), Dunnett's post-hoc test was used to compare multiple treatment groups to the control.

3.2.7.2 Mutant Frequency Analysis

A generalized mixed model was applied using the GLIMMIX macro [175] from SAS (SAS/STAT, SAS Institute, Cary, NC, USA) that models binary outcomes through the SAS PROC MIXED procedure with a logit link function for the binomial distribution. The GLIMMIX macro uses a penalized quasi-likelihood approach [176; 177] to approximate maximum likelihood estimators for the model parameters and variance components.

The generalized mixed model used in this analysis consisted of two fixed effects (tissue, exposure), a tissue by exposure interaction term, and the three random effects for animal, plate and packaging. The exposure effect consisted of five groups with different treatment and sampling times and either 3 or 4 doses. The tissue effect consisted of 3 levels, bone marrow (BM), liver (LV) and small intestine (SI).

Hypothesis testing for the variance components or random effects for packaging and plate effects were conducted using the likelihood ratio test. From this analysis there was a significant packaging effect (p-value <0.0001) but no significant plate effect (p-value of 1). The analysis of the dose response and the comparative analyses within tissues were carried out using an F-test, which accounts for the variance-covariance of the model. A Bonferroni-Holm step-wise method [178; 179] was used to control the family-wise error rate resulting from multiple comparisons to prevent it from exceeding the selected alpha level of 0.05. This technique employs a sequential approach where the pairwise comparisons are ranked in order of increasing p-value, and a step-down nominal significance level for each comparison is applied (i.e. α/rank position).

Dose-response trends were also modelled using a generalized mixed model. This model consisted of dose as a fixed effect and two random effects, animal and package. Random plate effects were not included in an effort to avoid fitting a saturated model and because there were no significant plate effects in the full model. The Wald statistics for fixed effects, testing the null hypothesis that the slope of the dose response is equal to zero, were used to determine one tailed p-values, given that this statistic is approximately distributed as a standard normal [180]. The selected alpha level was 0.05.

The least-square means [181; 182], standard errors and confidence intervals were reported for each exposure group by tissue. Since the generalized mixed model uses a log link function, these statistics were back transformed to the original scale.

3.3 Results

3.3.1 Dose Range-finding Study

One animal in the control group died at the beginning of the second week of treatment from an unknown cause and one animal in the low dose (100 mg/kg/day) group died due to mis-dosing. One animal from the mid-dose (200 mg/kg/day) group was found dead on day 26 after dosing was suspended on day 12 because of poor general condition. Only two animals from the high-dose (450 mg/kg/day) group survived to the end of the study period; three high-dose animals were removed from the study on days 8, 11 or 22 because of poor condition. Relative to the control, the terminal bodyweights of the low, mid- and high dose groups were depressed 6.4%, 15.1% or 22.3%, respectively. This difference was statistically significant for the mid- and high dose groups. Based on these results, the MTD expected for a 56 day study was estimated to be approximately 100 mg/kg/day.

3.3.2 Dose Response

The results from all groups and tissues are summarized in Table 3-2. The bone marrow dose response is shown in Figure 3-1. Spontaneous mutant frequencies ranged from 3.53×10^{-5} to 5.65×10^{-5}. For animals in the 7/3 treatment/sampling time group, mean mutant frequency increased negligibly with increasing dose. In the four other groups, a general dose-related increase in mutant frequency was observed. There was a statistically significant dose-related trend within the 28/3, 28/28 and 56/6 groups.

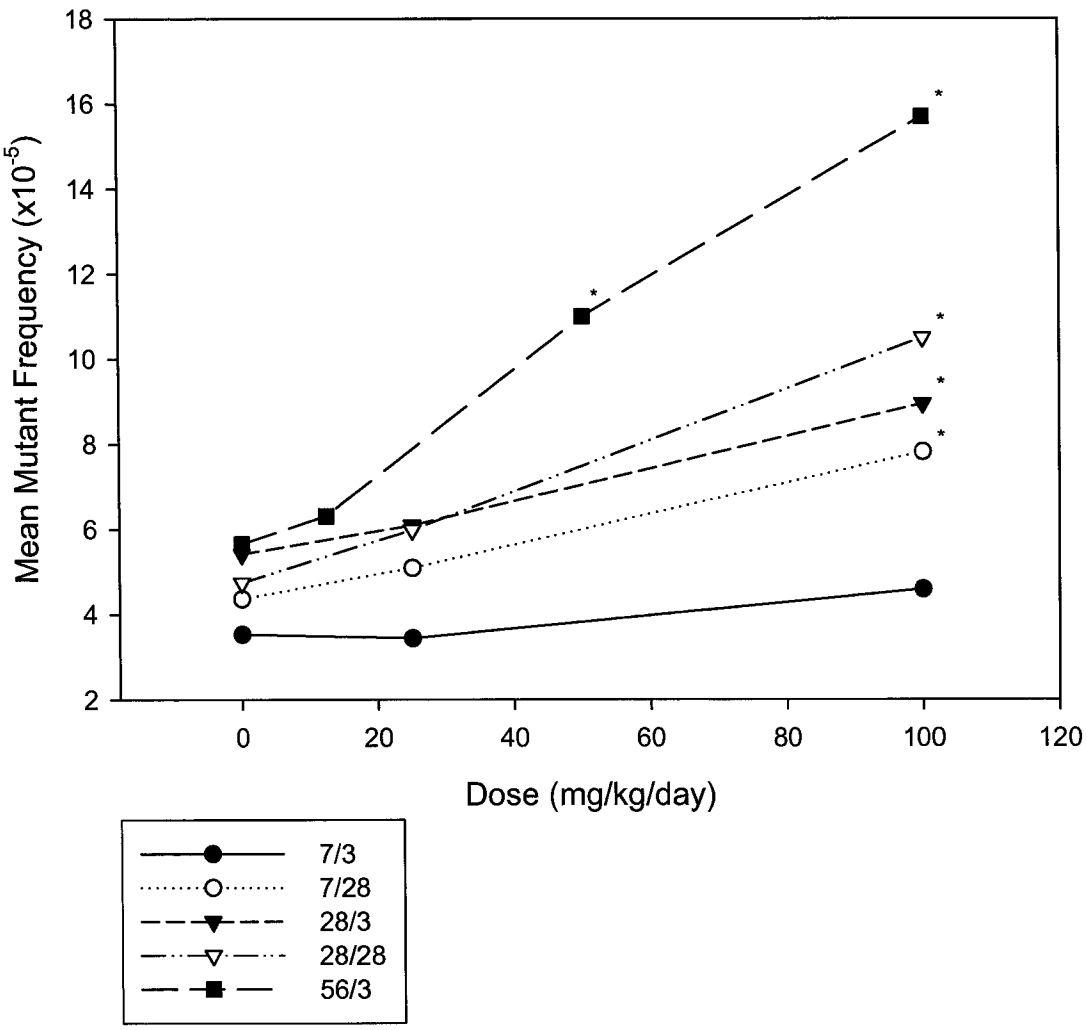
Table 3-2: Summary of results by tissue, group and dose

Tissue	Treatment /sampling time	Dose	Number of Animals	Total pfu screened ($x10^6$)	Mean MF ($x10^{-5}$)	MF 95% CI ($x10^{-5}$)
Bone marrow	7/3	0	5	1.17	3.53	2.37-5.26
	7/3	25	5	1.98	3.44	2.41-4.92
	7/3	100	6	1.74	4.54	3.24-6.38
	7/3	ENU	1	0.0817	172	104-284
	7/28	0	5	2.06	4.37	2.90-6.57
	7/28	25	5	1.81	5.09	3.40-7.64
	7/28	100	5	1.69	7.82	5.49-11.1
	7/28	ENU	1	0.294	131	77.8-220
	28/3	0	6	2.38	5.41	3.53-8.27
	28/3	25	5	1.83	6.09	3.93-9.44
	28/3	100	5	1.87	8.95	5.85-13.7
	28/3	ENU	1	0.371	45.0	25.6-79.2
	28/28	0	6	2.11	4.74	3.34-6.73
	28/28	25	7	1.97	5.98	4.30-8.32
	28/28	100	6	2.02	10.5	7.68-14.3
	28/28	ENU	1	0.249	111	68.0-180
	56/3	0	6	1.38	5.65	3.96-8.06
	56/3	12.5	6	2.04	6.30	4.56-8.72
	56/3	50	7	1.98	11.0	8.23-14.8
	56/3	100	7	1.69	15.7	11.9-20.9
	56/3	ENU	1	[a]		
Liver	7/3	0	5	0.805	4.54	2.93-7.06
	7/3	25	5	1.03	3.88	2.42-6.22
	7/3	100	6	1.67	4.94	3.48-7.02
	7/3	ENU	1	0.128	12.8	6.15-26.5
	7/28	0	5	1.72	6.23	4.42-8.77
	7/28	25	5	1.59	5.81	4.12-8.21
	7/28	100	5	1.41	7.23	5.12-10.2
	7/28	ENU	1	0.516	9.95	5.75-17.2
	28/3	0	6	1.61	4.26	3.02-6.01
	28/3	25	5	1.38	6.61	4.77-9.17
	28/3	100	5	1.52	10.1	7.52-13.5
	28/3	ENU	1	0.219	9.99	5.43-18.4
	28/28	0	6	1.85	6.21	4.54-8.48
	28/28	25	7	2.09	9.91	7.48-13.1
	28/28	100	6	1.24	14.1	10.4-18.9
	28/28	ENU	1	0.124	6.86	3.00-15.7
	56/3	0	6	0.979	7.88	5.66-11.0
	56/3	12.5	6	0.750	11.3	8.14-15.7
	56/3	50	7	1.43	13.1	9.93-17.3
	56/3	100	7	1.12	18.5	13.9-24.4
	56/3	ENU	1	0.258	8.41	4.52-15.6

Tissue	Treatment /sampling time	Dose	Number of Animals	Total pfu screened ($x10^6$)	Mean MF ($x10^{-5}$)	MF 95% CI ($x10^{-5}$)
Small intestine	7/3	0	5	0.340	10.5	6.59-16.9
	7/3	25	5	0.122	6.60	3.04-14.3
	7/3	100	6	0.501	10.4	6.78-15.9
	7/3	ENU	1	0.00547	365	190-704
	7/28	0	5	0.325	15.9	10.7-23.7
	7/28	25	5	0.240	12.3	7.73-19.7
	7/28	100	5	0.0832	23.6	13.9-40.4
	7/28	ENU	1	0.0620	144	85.4-244
	28/3	0	6	0.374	9.33	5.52-15.8
	28/3	25	5	0.280	9.11	5.10-16.3
	28/3	100	5	1.80	16.8	9.61-29.4
	28/3	ENU	1	0.00928	37.7	11.1-128
	28/28	0	6	1.06	9.34	6.60-13.2
	28/28	25	7	1.76	11.9	8.83-16.0
	28/28	100	6	0.862	19.7	14.4-26.9
	28/28	ENU	1	0.171	167	103-269
	56/3	0	6	1.17	13.5	9.57-19.0
	56/3	12.5	6	1.18	9.13	6.38-13.1
	56/3	50	7	1.91	22.0	16.1-30.0
	56/3	100	7	1.12	26.7	19.4-36.7
	56/3	ENU	1	0.153	108	64.4-181

[a] sample was lost

Figure 3-1: Dose-response relationships for bone marrow from the five treatment condition groups. The mutant frequencies shown are least-squares means from each group at the dose level indicated. Points annotated with an asterisk are significantly different from the corresponding group control ($p<0.05$).



The mean mutant frequency of animals in the 7/28, 28/3 and 28/28 groups receiving the maximum tolerated dose increased 1.8-fold, 1.7-fold and 2.2-fold, respectively, compared with the corresponding vehicle control. In the 56/3 group, a 1.9-fold increase was observed at ½ MTD and a 2.8-fold increase was observed at the MTD. There were no significant differences between mean mutant frequencies of the five controls. Significant differences in mean mutant frequency were observed in the 100 mg/kg/day group, relative to the corresponding control group, for the 7/28, 28/3, 28/28 and 56/3 groups, and in the 50 mg/kg/day group for the 56/3 group.

Figure 3-2 shows the dose-response for mutant frequency in the liver. Spontaneous mutant frequencies ranged from 4.26×10^{-5} to 7.88×10^{-5}. The group mean MF from animals in the 7/3 and 7/28 groups was similar to the controls. However, a significant dose-related increase in MF occurred in the 28/3, 28/28 and 56/3 groups. At the MTD, mutant frequencies were increased 2.4, 2.3 and 2.3-fold in the 28/3, 28/28 and 56/3 groups, respectively, compared with the corresponding controls. In the 56/3 group at ½ MTD, the mean MF was increased 1.7-fold over the control. There was a significant difference between the mean MF of the 28/3 and 56/3 controls. Significant differences from the corresponding control were observed for the 28/3, 28/28 and 56/3 groups at 100 mg/kg/day.

The dose-response relationships for the small intestine are shown in Figure 3-3. DNA from the 7/3, 7/28 and 28/3 groups did not package effectively, so the total number of pfu screened in these groups is quite small (122 000 to 832 000 for all groups except the high dose group for 28/3, in which 1 800 000 pfu were screened).

Figure 3-2: Dose-response relationships for liver from the five treatment condition groups. The mutant frequencies shown are least-squares means from each group at the dose level indicated. Points annotated with an asterisk are significantly different from the corresponding group control ($p<0.05$).

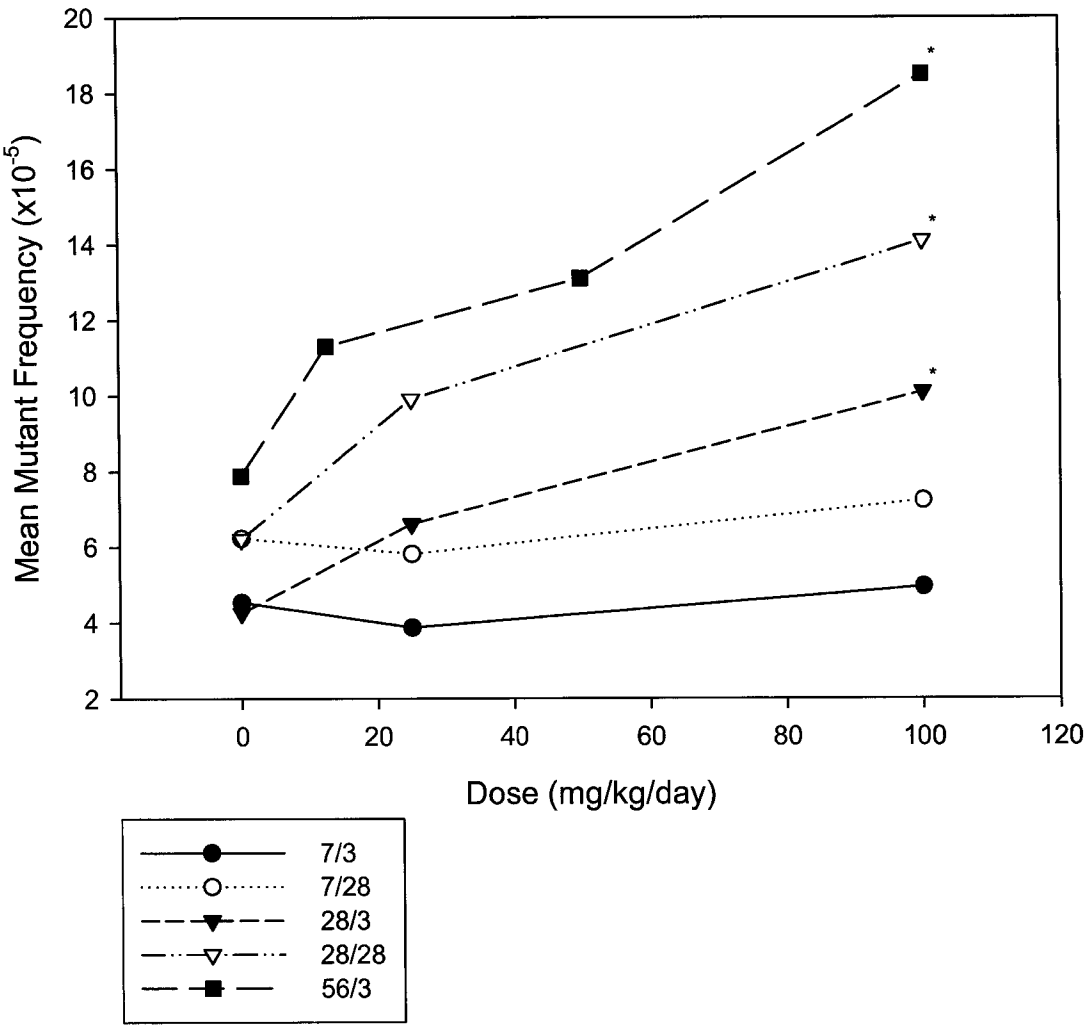
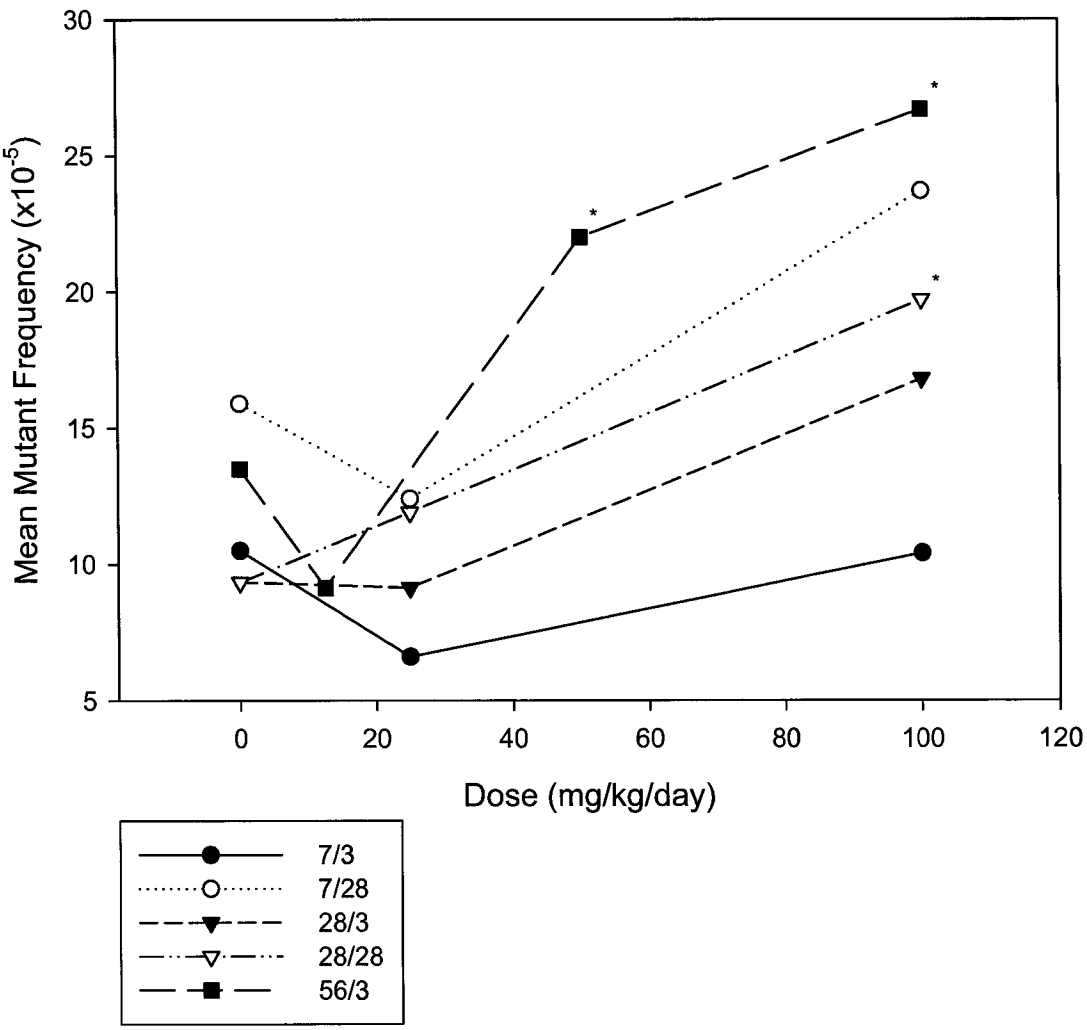


Figure 3-3: Dose-response relationships for small intestine from the five treatment condition groups. The mutant frequencies shown are least-squares means from each group at the dose level indicated. Points annotated with an asterisk are significantly different from the corresponding group control ($p<0.05$).



In contrast, the number of pfu screened in the 28/28 and 56/3 groups was considerably higher, ranging from 862 000 per group to 1 910 000 per group. Spontaneous MFs in the small intestine ranged from 9.33 $x10^{-5}$ to 15.9 $x10^{-5}$. Mean MF appeared to decrease slightly at the lowest dose (1/4 or 1/8 MTD) in all conditions except 28/3, then increased to 1.8, 2.1 or 2.0 times the spontaneous MF at the MTD for the 28/3, 28/28 and 56/3 groups, respectively. The group mean MF from animals in all of the dose groups for the 7/3 and 7/28 regimes was similar to the vehicle controls. There was a statistically significant dose-related trend within the 28/3, 28/28 and 56/3 groups. A significant increase in mean MF compared with the vehicle control occurred in the 28/28 and 56/3 groups at the MTD and in the 56/3 group at ½ MTD.

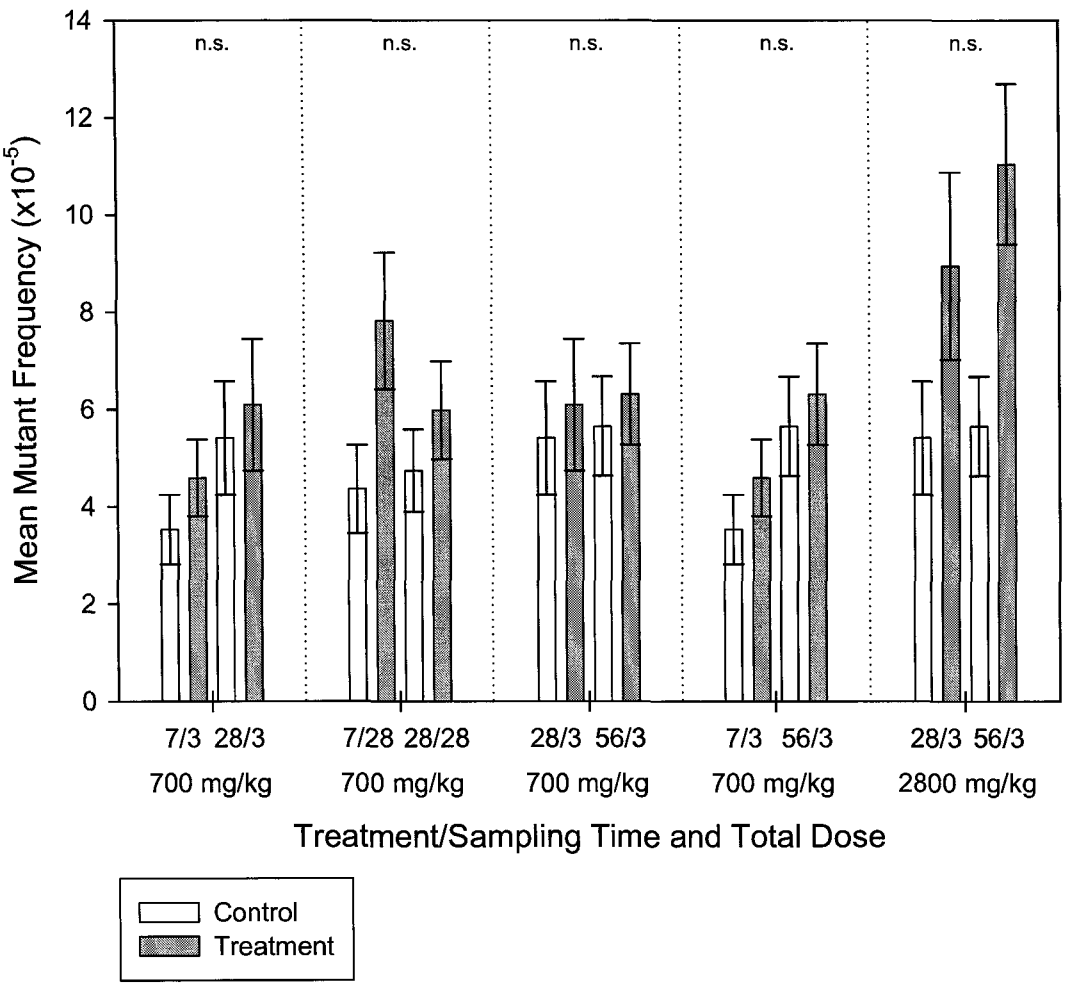
3.3.3 Effects of Treatment Duration and Sampling Time in Cases Where the Total Dose Administered was Equivalent

3.3.3.1 Effect of Treatment Duration

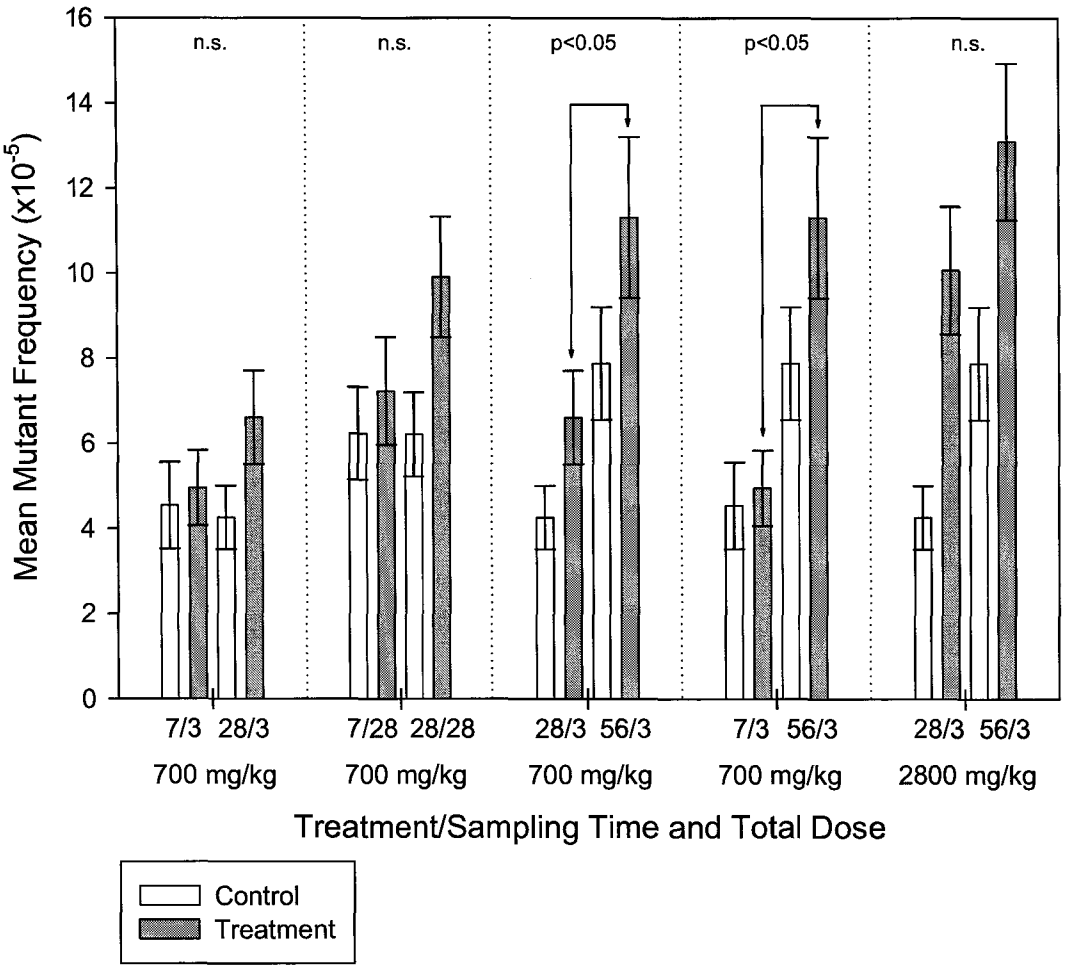
The effect of treatment period length on group mean MF for groups receiving the same total dose (variable dose rate) for bone marrow, liver and small intestine is shown in Figure 3-4. In the bone marrow and small intestine, there were no significant differences between groups where the same total dose was administered over treatment periods differing in length (but identical sampling time). In the liver, there was a significant difference in group mean MF between animals receiving 100 mg/kg/day over 7 days (7/3) and animals receiving 12.5 mg/kg/day over 56 days (56/3), and between animals administered 25 mg/kg/day over 28 days (28/3) and animals receiving 12.5 mg/kg/day over 56 days (56/3).

Figure 3-4: Effect of treatment duration on mutant frequency in the bone marrow (A), liver (B) and small intestine (C). Pairwise comparisons shown are between groups that were administered the same total dose over a different treatment duration (sampling times are identical for both groups). The x-axis indicates the treatment/sampling time (days), as well as the total dose administered. The error bars indicate standard error of the mean MF. There were no significant differences (n.s.) between any of the pairs for bone marrow or small intestine. In liver, the mean MF of 7/3 animals administered 100 mg/kg/day was significantly different ($p<0.05$) than 56/3 animals administered 12.5 mg/kg/day, and the mean MF of 28/3 animals administered 25 mg/kg/day was significantly different than 56/3 animals administered 12.5 mg/kg/day.

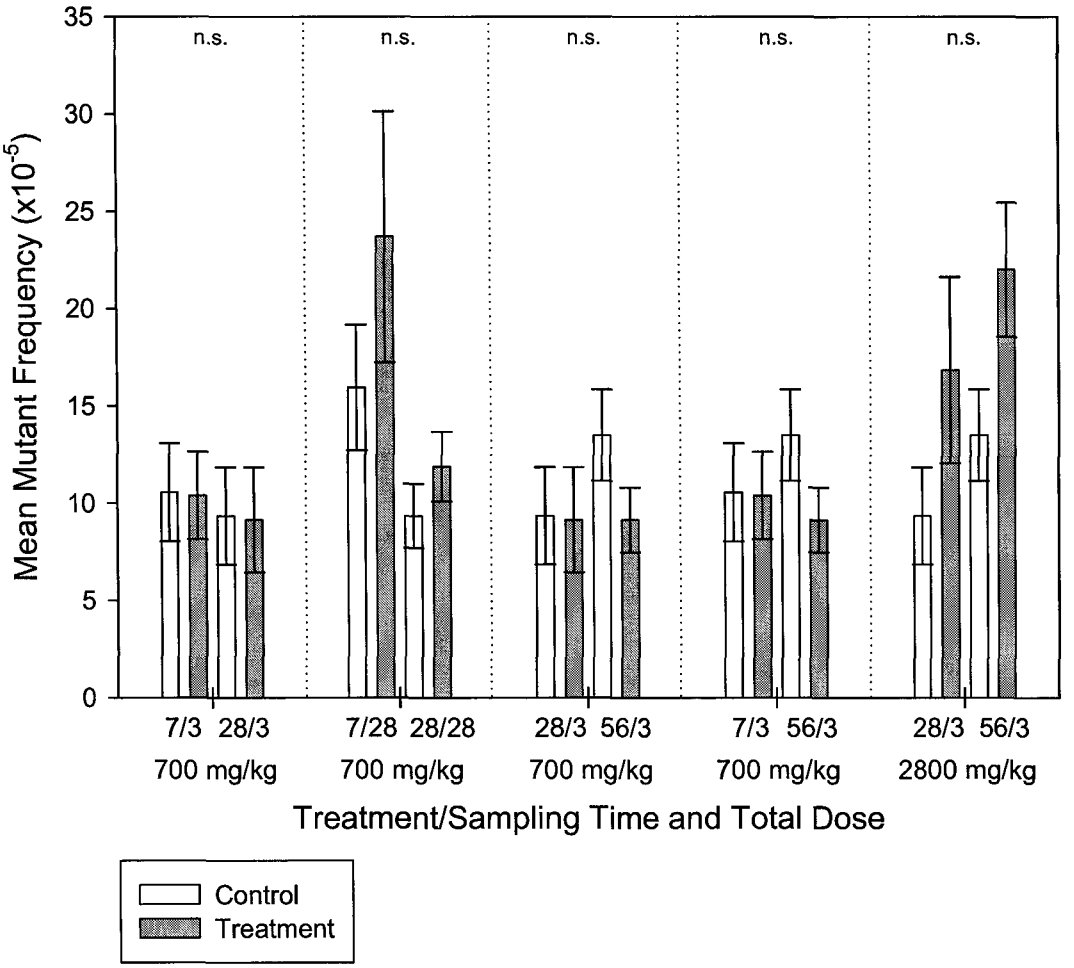
A



B



C



This indicates that the length of the treatment period in which a fixed total dose is administered was not a significant factor influencing MF for the bone marrow and small intestine, but was a factor contributing to differences in MF between the 7 day and 56 day, and the 28 day and 56 day treatment periods in the liver.

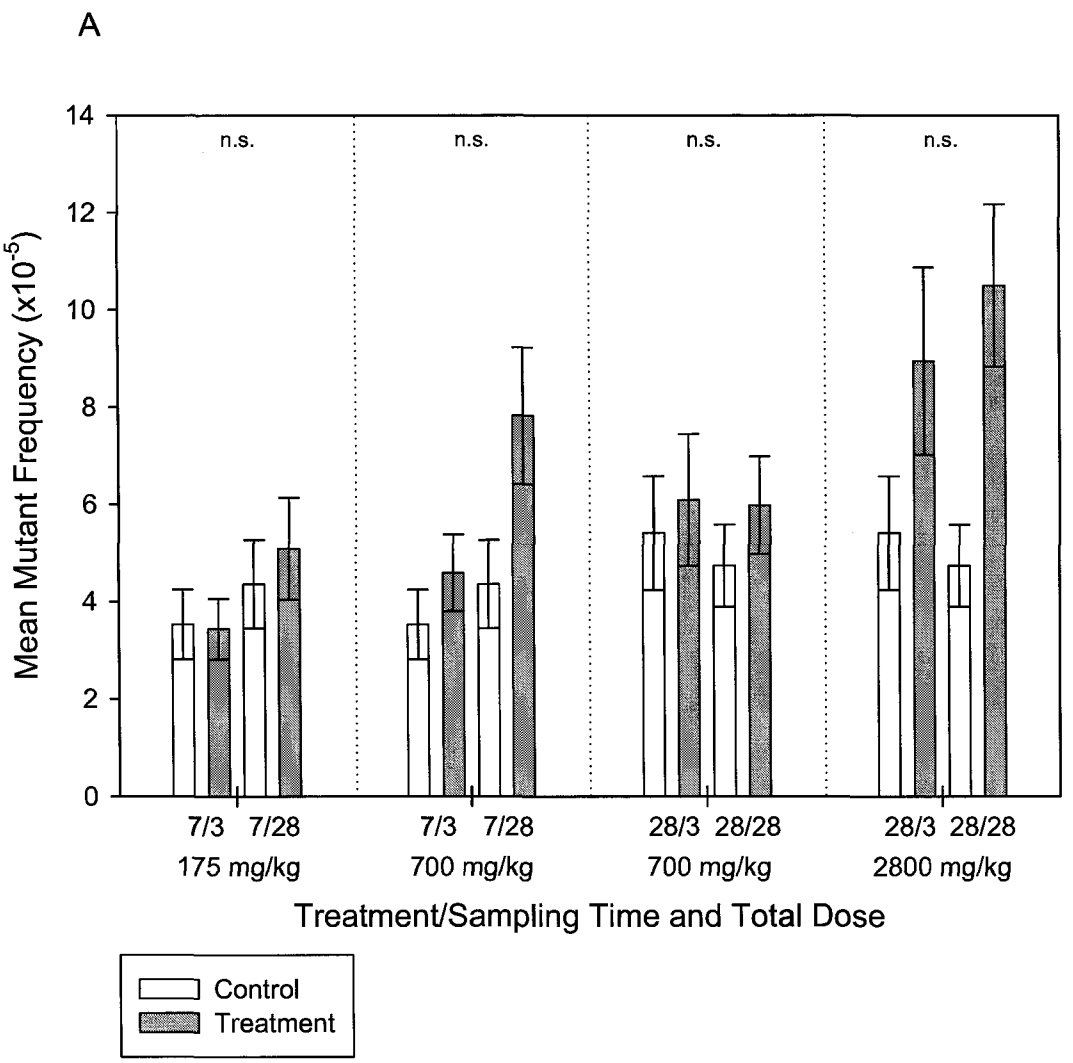
3.3.3.2 Effect of Sampling Time

Figure 3-5 shows the impact of differing sampling times on the group mean MF in situations where the total dose administered is equal. In the bone marrow and liver, no significant differences in mean MF occurred when the sampling time was increased from 3 days to 28 days for groups administered 175, 700 or 2800 mg/kg. In the small intestine, there was a significant difference in MF between the 3 day and 28 day sampling time for animals administered a total dose of 700 mg/kg/day over 7 days (7/3 and 7/28). This suggests that for the bone marrow and liver, extending the sampling time from 3 days to 28 days did not have an appreciable effect on the MF. In the small intestine, an inconsistent pattern emerged, where a significant difference in mean MF between the 3 day and 28 day sampling times occurred among animals administered 700 mg/kg over 7 days, but not among animals administered 700 mg/kg over 28 days, or animals administered 2800 mg/kg over 28 days.

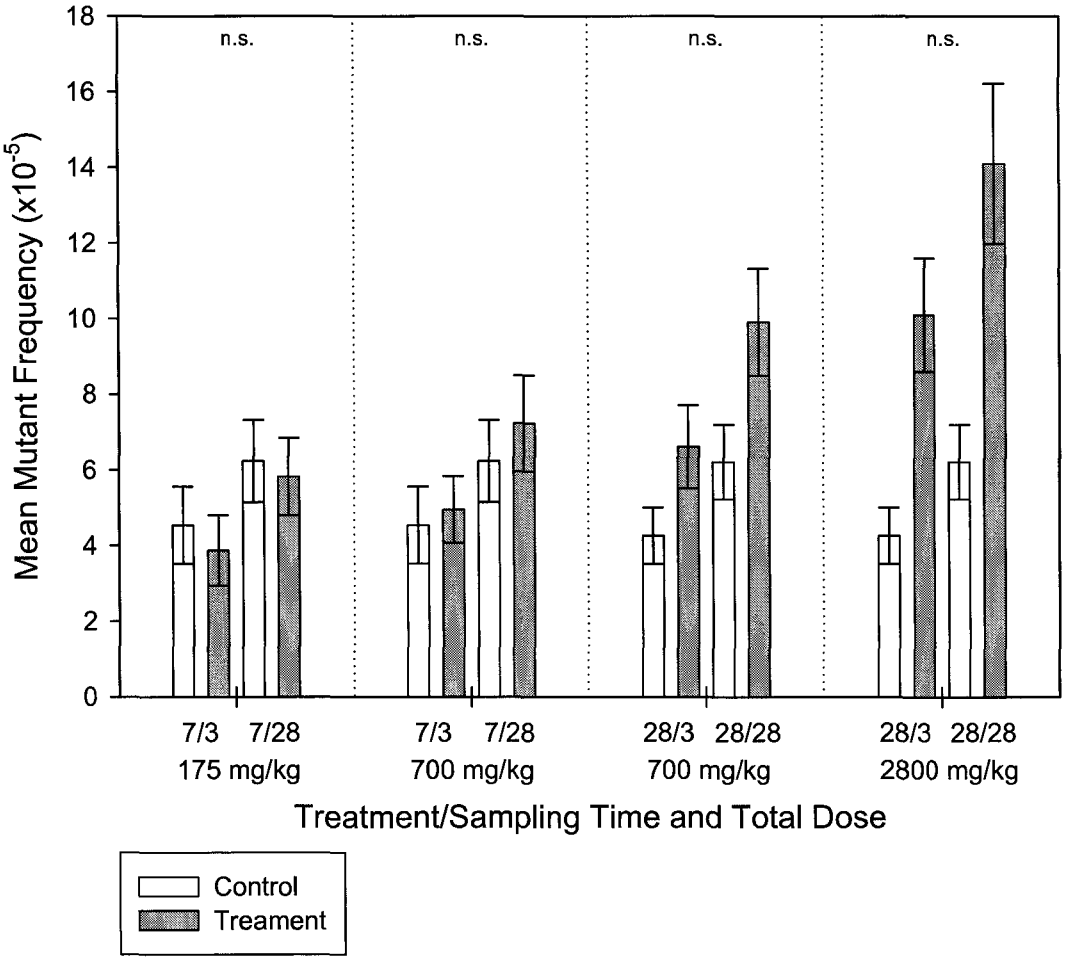
3.3.4 Effects of Dose Rate and Evidence of Additivity

The effects on mean MF of administering varying total doses as a result of providing the same daily dose rate over treatment periods differing in length are shown in Figure 3-6. If the effects of multiple doses are additive, the MF in groups administered a higher total dose will be greater than the MF in groups administered lower total doses.

Figure 3-5: Effect of sampling time on mutant frequency in the bone marrow (A), liver (B) and small intestine (C). Pairwise comparisons shown are between groups that were administered the same total dose over an identical treatment duration. The x-axis indicates the treatment/sampling time (days), as well as the total dose administered. The error bars indicate standard error of the mean MF. There were no significant differences (n.s.) between any of the pairs for bone marrow or liver. In the small intestine, there was a significant difference ($p<0.05$) in mean MF between the 7/3 and 7/28 animals administered a total dose of 700 mg/kg.



B



C

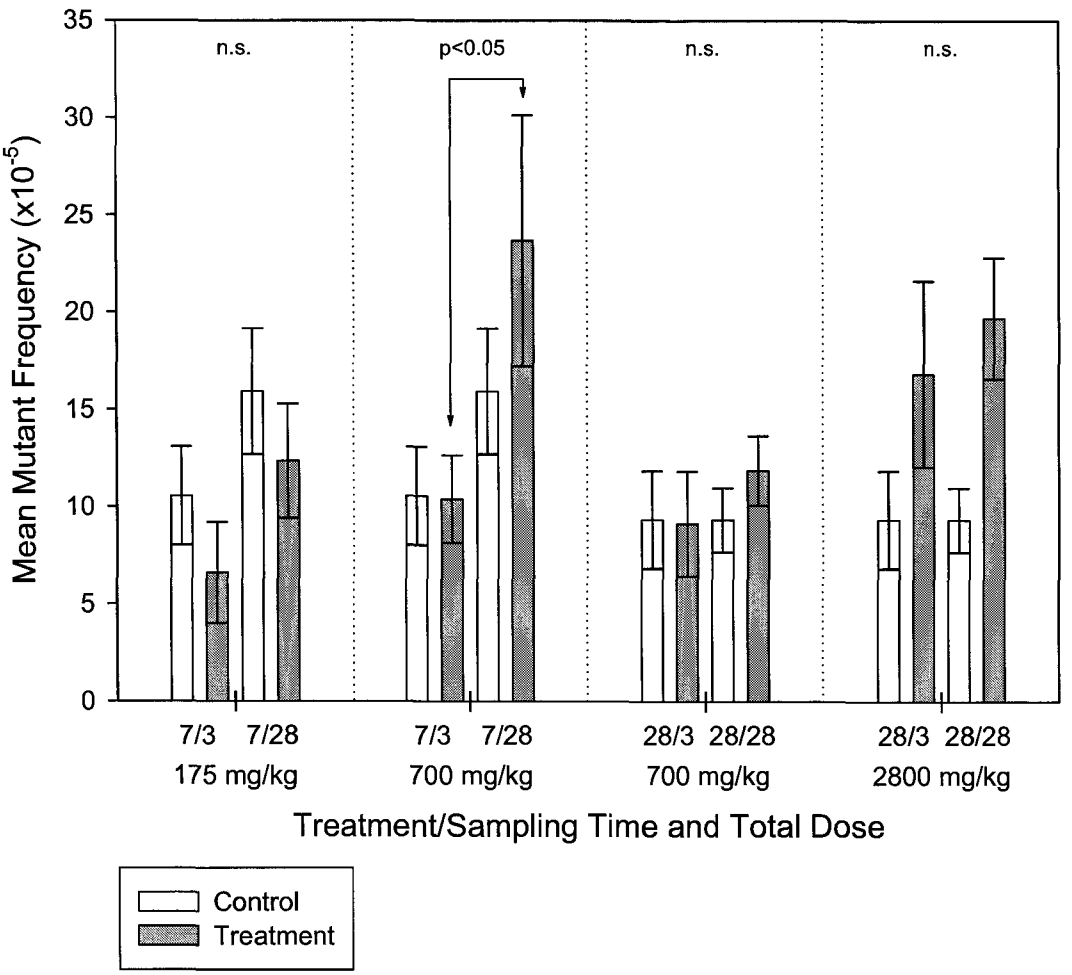
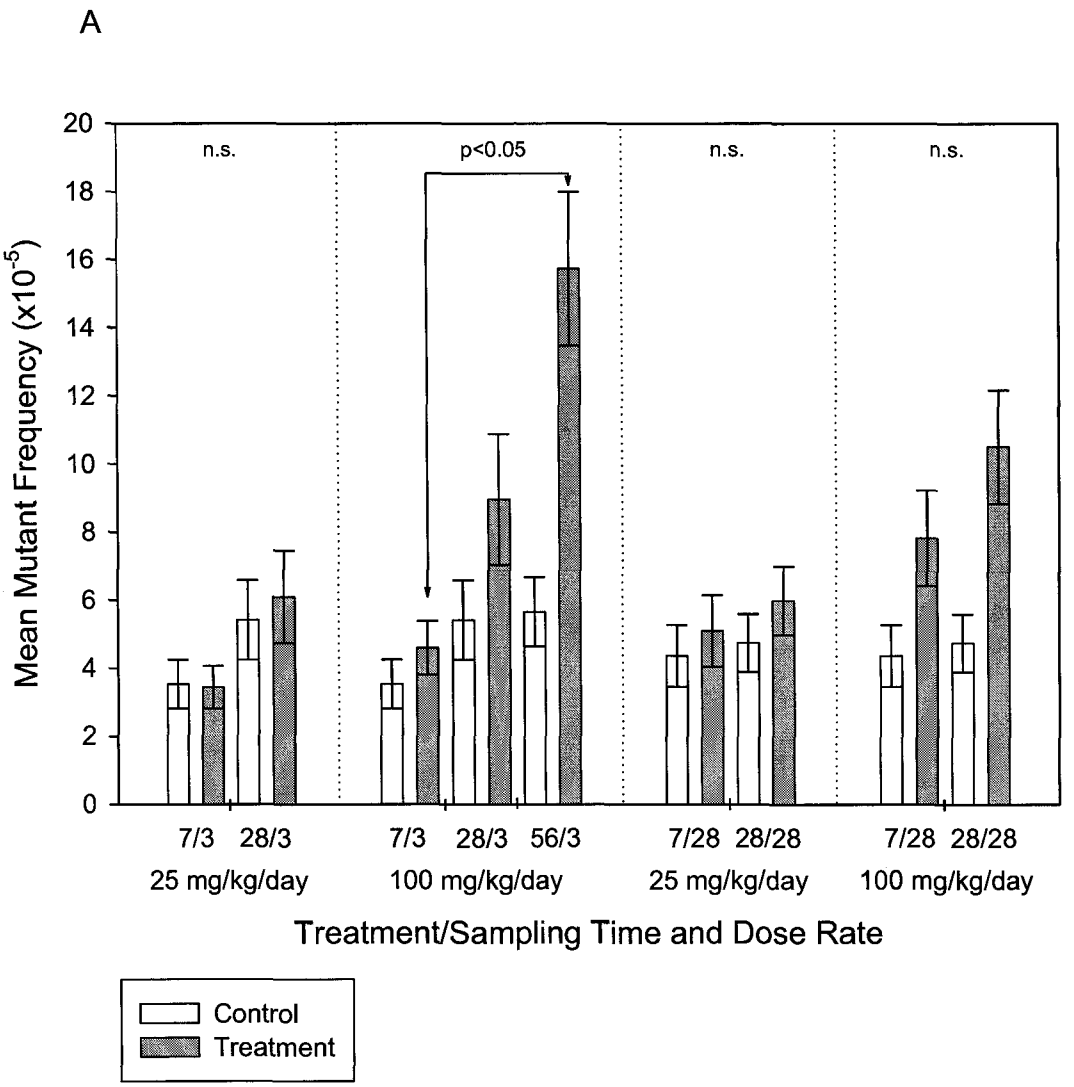
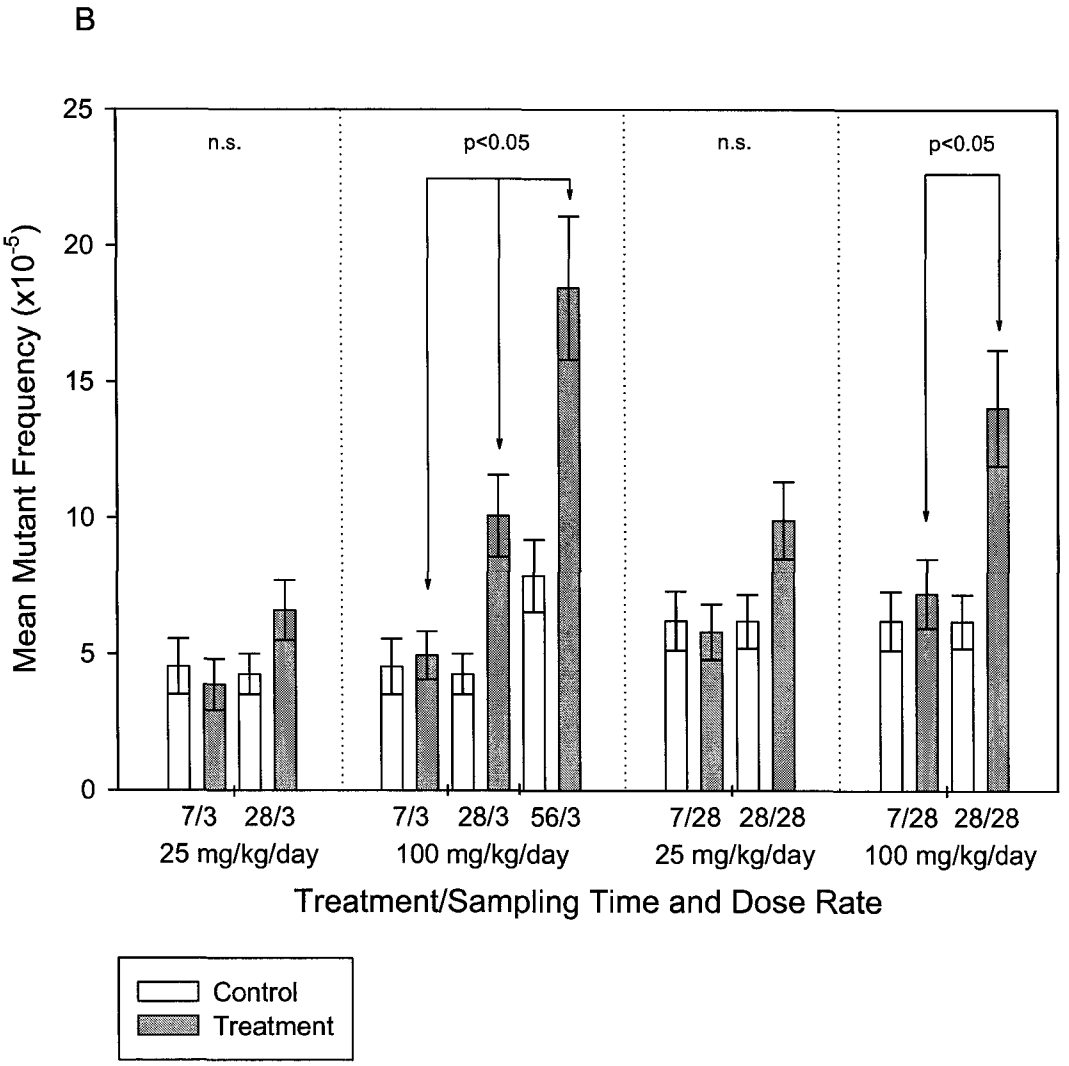
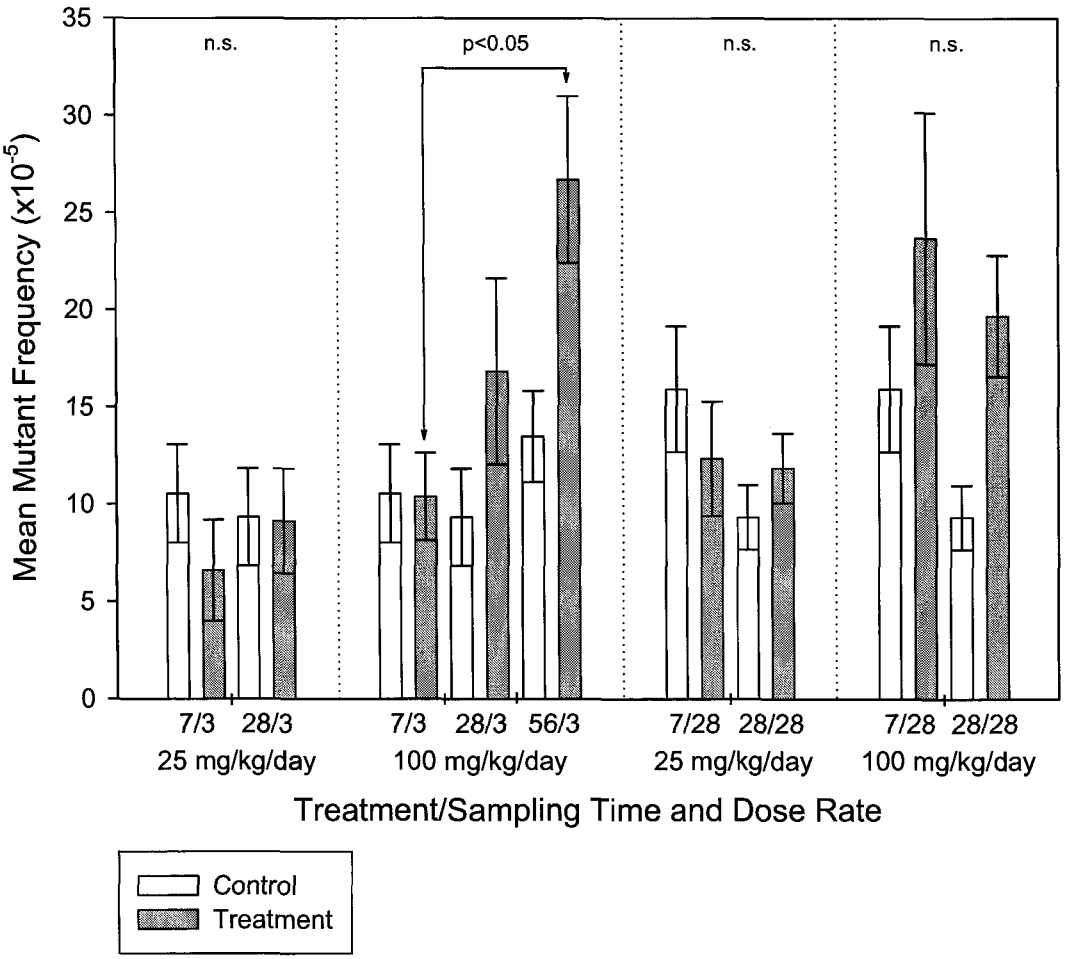


Figure 3-6: The effects of administering varying total doses as a result of providing the same daily dose rate over treatment periods differing in length. The mutant frequency is shown for groups administered equivalent dose rates in the bone marrow (A), liver (B) and small intestine (C). The x-axis indicates the treatment/sampling time (days) and dose rate (in mg/kg/day). The error bars indicate the standard error of the mean MF. In the groups receiving 100 mg/kg/day, there was a significant difference in mean MF in bone marrow between 7/3 and 56/3. In groups receiving 100 mg/kg/day, there was a significant difference in mean MF in the liver between 7/3, 28/3 and 56/3. For groups receiving 100 mg/kg/day, there was a significant difference in mean MF in the small intestine between 7/3 and 56/3. These data indicate an additive response is associated with an increase in the length of the treatment period and the administration of a greater total dose.





C



In the bone marrow of animals administered 100 mg/kg/day, the mean MF of the 56/3 group was significantly higher than the 7/3 group, but not the 28/3 group. There were no significant differences between the 7/3 and 28/3 groups administered 25 mg/kg/day or between the 7/28 and 28/28 groups administered 25 or 100 mg/kg/day. In the liver of animals administered 100 mg/kg/day, the mean MF of the 56/3 group was significantly higher than the 28/3 and 7/3 groups, and the mean MF of the 28/3 group was significantly greater than the 7/3 group; the mean MF of the 28/28 group was also significantly greater than the 7/28 group. There were no significant differences between the 7/3 and 28/3 groups or between the 7/28 and 28/28 groups administered 25 mg/kg/day. In the small intestine groups administered 100 mg/kg/day, the mean MF of the 56/3 group was significantly greater than the 7/3 group. There were no significant differences between the 7/3 and 28/3 groups administered 25 mg/kg/day or between the 7/28 and 28/28 groups administered 25 or 100 mg/kg/day.

3.4 Discussion

The purpose of this experiment was to examine the effects of treatment time and sampling time on the mutant frequencies induced by repeated doses of a weak mutagen in tissues with a range of proliferation rates. Determining the most appropriate treatment and sampling times is critical to the formation of a standardized test protocol suitable for testing chemical agents for the purposes of identifying genotoxic hazards. The goal for protocol development is to determine the treatment and sampling times that can be expected to yield detectable (if not the maximum) increases in mutant frequencies in a range of potential target tissues for mutagens of various potencies.

In theory, the rate at which pre-mutagenic lesions become fixed is a function of the turnover time of the tissue. DNA lesions induced in rapidly proliferating tissues, such as bone marrow, would be expected to become fixed relatively soon after induction, while those induced in more slowly proliferating tissues such as liver would require substantially more time to become fixed as mutations [173; 174; 183]. The time required for turnover in the rodent has been estimated at 2-3 days for bone marrow and 480-620 days for liver [184]. However, the rate of proliferation of cells within the liver is considerably more rapid following cell damage. The small intestine has an intermediate rate of cell proliferation, with a tissue turnover time of approximately 7 days [49; 185].

The vast majority of time course experiments reported in the literature have investigated the effects of prolonging the sampling time following the administration of a single dose of a strong mutagen. Following administration of five consecutive daily doses of N-ethyl-N-nitrosourea (ENU), the *lacZ* MF in the bone marrow rose rapidly within five days after the last treatment, and thereafter declined to a plateau where it remained past 55 days [174]. A similar trend of a rapid increase followed by a decrease to a stable plateau for at least 120 days was observed for the *cII* locus in bone marrow of mice following administration of a single dose of 120 mg/kg ENU [186]. In the liver, administration of five consecutive daily doses of ENU to mice caused the *lacZ* MF to rise 4-fold over the control within five days after the last treatment, remain fairly constant to day 20, then rise to a plateau by day 35; subsequently, the MF declined slowly to the last measurement at 55 days [174]. The MF at the *cII* locus in the liver of mice administered a single 120 mg/kg dose of ENU rose sharply between 15 and 30 days after treatment, then continued to increase beyond 120 days [186]. However, a markedly different

pattern emerged following treatment of female *lacI* transgenic rats with a single dose of 100 mg/kg cyproterone acetate, whereby the MF rose rapidly within 3 days after treatment, continued to increase to a maximum by 2 weeks, then dropped sharply to 40% of the initial post-treatment value by 4 weeks [187]. Although compounds such as ENU appear to induce a similar time-course effect at various loci following the administration of single doses, the response of the liver to cyproterone acetate suggests there may be significant differences between compounds, perhaps reflecting toxicokinetic factors.

Based upon the apparent neutrality of the transgenic loci, the effects of multiple treatments are presumed to be additive [172; 183; 185]; however, comparatively few studies have addressed the effects of multiple treatments. Low dose treatment of *lacZ* transgenic mice with ENU (94 μg/mL/day in drinking water) for up to 90 days, or *gpt* delta transgenic mice for up to 30 days has been shown to accumulate mutations in the small intestine in a dose-dependent manner [188; 189]. This pattern was supported by additional data indicating the effects of chronic (up to 480 days) treatment of *lacZ* transgenic mice with very low dose ENU (0.28-2.8 μg/mL/day in drinking water) also lead to a dose-dependent accumulation of mutations in the small intestine[190]. Further evidence of additivity at the *lacI* locus was demonstrated by the observation that the MF induced by ten weekly treatments of ENU, or the less potent gene mutagens 1,2-dimethylhydrazine and methyl methanesulfonate, was ten times greater than the MF induced by a single treatment [185].

In the current study, urethane was used as a representative weak mutagen. Urethane was shown previously to be mutagenic to *Salmonella typhimurium* TA1535 in the presence of a hamster liver S9 fraction, but was not mutagenic under other conditions

or to other strains [191]. Urethane induced sister chromatid exchanges in cultured Chinese hamster ovary (CHO) cells with and without S9; however, it did not induce chromosomal aberrations in CHO cells. *In vivo*, significantly increased frequencies of micronucleated erythrocytes were observed in peripheral blood obtained from male and female mice after 45 days of exposure and in bone marrow and peripheral blood obtained after 13 weeks of exposure in drinking water [192]. Numerous other studies have confirmed the clastogenic activity of urethane [193-195]. In the *lacZ* transgenic mouse, urethane has been previously found to be mutagenic to the liver, lung, spleen and bone marrow following a single i.p. injection of 900 mg/kg [196]. Urethane was also mutagenic at the *lacI* locus in the liver, lung and forestomach of mice administered 130 mg/kg/day in the diet for 105 days [197] and at the *cII* locus in the lung and spleen, but not the liver, after gavage administration of 50 mg/kg/day for 28 days [198].

In the current study, the dose response analysis (Figures 3-1, 3-2, and 3-3) indicated there was a reasonable degree of similarity between tissues with respect to the treatment conditions required to induce a significant increase in MF. For all three tissues, it appears that treatment at the MTD was required to produce a detectable increase, and at least 28 days of treatment was required to produce this response (except bone marrow where MF was significantly increased at 100 mg/kg/day in the 7/28 group). In bone marrow and small intestine, significant differences from the control were also induced in the 56/3 group by treatment at 50 mg/kg/day, which in terms of total dose administered was equivalent to the total dose received by the 28/3 and 28/28 groups administered 100 mg/kg/day. Interestingly, this equivalency in response was not observed in the liver, perhaps due to a mutation-inducing mitogenic effect that occurred at the MTD but not at

doses below. Consistent with the premise of additivity, groups administered a greater total dose had a greater induced MF. Significant dose related increasing trends were observed in the 28/3, 28/28 and 56/3 groups for all three tissues.

Toxicity occurring at doses at or near the MTD may cause mitogenic effects [199]. Compared with the more rapidly and continually proliferating bone marrow or small intestine, only a small proportion of liver cells are actively proliferating at any time in the absence of cell damage. In general, the effects of mitogens on mutation in the bone marrow or small intestine should be less significant because these tissues are undergoing continual cell proliferation. Daily administration of urethane at the MTD may have provided the liver with a continuous mitogenic stimulus (otherwise absent) that promoted the fixation of induced mutagenic lesions. Administration of lower daily doses may not have provided the same mitogenic stimulus, even if the total dose administered over the treatment period was the same. This could potentially account for the differing results between the 56/3 group administered 50 mg/kg/day (no significantly increased MF) and the 28/3 and 28/28 groups administered 100 mg/kg/day (significantly increased MF). Future investigation of the potential mitogenic effect on the liver of high dose urethane treatment could include detailed measurements of liver weights at sacrifice, and the measurement of bromodeoxyuridine incorporation into liver cell DNA.

Criteria for the interpretation of results from TGR mutation assays indicate a positive response occurs when the data exhibit a statistically significant dose-response relationship and/or a statistically significant increase in any dose group as compared to concurrent negative controls [64]. Based upon these criteria, positive responses occurred in the 7/28, 28/3, 28/28 and 56/3 groups for bone marrow, the 28/3, 28/28 and 56/3

groups for liver, and the 28/28 and 56/3 groups for small intestine. These results support the recommendations provided by the IWGT expert panel [65], which suggested a treatment duration of 28 days should be sufficient to detect weak mutagens.

The low number of pfu recovered in the small intestine from the 7/3, 7/28 and 28/3 groups could potentially explain some of the variable dose-responses shown in Figure 3-3. The original methodology used to isolate DNA from the small intestine may have contributed to the degradation of the DNA by causing shearing and by promoting the access of endogenous or exogenous nucleases to genomic DNA as a result of cell lysis. For the 28/28 and 56/3 groups, the modified approach using forceps to gently dislodge the epithelial cell layer from the underlying tissue, instead of drawing the tissue in and out of a syringe, may have limited cell lysis and reduced the extent of DNA attack by nucleases present within the sample. In addition, using low temperature to facilitate DNA precipitation and recovery may also have exposed the isolated DNA to lower shearing forces.

This experiment is based on an MTD derived from a 28-day preliminary study. The total dose administered was found to be a crucial factor determining the magnitude of the induced MF and the total dose administered is a function of the MTD. In order to allow for these comparisons between groups, it was necessary to match total doses between groups administered urethane for different treatment durations. Since MTD levels were not specifically determined at each treatment duration, it is possible (and quite likely) that higher doses could have been administered to animals treated for shorter periods. Consequently, it is possible that detectable increases in MF could be induced following high dose treatment of animals over intervals as short or shorter than 7 days.

Administration of the same total dose over treatment periods of differing length has not previously been shown to significantly affect MF at transgenic loci [190]. However, in the current study, the MF induced in the 7/3 group was significantly lower than the MF induced in the 56/3 group by a total dose of 700 mg/kg/day (but only in the liver, not in the bone marrow or small intestine). This could be a function of the longer sampling time afforded the initial doses in the 56/3 group compared with the 7/3 group, which may have resulted in the fixation of a larger proportion of the induced lesions.

For more slowly dividing tissues, such as liver, longer sampling times should allow the fixation of more mutagenic lesions and subsequently result in a higher MF. However, in the current study, no significant differences in MF in the liver occurred between short (3 day) and longer (28 day) sampling times among groups receiving the same total dose. There are several possible explanations for this phenomenon. One, the impact of lengthening the sampling time is not as great as anticipated, at least for urethane. More likely, the sampling time required for mutant manifestation in the liver is significantly longer than the 28 day maximum sampling time employed in this study. Nevertheless, there were significant differences between treatment and control MF in the liver among animals receiving the MTD, which suggests that, despite the failure to identify the maximum MF, detectable increases in MF did occur.

Additivity in MF resulting from longer treatment periods (higher total dose) occurred in all three tissues for groups treated at the MTD. However, the increases in MF did not occur in a perfectly additive manner in any tissue (i.e. the ratio of induced MFs did not correspond to the ratio of total doses). Qualitatively, the increases in MF across the 7/3, 28/3 and 56/3 groups appeared to be relatively linear in the bone marrow and

liver, but not the small intestine. It was also noteworthy that at 25 mg/kg/day (equivalent to ¼ the MTD), there were no significant differences between MFs induced by treatment for 7 or 28 days, suggesting additivity was dose-dependent.

In conclusion, these results have demonstrated that for the weak mutagen urethane, detectable positive responses were observed in the bone marrow, liver and small intestine of animals treated for a minimum of 28 days. The total dose administered was perhaps the most significant factor – generally, treatment at the MTD was required to produce a significant increase in MF. This would suggest that increases in MFs induced by exposures to low doses of weak mutagens may not be detectable using transgenic systems. However, when care is taken for appropriate dose selection, these results support the protocol recommendations of the IWGT working group.

Chapter 4

Conclusions and Implications

4.1 Operational Characteristics of TGR Assays

The analysis of the performance of TGR assays described in Chapter 2 has lead to the following conclusions:

1. The results of TGR assays incorporating sequence analysis suggest that the assays are capable of accurately and reliably identifying compounds that cause gene mutations. This ability has been demonstrated more frequently for strong mutagens.
2. Since there is no gene mutation assay that provides definitive genotoxicity results, comparison of TGR assays with other tests known to detect gene mutations provides a strong indication of the value of TGR assays as a genotoxicity test. The *Salmonella* reverse mutation assay was the only test examined that specifically identifies point mutations. The agreement of TGR assays with *Salmonella* was significantly better than expected by chance, suggesting that TGR assays can identify mutagens at least as well as an accepted gene mutation test. TGR assays also had agreement significantly better than expected with several other assays, likely because many strong mutagens induce both gene mutations and chromosomal aberrations.
3. Using the results of genotoxicity tests as predictors of rodent carcinogenicity is problematic in some cases. However, when used in this predictive capacity, TGR assays exhibit high sensitivity, and relatively low specificity, consistent with most other genotoxicity tests. Predictive values (PPV and NPV) are highly dependent on the prevalence of carcinogens within the population of chemicals studied. In cases where prevalence is high, TGR assays have high PPV and low NPV; when

prevalence is low, the PPV is substantially lower and NPV substantially higher. Of the seven genotoxicity assays examined, only TGR assays provided PPV significantly better than expected by chance and only TGR assays and *Salmonella* provided NPV significantly better than expected by chance.

4. When combining two genotoxicity tests in a test battery, the outcome decision rule can take two forms: (1) the *or* scenario, requiring only one positive test to lead to a 'positive' test battery, or (2) the *and* scenario, requiring two positive test results to lead to a 'positive' test battery. Considering all the test batteries and single assays examined using the dataset described in Table 2-1, the 'TGR or *Salmonella*' battery, TGR alone and *Salmonella* alone had carcinogenicity predictive values that, in general, were significantly better than expected by chance. Despite the lack of substantial increases in predictive values of the test batteries compared with the component assays alone, the *or* test batteries had higher false positive rates and lower false negative rates than the single tests.
5. The *in vivo* MN assay is the typical *in vivo* component of the standard test battery. When considered in a battery with *Salmonella* and *in vitro* CA, both TGR assays and *in vivo* MN performed similarly as predictors of carcinogenicity, suggesting there was no clear advantage of one assay over the other in this test battery scenario.

4.2 Integration of TGR Assays into Genotoxicity Test Batteries

Detailed analysis of the merits of including TGR assays in batteries with various other tests is beyond the scope of this work. However, it is informative to consider two

of the possible ways in which TGR assays could be incorporated into a testing strategy. It is assumed that the core battery consists of *Salmonella*, *in vitro* CA and *in vivo* MN, with the two *in vitro* tests conducted in all cases. The purpose of the test battery is to determine if the compound is genotoxic, not necessarily if it is carcinogenic. As described in Chapter 1, there is less uncertainty regarding human relevance associated with *in vivo* tests, and the results of these assays typically are given more weight than any of the *in vitro* assays.

4.2.1 Selective Replacement of the *In Vivo* Micronucleus Assay

In cases where a chemical is mutagenic to *Salmonella* but not clastogenic in the *in vitro* CA assay, the appropriate follow-up *in vivo* test is not a chromosomal aberration assay, but instead a gene mutation assay. TGR assays could be used in this case as a replacement for the *in vivo* MN assay to provide a model that would allow for further *in vivo* investigation of the same genetic endpoint examined using *Salmonella.* If the compound is not subsequently mutagenic in the TGR assay, the *Salmonella* results may have less overall relevance, as the compound is only an *in vitro* mutagen. If the compound is both mutagenic and clastogenic in *in vitro* testing, both *in vivo* tests may be required to determine fully its *in vivo* genotoxicity. Criteria for determining which *in vivo* test should be selected first could include cost, time required for testing, availability of suitable research facilities, etc. If the first *in vivo* test is positive, there may be no need to continue to the second *in vivo* test.

4.2.2 Addition to an Existing Test Battery

In situations where a compound is mutagenic to *Salmonella*, but not clastogenic in the *in vivo* MN assay, there is an unresolved issue of whether the compound is mutagenic

in vivo. The TGR assay could be used as a fourth assay to provide some resolution to this question. If the compound is not mutagenic in the TGR assay, the compound remains an *in vitro* mutagen.

4.3 Protocol for TGR Assays

The following conclusions are apparent based on the results of the investigations presented in Chapter 3:

1. For bone marrow, liver and small intestine, it appears that treatment at the MTD was required to produce a detectable increase in MF, and at least 28 days of treatment was generally required to produce this response. These results support the recommendations provided by the IWGT expert panel [65], which suggested a treatment duration of 28 days should be sufficient to detect weak mutagens.
2. Consistent with the premise of additivity, groups administered a greater total dose had a greater induced MF.
3. Mutant frequency was unaffected by administration of the same total dose over treatment periods of differing length, except in the liver (in which the MF in the 7/3 group was significantly lower than in the 56/3 group for a total dose of 700 mg/kg).
4. There were no significant differences in MF in the liver between short (3 day) and longer (28 day) sampling times among groups receiving the same total dose. The sampling time required for maximum mutant manifestation in the liver may be

significantly longer than the 28 day maximum sampling time employed in this study.

4.4 Opportunities for Further Experimentation

4.4.1 Testing of Additional Non-Genotoxic, Non-Carcinogenic Compounds

The database presented in Table 2-1 is comprised disproportionately of genotoxic carcinogens. The coordinated multi-laboratory testing of additional compounds that are non-genotoxic and non-carcinogenic would provide a more balanced database better capable of providing an accurate assessment of specificity and NPV.

4.4.2 Testing of Additional Compounds Using the IWGT Recommended Protocol

Further testing of other weak mutagenic compounds using an experimental design similar to that described in Chapter 3 would provide evidence as to whether the responses and trends observed for urethane hold constant. A series of compounds tested in a multi-laboratory trial by contract laboratories under Good Laboratory Practice conditions would also provide an indication of the interlaboratory reproducibility of the results of these assays when testing weak mutagens.

4.4.3 Comparison of Spontaneous and Induced MF in the Male Germline of Transgenic Rodents with MF Derived From Existing Male Germline Mutation Assays

The current work focused exclusively on somatic mutation. A significant benefit of the TGR assays is their ability to permit the investigation of mutations arising in any

tissue, including the germline. Further use of the TGR models to investigate germline mutation would be facilitated by experiments designed to address the comparability of the TGR models with the existing male germline mutation assays (morphological specific locus test, heritable translocation assay and dominant lethal assay).

References

1. OECD. Test Guideline 473: *In vitro* mammalian chromosomal aberration test. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1997.

2. Kirsch-Volders M., T. Sofuni, M. Aardema, S. Albertini, D. Eastmond, M. Fenech, M. Ishidate Jr, E. Lorge, H. Norppa, J. Surralles, W. von der Hude and A. Wakata. 2000. Report from the In Vitro Micronucleus Assay Working Group. Environ Mol Mutagen. 35 (3): 167-72.

3. Kirsch-Volders M., T. Sofuni, M. Aardema, S. Albertini, D. Eastmond, M. Fenech, M. Ishidate Jr, S. Kirchner, E. Lorge, T. Morita, H. Norppa, J. Surralles, A. Vanhauwaert and A. Wakata. 2003. Report from the *in vitro* micronucleus assay working group. Mutat Res. 540 (2): 153-63.

4. OECD. Test Guideline 471: Bacterial reverse mutation test. in. OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1997.

5. OECD. Test Guideline 476: *In vitro* mammalian cell gene mutation test. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1997.

6. OECD. Test Guideline 474: Mammalian Erythrocyte Micronucleus Test. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1997.

7. Heddle J.A., M.C. Cimino, M. Hayashi, F. Romagna, M.D. Shelby, J.D. Tucker, P. Vanparys and J.T. MacGregor. 1991. Micronuclei as an index of cytogenetic damage: past, present, and future. Environ Mol Mutagen. 18 (4): 277-91.

8. Mavournin K.H., D.H. Blakey, M.C. Cimino, M.F. Salamone and J.A. Heddle. 1990. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res. 239 (1): 29-80.

9. Krishna G. and M. Hayashi. 2000. *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. Mutat Res. 455 (1-2): 155-66.

10. Salamone M.F. and K.H. Mavournin. 1994. Bone marrow micronucleus assay: a review of the mouse stocks used and their published mean spontaneous micronucleus frequencies. Environ Mol Mutagen. 23 (4): 239-73.

11. Waters M.D., H.F. Stack, M.A. Jackson, B.A. Bridges and I.D. Adler. 1994. The performance of short-term tests in identifying potential germ cell mutagens: a qualitative and quantitative analysis. Mutat Res. 341 (2): 109-31.

12. Witt K.L., L.A. Hughes, L.T. Burka, A.F. McFee, J.M. Mathews, S.L. Black and J.B. Bishop. 2003. Mouse bone marrow micronucleus test results do not predict the germ cell mutagenicity of N-hydroxymethylacrylamide in the mouse dominant lethal assay. Environ Mol Mutagen. 41 (2): 111-20.

13. OECD. Test Guideline 475: Mammalian Bone Marrow Chromosomal Aberration Test. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1997.

14. OECD. Test Guideline 484: Mouse Spot Test. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1986.

15. Russell L.B. and M.H. Major. 1957. Radiation-induced presumed somatic mutations in the house mouse. Genetics. 42 (2): 161-75.

16. Fahrig R. 1975. A mammalian spot test: induction of genetic alterations in pigment cells of mouse embryos with x-rays and chemical mutagens. Mol Gen Genet. 138 (4): 309-14.

17. Russell L.B. 1977. Validation of the *in vivo* somatic mutation method in the mouse as a prescreen for germinal point mutations. Arch Toxicol. 38 (1-2): 75-85.

18. Fahrig, R. The mouse spot test. Phillips, D.H. and S. Venitt, eds. In: Environmental Mutagenesis. Oxford: Bios Scientific Publishers; 1995. pp. 180-99.

19. Fahrig R. and A. Neuhauser-Klaus. 1985. Similar pigmentation characteristics in the specific-locus and the mammalian spot test. A way to distinguish between induced mutation and recombination. J Hered. 76 (6): 421-6.

20. Gondo Y., J.M. Gardner, Y. Nakatsu, D. Durham-Pierre, S.A. Deveau, C. Kuper and M.H. Brilliant. 1993. High-frequency genetic reversion mediated by a DNA duplication: the mouse pink-eyed unstable mutation. Proc Natl Acad Sci U S A. 90 (1): 297-301.

21. Searle A.G. 1977. The use of pigment loci for detecting reverse mutations in somatic cells of mice. Arch Toxicol. 38 (1-2): 105-8.

22. Reliene R., A.J. Bishop, J. Aubrecht and R.H. Schiestl. 2004. *In vivo* DNA deletion assay to detect environmental and genetic predisposition to cancer. Methods Mol Biol. 262: 125-39.

23. Schiestl R.H., J. Aubrecht, F. Khogali and N. Carls. 1997. Carcinogens induce reversion of the mouse pink-eyed unstable mutation. Proc Natl Acad Sci U S A. 94 (9): 4576-81.

24. Schiestl R.H., J. Aubrecht, W.Y. Yap, S. Kandikonda and S. Sidhom. 1997. Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin induce intrachromosomal recombination *in vitro* and *in vivo*. Cancer Res. 57 (19): 4378-83.

25. Jalili T., G.G. Murthy and R.H. Schiestl. 1998. Cigarette smoke induces DNA deletions in the mouse embryo. Cancer Res. 58 (12): 2633-8.

26. Bishop A.J., B. Kosaras, R.L. Sidman and R.H. Schiestl. 2000. Benzo(a)pyrene and X-rays induce reversions of the pink-eyed unstable mutation in the retinal pigment epithelium of mice. Mutat Res. 457 (1-2): 31-40.

27. Bodenstein L. and R.L. Sidman. 1987. Growth and development of the mouse retinal pigment epithelium. I. Cell and tissue morphometrics and topography of mitotic activity. Dev Biol. 121 (1): 192-204.

28. Tates A.D., F.J. van Dam, F.A. de Zwart, C.M. van Teylingen and A.T. Natarajan. 1994. Development of a cloning assay with high cloning efficiency to detect induction of 6-thioguanine-resistant lymphocytes in spleen of adult mice following *in vivo* inhalation exposure to 1,3-butadiene. Mutat Res. 309 (2): 299-306.

29. Van Sloun P.P., S.W. Wijnhoven, H.J. Kool, R. Slater, G. Weeda, A.A. van Zeeland, P.H. Lohman and H. Vrieling. 1998 . Determination of spontaneous loss of heterozygosity mutations in Aprt heterozygous mice. Nucleic Acids Res. 26 (21): 4888-94.

30. Lommel L., C. Carswell-Crumpton and P.C. Hanawalt. 1995. Preferential repair of the transcribed DNA strand in the dihydrofolate reductase gene throughout the cell cycle in UV-irradiated human cells. Mutat Res. 336 (2): 181-92.

31. Dobrovolsky V.N., T. Chen and R.H. Heflich. 1999. Molecular analysis of *in vivo* mutations induced by N-ethyl-N-nitrosourea in the autosomal *Tk* and the X-linked *Hprt* genes of mouse lymphocytes. Environ Mol Mutagen. 34 (1): 30-8.

32. Deubel W., I.D. Bassukas, W. Schlereth, R. Lorenz and K. Hempel. 1996. Age dependent selection against HPRT deficient T lymphocytes in the HPRT+/- heterozygous mouse. Mutat Res. 351 (1): 67-77.

33. Meng Q., R.F. Henderson, D.M. Walker, M.J. Bauer, A.A. Reilly and V.E. Walker. 1999. Mutagenicity of the racemic mixtures of butadiene monoepoxide and butadiene diepoxide at the *Hprt* locus of T-lymphocytes following inhalation exposures of female mice and rats. Mutat Res. 429 (1): 127-40.

34. Dush M.K., J.A. Tischfield, S.A. Khan, E. Feliciano, J.M. Sikela, C.A. Kozak and P.J. Stambrook. 1986. An unusual adenine phosphoribosyltransferase pseudogene is syntenic with its functional gene and is flanked by highly polymorphic DNAs. Mol Cell Biol. 6 (12): 4161-7.

35. Fratini A., R.N. Simmers, D.F. Callen, V.J. Hyland, J.A. Tischfield, P.J. Stambrook and G.R. Sutherland. 1986. A new location for the human adenine phosphoribosyltransferase gene (APRT) distal to the haptoglobin (HP) and fra(16)(q23)(FRA16D) loci. Cytogenet Cell Genet. 43 (1-2): 10-3.

36. Tischfield J.A. 1997. Loss of heterozygosity or: how I learned to stop worrying and love mitotic recombination. Am J Hum Genet. 61 (5): 995-9.

37. Shao C., L. Deng, O. Henegariu, L. Liang, N. Raikwar, A. Sahota, P.J. Stambrook and J.A. Tischfield. 1999. Mitotic recombination produces the majority of recessive fibroblast variants in heterozygous mice. Proc Natl Acad Sci U S A. 96 (16): 9230-5.

38. Ponomareva O.N., J.A. Rose, M. Lasarev, J. Rasey and M.S. Turker. 2002. Tissue-specific deletion and discontinuous loss of heterozygosity are signatures for the mutagenic effects of ionizing radiation in solid tissues. Cancer Res. 62 (5): 1518-23.

39. Stambrook P.J., C. Shao, M. Stockelman, G. Boivin, S.J. Engle and J.A. Tischfield. 1996. APRT: a versatile *in vivo* resident reporter of local mutation and loss of heterozygosity. Environ Mol Mutagen. 28 (4): 471-82.

40. Dobrovolsky V.N., D.A. Casciano and R.H. Heflich. 1999. *Tk*+/- mouse model for detecting *in vivo* mutation in an endogenous, autosomal gene. Mutat Res. 423 (1-2): 125-36.

41. Dobrovolsky V.N., D.A. Casciano and R.H. Heflich. 1996. Development of a novel mouse *tk*+/- embryonic stem cell line for use in mutagenicity studies. Environ Mol Mutagen. 28 (4): 483-9.

42. Hozier J., J. Scalzi, J. Sawyer, N. Carley, M. Applegate, D. Clive and M.M. Moore. 1991. Localization of the mouse thymidine kinase gene to the distal portion of chromosome 11. Genomics. 10 (3): 827-30.

43. Dobrovolsky V.N., J.G. Shaddock and R.H. Heflich. 2000. 7,12-dimethylbenz[a]anthracene-induced mutation in the *Tk* gene of *Tk*(+/-) mice: automated scoring of lymphocyte clones using a fluorescent viability indicator. Environ Mol Mutagen. 36 (4): 283-91.

44. Dobrovolsky V.N., J.G. Shaddock and R.H. Heflich. 2002. Mutagenicity of gamma-radiation, mitomycin C, and etoposide in the *Hprt* and *Tk* genes of *Tk*(+/-) mice. Environ Mol Mutagen. 39 (4): 342-7.

45. Moore M.M. and D. Clive. 1982. The quantitation of TK-/- and HGPRT- mutants of L5178Y/TK+/- mouse lymphoma cells at varying times post-treatment. Environ Mutagen. 4 (4): 499-519.

46. Winton D.J., M.A. Blount and B.A. Ponder. 1988. A clonal marker induced by mutation in mouse intestinal epithelium. Nature. 333 (6172): 463-6.

47. Winton D.J., N.J. Gooderham, A.R. Boobis, D.S. Davies and B.A. Ponder. 1990. Mutagenesis of mouse intestine *in vivo* using the *Dlb-1* specific locus test: studies with 1,2-dimethylhydrazine, dimethylnitrosamine, and the dietary mutagen 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline. Cancer Res. 50 (24): 7992-6.

48. Uiterdijk H.G., B.A. Ponder, M.F. Festing, J. Hilgers, L. Skow and R. Van Nie. 1986. The gene controlling the binding sites of Dolichos biflorus agglutinin, Dlb-1, is on chromosome 11 of the mouse. Genet Res. 47 (2): 125-9.

49. Tao K.S., C. Urlando and J.A. Heddle. 1993. Comparison of somatic mutation in a transgenic versus host locus. Proc Natl Acad Sci U S A. 90 (22): 10681-5.

50. OECD. Test Guideline 479: *In vitro* sister chromatid exchange assay in mammalian cells. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1986.

51. Rodriguez-Reyes R. and P. Morales-Ramirez. 2003. Sister chromatid exchange induction and the course of DNA duplication, two mechanisms of sister chromatid exchange induction by ENU and the role of BrdU. Mutagenesis. 18 (1): 65-72.

52. Perry P. and H.J. Evans. 1975. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature. 258 (5531): 121-5.

53. OECD. Test Guideline 486: Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vitro*. In: OECD Guidelines for Testing of Chemicals. Paris: Organization for Economic Cooperation and Development; 1997.

54. Madle S., S.W. Dean, U. Andrae, G. Brambilla, B. Burlinson, D.J. Doolittle, C. Furihata, T. Hertner, C.A. McQueen and H. Mori. 1994. Recommendations for the performance of UDS tests *in vitro* and *in vivo*. Mutat Res. 312 (3): 263-85.

55. Sega G.A. 1974. Unscheduled DNA synthesis in the germ cells of male mice exposed *in vivo* to the chemical mutagen ethyl methanesulfonate. Proc Natl Acad Sci U S A. 71 (12): 4955-9.

56. Sega G.A. 1979. Unscheduled DNA synthesis (DNA repair) in the germ cells of male mice--its role in the study of mammalian mutagenesis. Genetics. 92 (1 Pt 1 Suppl): s49-58.

57. Working P.K. and B.E. Butterworth. 1984. An assay to detect chemically induced DNA repair in rat spermatocytes. Environ Mutagen. 6 (3): 273-86.

58. Gossen J.A., W.J. de Leeuw, C.H. Tan, E.C. Zwarthoff, F. Berends, P.H. Lohman, D.L. Knook and J. Vijg. 1989. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. Proc Natl Acad Sci U S A. 86 (20): 7971-5.

59. Kohler S.W., G.S. Provost, P.L. Kretz, A. Fieck, J.A. Sorge and J.M. Short. 1990. The use of transgenic mice for short-term, *in vivo* mutagenicity testing. Genet Anal Tech Appl. 7 (8): 212-18.

60. Vijg, J. and G.R. Douglas. Bacteriophage lambda and plasmid lacZ transgenic mice for studying mutations *in vivo*. In: Pfeifer, G.P., ed. Technologies for Detection of DNA Damage and Mutations. New York: Plenum Press; 1996. pp. 391-410 .

61. Ikehata H., M. Takatsu, Y. Saito and T. Ono. 2000. Distribution of spontaneous CpG-associated G:C --> A:T mutations in the *lacZ* gene of Muta mice: effects of CpG methylation, the sequence context of CpG sites, and severity of mutations on the activity of the lacZ gene product. Environ Mol Mutagen. 36 (4): 301-11.

62. Monroe J.J., M.G. Manjanatha and T.R. Skopek. 2001. Extent of CpG methylation is not proportional to the *in vivo* spontaneous mutation frequency at transgenic loci in Big Blue rodents. Mutat Res. 476 (1-2): 1-11.

63. You Y.H., A. Halangoda, V. Buettner, K. Hill, S. Sommer and G. Pfeifer. 1998. Methylation of CpG dinucleotides in the *lacI* gene of the Big Blue transgenic mouse. Mutat Res. 420 (1-3): 55-65.

64. Heddle J.A., S. Dean, T. Nohmi, M. Boerrigter, D. Casciano, G.R. Douglas, B.W. Glickman, N.J. Gorelick, J.C. Mirsalis, H.J. Martus, T.R. Skopek, V. Thybaud, K.R. Tindall and N. Yajima. 2000. *In vivo* transgenic mutation assays. Environ Mol Mutagen. 35 (3): 253-9.

65. Thybaud V., S. Dean, T. Nohmi, J. de Boer, G.R. Douglas, B.W. Glickman, N.J. Gorelick, J.A. Heddle, R.H. Heflich, I. Lambert, H.J. Martus, J.C. Mirsalis, T. Suzuki and N. Yajima. 2003. *In vivo* transgenic mutation assays. Mutat Res. 540 (2): 141-51.

66. Blakey D.H., G.R. Douglas, K.C. Huang and H.J. Winter. 1995. Cytogenetic mapping of lambda gt10 *lacZ* sequences in the transgenic mouse strain 40.6 (Muta Mouse). Mutagenesis. 10 (2): 145-48.

67. Gossen J.A. and J. Vijg. 1988. *E. coli* C: a convenient host strain for rescue of highly methylated DNA. Nucleic Acids Res. 16 (19): 9343.

68. Gossen J.A., W.J. de Leeuw, A. Verwest, P.H. Lohman and J. Vijg. 1991. High somatic mutation frequencies in a *LacZ* transgene integrated on the mouse X-chromosome. Mutat Res. 250 (1-2): 423-29.

69. Mientjes E.J., J.H.M. van Delft, B.M. op't Hof, J.A. Gossen, J. Vijg, P.H.M. Lohman and R.A. Baan. 1994. An improved selection method for lambda *lacZ* phages based on galactose sensitivity. Transgenic Res. 3 (1): 67-9.

70. Lambert I.B., T.M. Singer, S.E. Boucher and G.R. Douglas. 2005. Detailed review of transgenic rodent mutation assays. Mutat Res. 590 (1-3): 1-280.

71. Kohler S.W., G.S. Provost, A. Fieck, P.L. Kretz, W.O. Bullock, J.A. Sorge, D.L. Putman and J.M. Short. 1991. Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc Natl Acad Sci U S A. 88 (18): 7958-62.

72. Kohler S.W., G.S. Provost, A. Fieck, P.L. Kretz, W.O. Bullock, D.L. Putman, J.A. Sorge and J.M. Short. 1991. Analysis of spontaneous and induced mutations in transgenic mice using a lambda ZAP/*lacI* shuttle vector. Environ Mol Mutagen. 18 (4): 316-21.

73. Dycaico M.J. , G.S. Provost, P.L. Kretz, S.L. Ransom, J.C. Moores and J.M. Short. 1994. The use of shuttle vectors for mutation analysis in transgenic mice and rats. Mutat Res. 307 (2): 461-78.

74. Vijg J., M.E. Dolle, H.J. Martus and M.E. Boerrigter. 1997. Transgenic mouse models for studying mutations *in vivo*: applications in aging research. Mech Ageing Dev. 99 (3): 257-71.

75. Gossen J.A., W.J. de Leeuw, A.C. Molijn and J. Vijg. 1993. Plasmid rescue from transgenic mouse DNA using *LacI* repressor protein conjugated to magnetic beads. Biotechniques. 14 (4): 624-9.

76. Masumura K., M. Matsui, M. Katoh, N. Horiya, O. Ueda, H. Tanabe, M. Yamada, H. Suzuki, T. Sofuni and T. Nohmi. 1999. Spectra of *gpt* mutations in ethylnitrosourea-treated and untreated transgenic mice. Environ Mol Mutagen. 34 (1): 1-8.

77. Hayashi H., H. Kondo, K. Masumura, Y. Shindo and T. Nohmi. 2003. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and *Spi*-selection. Environ Mol Mutagen. 41 (4): 253-9.

78. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals (S2B). International Conference on Harmonisation; 1997.

79. Waters M.D., H.F. Stack, N.E. Garrett and M.A. Jackson. 1991. The Genetic Activity Profile database. Environ Health Perspect. 96: 41-5.

80. Cohen J. 1960. A coefficient of agreement for nominal scales. Educational and Psychological Measurement. 20 (1): 37-46.

81. Kim B.S. and B.H. Margolin. 1999. Prediction of rodent carcinogenicity utilizing a battery of *in vitro* and *in vivo* genotoxicity tests. Environ Mol Mutagen. 34 (4): 297-304.

82. Zeiger E. 1998. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: premises, promises, and performance. Regul Toxicol Pharmacol. 28 (2): 85-95.

83. Zeiger E., J.K. Haseman, M.D. Shelby, B.H. Margolin and R.W. Tennant. 1990. Evaluation of four *in vitro* genetic toxicity tests for predicting rodent carcinogenicity: confirmation of earlier results with 41 additional chemicals. Environ Mol Mutagen. 16 Suppl 18: 1-14.

84. Tennant R.W. , B.H. Margolin, M.D. Shelby, E. Zeiger, J.K. Haseman, J. Spalding, W. Caspary, M. Resnick, S. Stasiewicz, B. Anderson *et al.* 1987. Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. Science. 236 (4804): 933-41.

85. Klopman G. and H.S. Rosenkranz. 1991. Quantification of the predictivity of some short-term assays for carcinogenicity in rodents. Mutat Res. 253 (3): 237-40.

86. Culp S.J., F.A. Beland, R.H. Heflich, R.W. Benson, L.R. Blankenship, P.J. Webb, P.W. Mellick, R.W. Trotter, S.D. Shelton, K.J. Greenlees and M.G. Manjanatha. 2002. Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green. Mutat Res. 506-507: 55-63.

87. Shane B.S., D.L. Smith-Dunn, J.G. deBoer, B.W. Glickman and M.L. Cunningham. 2000. Subchronic administration of phenobarbital alters the mutation spectrum of *lacI* in the livers of Big Blue transgenic mice. Mutat Res. 448 (1): 69-80.

88. Singh V.K., L. Ganesh, M.L. Cunningham and B.S. Shane. 2001. Comparison of the mutant frequencies and mutation spectra of three non-genotoxic carcinogens, oxazepam, phenobarbital, and Wyeth 14,643, at the □c*II* locus in Big Blue transgenic mice. Biochem Pharmacol. 62 (6): 685-92.

89. Shane B.S., J.G. deBoer, B.W. Glickman and M.L. Cunningham. 1999. Oxazepam is mutagenic *in vivo* in Big Blue transgenic mice. Carcinogenesis. 20 (7): 1315-21.

90. Ennever F.K. and H.S. Rosenkranz. 1988. The influence of the proportion of carcinogens on the cost-effectiveness of short-term tests. Mutat Res. 197 (1): 1-13.

91. Fung V.A., J.C. Barrett and J. Huff. 1995. The carcinogenesis bioassay in perspective: application in identifying human cancer hazards. Environ Health Perspect. 103 (7-8): 680-3.

92. Kirkland D., M. Aardema, L. Henderson and L. Muller. 2005. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. Mutat Res. 584 (1-2): 1-256.

93. McCann J., E. Choi, E. Yamasaki and B.N. Ames. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc Natl Acad Sci U S A. 72 (12): 5135-9.

94. Purchase I.F., E. Longstaff, J. Ashby, J.A. Styles, D. Anderson, P.A. Lefevre and F.R. Westwood. 1978. An evaluation of 6 short-term tests for detecting organic chemical carcinogens. Br J Cancer. 37 (6): 873-903.

95. Kociba R.J., D.G. Keyes, J.E. Beyer, R.M. Carreon, C.E. Wade, D.A. Dittenber, R.P. Kalnins, L.E. Frauson, C.N. Park, S.D. Barnard, R.A. Hummel and C.G. Humiston. 1978. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. Toxicol Appl Pharmacol. 46 (2): 279-303.

96. National Toxicology Program. 1982. Carcinogenesis Bioassay of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (CAS No. 1746-01-6) in Osborne-Mendel Rats and B6C3F1 Mice (Gavage Study). Natl Toxicol Program Tech Rep Ser. 209: 1-195.

97. Whysner J. and G.M. Williams. 1996 . 2,3,7,8-Tetrachlorodibenzo-p-dioxin mechanistic data and risk assessment: gene regulation, cytotoxicity, enhanced cell proliferation, and tumor promotion. Pharmacol Ther. 71 (1-2): 193-223.

98. Shu H.P., D.J. Paustenbach and F.J. Murray. 1987. A critical evaluation of the use of mutagenesis, carcinogenesis, and tumor promotion data in a cancer risk assessment of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Regul Toxicol Pharmacol. 7 (1): 57-88.

99. Gunz D., S.E. Shephard and W.K. Lutz. 1993. Can nongenotoxic carcinogens be detected with the lacI transgenic mouse mutation assay? Environ Mol Mutagen. 21 (3): 209-11.

100. Flaks A. and B. Flaks. 1983. Induction of liver cell tumours in IF mice by paracetamol. Carcinogenesis. 4 (4): 363-8.

101. Flaks B., A. Flaks and A.P. Shaw. 1985. Induction by paracetamol of bladder and liver tumours in the rat. Effects on hepatocyte fine structure. Acta Pathol Microbiol Immunol Scand [A]. 93 (6): 367-77.

102. National Toxicology Program. 1993. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 394: 1-274.

103. Bergman K., L. Muller and S.W. Teigen. 1996. Series: current issues in mutagenesis and carcinogenesis, No. 65. The genotoxicity and carcinogenicity of paracetamol: a regulatory (re)view. Mutat Res. 349 (2): 263-88.

104. Clements, J. Study to determine the ability of paracetamol to induce mutations at the thymidine kinase (*tk*) locus in mouse lymphoma L5178Y cells using a fluctuation assay.: Hazleton Microtest; 1992 Mar 31. Report No.: Report No. ZTKREUBF.001.

105. Kanki K., A. Nishikawa, K. Masumura, T. Umemura, T. Imazawa, Y. Kitamura, T. Nohmi and M. Hirose. 2005. *In vivo* mutational analysis of liver DNA in *gpt* delta transgenic rats treated with the hepatocarcinogens N-nitrosopyrrolidine, 2-amino-3-methylimidazo[4,5-f]quinoline, and di(2-ethylhexyl)phthalate. Mol Carcinog. 42 (1): 9-17.

106. International Programme on Chemical Safety Carbon tetrachloride. Geneva: World Health Organization; 1999.

107. Weisburger E.K. 1977. Carcinogenicity studies on halogenated hydrocarbons. Environ Health Perspect. 21: 7-16.

108. Reuber M.D. and E.L. Glover. 1970 . Cirrhosis and carcinoma of the liver in male rats given subcutaneous carbon tetrachloride. J Natl Cancer Inst. 44 (2): 419-27.

109. McGregor D. and M. Lang. 1996. Carbon tetrachloride: genetic effects and other modes of action. Mutat Res. 366 (3): 181-95.

110. International Agency for Research on Cancer. 1999. Carbon tetrachloride. IARC Monogr Eval Carcinog Risks Hum. 71 Pt 2: 401-32.

111. Tafazoli M. , A. Baeten, P. Geerlings and M. Kirsch-Volders. 1998. *In vitro* mutagenicity and genotoxicity study of a number of short-chain chlorinated hydrocarbons using the micronucleus test and the alkaline single cell gel electrophoresis technique (Comet assay) in human lymphocytes: a structure-activity relationship (QSAR) analysis of the genotoxic and cytotoxic potential. Mutagenesis. 13 (2): 115-26.

112. Tombolan F. , D. Renault, D. Brault, M. Guffroy, F. Perin and V. Thybaud. 1999. Effect of mitogenic or regenerative cell proliferation on lacz mutant frequency in the liver of Muta™Mice treated with 5, 9-dimethyldibenzo[c,g]carbazole. Carcinogenesis. 20 (7): 1357-62.

113. Hachiya N. and Y. Motohashi. 2000 . Examination of *lacZ* mutant induction in the liver and testis of Muta Mouse following injection of halogenated aliphatic hydrocarbons classified as human carcinogens. Ind Health. 38 (2): 213-20.

114. Henderson R.F., F.F. Hahn, E.B. Barr, S.A. Belinsky, M.G. Menache and J.M. Benson. 1999. Carcinogenicity of inhaled butadiene diepoxide in female B6C3F1 mice and Sprague-Dawley rats. Toxicol Sci. 52 (1): 33-44.

115. Jackson M.A., H.F. Stack, J.M. Rice and M.D. Waters. 2000. A review of the genetic and related effects of 1,3-butadiene in rodents and humans. Mutat Res. 463 (3): 181-213.

116. Abu-Shakra A., E.T. McQueen and M.L. Cunningham. 2000. Rapid analysis of base-pair substitutions induced by mutagenic drugs through their oxygen radical or epoxide derivatives. Mutat Res. 470 (1): 11-8.

117. Adler I.D., U. Kliesch, L. Nylund and K. Peltonen. 1997. *In vitro* and *in vivo* mutagenicity of the butadiene metabolites butadiene diolepoxide, butadiene monoepoxide and diepoxybutane. Mutagenesis. 12 (5): 339-45.

118. Steen A.M., K.G. Meyer and L. Recio. 1997. Analysis of *hprt* mutations occurring in human TK6 lymphoblastoid cells following exposure to 1,2,3,4-diepoxybutane. Mutagenesis. 12 (2): 61-7.

119. Vlachodimitropoulos D., H. Norppa, K. Autio, J. Catalan, A. Hirvonen, G. Tasa, M. Uuskula, N.A. Demopoulos and M. Sorsa. 1997. GSTT1-dependent induction of centromere-negative and -positive micronuclei by 1,2:3,4-diepoxybutane in cultured human lymphocytes. Mutagenesis. 12 (5): 397-403.

120. Xi L., L. Zhang, Y. Wang and M.T. Smith. 1997. Induction of chromosome-specific aneuploidy and micronuclei in human lymphocytes by metabolites of 1,3-butadiene. Carcinogenesis. 18 (9): 1687-93.

121. Marx M.P., S. Smith, A.D. Heyns and I.Z. van Tonder. 1983. Fanconi's anemia: a cytogenetic study on lymphocyte and bone marrow cultures utilizing 1,2:3,4-diepoxybutane. Cancer Genet Cytogenet. 9 (1): 51-9.

122. Walk R.A., J. Jenderny, G. Rohrborn and U. Hackenberg. 1987. Chromosomal abnormalities and sister-chromatid exchange in bone marrow cells of mice and Chinese hamsters after inhalation and intraperitoneal administration: I. Diepoxybutane. Mutat Res. 182 (6): 333-42.

123. Komulainen H., V.M. Kosma, S.L. Vaittinen, T. Vartiainen, E. Kaliste-Korhonen, S. Lotjonen, R.K. Tuominen and J. Tuomisto. 1997. Carcinogenicity of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone in the rat. J Natl Cancer Inst. 89 (12): 848-56.

124. McDonald T.A. and H. Komulainen. 2005. Carcinogenicity of the chlorination disinfection by-product MX. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 23 (2): 163-214.

125. Daniel F.B. , G.R. Olson and J.A. Stober. 1991. Induction of gastrointestinal tract nuclear anomalies in B6C3F1 mice by 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone and 3,4-(dichloro)-5-hydroxy-2[5H]-furanone, mutagenic byproducts of chlorine disinfection. Environ Mol Mutagen. 17 (1): 32-9.

126. Maki-Paakkanen J. and K. Jansson. 1995. Cytogenetic effects in the peripheral lymphocytes and kidney cells of rats exposed to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) orally on three consecutive days. Mutat Res. 343 (2-3): 151-6.

127. Nishikawa A., K. Sai, K. Okazaki, H.Y. Son, K. Kanki, M. Nakajima, N. Kinae, T. Nohmi, J.E. Trosko, T. Inoue and M. Hirose. 2006. MX, a by-product of water chlorination, lacks *in vivo* genotoxicity in *gpt* delta mice but inhibits gap junctional intercellular communication in rat WB cells. Environ Mol Mutagen. 47 (1): 48-55.

128. National Cancer Institute Carcinogenesis bioassay of chloroform. Bethesda, MD: National Cancer Institute; 1976. Report No.: NTIS No. PB264018/AS.

129. Jorgenson T.A., E.F. Meierhenry, C.J. Rushbrook, R.J. Bull and M. Robinson. 1985. Carcinogenicity of chloroform in drinking water to male Osborne-Mendel rats and female B6C3F1 mice. Fundam Appl Toxicol. 5 (4): 760-9.

130. Tumasonis C.F., D.N. McMartin and B. Bush. 1985. Lifetime toxicity of chloroform and bromodichloromethane when administered over a lifetime in rats. Ecotoxicol Environ Saf. 9 (2): 233-40.

131. Golden R.J. , S.E. Holm, D.E. Robinson, P.H. Julkunen and E.A. Reese. 1997. Chloroform mode of action: implications for cancer risk assessment. Regul Toxicol Pharmacol. 26 (2): 142-55.

132. Butterworth B.E., M.V. Templin, A.A. Constan, C.S. Sprankle, B.A. Wong, L.J. Pluta, J.I. Everitt and L. Recio. 1998. Long-term mutagenicity studies with chloroform and dimethylnitrosamine in female lacI transgenic B6C3F1 mice. Environ Mol Mutagen. 31 (3): 248-56.

133. Robbiano L., E. Mereto, A. Migliazzi Morando, P. Pastore and G. Brambilla. 1998 . Increased frequency of micronucleated kidney cells in rats exposed to halogenated anaesthetics. Mutat Res. 413 (1): 1-6.

134. Fujie K., T. Aoki and M. Wada. 1990. Acute and subacute cytogenetic effects of the trihalomethanes on rat bone marrow cells *in vivo*. Mutat Res. 242 (2): 111-9.

135. Mirsalis J.C., C.K. Tyson, K.L. Steinmetz, E.K. Loh, C.M. Hamilton, J.P. Bakke and J.W. Spalding. 1989. Measurement of unscheduled DNA synthesis and S-phase synthesis in rodent hepatocytes following *in vivo* treatment: testing of 24 compounds. Environ Mol Mutagen. 14 (3): 155-64.

136. Fujimoto N. , H. Watanabe, T. Nakatani, G. Roy and A. Ito. 1998. Induction of thyroid tumours in (C57BL/6N x C3H/N)F1 mice by oral administration of kojic acid. Food Chem Toxicol. 36 (8): 697-703.

137. Nohynek G.J., D. Kirkland, D. Marzin, H. Toutain, C. Leclerc-Ribaud and H. Jinnai. 2004. An assessment of the genotoxicity and human health risk of topical use of kojic acid. Food Chem Toxicol. 42 (1): 93-105.

138. Wei C.I., T.S. Huang, S.Y. Fernando and K.T. Chung. 1991. Mutagenicity studies of kojic acid. Toxicol Lett. 59 (1-3): 213-20.

139. Shibuya T., T. Murota, K. Sakamoto, S. Iwahara and M. Ikeno. 1982. Mutagenicity and dominant lethal test of kojic acid--Ames test, forward mutation test in cultured Chinese hamster cells and dominant lethal test in mice. J Toxicol Sci. 7 (4): 255-62.

140. Bjeldanes L.F. and H. Chew. 1979. Mutagenicity of 1,2-dicarbonyl compounds: maltol, kojic acid, diacetyl and related substances. Mutat Res. 67 (4): 367-71.

141. Suzuki H., N. Ikeda, K. Kobayashi, Y. Terashima, Y. Shimada, T. Suzuki, T. Hagiwara, S. Hatakeyama, K. Nagaoka, J. Yoshida, Y. Saito, J. Tanaka and M. Hayashi. 2005. Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats. A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS). Mutat Res. 583 (2): 133-45.

142. Danse L.H., F.L. van Velsen and C.A. van der Heijden. 1984. Methylbromide: carcinogenic effects in the rat forestomach. Toxicol Appl Pharmacol. 72 (2): 262-71.

143. International Programme on Chemical Safety. Methyl Bromide. Geneva: World Health Organization; 1995.

144. Boorman G.A., H.L. Hong, C.W. Jameson, K. Yoshitomi and R.R. Maronpot. 1986. Regression of methyl bromide-induced forestomach lesions in the rat. Toxicol Appl Pharmacol. 86 (1): 131-9.

145. Mitsumori K., K. Maita, T. Kosaka, T. Miyaoka and Y. Shirasu. 1990. Two-year oral chronic toxicity and carcinogenicity study in rats of diets fumigated with methyl bromide. Food Chem Toxicol. 28 (2): 109-19.

146. National Toxicology Program. 1992. NTP Toxicology and Carcinogenesis Studies of Methyl Bromide (CAS: 74-83-9) in B6C3F1 Mice (Inhalation Studies). Natl Toxicol Program Tech Rep Ser. 385: 1-212.

147. Reuzel P.G. , H.C. Dreef-van der Meulen, V.M. Hollanders, C.F. Kuper, V.J. Feron and C.A. van der Heijden. 1991. Chronic inhalation toxicity and carcinogenicity study of methyl bromide in Wistar rats. Food Chem Toxicol. 29 (1): 31-9.

148. International Agency for Research on Cancer. 1999. Methyl Bromide. IARC Monogr Eval Carcinog Risks Hum. 71 Pt 2: 721-35.

149. Pletsa V., M.J. Steenwinkel, J.H. van Delft, R.A. Baan and S.A. Kyrtopoulos. 1999. Methyl bromide causes DNA methylation in rats and mice but fails to induce somatic mutations in lambda *lacZ* transgenic mice. Cancer Lett. 135 (1): 21-7.

150. Bolt H.M. and B. Gansewendt. 1993 . Mechanisms of carcinogenicity of methyl halides. Crit Rev Toxicol. 23 (3): 237-53.

151. National Toxicology Program. 1996. NTP Toxicology and Carcinogenesis Studies of Nickel Subsulfide (CAS No. 12035-72-2) in F344 Rats and B6C3F1 Mice (Inhalation Studies). Natl Toxicol Program Tech Rep Ser. 453: 1-365.

152. International Programme on Chemical Safety. Nickel. Geneva: World Health Organization; 1991.

153. Mayer C., R.G. Klein, H. Wesch and P. Schmezer. 1998. Nickel subsulfide is genotoxic *in vitro* but shows no mutagenic potential in respiratory tract tissues of BigBlue rats and Muta Mouse mice *in vivo* after inhalation. Mutat Res. 420 (1-3): 85-98.

154. Arrouijal F.Z., H.F. Hildebrand, H. Vophi and D. Marzin. 1990. Genotoxic activity of nickel subsulphide alpha-Ni3S2. Mutagenesis. 5 (6): 583-9.

155. Kawanishi S., S. Oikawa, S. Inoue and K. Nishino. 2002. Distinct mechanisms of oxidative DNA damage induced by carcinogenic nickel subsulfide and nickel oxides. Environ Health Perspect. 110 Suppl 5: 789-91.

156. Lee Y.W., C.B. Klein, B. Kargacin, K. Salnikow, J. Kitahara, K. Dowjat, A. Zhitkovich, N.T. Christie and M. Costa. 1995. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. Mol Cell Biol. 15 (5): 2547-57.

157. National Cancer Institute. 1976. Carcinogenesis bioassay of trichloroethylene. Natl Cancer Inst Carcinog Tech Rep Ser. 2: 1-215.

158. National Toxicology Program. 1990. NTP Carcinogenesis Studies of Trichloroethylene (Without Epichlorohydrin) (CAS No. 79-01-6) in F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser. 243: 1-174.

159. Douglas G.R., J.D. Gingerich, L.M. Soper, M. Potvin and S. Bjarnason. 1999. Evidence for the lack of base-change and small-deletion mutation induction by trichloroethylene in *lacZ* transgenic mice. Environ Mol Mutagen. 34 (2-3): 190-4.

160. Fahrig R., S. Madle and H. Baumann. 1995. Genetic toxicology of trichloroethylene (TCE). Mutat Res. 340 (1): 1-36.

161. Moore M.M. and K. Harrington-Brock. 2000. Mutagenicity of trichloroethylene and its metabolites: implications for the risk assessment of trichloroethylene. Environ Health Perspect. 108 Suppl 2: 215-23.

162. National Toxicology Program. 1982. Carcinogenesis Bioassay of Di(2-ethylhexyl)phthalate (CAS No. 117-81-7) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 217: 1-127.

163. Cattley R.C., J.G. Conway and J.A. Popp. 1987. Association of persistent peroxisome proliferation and oxidative injury with hepatocarcinogenicity in female F-344 rats fed di(2-ethylhexyl)phthalate for 2 years. Cancer Lett. 38 (1-2): 15-22.

164. Rao M.S., A.V. Yeldandi and V. Subbarao. 1990. Quantitative analysis of hepatocellular lesions induced by di(2-ethylhexyl)phthalate in F-344 rats. J Toxicol Environ Health. 30 (2): 85-9.

165. Schmezer P. , B.L. Pool, R.G. Klein, D. Komitowski and D. Schmahl. 1988. Various short-term assays and two long-term studies with the plasticizer di(2-ethylhexyl)phthalate in the Syrian golden hamster. Carcinogenesis. 9 (1): 37-43.

166. Melnick R.L. 1992. Does chemically induced hepatocyte proliferation predict liver carcinogenesis? FASEB J. 6 (9): 2698-706.

167. International Programme on Chemical Safety Diethylhexyl phthalate. Geneva: World Health Organization; 1992.

168. Doull J., R. Cattley, C. Elcombe, B.G. Lake, J. Swenberg, C. Wilkinson, G. Williams and M. van Gemert. 1999. A cancer risk assessment of di(2-ethylhexyl)phthalate: application of the new U.S. EPA Risk Assessment Guidelines. Regul Toxicol Pharmacol. 29 (3): 327-57.

169. National Toxicology Program. 1993. NTP Toxicology and Carcinogenesis Studies of Oxazepam (CAS No. 604-75-1) in Swiss-Webster and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser. 443: 1-321.

170. National Toxicology Program. 1998. NTP Toxicology and Carcinogenesis Studies of Oxazepam (CAS No. 604-75-1) in F344/N Rats (Feed Studies). Natl Toxicol Program Tech Rep Ser. 468: 1-189.

171. Giri A.K. and S. Banerjee. 1996. Genetic toxicology of four commonly used benzodiazepines: a review. Mutat Res. 340 (2-3): 93-108.

172. Cosentino L. and J.A. Heddle. 1996. A test for neutrality of mutations of the *lacZ* transgene. Environ Mol Mutagen. 28 (4): 313-6.

173. Suzuki T., S. Itoh, M. Nakajima, N. Hachiya and T. Hara. 1999. Target organ and time-course in the mutagenicity of five carcinogens in MutaMouse: a summary report of the second collaborative study of the transgenic mouse mutation assay by JEMS/MMS. Mutat Res. 444 (2): 259-68.

174. Douglas G.R., J. Jiao, J.D. Gingerich, L.M. Soper and J.A. Gossen. 1996. Temporal and molecular characteristics of *lacZ* mutations in somatic tissues of transgenic mice. Environ Mol Mutagen. 28 (4): 317-24.

175. Littell, R.C.; G.A. Milliken; W. Stroup and R.D. Wolfinger. SAS System for Mixed Models. Cary, North Carolina: SAS Institute Inc.; 1996.

176. Breslow N.E. and D.G. Clayton. 1993. Approximate inference in generalized linear mixed models. J Am Stat Assoc. 88 (421): 9-25.

177. Wolfinger R.D. and M. O'Connell. 1993. Generalized linear models: A pseudo-likelihood approach. J Stat Comput Simul. 48 (3-4): 233-43.

178. Holm S. 1979. A Simple Sequentially Rejective Bonferroni Test Procedure. Scand J Statist. 6: 65-70.

179. Hochberg, Y.; Tamhane, A. C. Multiple Comparison Procedures. New York: John Wiley & Sons, Inc.; 1987.

180. Fung K.Y., X. Lin and D. Krewski. 1998. Use of generalized linear mixed models in analyzing mutant frequency data from the transgenic mouse assay. Environ Mol Mutagen. 31 (1): 48-54.

181. Searle S.R. , F.M. Speed and G.A. Milliken. 1980. The population marginal means in the linear model: An alternative to least squares means. Am Stat. 34 (4): 216-21.

182. Goodnight, J. H. and W.R. Harvey Least-Squares Means in the Fixed-Effects General Linear Models. Cary, North Carolina: SAS Institute Inc.; 1978.

183. Heddle J.A. , H.J. Martus and G.R. Douglas. 2003. Treatment and sampling protocols for transgenic mutation assays. Environ Mol Mutagen. 41 (1): 1-6.

184. Cameron, I.L. Cell proliferation and renewal in the mammalian body. Cameron, I.L. and J.D. Thrasher, Eds. Cellular and Molecular Renewal in the Mammalian Body. New York: Academic Press; 1971. pp. 45-85.

185. Tao K.S. and J.A. Heddle. 1994. The accumulation and persistence of somatic mutations *in vivo*. Mutagenesis. 9 (3): 187-91.

186. Wang J., X. Liu, R.H. Heflich and T. Chen. 2004. Time course of *cII* gene mutant manifestation in the liver, spleen, and bone marrow of N-ethyl-N-nitrosourea-treated Big Blue transgenic mice. Toxicol Sci. 82 (1): 124-8.

187. Topinka J., D. Oesterle, R. Reimann and T. Wolff. 2004. No-effect level in the mutagenic activity of the drug cyproterone acetate in rat liver. Part I. Single dose treatment. Mutat Res. 550 (1-2): 89-99.

188. Cosentino L. and J.A. Heddle. 2000. Differential mutation of transgenic and endogenous loci *in vivo*. Mutat Res. 454 (1-2): 1-10.

189. Swiger R.R. , L. Cosentino, K.I. Masumura, T. Nohmi and J.A. Heddle. 2001. Further characterization and validation of *gpt* delta transgenic mice for quantifying somatic mutations *in vivo*. Environ Mol Mutagen. 37 (4): 297-303.

190. Cosentino L. and J.A. Heddle. 1999. Effects of extended chronic exposures on endogenous and transgenic loci: implications for low-dose extrapolations. Environ Mol Mutagen. 34 (2-3): 208-15.

191. Zeiger E., B. Anderson, S. Haworth, T. Lawlor and K. Mortelmans. 1992. Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. Environ Mol Mutagen. 19 Suppl 21: 2-141.

192. Chan P.C. 1996. NTP technical report on toxicity studies of urethane in drinking water and urethane in 5% ethanol administered to F344/N rats and B6C3F1 mice. Toxic Rep Ser. (52): 1-91, A1-9, B1-9 passim.

193. Balansky R. , P. Blagoeva and Z. Mircheva. 1992. Clastogenic activity of urethane in mice. Mutat Res. 281 (2): 99-103.

194. Westmoreland C., M. Plumstead and D. Gatehouse. 1991. Activity of urethane in rat and mouse micronucleus tests after oral administration. Mutat Res. 262 (4): 247-51.

195. Sanderson B.J. and A.M. Clark. 1993. Micronuclei in adult and foetal mice exposed *in vivo* to heliotrine, urethane, monocrotaline and benzidine. Mutat Res. 285 (1): 27-33.

196. Williams C.V., K. Fletcher, H. Tinwell and J. Ashby. 1998. Mutagenicity of ethyl carbamate to lacZ- transgenic mice. Mutagenesis. 13 (2): 133-7.

197. Shephard S.E., D. Gunz and C. Schlatter. 1995. Genotoxicity of agaritine in the lacI transgenic mouse mutation assay: evaluation of the health risk of mushroom consumption. Food Chem Toxicol. 33 (4): 257-64.

198. Chang P.Y., J. Mirsalis, E.S. Riccio, J.P. Bakke, P.S. Lee, J. Shimon, S. Phillips, D. Fairchild, Y. Hara and J.A. Crowell. 2003. Genotoxicity and toxicity of the potential cancer-preventive agent polyphenon E. Environ Mol Mutagen. 41 (1): 43-54.

199. Ames B.N. and L.S. Gold. 1990. Chemical carcinogenesis: too many rodent carcinogens. Proc Natl Acad Sci U S A. 87 (19): 7772-6.

Appendix 1

Individual Animal Mutant Frequency Data from Chapter 3

Bone marrow

Animal	Dose (mg/kg/day)	Group	Number of packaging reactions	Total mutants	Total pfu ($x10^5$)	Mean MF ($x10^{-5}$)	Standard Error ($x10^{-5}$)	95% C.I. ($x10^{-5}$)
1	0	7/3	2	18	2.59	6.67	1.89	3.82-11.6
2	0	7/3	2	8	2.25	3.43	1.32	1.61-7.31
3	0	7/3	2	7	2.13	3.14	1.29	1.41-7.01
4	0	7/3	3	6	2.39	2.39	1.04	1.02-5.62
5	0	7/3	2	7	2.37	2.86	1.17	1.28-6.38
6	0	7/28	1	8	3.66	2.08	0.828	0.955-4.54
7	0	7/28	1	23	5.30	4.14	1.14	2.40-7.12
8	0	7/28	1	31	2.17	13.6	3.48	8.24-22.5
9	0	7/28	1	4	2.51	1.52	0.808	0.535-4.31
10	0	7/28	1	17	6.97	2.32	0.705	1.28-4.21
11	0	28/3	1	35	5.02	6.97	1.84	4.16-11.7
12	0	28/3	1	17	4.07	4.18	1.32	2.25-7.76
13	0	28/3	1	28	4.47	6.27	1.74	3.64-10.8
14	0	28/3	1	19	4.92	3.86	1.18	2.12-7.04
15	0	28/3	1	8	2.56	3.13	1.27	1.41-6.95
16	0	28/3	1	22	2.80	7.87	2.31	4.42-14.0
17	25	7/3	2	17	3.26	5.05	1.46	2.87-8.89
18	25	7/3	2	11	2.64	3.91	1.34	2.00-7.64
19	25	7/3	3	20	7.85	2.55	0.710	1.48-4.41
20	25	7/3	2	7	3.05	2.12	0.875	0.946-4.76
21	25	7/3	2	16	3.00	5.09	1.51	2.85-9.09
22	25	7/28	1	21	5.57	3.60	1.02	2.06-6.28
23	25	7/28	1	16	2.97	5.14	1.59	2.80-9.42
24	25	7/28	1	28	3.07	8.68	2.28	5.19-14.5
25	25	7/28	1	8	3.61	2.11	0.840	0.969-4.60
26	25	7/28	1	13	2.86	4.34	1.44	2.26-8.31
27	25	28/3	1	7	2.61	2.68	1.15	1.16-6.21
28	25	28/3	1	27	4.02	6.72	1.88	3.89-11.6
29	25	28/3	1	28	5.84	4.79	1.33	2.78-8.25
30	25	28/3	1	27	2.66	10.1	2.83	5.86-17.5
31	25	28/3	1	21	3.21	6.55	1.95	3.65-11.7
32	100	7/3	2	10	2.66	3.57	1.26	1.78-7.14
33	100	7/3	2	16	2.87	5.33	1.58	2.98-9.52
34	100	7/3	4	0	0.0547	0		
35	100	7/3	2	16	2.86	5.38	1.59	3.02-9.60
36	100	7/28	1	10	2.02	4.72	1.72	2.31-9.65
37	100	7/3	3	28	6.50	4.07	0.980	2.54-6.53
38	100	7/28	3	26	2.09	12.5	3.04	7.78-20.2
39	100	7/28	2	38	3.81	9.77	2.20	6.28-15.2
40	100	7/28	1	15	4.14	3.46	1.09	1.86-6.42
42	100	7/28	1	37	4.84	7.29	1.79	4.51-11.8
43	100	7/3	2	21	2.38	8.29	2.25	4.87-14.1
45	100	28/3	1	35	3.46	10.1	2.67	6.03-17.0
46	100	28/3	1	22	4.51	4.88	1.44	2.74-8.69
47	100	28/3	1	23	3.19	7.20	2.09	4.07-12.7
48	100	28/3	1	36	3.03	11.9	3.12	7.11-19.9

Animal	Dose (mg/kg/day)	Group	Number of packaging reactions	Total mutants	Total pfu ($x10^5$)	Mean MF ($x10^{-5}$)	Standard Error ($x10^{-5}$)	95% C.I. ($x10^{-5}$)
49	100	28/3	1	75	6.16	11.3	2.79	6.99-18.3
50	0	28/28	1	16	2.52	6.87	2.11	3.77-12.5
51	0	28/28	1	13	3.31	4.24	1.40	2.23-8.09
52	0	28/28	1	17	4.77	3.85	1.16	2.14-6.95
53	0	28/28	2	10	2.89	3.49	1.23	1.74-6.98
54	0	28/28	1	18	2.32	8.40	2.48	4.71-15.0
55	0	28/28	1	17	5.33	3.45	1.04	1.91-6.22
56	0	56/3	2	3	0.818	3.67	2.24	1.11-12.2
57	0	56/3	1	0	2.08	0		
58	0	56/3	1	0	0	0		
59	0	56/3	1	46	3.57	13.7	2.98	8.97-21.0
60	0	56/3	1	11	3.62	3.24	1.10	1.66-6.31
61	0	56/3	2	19	3.73	5.02	1.38	2.93-8.60
62	12.5	56/3	1	19	2.84	7.14	2.00	4.13-12.3
63	12.5	56/3	2	17	3.09	5.39	1.54	3.08-9.44
64	12.5	56/3	1	19	2.20	9.22	2.58	5.34-15.9
65	12.5	56/3	1	24	3.95	6.48	1.68	3.90-10.8
66	12.5	56/3	2	25	5.22	4.62	1.16	2.82-7.55
67	12.5	56/3	2	27	3.06	8.52	2.09	5.27-13.8
68	25	56/3	2	14	3.02	4.72	1.47	2.56-8.70
69	25	28/28	1	20	3.55	6.09	1.74	3.48-10.7
70	25	28/28	1	18	2.09	9.30	2.75	5.21-16.6
71	25	28/28	1	17	3.11	5.90	1.78	3.28-10.6
72	25	28/28	1	10	2.25	4.80	1.74	2.36-9.78
73	25	28/28	1	16	3.51	4.93	1.51	2.70-8.99
74	25	28/28	2	16	2.14	7.52	2.23	4.20-13.4
75	50	28/28	1	19	2.37	8.54	2.39	4.94-14.8
76	50	56/3	2	42	3.55	11.5	2.49	7.53-17.6
77	50	56/3	1	42	2.89	15.5	3.44	10.0-23.9
78	50	56/3	1	29	3.25	9.50	2.33	5.88-15.4
79	50	56/3	3	52	2.77	18.8	3.85	12.6-28.1
80	50	56/3	2	30	2.74	10.8	2.57	6.81-17.2
81	50	56/3	3	14	2.17	6.34	1.92	3.51-11.5
82	100	56/3	1	32	3.09	11.2	2.81	6.86-18.3
83	100	28/28	2	45	3.49	12.7	2.79	8.29-19.6
84	100	28/28	1	34	4.36	8.44	2.08	5.20-13.7
85	100	28/28	1	34	3.06	12.0	2.97	7.40-19.5
86	100	28/28	2	21	1.97	11.0	3.01	6.48-18.9
87	100	28/28	1	30	4.21	7.70	1.96	4.67-12.7
88	100	28/28	4	23	2.24	10.3	2.56	6.34-16.8
89	100	56/3	1	30	2.19	14.6	3.53	9.07-23.5
90	100	56/3	1	50	2.52	21.1	4.50	13.9-32.1
91	100	56/3	2	68	2.82	23.4	4.54	16.0-34.2
92	100	56/3	3	39	2.08	18.8	4.01	12.3-28.5
93	100	56/3	1	28	3.07	9.71	2.40	5.98-15.8
94	100	56/3	1	30	2.00	16.0	3.87	9.94-25.7

Liver

Animal	Dose (mg/kg/day)	Group	Number of packaging reactions	Number of mutants	Total pfu ($x10^5$)	Mean MF ($x10^{-5}$)	Standard Error ($x10^{-5}$)	95% C.I. ($x10^{-5}$)
1	0	7/3	2	5	1.46	3.64	1.71	1.45-9.13
2	0	7/3	2	8	1.38	6.11	2.33	2.90-12.9
3	0	7/3	2	11	1.72	7.63	2.58	3.93-14.8
4	0	7/3	3	3	1.05	2.85	1.70	0.885-9.16
5	0	7/3	2	4	2.44	2.37	1.26	0.837-6.70
6	0	7/28	1	14	2.53	5.81	1.83	3.14-10.8
7	0	7/28	1	15	3.28	4.81	1.47	2.63-8.76
8	0	7/28	1	24	3.61	6.45	1.65	3.90-10.6
9	0	7/28	1	30	4.43	6.45	1.55	4.02-10.3
10	0	7/28	1	32	3.37	9.99	2.42	6.22-16.1
11	0	28/3	1	21	3.84	5.20	1.36	3.12-8.68
12	0	28/3	1	9	2.03	4.14	1.50	2.03-8.43
13	0	28/3	1	13	3.76	3.28	1.02	1.78-6.03
14	0	28/3	1	11	2.19	4.72	1.58	2.46-9.08
15	0	28/3	1	10	2.25	4.36	1.51	2.21-8.59
16	0	28/3	1	8	2.00	3.83	1.46	1.81-8.08
17	25	7/3	2	10	2.27	4.64	1.64	2.32-9.29
18	25	7/3	2	5	2.21	3.27	1.57	1.27-8.40
19	25	7/3	3	7	2.54	3.97	1.66	1.75-9.00
20	25	7/3	2	5	1.54	4.67	2.25	1.82-12.0
21	25	7/3	2	2	1.71	1.69	1.23	0.404-7.05
22	25	7/28	1	17	3.27	5.46	1.60	3.07-9.70
23	25	7/28	1	9	1.98	4.78	1.78	2.31-9.91
24	25	7/28	1	50	5.98	8.10	1.70	5.38-12.2
25	25	7/28	1	13	3.29	4.04	1.26	2.19-7.46
26	25	7/28	1	12	1.36	9.28	3.09	4.84-17.8
27	25	28/3	1	23	2.13	10.9	2.69	6.71-17.7
28	25	28/3	1	4	1.75	2.14	1.11	0.773-5.94
29	25	28/3	1	30	4.89	6.16	1.41	3.94-9.66
30	25	28/3	1	19	3.52	5.50	1.47	3.25-9.30
31	25	28/3	1	15	1.46	10.2	2.97	5.78-18.1
32	100	7/3	2	3	1.21	3.57	2.16	1.09-11.7
33	100	7/3	2	6	2.62	3.31	1.47	1.38-7.92
34	100	7/3	5	15	3.47	4.25	1.31	2.33-7.76
35	100	7/3	2	4	2.37	1.87	0.976	0.669-5.20
36	100	7/28	1	18	2.12	8.92	2.57	5.07-15.7
37	100	7/3	3	39	3.29	11.2	2.63	7.08-17.7
38	100	7/28	3	11	1.54	11.1	4.04	5.41-22.6
39	100	7/28	2	33	3.48	9.98	2.28	6.38-15.6
40	100	7/28	1	13	2.41	5.66	1.83	3.01-10.7
42	100	7/28	1	29	5.24	5.33	1.29	3.31-8.56
43	100	7/3	2	11	3.71	4.28	1.50	2.15-8.49
45	100	28/3	1	30	3.17	9.29	2.17	5.87-14.7
46	100	28/3	1	36	2.58	13.1	2.87	8.50-20.1
47	100	28/3	1	34	2.25	14.8	3.29	9.53-22.9
48	100	28/3	1	10	2.28	4.29	1.48	2.18-8.43

Animal	Dose (mg/kg/day)	Group	Number of packaging reactions	Number of mutants	Total pfu ($x10^5$)	Mean MF ($x10^{-5}$)	Standard Error ($x10^{-5}$)	95% C.I. ($x10^{-5}$)
49	100	28/3	1	46	4.94	9.29	1.87	6.27-13.8
50	0	28/28	1	16	3.02	5.27	1.50	3.02-9.22
51	0	28/28	1	20	3.13	6.38	1.71	3.78-10.8
52	0	28/28	1	22	3.69	6.16	1.61	3.69-10.3
53	0	28/28	2	14	2.24	6.45	1.94	3.57-11.6
54	0	28/28	1	27	3.50	7.73	1.87	4.82-12.4
55	0	28/28	1	13	2.88	4.67	1.48	2.51-8.68
56	0	56/3	2	18	1.61	11.7	3.20	6.86-20.0
57	0	56/3	1	9	1.33	6.46	2.33	3.18-13.1
58	0	56/3	1	10	1.25	8.38	2.89	4.26-16.5
59	0	56/3	1	17	2.47	7.21	2.01	4.18-12.5
60	0	56/3	1	15	1.30	12.1	3.53	6.79-21.4
61	0	56/3	2	10	1.83	5.30	1.82	2.70-10.4
62	12.5	56/3	1	14	1.36	10.8	3.26	6.01-19.5
63	12.5	56/3	2	10	0.875	12.0	4.16	6.13-23.7
64	12.5	56/3	1	7	0.997	7.34	2.95	3.33-16.2
65	12.5	56/3	1	32	2.21	15.3	3.43	9.85-23.7
66	12.5	56/3	2	19	1.45	13.8	3.69	8.16-23.3
67	12.5	56/3	2	4	0.608	6.49	3.36	2.35-17.9
68	25	28/28	2	23	3.29	7.02	1.78	4.27-11.6
69	25	28/28	1	24	2.76	9.00	2.29	5.46-14.8
70	25	28/28	1	11	2.42	4.51	1.51	2.35-8.68
71	25	28/28	1	49	3.97	12.4	2.52	8.30-18.5
72	25	28/28	1	18	1.50	12.3	3.35	7.18-20.9
73	25	28/28	1	19	4.48	4.38	1.21	2.55-7.52
74	25	28/28	2	57	2.47	23.5	4.47	16.2-34.1
75	50	56/3	1	33	1.87	18.6	4.13	12.0-28.7
76	50	56/3	2	14	1.11	12.4	3.71	6.91-22.3
77	50	56/3	1	32	2.77	12.2	2.73	7.84-18.9
78	50	56/3	1	54	3.54	16.1	3.11	11.0-23.5
79	50	56/3	3	23	1.63	13.8	3.42	8.48-22.4
80	50	56/3	2	20	1.58	13.3	3.49	7.92-22.2
81	50	56/3	3	14	1.74	7.82	2.34	4.35-14.1
82	100	28/28	1	24	1.78	13.9	3.54	8.45-22.9
83	100	28/28	2	31	1.89	16.2	3.73	10.4-25.5
84	100	28/28	1	16	1.44	11.4	3.35	6.45-20.3
85	100	28/28	1	29	2.35	12.7	3.06	7.96-20.4
86	100	28/28	2	28	2.53	11.0	2.57	6.93-17.4
87	100	28/28	1	41	2.45	16.3	3.48	10.8-24.8
88	100	56/3	4	41	2.45	17.6	3.66	11.7-26.4
89	100	56/3	1	0	0	0		
90	100	56/3	1	0	0	0		
91	100	56/3	2	41	1.53	26.1	5.39	17.4-39.2
92	100	56/3	3	16	1.41	11.9	3.39	6.79-20.8
93	100	56/3	1	33	2.57	13.5	2.99	8.72-20.8
94	100	56/3	1	64	3.24	20.8	3.88	14.5-30.0

Small Intestine

Animal	Dose (mg/kg/day)	Group	Number of packaging reactions	Number of mutants	Total pfu ($x10^5$)	Mean MF ($x10^{-5}$)	Standard Error ($x10^{-5}$)	95% C.I. ($x10^{-5}$)
1	0	7/3	2	19	1.50	12.9	3.65	7.45-22.5
2	0	7/3	2	8	0.550	14.8	5.76	6.91-31.7
3	0	7/3	2	2	0.0381	53.5	38.8	12.9-222
4	0	7/3	2	7	1.20	6.00	2.47	2.67-13.5
5	0	7/3	2	1	0.111	9.18	9.30	1.26-66.8
6	0	7/28	3	12	1.26	9.41	3.05	4.99-17.7
7	0	7/28	3	14	0.719	19.1	5.84	10.5-34.8
8	0	7/28	3	7	0.285	24.0	9.76	10.8-53.3
9	0	7/28	3	8	0.396	19.5	7.55	9.12-41.7
10	0	7/28	3	6	0.586	9.97	4.34	4.25-23.4
11	0	28/3	1	2	0.323	6.19	4.55	1.46-26.2
12	0	28/3	1	3	0.341	8.79	5.38	2.65-29.2
13	0	28/3	1	3	0.368	8.16	4.99	2.46-27.1
14	0	28/3	1	2	0.343	5.83	4.29	1.38-24.7
15	0	28/3	1	13	0.835	15.6	5.35	7.94-30.5
16	0	28/3	1	5	0.548	9.12	4.48	3.48-23.9
17	25	7/3	2	1	0.0663	0.00		
18	25	7/3	2	2	0.126	16.3	11.8	3.92-67.6
19	25	7/3	2	3	0.535	5.77	3.47	1.78-18.7
20	25	7/3	2	0	0.0232	0.00	0.00	
21	25	7/3	2	3	0.467	6.61	3.97	2.04-21.5
22	25	7/28	3	10	0.929	10.5	3.68	5.27-20.8
23	25	7/28	3	4	0.166	23.2	12.2	8.30-65.0
24	25	7/28	3	4	0.457	8.41	4.42	3.00-23.5
25	25	7/28	3	6	0.588	9.93	4.33	4.23-23.3
26	25	7/28	3	5	0.257	15.6	8.16	5.58-43.5
27	25	28/3	1	1	0.161	6.22	6.35	0.842-46.0
28	25	28/3	1	5	0.497	10.1	4.94	3.84-26.3
29	25	28/3	1	5	0.379	13.2	6.47	5.04-34.5
30	25	28/3	1	6	0.560	10.7	4.88	4.39-26.2
31	25	28/3	1	3	0.413	7.27	4.45	2.19-24.1
32	100	7/3	2	2	0.533	3.81	2.76	0.918-15.8
33	100	7/3	2	7	0.487	14.5	5.95	6.48-32.4
34	100	7/3	2	7	0.487	14.6	5.98	6.51-32.6
35	100	7/3	2	0	0.138	0.00	0.00	
36	100	7/28	3	4	0.177	21.6	11.4	7.70-60.7
37	100	7/3	2	3	0.124	24.4	14.6	7.54-79.0
38	100	7/28	3	2	0.147	13.4	9.69	3.26-55.2
39	100	7/28	3	2	0.0977	9.71	9.86	1.33-71.0
40	100	7/28	3	6	0.0547	108	47.0	46.3-253
42	100	7/28	3	6	0.355	16.4	7.17	6.95-38.6
43	100	7/3	2	37	3.25	11.5	2.65	7.34-18.1
45	100	28/3	1	1	0.353	2.83	2.89	0.384-20.9
46	100	28/3	1	6	0.205	29.2	13.3	12.0-71.4
47	100	28/3	1	6	0.177	33.9	15.4	13.9-82.7
48	100	28/3	1	4	0.0729	54.9	29.6	19.1-158

Animal	Dose (mg/kg/day)	Group	Number of packaging reactions	Number of mutants	Total pfu ($x10^5$)	Mean MF ($x10^{-5}$)	Standard Error ($x10^{-5}$)	95% C.I. ($x10^{-5}$)
49	100	28/3	1	6	0.452	13.3	6.05	5.43-32.4
50	0	28/28	2	10	1.82	6.21	2.17	3.12-12.3
51	0	28/28	2	20	2.22	10.4	2.80	6.11-17.6
52	0	28/28	1	27	3.70	8.67	2.20	5.28-14.2
53	0	28/28	2	0	0.0331	0.00	0.00	
54	0	28/28	3	14	1.05	13.4	4.08	7.39-24.4
55	0	28/28	1	15	1.76	10.1	3.10	5.56-18.5
56	0	56/3	2	15	2.24	6.28	1.91	3.46-11.4
57	0	56/3	2	55	2.76	18.6	3.90	12.3-28.0
58	0	56/3	1	20	1.50	15.6	4.34	9.06-26.9
59	0	56/3	1	16	1.93	9.67	2.90	5.38-17.4
60	0	56/3	1	14	1.92	8.55	2.69	4.62-15.8
61	0	56/3	1	29	1.36	24.9	6.20	15.3-40.6
62	12.5	56/3	1	14	1.31	12.5	3.92	6.74-23.1
63	12.5	56/3	2	12	2.03	5.67	1.87	2.97-10.8
64	12.5	56/3	2	29	3.26	8.38	2.06	5.18-13.6
65	12.5	56/3	1	18	1.44	14.6	4.21	8.33-25.7
66	12.5	56/3	1	17	1.36	14.6	4.29	8.24-26.0
67	12.5	56/3	2	14	2.41	5.59	1.74	3.04-10.3
68	25	28/28	2	35	2.98	12.9	2.99	8.17-20.3
69	25	28/28	1	28	3.02	11.0	2.76	6.75-18.0
70	25	28/28	3	8	1.42	5.33	2.04	2.52-11.3
71	25	28/28	2	21	2.11	11.2	2.98	6.68-18.9
72	25	28/28	3	15	1.33	10.7	3.16	5.96-19.1
73	25	28/28	1	49	5.43	10.7	2.33	6.99-16.4
74	25	28/28	2	24	1.30	21.4	5.46	13.0-35.3
75	50	56/3	2	46	3.35	12.7	2.77	8.26-19.5
76	50	56/3	2	44	2.03	20.3	4.47	13.1-31.2
77	50	56/3	2	102	3.60	25.7	4.88	17.7-37.3
78	50	56/3	2	72	2.60	24.5	4.94	16.5-36.4
79	50	56/3	2	23	2.11	10.7	2.80	6.38-17.9
80	50	56/3	2	142	2.80	47.0	8.55	32.9-67.2
81	50	56/3	2	39	2.60	14.4	3.26	9.24-22.5
82	100	28/28	1	32	2.26	16.8	4.06	10.5-27.0
83	100	28/28	3	11	0.817	13.6	4.55	7.09-26.2
84	100	28/28	1	23	1.84	14.9	3.95	8.84-25.0
85	100	28/28	3	39	1.44	27.6	5.92	18.1-42.0
86	100	28/28	3	39	1.46	28.1	6.06	18.5-42.9
87	100	28/28	3	10	0.812	12.5	4.34	6.33-24.7
88	100	56/3	1	29	1.26	26.9	6.68	16.5-43.8
89	100	56/3	2	56	1.49	36.0	7.52	23.9-54.2
90	100	56/3	1	40	1.63	28.7	6.56	18.3-44.9
91	100	56/3	1	35	1.34	30.5	7.21	19.2-48.5
92	100	56/3	2	20	1.42	14.0	3.84	8.14-23.9
93	100	56/3	2	50	2.36	20.6	4.39	13.5-31.2
94	100	56/3	1	48	1.71	32.8	7.19	21.3-50.4

Appendix 2

Adjusted P-values from the Pairwise Comparisons and P-values from the Trend Analysis of Mutant Frequency Data in Chapter 3

Dose Related Differences from the Vehicle Control

Tissue	Comparison	Adjusted p-value *
Bone marrow	56/3 (vehicle) versus 56/3 (100 mg/kg/day)	**<0.0001**
Liver	56/3 (vehicle) versus 56/3 (100 mg/kg/day)	**<0.0002**
Liver	28/28 (vehicle) versus 28/28 (100 mg/kg/day)	**<0.0003**
Bone marrow	28/28 (vehicle) versus 28/28 (100 mg/kg/day)	**<0.0004**
Liver	28/3 (vehicle) versus 28/3 (100 mg/kg/day)	**<0.0005**
Small intestine	56/3 (vehicle) versus 56/3 (100 mg/kg/day)	**<0.0006**
Small intestine	28/28 (vehicle) versus 28/28 (100 mg/kg/day)	**<0.0007**
Bone marrow	56/3 (vehicle) versus 56/3 (50 mg/kg/day)	**0.0016**
Small intestine	56/3 (vehicle) versus 56/3 (50 mg/kg/day)	**0.016**
Bone marrow	7/28 (vehicle) versus 7/28 (100 mg/kg/day)	**0.039**
Bone marrow	28/3 (vehicle) versus 28/3 (100 mg/kg/day)	**0.045**
Liver	56/3 (vehicle) versus 56/3 (50 mg/kg/day)	0.060
Liver	28/28 (vehicle) versus 28/28 (25 mg/kg/day)	0.073
Small intestine	56/3 (vehicle) versus 56/3 (12.5 mg/kg/day)	0.42
Liver	28/3 (vehicle) versus 28/3 (25 mg/kg/day)	0.58
Small intestine	28/3 (vehicle) versus 28/3 (100 mg/kg/day)	0.95
Liver	56/3 (vehicle) versus 56/3 (12.5 mg/kg/day)	1.0
Small intestine	28/28 (vehicle) versus 28/28 (25 mg/kg/day)	1.0
Small intestine	7/28 (vehicle) versus 7/28 (100 mg/kg/day)	1.0
Bone marrow	28/28 (vehicle) versus 28/28 (25 mg/kg/day)	1.0
Small intestine	7/3 (vehicle) versus 7/3 (25 mg/kg/day)	1.0
Bone marrow	7/3 (vehicle) versus 7/3 (100 mg/kg/day)	1.0
Small intestine	7/28 (vehicle) versus 7/28 (25 mg/kg/day)	1.0
Liver	7/28 (vehicle) versus 7/28 (100 mg/kg/day)	1.0
Bone marrow	7/28 (vehicle) versus 7/28 (25 mg/kg/day)	1.0
Bone marrow	28/3 (vehicle) versus 28/3 (25 mg/kg/day)	1.0
Bone marrow	56/3 (vehicle) versus 56/3 (12.5 mg/kg/day)	1.0
Liver	7/3 (vehicle) versus 7/3 (25 mg/kg/day)	1.0
Liver	7/28 (vehicle) versus 7/28 (25 mg/kg/day)	1.0
Liver	7/3 (vehicle) versus 7/3 (100 mg/kg/day)	1.0
Bone marrow	7/3 (vehicle) versus 7/3 (25 mg/kg/day)	1.0
Small intestine	28/3 (vehicle) versus 28/3 (25 mg/kg/day)	1.0
Small intestine	7/3 (vehicle) versus 7/3 (100 mg/kg/day)	1.0

* Bonferroni-Holm correction for multiple comparisons

Effects of Treatment Duration (administration of the same total dose)

Tissue	Comparison	Adjusted p-value *
Liver	Total dose 700 mg/kg - 7/3 versus 56/3	**0.0008**
Liver	Total dose 700 mg/kg - 28/3 versus 56/3	**0.047**
Small intestine	Total dose 700 mg/kg - 7/28 versus 28/28	0.082
Liver	Total dose 700 mg/kg - 7/28 versus 28/28	0.66
Bone marrow	Total dose 700 mg/kg - 7/3 versus 56/3	0.86
Liver	Total dose 2800 mg/kg - 28/3 versus 56/3	1.0
Liver	Total dose 700 mg/kg - 7/3 versus 28/3	1.0
Bone marrow	Total dose 700 mg/kg - 7/28 versus 28/28	1.0
Bone marrow	Total dose 700 mg/kg - 7/3 versus 28/3	1.0
Small intestine	Total dose 2800 mg/kg - 28/3 versus 56/3	1.0
Bone marrow	Total dose 2800 mg/kg - 28/3 versus 56/3	1.0
Small intestine	Total dose 700 mg/kg - 7/3 versus 56/3	1.0
Small intestine	Total dose 700 mg/kg - 7/3 versus 28/3	1.0
Bone marrow	Total dose 700 mg/kg - 28/3 versus 56/3	1.0
Small intestine	Total dose 700 mg/kg - 28/3 versus 56/3	1.0

* Bonferroni-Holm correction for multiple comparisons

Effects of Sampling Time (administration of the same total dose)

Tissue	Comparison	Adjusted p-value *
Small intestine	Total dose 700 mg/kg - 7/3 versus 7/28	**0.0183**
Bone marrow	Total dose 700 mg/kg - 7/3 versus 7/28	0.0598
Liver	Total dose 700 mg/kg - 28/3 versus 28/28	0.1977
Liver	Total dose 2800 mg/kg - 28/3 versus 28/28	0.4712
Liver	Total dose 700 mg/kg - 7/3 versus 7/28	0.6515
Bone marrow	Total dose 175 mg/kg - 7/3 versus 7/28	0.9294
Small intestine	Total dose 175 mg/kg - 7/3 versus 7/28	1.0
Liver	Total dose 175 mg/kg - 7/3 versus 7/28	1.0
Small intestine	Total dose 700 mg/kg - 28/3 versus 28/28	1.0
Bone marrow	Total dose 2800 mg/kg - 28/3 versus 28/28	1.0
Small intestine	Total dose 2800 mg/kg - 28/3 versus 28/28	1.0
Bone marrow	Total dose 700 mg/kg - 28/3 versus 28/28	1.0

* Bonferroni-Holm correction for multiple comparisons

Effects of Administration of Different Total Doses

Tissue	Comparison	Adjusted p-value *
Liver	7/3 (700 mg/kg) versus 56/3 (5600 mg/kg)	**<0.0001**
Bone marrow	7/3 (700 mg/kg) versus 56/3 (5600 mg/kg)	**<0.0002**
Liver	7/3 (700 mg/kg) versus 28/28 (2800 mg/kg)	**<0.0003**
Liver	7/28 (700 mg/kg) versus 56/3 (5600 mg/kg)	**<0.0004**
Bone marrow	7/3 (700 mg/kg) versus 28/28 (2800 mg/kg)	**0.0020**
Small intestine	7/3 (700 mg/kg) versus 56/3 (5600 mg/kg)	**0.0030**
Liver	7/3 (175 mg/kg) versus 28/28 (700 mg/kg)	**0.0056**
Liver	7/3 (700 mg/kg) versus 28/3 (2800 mg/kg)	**0.018**
Bone marrow	7/28 (700 mg/kg) versus 56/3 (5600 mg/kg)	**0.023**
Liver	28/3 (2800 mg/kg) versus 56/3 (5600 mg/kg)	**0.035**
Liver	7/28 (700 mg/kg) versus 28/28 (2800 mg/kg)	**0.046**
Bone marrow	7/3 (700 mg/kg) versus 28/3 (2800 mg/kg)	0.17
Small intestine	7/3 (700 mg/kg) versus 28/28 (2800 mg/kg)	0.22
Liver	7/28 (175 mg/kg) versus 28/28 (700 mg/kg)	0.27
Bone marrow	7/3 (175 mg/kg) versus 28/28 (700 mg/kg)	0.39
Bone marrow	28/3 (2800 mg/kg) versus 56/3 (5600 mg/kg)	0.48
Bone marrow	7/3 (175 mg/kg) versus 28/3 (700 mg/kg)	0.82
Bone marrow	28/28 (2800 mg/kg) versus 56/3 (5600 mg/kg)	1.0
Liver	7/3 (175 mg/kg) versus 28/3 (700 mg/kg)	1.0
Liver	7/28 (700 mg/kg) versus 28/3 (2800 mg/kg)	1.0
Small intestine	28/3 (2800 mg/kg) versus 56/3 (5600 mg/kg)	1.0
Small intestine	7/3 (175 mg/kg) versus 28/28 (700 mg/kg)	1.0
Small intestine	7/3 (700 mg/kg) versus 28/3 (2800 mg/kg)	1.0
Small intestine	28/28 (2800 mg/kg) versus 56/3 (5600 mg/kg)	1.0
Liver	28/28 (2800 mg/kg) versus 56/3 (5600 mg/kg)	1.0
Bone marrow	7/28 (700 mg/kg) versus 28/28 (2800 mg/kg)	1.0
Small intestine	7/28 (700 mg/kg) versus 28/3 (2800 mg/kg)	1.0
Small intestine	7/28 (175 mg/kg) versus 28/3 (700 mg/kg)	1.0
Small intestine	7/3 (175 mg/kg) versus 28/3 (700 mg/kg)	1.0
Bone marrow	7/28 (175 mg/kg) versus 28/28 (700 mg/kg)	1.0
Bone marrow	7/28 (175 mg/kg) versus 28/3 (700 mg/kg)	1.0
Small intestine	7/28 (700 mg/kg) versus 28/28 (2800 mg/kg)	1.0
Liver	7/28 (175 mg/kg) versus 28/3 (700 mg/kg)	1.0
Bone marrow	7/28 (700 mg/kg) versus 28/3 (2800 mg/kg)	1.0
Small intestine	7/28 (700 mg/kg) versus 56/3 (5600 mg/kg)	1.0
Small intestine	7/28 (175 mg/kg) versus 28/28 (700 mg/kg)	1.0

* Bonferroni-Holm correction for multiple comparisons

Analysis of Dose Related Increasing Trends

Tissue	Group	P-value
Bone marrow	7/3	0.050
	7/28	0.051
	28/3	**0.0089**
	28/28	**<0.0001**
	56/3	**<0.0001**
Liver	7/3	0.44
	7/28	0.22
	28/3	**0.0005**
	28/28	**0.001**
	56/3	**<0.0001**
Small intestine	7/3	0.39
	7/28	0.073
	28/3	**0.015**
	28/28	**0.0002**
	56/3	**0.0001**

Comparison of Vehicle Controls with Positive Controls

Tissue	Comparison	Adjusted p-value *
Bone marrow	7/3 (vehicle) versus 7/3 (ENU)	**<0.0001**
Bone marrow	7/28 (vehicle) versus 7/28 (ENU)	**<0.0002**
Bone marrow	28/28 (vehicle) versus 28/28 (ENU)	**<0.0003**
Small intestine	28/28 (vehicle) versus 28/28 (ENU)	**<0.0004**
Small intestine	7/3 (vehicle) versus 7/3 (ENU)	**<0.0005**
Bone marrow	28/3 (vehicle) versus 28/3 (ENU)	**<0.0006**
Small intestine	56/3 (vehicle) versus 56/3 (ENU)	**<0.0007**
Small intestine	7/28 (vehicle) versus 7/28 (ENU)	**<0.0008**
Liver	28/3 (vehicle) versus 28/3 (ENU)	0.11
Liver	7/3 (vehicle) versus 7/3 (ENU)	0.15
Small intestine	28/3 (vehicle) versus 28/3 (ENU)	0.44
Liver	7/28 (vehicle) versus 7/28 (ENU)	1.0
Liver	28/28 (vehicle) versus 28/28 (ENU)	1.0
Liver	56/3 (vehicle) versus 56/3 (ENU)	1.0

* Bonferroni-Holm correction for multiple comparisons

Appendix 3

List of Abbreviations

Aprt	adenine phosphoribosyl transferase
BrdU	5-bromo-2'-deoxyuridine
CA	chromosomal aberration
CCl_4	carbon tetrachloride
DEB	1,2:3,4-diepoxybutane
DEHP	di(2-ethylhexyl)phthalate
Dlb-1	beta-1,4-N-acetyl-galactosaminyl transferase 2
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosourea
Hprt	hypoxanthine guanine phosphoribosyl transferase
IWGT	International Workshops on Genotoxicity Testing
KA	kojic acid
MF	mutant frequency
MLA	mouse lymphoma assay
MN	micronucleus
MTD	maximum tolerated dose
MX	3-chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone
NPV	negative predictive value
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
PCE	polychromatic erythrocyte
pfu	plaque-forming unit

PPV	positive predictive value
RPE	retinal pigment epithelium
SCE	sister chromatid exchange
SDS	sodium dodecyl sulfate
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCE	trichloroethylene
TGR	transgenic rodent
Tk	thymidine kinase
TRAID	Transgenic Rodent Assay Information Database
UDS	unscheduled DNA synthesis