3.0 EXISTING GENOTOXICITY ASSAYS

3.1 Introduction

The utility of TGR mutation assays in genotoxic risk assessment may be considered in the context of currently available assays. Typically, genotoxic effects can occur through two mechanisms: via gene mutations or via chromosomal mutations. For the purposes of this chapter, gene mutations are considered to be permanent single mutations involving only one gene, whereas 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 aberrations 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 the advantages 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 toxicokinetics and DNA repair – that are relevant when considering potential effects in 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.

In vivo genotoxicity assays offer the advantage that mammalian DNA repair processes, the status of cell cycle checkpoint genes and toxicokinetic factors in the model system share greater similarity with humans, the species of interest for risk assessment; consequently, the degree of uncertainty in extrapolating to humans is lower than when considering most *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 are also greater than for *in vitro* assays, and this is reflected in their increased cost.

The testing of chemicals for the purposes of determining safety requires reproducibility between laboratories and standardisation in test methods. For this reason, testing protocols have been developed under the auspices of international organisations, such as the 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, existing genetic toxicology tests are reviewed and their benefits and limitations described (see also Table 3-1). For the purposes of this discussion, assays are grouped into four categories: *in vitro* mutation assays (briefly reviewed), *in vivo* somatic cell gene mutation assays, *in vivo* somatic cell chromosomal aberration assays and *in vivo* indicator assays.

3.2 *In vitro* genotoxicity assays

3.2.1 In vitro *chromosomal aberration assays*

Chemicals causing chromosomal aberrations may be identified with an *in vitro* cytogenetics assay (OECD, 1997b). Mammalian cell cultures, such as those derived from the Chinese hamster (*i.e.* Chinese hamster ovary or V79 lines), are treated with the test chemical; after an appropriate exposure period, mitosis in the cultures is arrested in metaphase with an

Table 3-1. Comparison of existing tests used to examine somatic cell genotoxicity *in vivo*

A.

	Rodent erythrocyte micronucleus	Bone marrow chromosomal aberration	Mouse spot test	Retinoblast[a]	*Hprt*[a]
Species	Rat or mouse	Rat or mouse	Mouse	Mouse	Rat or mouse
Number and sex	Minimum 5 per sex per dose	Minimum 5 per sex per dose	Sufficient number of females (~50) to produce about 300 F_1 mice per dose	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient
Dose levels	At least three, plus controls; maximum should be MTD; limit test acceptable where no toxicity observed at 2 000 mg/kg bw	At least three, plus controls; maximum should be MTD; limit test acceptable where no toxicity observed at 2 000 mg/kg bw	At least two doses (plus controls), one of which should induce mild toxicity or reduced litter size	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity
Route of administration	Oral or intraperitoneal; others where justified	Oral or intraperitoneal; others where justified	Oral or intraperitoneal; others where justified	Route relevant to human exposure (oral, intra-peritoneal or inhalation)	Route considered most relevant to human expo-sure
Treatment schedule	Single administration; multiple dosing where justified	Single administration	Single administration on the 8th, 9th or 10th day of gestation	Single administration on the 10th day of gestation	Single or multiple admin-istration
Tissue analysed	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	1.5 times 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	Mutation frequency deter-mined after a selection period of several days in 6-TG
Analysis	At least 2 000 PCEs per animal	At least 1 000 cells per animal for mitotic index; at least 100 cells per animal for chromosomal aberra-tions	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
In-life phase	About 1 week	About 2 weeks	About 6 weeks	About 2–3 months	About 2 months

Table 3-1 (continued)

B.

	Aprt[a]	*Tk*$^{+/-}$ [a]	*Dlb-1*[a]	Sister chromatid exchange[a]	Unscheduled DNA synthesis	Comet assay[a]
Species	*Aprt*$^{+/-}$ or *Aprt*$^{-/-}$ mouse	*Tk* heterozygous mouse	*Dlb-1* heterozygous mouse	Rat or mouse	Rat; also mouse	Rat or mouse
Number and sex	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient	At least three per dose; usually males are sufficient	4–5 per sex per dose should be sufficient
Dose levels	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD
Route of administration	Route considered most relevant to human exposure	Route considered most relevant to human exposure	Oral administration	Gavage or intra-peritoneal injection are most common	Oral (intraperitoneal injection is not recommended)	Route considered most relevant to human exposure
Treatment schedule	Single or multiple administration	Single or multiple administration	Single or multiple administration	Single administration	Single administration	Single or multiple administration
Tissue analysed	Splenic T-lymphocytes or skin fibroblasts (primarily)	Splenic T-lymphocytes (primarily)	Intestinal epithelial cells	Bone marrow (other cell types may be used)	Liver	Any from which a single-cell suspension can be prepared
Sampling times	Mutation frequency determined after a selection period of several days in 8-AA or DAP	Mutation frequency determined after a selection period of 10–12 days in BrdU	14 days	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	2–6 hours and 16–26 hours after single dosing, or 2–6 hours after the last treat-ment following multiple dosing
Analysis	Calculation of induced mutant frequency	Calculation of induced mutant frequency	Calculation of induced mutant frequency	Scoring sister chromatid exchanges by examination using light microscopy	Determination of net nuclear grain count by autoradiography in at least 100 cells per animal	Computer image analysis of comets
In-life phase	About 2 months	About 2 months	About 2 months	About 1 week	About 1 week	About 1 week

8-AA, 8-azaadenine; BrdU, 5-bromo-2′-deoxyuridine; bw, body weight; DAP, 2,6-diaminopurine; MTD, maximum tolerable dose; PCE, polychromatic erythrocyte; TdR, thymidine; 6-TG, 6-thioguanine

[a] Non-guideline study.

inhibitor, such as colcemid or colchicine. The stained metaphase spreads are examined by light microscopy to detect chromosome or chromatid aberrations. A biologically significant increase in the frequency of cells with structural aberrations compared with that of the concurrent control group indicates that the chemical is clastogenic. Major drawbacks of this assay, in comparison with some of the other *in vitro* assays, are the subjectivity and cost of having the metaphase spreads scored by an observer.

A harmonised protocol for the *in vitro* micronucleus test has been developed recently by an IWGT working group (Kirsch-Volders *et al*., 2000, 2003). The European Centre for the Validation of Alternative Methods has determined that the *in vitro* micronucleus test is a valid alternative to the *in vitro* chromosomal aberration test (European Centre for the Validation of Alternative Methods Scientific Advisory Committee, 2006), and the OECD is developing a new Test Guideline (OECD, 2007); however, the transition of this assay from the development stage to routine regulatory use has not yet occurred.

3.2.2 **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 *E. coli* (*e.g.* WP2*uvr*A) (OECD, 1997a). Revertant cells grow on minimal agar containing trace amounts of histidine or tryptophan, whereas 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 results of the concurrent negative control (typically considered a two-fold or greater increase), the chemical is concluded to be mutagenic.

Mammalian forward mutation assays, such as the thymidine kinase (*Tk*) assay or the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) assay, detect mutations at the heterozygous *Tk* or hemizygous *Hprt* gene (OECD, 1997e). Cells such as mouse lymphoma L5178Y 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, whereas wild-type cells accumulate a toxic metabolite and do not proliferate. Comparison of the mutant frequency of the treatment groups with that of the concurrent negative control group allows the identification of a mutagenic chemical.

3.3 *In vivo* assays for somatic cell chromosomal aberrations

In vivo chromosomal aberration assays assess the potential of a test 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.

3.3.1 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 acceptance, and a test method has been described in OECD Test Guideline 474 (OECD, 1997c).

3.3.1.1 Principles

The micronucleus assay detects chromosome damage and whole chromosome loss in polychromatic erythrocytes (PCEs) and eventually in normochromatic erythrocytes in peripheral blood as the red cells mature. A micronucleus is a small structure (5–20% of 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 form only in dividing cells.

There are four generally accepted mechanisms through which micronuclei can form: 1) the mitotic loss of acentric chromosome fragments (forming structural aberrations); 2) mechanical consequences of chromosomal breakage and exchange, such as from lagging chromosomes, an inactive centromere or tangled chromosomes (forming structural aberrations); 3) mitotic loss of whole chromosomes (forming numerical aberrations); and 4) apoptosis (Heddle *et al*., 1991). 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, whereas 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 PCE (a period of about 6 hours following the final mitosis), the nucleus is extruded, making detection of micronuclei easier (Mavournin *et al*., 1990). In addition, the PCE still contains ribonucleic acid (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 (Krishna and Hayashi, 2000). 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 (Mavournin *et al*., 1990).

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 (OECD, 1997c). 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 (MTD) 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 extrusion of the erythroblast nucleus (Mavournin *et al*., 1990).

The incidence of micronucleated PCEs is low in untreated animals. To allow for appropriate statistical power, a large number of PCEs (usually at least 2 000 PCEs per animal) must be scored for the incidence of micronuclei; the proportion of PCEs among total erythrocytes is also determined as a measure of cytotoxicity (OECD, 1997c).

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, which offer tremendous potential for improving the sensitivity and the efficiency of the assay (Hayashi *et al*., 2007).

Any test chemical that induces an increase in the frequency of micronucleated PCEs is concluded to have 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 one of two molecular cytogenetic methods: immunofluorescent antikinetochore (CREST) staining or fluorescence *in situ* hybridisation (FISH) with pancentromeric DNA probes (see Heddle *et al*., 1991; Krishna and Hayashi, 2000).

3.3.1.2 Benefits and 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 PCEs/1 000 PCEs), which provides fairly high sensitivity to small test chemical–induced increases in micronucleus frequency (Salamone and Mavournin, 1994). 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. The assay is widely accepted by regulatory agencies internationally.

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 assessed. Despite evidence suggesting that the bone marrow micronucleus assay can detect most germ cell clastogens (Waters *et al*., 1994), 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 PCEs, highlights a potential drawback of mutation assays exclusively involving somatic cells (Witt *et al*., 2003). 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.

3.3.2 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. The recommended methodology has been published in OECD Test Guideline 475 (OECD, 1997d).

3.3.2.1 Principles

The assay is based on the ability of a test agent to induce chromosome structural or numerical alterations that can be visualised microscopically. The target tissue for the chromosomal aberration assay is the bone marrow, because it is a rapidly dividing, well-vascularised 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 MTD to that which

does not induce appreciable toxicity. The MTD 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 1.5 times the normal cell cycle length (usually 12–18 hours for most rodent species), animals are euthanised, 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 1 000 cells per animal are scored to determine the mitotic index, and a minimum of 100 metaphase cells per 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.

3.3.2.2 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. The assay has been in use for many years, it is accepted by regulatory agencies globally and a large database exists for comparisons. Although bone marrow cells are the usual target, the assay can be adapted (*e.g.* by altering sampling times) to allow examination of other cell types, including spermatogonia. This allows the assay to be used to evaluate the potential for germ cell chromosomal aberrations that could lead to heritable genetic effects.

However, conducting a chromosomal aberration assay is much more time consuming than conducting 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 or chromosomal damage during slide preparation, the number of scoreable metaphases may be low; this could make it difficult to find a sufficient number of intact metaphases per animal, which would reduce the sensitivity of the test to detect small increases. Furthermore, like the micronucleus assay, the chromosomal aberration assay does not, by design, provide information regarding whether the test chemical induced gene mutations in the target cells or other tissues besides the bone marrow within the animal.

3.4 *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* (adenine phosphoribosyltransferase), $Tk^{+/-}$ and *Dlb-1*.

3.4.1 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. The recommended methodology is described in OECD Test Guideline 484 (OECD, 1986b).

3.4.1.1 Principles

Although coat colour spots were induced experimentally by X-irradiation in 1957 (Russell and Major, 1957), the ability of chemicals to induce these genetic changes was not recognised until 1975, when colour spots were induced by treatment of mice with *N*-ethyl-*N*-nitrosourea (ENU) (Fahrig, 1975). 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 (Russell, 1977). 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 (Fahrig, 1995). 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 day 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: 1) white ventral spots that are presumed to be the result of chromosomal aberrations leading to cell death; 2) yellow, agouti-like spots that are likely to be a result of misdifferentiations; and 3) 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 (OECD, 1986b).

By examining fur from the spot using fluorescence microscopy, it is possible to distinguish the different classes of spots and to identify, from somatic mutation spots, the gene loci affected (Fahrig and Neuhauser-Klaus, 1985). 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) (Fahrig, 1995).

3.4.1.2 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, it does not require specialised 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 detected within the small melanocyte population only very early in development. 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 have greatly limited the use of this assay.

3.4.2 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.

3.4.2.1 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 (Gondo *et al.*, 1993). 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 wild-type *p* in a retinal pigment epithelial precursor cell and leads to the production of a retinal pigment epithelial cell with black pigmentation, which is visible against the remaining non-pigmented retinal pigment epithelial cells (Searle, 1977). 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, is 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 (Reliene *et al.*, 2004).

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 (Schiestl *et al.*, 1997a).

3.4.2.2 Benefits and limitations

A number of carcinogens have been found to induce intrachromosomal recombinations (Schiestl *et al.*, 1997a, 1997b; Jalili, Murthy and Schiestl, 1998; Bishop *et al.*, 2000), 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 (Bishop *et al.*, 2000), and it allows for direct examination at the single-cell level. However, the target cells in this assay are retinal pigment epithelial precursor cells, which proliferate only during the period starting at about embryonic day 9 until shortly after birth (Bodenstein and Sidman, 1987). Because the assay is used to determine the frequency of deletions occurring in embryos, it may not necessarily be representative of other life stages.

3.4.3 Gene mutation assays using endogenous genes with selectable phenotypes

3.4.3.1 Hprt

The *Hprt* assay uses one of the few genes that are 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.

3.4.3.1.1 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 administered mutagen compared with 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*. (1994). 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. Cloning efficiency plates are scored after 8–9 days, whereas mutant frequency plates are scored after a 10- to 12-day expression period. The mutant frequency is calculated as the ratio between the cloning efficiencies in selective media versus those in cloning media. A significant increase in mutant frequency in treatment cultures compared with controls indicates that 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} (Van Sloun *et al*., 1998). Using standard techniques, further molecular analysis of *Hprt* mutations can be performed, if desired.

3.4.3.1.2 Benefits and limitations

The *Hprt* assay detects point mutations, frameshifts, small insertions and small deletions. As an endogenous gene, *Hprt* is transcriptionally active and thus subject to transcription-coupled DNA repair (Lommel, Carswell-Crumpton and Hanawalt, 1995). However, because *Hprt* is an X-linked gene and therefore functionally hemizygous, it is not particularly efficient at detecting large deletions, chromosomal recombination and non-disjunction events that may disrupt essential flanking genes and are more effectively identified with assays using endogenous autosomal genes (Dobrovolsky, Chen and Heflich, 1999). 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 (Deubel *et al*., 1996). 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 (Walker *et al*., 1999b). 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 assessed only in T-lymphocytes, which prevents identification of mutagenic effects that may arise preferentially in other target tissues.

3.4.3.2 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.

3.4.3.2.1 Principles

Aprt is the gene coding for an enzyme (adenine phosphoribosyltransferase) that catalyses the conversion of adenine to adenosine monophosphate (AMP) in the purine salvage pathway; it is expressed in all tissues. The mouse *Aprt* gene is located on chromosome 8 (Dush *et al*., 1986), whereas the human gene is located on chromosome 16 (Fratini *et al*., 1986). In the mouse, the *Aprt* gene, because of its location near the telomere, is a large target for chromosomal events such as translocation and mitotic recombination (Tischfield, 1997).

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 (Van Sloun *et al*., 1998). 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 (Shao *et al*., 1999) and epithelial cells from the kidney (Ponomareva *et al*., 2002). 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 or 2,6-diaminopurine, which are toxic to *Aprt*-proficient cells. After a 6- to 8-day expression period, the cloning frequency and mutant frequency are determined using methods similar to those used for the *Hprt* model (Tates *et al*., 1994). The spontaneous mutant frequency at the *Aprt* locus in heterozygous mice is approximately 8.7×10^{-6} in T-lymphocytes (Van Sloun *et al*., 1998) and 1.7×10^{-4} in skin fibroblasts (Tischfield, 1997). Using standard techniques, further molecular analysis of *Aprt* mutations can be performed, if desired.

An $Aprt^{-/-}$ homozygous knockout mouse has also been developed, which is capable of detecting chemicals that cause point mutations (Stambrook *et al*., 1996). 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 (Stambrook *et al*., 1996).

3.4.3.2.2 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 because of 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 mutation frequency arising from dilution as the T-lymphocyte pool is renewed.

3.4.3.3 Tk$^{+/-}$

A *Tk* heterozygous mouse model has been constructed (Dobrovolsky, Casciano and Heflich, 1999). This model is currently limited to basic research applications, as it remains in an early stage of development and has not yet been used for routine testing.

3.4.3.3.1 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 (Dobrovolsky, Casciano and Heflich, 1996, 1999). *Tk* is generally expressed only in dividing cells and encodes thymidine kinase, which is involved in pyrimidine salvage, catalysing phosphorylation of thymine deoxyriboside to form thymidylate. The *Tk* gene is located on the distal portion of mouse chromosome 11 (Hozier *et al*., 1991). 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), whereas 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, Casciano and Heflich (1999). Cells are incubated in microwell culture plates with the selective agent BrdU, which 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 that the test chemical induced mutation at the *Tk* locus. Using standard techniques, further molecular analysis of *Tk* mutations can be performed, if desired. The spontaneous mutant frequency is approximately 2×10^{-5} (Takahashi, Kubota and Sato, 1998).

3.4.3.3.2 Benefits and limitations

The *Tk*$^{+/-}$ mouse is the analogous *in vivo* model to the commonly used 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, non-disjunction and large deletions that often lead to loss of heterozygosity, for which it is particularly sensitive (Dobrovolsky, Casciano and Heflich, 1999; Dobrovolsky, Shaddock and Heflich, 2000, 2002). 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, Casciano and Heflich (1999) suggest that this potential can be minimised by keeping cultures under tight selection pressure during the culture phase, since studies with *Tk*$^{+/-}$ mouse lymphoma cells have indicated that several cell divisions in the absence of the selective agent are required in order to fix mutations (Moore and Clive, 1982).

3.4.3.4 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 (Winton, Blount and Ponder, 1988; Winton *et al*., 1990), but it remains in the development stage and has not been widely used in the research community.

3.4.3.4.1 Principles

The *Dlb-1* specific locus test identifies mutations occurring in the small intestine of mice heterozygous at the *Dlb-1* locus. *Dlb-1* is a polymorphic genetic locus that exists on chromosome 11 in the mouse (Uiterdijk *et al*., 1986) and has two alleles. *Dlb-1*^{b} is an autosomal dominant gene that determines the expression of binding sites for the lectin *Dolichos biflorus* agglutinin in intestinal epithelium, whereas *Dlb-1*^{a} determines *Dolichos biflorus* agglutinin receptor expression in vascular endothelium (Winton, Blount and Ponder, 1988). Mice heterozygous at the *Dlb-1* locus (*Dlb-1*^{a}/*Dlb-1*^{b}) that develop a mutation of the *Dlb-1*^{b} allele in an intestinal stem cell can be detected by staining an intestinal epithelial cell preparation with a peroxidase conjugate of *Dolichos biflorus* agglutinin and scoring non-staining cell ribbons on the villus; these cell ribbons are cells derived from a stem cell carrying a *Dlb-1* mutation (Winton *et al*., 1990). The spontaneous mutation frequency of the *Dlb-1* gene has been reported to be approximately 1.6×10^{-5} mutants per villus per animal per week (Tao, Urlando and Heddle, 1993a).

3.4.3.4.2 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 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.

3.5 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 that some type of interaction occurred. The three most commonly conducted tests are the sister chromatid exchange assay, the unscheduled DNA synthesis assay and the single-cell gel electrophoresis (comet) assay.

3.5.1 Sister chromatid exchange assay

The sister chromatid exchange assay is used for assessing chromosome damage; more commonly, it has been conducted as an *in vitro* test. Methodology for the *in vitro* assay is described in OECD Test Guideline 479 (OECD, 1986a), but there is no guideline for the *in vivo* assay.

3.5.1.1 Principles

Sister chromatid exchanges 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 that the replication fork is the site of sister chromatid exchange production (Rodriguez-Reyes and Morales-Ramirez, 2003). However, sister chromatid exchange 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 sister chromatid exchange. It is also noteworthy that several strong clastogens, such as ionising radiation and bleomycin, have failed to produce an increase in sister chromatid exchanges (Perry and Evans, 1975). Because sister chromatid exchange induction does not, by itself, indicate that a

chemical is mutagenic, the interpretation of the toxicological relevance of sister chromatid exchange is often difficult.

Rodents are most commonly used for *in vivo* sister chromatid exchange assays. Bone marrow cells are usually sampled, because they contain a requisite 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 a single dose of BrdU, followed at 21 hours by an injection of the mitotic inhibitor colchicine. 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.

3.5.1.2 Benefits and limitations

The sister chromatid exchange 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 analysed. A significant number of sister chromatid exchange 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 sister chromatid exchange induction. Because factors other than direct DNA interaction can cause sister chromatid exchanges, an increase in sister chromatid exchange induction 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.

3.5.2 Unscheduled DNA synthesis assay

The unscheduled DNA synthesis 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 test chemical. A protocol is described in OECD Test Guideline 486 (OECD, 1997f).

3.5.2.1 Principles

The unscheduled DNA synthesis 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 is 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.

The larger the number of nucleotides excised and repaired, the greater the incorporation of detectable ^{3}H-TdR into DNA. For that reason, the unscheduled DNA synthesis assay is more sensitive in detecting DNA damage that is repaired through nucleotide excision repair (removal of up to 100 nucleotides) compared with base excision repair (removal of 1–3 nucleotides) (OECD, 1997f). Test chemicals more prone to inducing nucleotide excision repair, such as those that form bulky DNA adducts, have a greater potential to cause detectable unscheduled DNA synthesis. However, the unscheduled DNA synthesis 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 unscheduled DNA synthesis assay is usually conducted using rats, although 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. Chemicals inducing a significant increase in net nuclear grain count for at least one treatment group have induced unscheduled DNA synthesis (Madle *et al.*, 1994; OECD, 1997f).

3.5.2.2 Benefits and limitations

Theoretically, any tissue with a low proportion of cells in S-phase can be used for analysis. Although only liver is routinely used, an unscheduled DNA synthesis assay using spermatocytes has been developed (Sega, 1974, 1979; Working and Butterworth, 1984), allowing the measurement of DNA interactions that may be germ cell specific. Because unscheduled DNA synthesis is measured in the whole genome, it is potentially much more sensitive than assays examining only specific loci. However, the extent of unscheduled DNA synthesis gives no indication of the fidelity of the repair process. For that reason, unscheduled DNA synthesis does not provide specific information on the mutagenic potential of a test chemical, but only information suggesting that it does or does not induce excision repair.

3.5.3 Single-cell gel electrophoresis (comet) assay

The comet assay is a quantitative technique for measuring DNA damage in eukaryotic cells at the level of the single cell. This DNA damage may or may not lead to mutations. Under alkaline conditions (pH >13) first described by Singh *et al.* (1988), the assay can detect single- and double-strand breaks, incomplete repair sites and alkali-labile sites in nearly any single-cell suspension of eukaryotic cells. The presence of DNA–DNA and DNA–protein cross-linking can also be inferred in some cases. A growing body of work has demonstrated that the comet assay may have value in regulatory applications, and a number of questions related to the development of a standardised protocol have been addressed recently by various expert working groups (Tice *et al.*, 2000; Hartmann *et al.*, 2003; Burlinson *et al.*, 2007).

3.5.3.1 Principles

The alkaline comet assay is based on the observation that electrophoresis of DNA will cause it to migrate in an agarose gel matrix. Under a microscope, a cell with DNA damage subjected to these conditions will take a distinctive comet-like shape, with a nuclear (head) region and a tail containing the DNA fragments or DNA strands oriented towards the anode. Numerous parameters affect the detection of DNA damage, including pH, the agarose concentration and the conditions of electrophoresis (time, temperature, amperage and voltage).

Rats or mice can be treated with the test substance either once or multiple times at intervals of 24 hours. At least two doses are selected, with the highest dose being the MTD. Tissue samples are obtained from the organs of interest at 2–6 hours and 16–26 hours after single dosing, or 2–6 hours after the last treatment following multiple dosing (Hartmann *et al.*, 2003). Slides are prepared containing a cell layer imbedded in agarose; the cells are lysed, incubated in alkaline electrophoresis buffer and then electrophoresed under alkaline conditions. After neutralisation and staining, the slides are scored, preferably by computerised image analysis software (Burlinson *et al.*, 2007). Because of the association of cytotoxicity

and increased levels of DNA strand breaks, it is advisable to conduct a concurrent assessment of cytotoxicity and to exclude from analysis cells with a characteristic apoptotic/necrotic appearance (Hartmann *et al*., 2003).

A dose-related change in an appropriate parameter (*i.e.* tail moment or tail length) at a single sampling time or a change in an appropriate parameter in the treated group compared with the untreated control at a single sampling time constitutes a positive response. Increased DNA migration indicates the induction of DNA strand breaks or alkali-labile sites, whereas reduced migration suggests the presence of stabilising DNA–DNA or DNA–protein cross-links (Pfuhler and Wolf, 1996; Merk and Speit, 1999).

3.5.3.2 Benefits and limitations

For the most part, cells from any tissue from which a single-cell suspension can be obtained are amenable to comet assay analysis. Therefore, the comet assay has applications for both somatic cell and germ cell genotoxicity testing and has the flexibility to permit various routes of administration. In addition to applications in genotoxicity testing, the assay has uses in human biomonitoring of occupational or environmental exposures. However, some lesions detected by the comet assay, such as single-strand breaks, may also be correctly repaired without resulting in permanent genetic damage. As such, the assay does not directly detect mutations or identify aneugens.

3.6 Conclusion

In vivo assays are necessary components of any thorough genetic toxicity testing scheme. They are influenced by toxicokinetic factors, DNA repair processes and cell cycle checkpoint genes that may, in some cases, affect genotoxicity differently from the *in vitro* models. However, existing assays are seriously limited by a range of different factors, including assay cost, 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.