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Mutagenicity of chemicals in genetically modified animals

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Abstract

The strategy for assessing human health risks of chemicals consists of a large number of tests in different research disciplines. Tests include acute and chronic toxicity, genotoxicity, reproduction toxicity and carcinogenicity. Genotoxic properties of chemicals are assessed in short-term *in vitro* and *in vivo* genotoxicity tests. There are two main endpoints for genotoxicity: gene mutations and chromosome aberrations. Under *in vitro* conditions, there are sufficient assays for both endpoints. Testing under *in vivo* conditions is essential to confirm *in vitro* data since it is impossible to mimic, in a petri dish, all the complex factors determining whether a chemical will induce mutations in a specific tissue in animals *in vivo*. Moreover, in a regulatory context, a relevant negative *in vivo* result from an adequately performed test overrules positive *in vitro* results. There are appropriate assays in existence to investigate *in vivo* chromosome aberration; however, problems occur when a compound induces gene mutations *in vitro*. In the absence of reliable *in vivo* gene mutation assays, a justified assessment of the genotoxic potential of chemicals may be hampered. Introduced in this report, based open literature data up to August 2000, are several promising new *in vivo* gene mutation assays. The report is not restricted to assays with the commercially available transgenic models; all assays - whether using transgenes or endogenous genes as reporter genes – are incorporated. In reviewing the current state of the art in evaluating these assays, the advantages and the disadvantages of the assays are discussed. This is to determine the feasibility of the routine use of these new *in vivo* gene mutation tests for health risk estimation. Gene mutation assays with transgenic animals have already been used on a small scale for legislation of chemicals. However, to allow the routine use of these assays for regulatory purposes, they will have to be validated further and an official OECD guideline prepared.

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Summary

The assessment of the potential genotoxicity of chemicals *in vivo* plays an important role both for verification and confirmation of intrinsic mutagenicity and for establishing the mode of action of chemical carcinogens. Genotoxic endpoints measured *in vitro* include gene mutations and chromosome aberrations, but until recently reliable *in vivo* tests for gene mutations were lacking. The present paper gives an overview of the recently developed novel gene mutation tests for assessment of potential mutagenicity in animals *in vivo*: 1) assays using endogenous genes as reporter genes, e.g., *hprt, aprt*, *tk* or *Dlb-1* and 2) assays using transgenic animals, i.e., animals that possess an exogenous bacterial reporter gene, a so-called transgene, e.g., *lacZ* or *lacI*. Only a few endogenous genes and tissues are suitable for measuring mutations in animals *in vivo*. In contrast, the exogenous reporter genes, which are transmitted via the germ cells, can be measured in every tissue of the transgenic rodents. The transgenic animal assays include bacteriophage- and plasmid-vector based models. The bacteriophage-based models can detect point mutations, small deletions, and insertions, while the plasmid-based models also enable detection of large deletions.

The discussion on validation is restricted mainly to the commercially available Muta™Mouse (*lacZ*) and Big Blue® (*lacI*) models, since only for these two systems there are sufficient data for validation. Comparison of mutation induction in endogenous and exogenous genes simultaneously in the same animals and in the same tissues showed that, despite differences in mutation properties of the various model mutagens used, the responses of the exogenous loci (*lacI*, *lacZ* transgene) and the endogenous loci (*Dlb-1*, *hprt*) were generally comparable upon acute dosing, when expressed in terms of absolute increases in mutation frequency. However, when expressed as relative increase over controls, the tests with transgenic animals are generally less sensitive than those with endogenous genes as a consequence of a lower background mutation frequency in endogenous as compared to exogenous reporter genes. Several studies indicate that transgenic mice respond as expected for wild type mice with a similar genetic background, e.g., in terms of toxicokinetics and toxicodynamics. Moreover, it has been shown that the mutations in the *lacI* or *lacZ* genes are neutral, i.e., that the mutations do not give selective advantage or disadvantage.

In a recent validation study on the predictive value of the novel *in vivo* gene mutation tests for carcinogenicity, which included a total of 33 model compounds, it was shown that the Big Blue® (n = 21) and Muta™Mouse (n = 12) systems perform well with regard to positive predictivity, specificity, sensitivity, and overall accuracy. The negative predictivity for carcinogenicity was, however, very low (33-50%). It is not clear to what extent the results of this validation study are influenced by the selection of the chemicals and the limited set-up of this study. Moreover, the study did not assess questions such as target organ specificity for mutagenicity in the transgenic animals with respect to target organ specificity for carcinogenicity or on the possible relationship between genotoxicity and carcinogenicity profile in relation to the presumed mode of action of the carcinogen in question.

In conclusion, the results obtained until now indicate that the novel *in vivo* gene mutation assays are suitable for mechanistic and fundamental studies on the occurrence of (point) mutations. Although these novel assays are not yet fully validated and a test guideline is not yet available, the bacteriophage λ-based *lacI* and *lacZ* models may already be used for legislation. However, the assays should not be used on a routine base and only carried out with a clear purpose keeping in mind the uncertain negative predictivity for carcinogenicity.

Samenvatting

Het onderzoek naar mogelijke mutagene eigenschappen van chemische stoffen *in vivo* speelt een belangrijke rol zowel voor verificatie en/of bevestiging van intrinsieke mutagene eigenschappen als voor het vaststellen van het werkingsmechanisme van kanker verwekkende stoffen. De genotoxische eindpunten die *in vitro* worden gemeten zijn genmutaties en chromosoomafwijkingen. Tot voor kort waren er geen betrouwbare *in vivo* testen op genmutaties beschikbaar. Dit rapport geeft een overzicht van recent ontwikkelde *in vivo* genmutatietesten. Er wordt onderscheid gemaakt tussen: 1) toetsen met normale wild type muizen en een endogeen gen als reporter gen, bijvoorbeeld, *hprt, aprt*, *tk* of *Dlb-1* en 2) toetsen die gebruik maken van transgene dieren die over een exogeen, bacterieel reporter gen, een transgen, beschikken, zoals bijvoorbeeld *lacZ* of *lacI*. Slechts een paar endogene genen en weefsels zijn geschikt voor het meten van genmutaties *in vivo*. Exogene reporter genen, die via de geslachtscellen worden overgeërfd, kunnen daarentegen worden onderzocht in ieder willekeurig weefsel van transgene dieren. Transgene diermodellen zijn gebaseerd op het gebruik van bacteriofaag dan wel plasmide vectoren. De laatste modellen kunnen puntmutaties, kleine deleties en inserties aantonen terwijl modellen gebaseerd op een plasmide vector daarnaast ook grote deleties kunnen meten.

De validatie discussie beperkt zich hoofdzakelijk tot de commercieel verkrijgbare Muta^{TM}Mouse (*lacZ*) en Big Blue$^{®}$ (*lacI*) modellen. Bepaling van de mutatieinductie in endogene en exogene genen in hetzelfde dier, toonde aan dat ondanks verschillen in mutagene eigenschappen van de gebruikte modelstoffen, bij acute blootstelling de resultaten verkregen met exogene (*lacI*, *lacZ*) en endogene loci (*Dlb-1* en *hprt)* in termen van absolute toename in mutatie frequenties vergelijkbaar waren. Echter de toets met transgene muizen blijkt in het algemeen minder gevoelig als de resultaten worden uitgedrukt als een relatieve toename ten opzichte van onbehandelde dieren. De oorzaak is waarschijnlijk de lagere achtergrond mutatie frequentie in endogene dan in exogene reporter genen. In termen van toxicokinetiek en toxicodynamiek hebben verschillende studies aangetoond dat transgene muizen niet verschillen van wild type muizen met een identieke genetische achtergrond. Daarnaast is aangetoond dat de mutaties in de *lacI* en *lacZ* genen neutraal zijn; mutaties leiden niet tot een selectief voor- of nadeel.

In een recente validatie studie naar de voorspellende waarde van de nieuwe *in vivo* genmutatietesten voor carcinogeniteit voldeden de Big Blue$^{®}$ (n = 21) en Muta^{TM}Mouse (n = 12) modellen goed wat betreft de positieve voorspelbaarheid, specificiteit, gevoeligheid en nauwkeurigheid. De negatieve voorspelbaarheid voor carcinogeniteit was echter erg laag (33-50%). Het is onduidelijk in hoeverre de resultaten zijn beïnvloed door de keuze van de modelstoffen en de beperkte opzet van de studie. Bovendien werd er geen rekening gehouden met de doelorgaan specificiteit voor carcinogeniteit van de verschillende modelstoffen noch met de mogelijke relatie tussen genotoxiciteit en carcinogeniteit wat betreft het veronderstelde werkingsmechanisme van de onderzochte modelstof.

Er kan worden geconcludeerd dat de resultaten die tot dusver met de nieuwe *in vivo* genmutatietesten zijn verkregen aantonen dat deze toetsen geschikt zijn voor mechanistisch en fundamenteel onderzoek naar het voorkomen van mutaties. Hoewel deze nieuwe testsystemen nog niet volledig zijn gevalideerd en er nog geen OECD-richtlijn beschikbaar is, kunnen deze nieuwe toetsen reeds beperkt gebruikt worden voor beleidsdoeleinden. Vanwege de slechte negatieve voorspelbaarheid voor kanker, moeten ze echter nog niet routinematig maar alleen met een specifiek doel gebruikt worden.

1 Introduction

Governments are daily confronted with regulation of new chemicals, re-evaluation of existing chemicals and, increasingly more, with risk estimation of putative genotoxic or carcinogenic compounds. The strategy for the assessment of human health risks of chemicals consists of a large number of tests on different research disciplines. The potential genotoxicity of chemicals is assessed in short-term *in vitro* and *in vivo* genotoxicity tests. For genotoxicity two main endpoints exist, gene mutations and chromosome aberrations (Fig. 1). Most compounds have a preference for one of both, although there are no chemicals that induce exclusively either gene mutations or chromosome aberrations. This implies that one has to test compounds on both endpoints. Moreover, genotoxicity testing should be performed not only in somatic but also in germ cells to assess the potential genetic risks. Unfortunately, a quantitative assay for genetic risk assessment is not available.

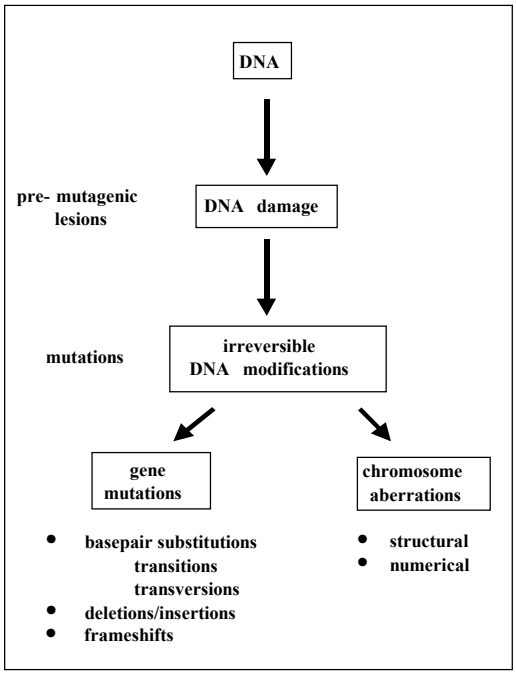

Figure 1: Endpoints in genotoxicity gene mutations and chromosome aberrations

Under *in vitro* conditions, there are sufficient assays for both endpoints (Table 1). Gene mutation assays in bacteria (Ames test) and mammalian cells as well as assays for chromosome aberrations in mammalian cells are routinely performed. Next to these, there are different kinds of indicator tests such as tests for DNA damage and repair. However, the value of these latter tests is limited compared to those for mutagenicity, i.e., tests for gene mutations and chromosome aberrations.

Testing under *in vivo* conditions is essential to confirm *in vitro* data since it is impossible to mimic in a petridish all complex factors that determine whether a chemical will induce mutations in a specific tissue in animals *in vivo*. *In vivo* tests take into account whole animal processes like absorption, tissue distribution, metabolism and excretion of the chemical and its metabolites, and repair of lesions. Moreover in a regulatory context a relevant negative *in vivo* result from an adequately performed test

overrules positive *in vitro* results. It is obvious that *in vivo* confirmation makes only sense when in an animal model the endpoint is evaluated which showed positive results under *in vitro* conditions.

For *in vitro* clastogenic chemicals, an *in vivo* chromosome aberration test or a micronucleus test allows evaluation of this endpoint. However, an important limitation of these assays is that testing is predominantly done in cells collected from peripheral blood or bone marrow. Exposure to a compound does not automatically mean that the reporter cells, i.e., bone marrow cells, peripheral blood cells, or their predecessors, have been exposed. Besides, these assays suffer from a tissue restriction and the value of a negative result obtained in a non-target tissue is questionable.

Table 1: Available genotoxicty assays in mammalian cells or mammals

cytogenetic assay	gene mutation assay	DNA effects/indicator assays
in vitro:		
chromosome aberration assay	*hprt*-test mouse lymphoma assay	sister chromatid exchange assay unscheduled DNA synthesis assay
in vivo		
micronucleus test chromosome aberration assay	mouse spot test	sister chromatid exchange assay unscheduled DNA synthesis assay

Problems occur when a compound induces gene mutations *in vitro* (Table 1). To date, some gene mutation tests in *Drosophila melanogaster* are available. However, the value of these tests for risk assessment in humans is at least questionable. In mammals the mouse spot test exists, which is a rather insensitive, animal consuming and expensive type of test. Often as a surrogate an *in vivo* test for unscheduled DNA synthesis (UDS test) is performed. The occurrence of unscheduled DNA synthesis does not directly point to the induction of DNA mutations, but merely to an increase in DNA repair as a consequence of DNA damage. In other words, an increase in unscheduled DNA synthesis points to the occurrence of DNA damage, which does not result in DNA mutations per se.

Table 2: Promising novel in vivo gene mutation assays in mammals

Endogenous reporter genes	Transgenic reporter genes	
	Bacteriophage-based models	Plasmid-based models
aprt heterozygous mouse *tk* heterozygous mouse *hprt* somatic mutation assay *Dlb-1* specific locus assay	Muta™ Mouse (*lacZ*) Big Blue® mice (*lacI*) Big Blue® rat (*lacI)* *λsupF* transgenic mouse *phiX174* transgenic mouse *λcII/cI* transgenic mice *gptΔ* transgenic mouse	*lacZ* plasmid mouse *rpsL* transgenic mouse

This lack of a well-validated *in vivo* gene mutation test often hampers a justified assessment of the genotoxic potential of chemicals. Recently there have been a number of promising new developments, which may solve the problem of this lack of *in vivo* gene mutation tests. These novel gene mutation tests for assessment of potential mutagenicity in animals *in vivo* are listed in Table 2. They include i) assays using endogenous genes as reporter genes, e.g., *hprt, aprt*, *tk* or *Dlb-1* and ii) assays using transgenic animals, i.e., animals that possess an exogenous bacterial reporter gene, a so-called transgene, e.g., *lacZ* or *lacI*.

On a small scale, gene mutation assays with these models have been already used. However, to allow the use of these models for regulatory purposes they have to be validated further and an official OECD guideline has to be prepared. In this report, the various models are introduced and the actual state of the art concerning the evaluation of the models is reviewed. Finally, the advantages and the disadvantages of the models will be discussed in order to determine the feasibility of the routine use of these new *in vivo* gene mutation tests for health risk estimation.

2 Overview of novel gene mutation tests *in vivo*

The novel *in vivo* gene mutation tests all have in common the use of selectable reporter genes to determine the mutation frequency. Only few selectable reporter genes are endogenously available in mammalian cells, e.g. *hprt, aprt, tk, or Dlb-1*. Moreover, the endogenous reporter genes that are suitable for mutagenicity testing, demonstrate a certain tissue restriction; application of tests is, therefore, restricted to only a few specific target tissues. Next the mutation frequencies can be determined via selective growth on certain culture media *in vitro*. This selection procedure is hampered by the fact that the selective markers are only present twice; many copies of a reporter gene would dramatically improve the detection of gene mutations.

Modern molecular biological techniques enabled the development of transgenic animals, which carry many copies of a reporter gene. These reporter genes are transmitted by the germ cells, and thus present manifold in all cells including the germ cells. Consequently, these novel gene mutation tests are divided into models using endogenous genes and those using transgenes.

2.1 Models using endogenous genes

As already mentioned the models using endogenous reporter genes demonstrate a certain tissue restriction. Determination of the mutation frequency of *Dlb-1* is restricted to the small intestine and eventually the colon; determination of the mutation frequency of *hprt, aprt* and *tk* to those tissues which express the reporter gene and which can be subcultured *in vitro*. Against the disadvantage of endogenous reporter genes that selection is hampered by the presence of only one selective marker stands the advantage that these models detect not only point mutations, frameshifts, small insertions and small deletions but also intragenic, large deletions and loss of heterozygosity (LOH; *aprt* and *tk*).

2.1.1 Hypoxanthine-guanine phosphoribosyltransferase (*hprt*) mouse model

The *hprt* gene is one of the few genes that are suitable as a reporter gene for mutation induction in animals and humans and that can be performed in un-modified species. The *hprt* gene, which has a coding region of 657 bp (Skopek *et al.*, 1995), is located on the X chromosome and spans 32 kb in rodent and 46 kb in human cells. However, both male and female cells carry only a single active copy of the *hprt* gene. *Hprt* is a non-essential enzyme for cells in culture. Although *hprt* is an endogenous gene present in all tissues, mutant selection is predominantly performed in splenocytes (van Dam *et al.,* 1992; Skopek *et al.,* 1992; Tates *et al.,* 1994) or (human) peripheral T lymphocytes. Mutants are selected by culture in the presence of 6-thioguanine, which is a substrate for the enzyme. It is converted into the corresponding monophosphate, which in turn is toxic to cells. *Hprt* mutants have lost this enzyme activity, are consequently resistant to the toxic 6-thioguanine and can grow in medium containing 6-thioguanine.
Structural and theoretical considerations as well as experimental evidence indicate that the *hprt* gene is relatively deficient in recovering large genetic alterations (Dobrovolsky *et al.*, 1999a) due its location on the hemizygous X-chromosome.

2.1.2 *Dlb-1* specific locus test

An assay system which allows scoring of mutations *in vivo* in the small intestine (and possibly in the colon) of the mouse involves the *Dlb-1* locus (Winton *et al.,* 1990). *Dlb-1* is a polymorphic genetic locus on mouse chromosome 11 with two alleles*:* $Dlb\text{-}1^b$ (most mouse strains) which determines expression of a binding site for the lectin *Dolichos biflorus* agglutinin (DBA) in mouse intestinal epithelium and $Dlb\text{-}l^a$ (SWR mice) which determines expression in vascular endothelium. DBA binding is co-dominant. In heterozygote $Dlb\text{-}1^a/Dlb\text{-}1^b$ mice, mutations affecting the single $Dlb\text{-}1^b$ allele in an intestinal stem cell are recognised by the histochemical detection of its clonal descendants with a peroxidase conjugate of *Dolichos biflorus* agglutinin. The mutated clones, in contrast to most of the epithelium, do not stain. Because the *Dlb-1* gene has not yet been cloned, the molecular nature of the mutations cannot be determined in DNA sequences.

2.1.3 Adenine phosphoribosyl transferase (*aprt*) mouse model

The human *aprt* gene, which is involved in the nucleotide salvage pathway of DNA synthesis, is located on chromosome 16 and is 2.6 kilobases in length; the mouse *aprt* gene is located near the telomere at chromosome 8. It codes for a protein that converts adenine into AMP. In the C57Bl/6 *aprt* mouse model the gene was knocked out by homologous recombination in embryonic stem cells; a part of the promotor region as well as the ATG start codon were deleted (Engle *et al.,* 1996; van Sloun *et al.*, 1998). This location sensitises the model to proximal chromosomal events like mitotic recombination and translocations. Because of the recessive nature of *aprt* mutations, for genotoxicity testing heterozygous *aprt* mice are used. Mutants occur when the second allele is mutated or lost (LOH). *Aprt* mutants can be selected due to their resistance to the toxic purine analogues 8-azaadenine or 2,6-diaminopurine; only $aprt^{-/-}$ cells survive culture on medium containing these analogues. The *aprt* model detects small mutations, intragenic large deletions and LOH.

2.1.4 Thymidine kinase (*tk*) mouse model

Dobrovolsky *et al.* (1999a) reported the development of a novel *in vivo* gene mutation assay in mice using the autosomal, recessive thymidine kinase (*tk*) gene, which participates like *aprt* in the nucleotide salvage pathway of DNA synthesis. In the C57Bl/6 *tk* mouse the gene was knocked out by homologous recombination in embryonic stem cells (Dobrovolsky *et al.*, 1996). Due to the recessive nature of *tk* mutations, heterozygous *tk* mice have to be used for genotoxicity testing. Mutants occur when the second allele is mutated or lost (LOH).
Culture in the presence of trifluorothimidine (TFT) or 5-bromo-2'-deoxyuridine (BrdU), which are substrate for the enzyme, results in mutant selection. Exclusively $tk^{-/-}$ cells survive culture on medium containing these analogues.
The advantage of the *tk* model is its sensitivity for large deletions, large chromosomal alterations and LOH. A disadvantage of the *tk* model is the use of BrdU as selective agent. BrdU itself is a mutagen and it is therefore impossible to absolutely rule out the possibility that some BrdU resistant mutants were produced *ex vivo* due to exposure to BrdU.

2.2 Models using transgenes

To date, transgenic mice and rats are already used in *in vivo* gene mutation assay. These animals have in common that they contain multiple copies of a transgene in a shuttle vector as reporter gene, which is transmitted by the germ cells, and thus present manifold in all cells including the germ cells. The key problem for using transgenic animals in gene mutation assays was the rescue of the integrated vector from the genome and the detection of gene mutations *in vitro*.

Based on the shuttle vector used transgenic mice models for mutagenicity testing can be divided in two main approaches. The first using a transgene in a bacteriophage vector whereas in the second one the transgene is in a plasmid vector (Fig. 2).

2.2.1 Bacteriophage vector mice

These transgenic mice are developed by microinjection of a bacteriophage shuttle vector. Principally, the determination of the mutation frequency is identical in the various models. Mice, bearing the shuttle vector, are treated with a chemical and after a certain manifestation period in which the DNA damage is fixed into stable mutations, the mice are killed, the tissues dissected and the genomic DNA isolated (fig. 2). The next step is *in vitro* packaging into bacteriophages. The use of λ bacteriophages as a shuttle vector was first developed in mouse fibroblasts by Glazer *et al.* (1986) and was applied to transgenic mice by Gossen *et al.* (1989). Proteins in the packaging extract cleave the shuttle factor at the *cos* sites and package it in the phage heads. These phages are then used to infect *E. coli* deficient for the reporter gene to produce plaques. Mutants are quantified either by color selection or by selective growth on plates containing a medium composition on which non-mutated bacteria can not grow. An aliquot of the infected bacteria is plated on normal minimal plates for the measurement of packaging efficiency. The mutation frequency is calculated by dividing the total number of colored or resistant plaques for the tissue of the individual animal by the total number of plaques with rescued shuttle vectors from the same tissue or from the same DNA sample.

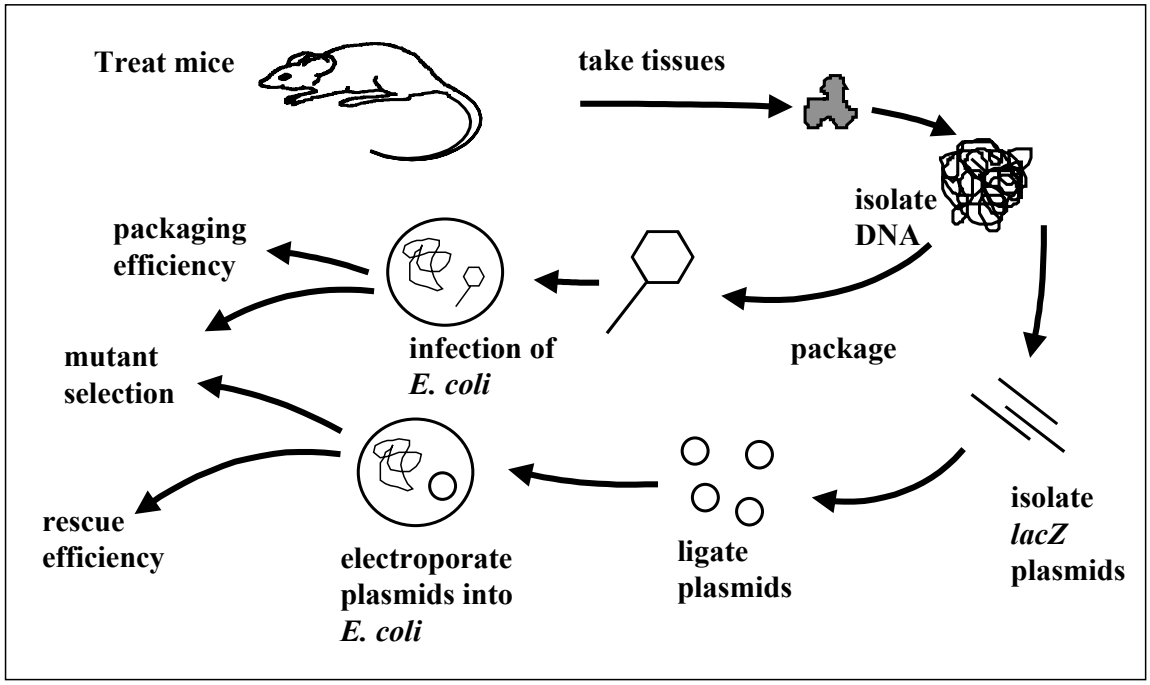

Figure 2: Transgenic mice in vivo gene mutation assays

These transgenic mice models developed with bacteriophage shuttle vectors are suitable for the detection of point mutations, insertions and small deletions. The models do not allow detection of large deletions due to the fact that the *cos*-sites, at the end of the vector, together with a restrictive length of the vector are essential for excision and packaging into phage heads. The models sometimes suffer from a low packaging efficiency, probably due to the large size of the shuttle vector, which should be isolated intact and the rather high copy number per haploid genome (about 2 million bp per phage DNA at one locus). This requires large amounts of packaging extracts to collect a sufficiently high number of reporter genes.

2.2.1.1 *lacI* transgenic mouse model

To date *lacI* models are commercially available; the Big Blue® mouse (B6C3F1) and Big Blue® rat (F344) from Stratagene. The *lacI* mouse model developed by Short and coworkers (Kohler *et al.*, 1991) contains about 30-40 copies of the λ LIZα shuttle vector (Fig. 3) in a head to tail fashion at a single locus on chromosome 4 without detectable rearrangements. In the rat model (Dycaico *et al.*, 1994) 15-20 copies are present per haploid genome (Gollapudi *et al.*, 1998). The λLIZα shuttle vector is 45.6 kb long whereas the reporter gene *lacI* counts 1080 bp. The vector contains the entire $lacI^q$ gene and the α*lacZ* gene (Kohler *et al.*, 1991). The *lacI* gene codes for a homotetrameric protein that binds to the *lacO* operator sequence, which negatively regulates *lacZ* expression. The α*lacZ* gene codes for the αamino portion of β galactosidase.

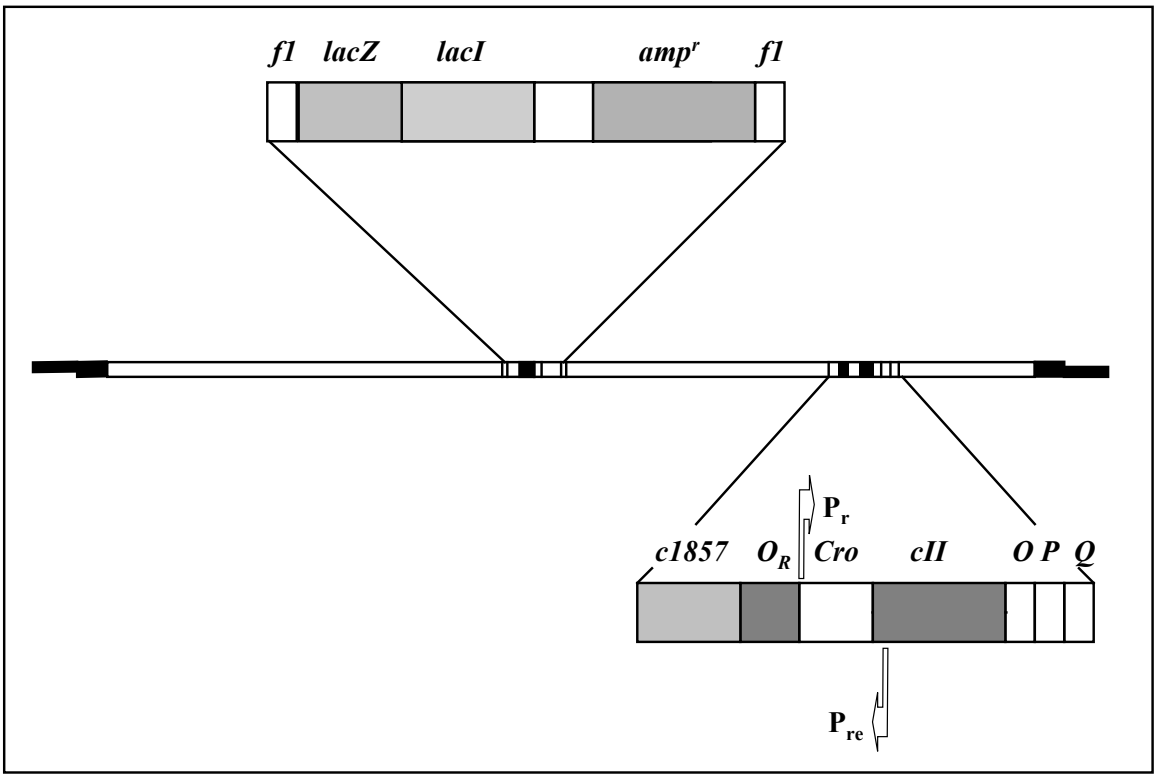

Figure 3: The λLizα shuttle factor with the lacI and cII reporter gene (Stratagene: http://www.stratagene.com/cellbio/toxicology/big_blue_system.htm#liz [December 15, 2000]

After packaging the phage is absorbed to *E. coli* SCS-8 cells (*lacZΔM15*). The *colE1* and amp^R genes allow replication and selection of the vector in *E. coli*. The bacteria are then seeded on a selection medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). The *lac I* gene allows

the *lac* repressor to bind to the *lac* operator which inhibits *αlacZ* expression and thus β-galactosidase activity resulting in white (colorless) plaques. If a mutation in *lacI* does occur the *lac* repressor protein is inactive or unable to bind the *lac* operator and transcription of the *αlacZ* gene will occur. The resulting functional β-galactosidase cleaves X-gal thus generating blue plaques. The ratio between blue and white plaques is a measure of the mutagenicity.

2.2.1.2 *lacZ* transgenic mouse model

The *lacZ* mouse model (Muta^{TM}Mouse) was developed by microinjection of the λgt10-*lacZ* shuttle vector (fig. 4) in fertilized CD2 oocytes of (BALB/c x DBA/2)CD2 F1 mice (Gossen *et al*, 1989). In contrast to the *lacI* model this shuttle vector contains the entire *lacZ* gene as reporter gene. The vector is about 47 kb long whereas the *lacZ* gene consists of about 3100 bp. The *lacZ* mouse model contains about 80 copies of the shuttle vector in a head to tail fashion at chromosome 3.

After *in vitro* packaging the bacteriophage is preabsorbed into *E. coli* C (*lacZ*) cells. The bacteria are seeded on a medium containing X-gal. Plaques containing a normal *lacZ* are β-galactosidase active and are blue, whereas plaques containing mutated *lacZ* will be white (colourless). In this case the ratio between colourless and blue plaques is a measure of the mutagenicity.

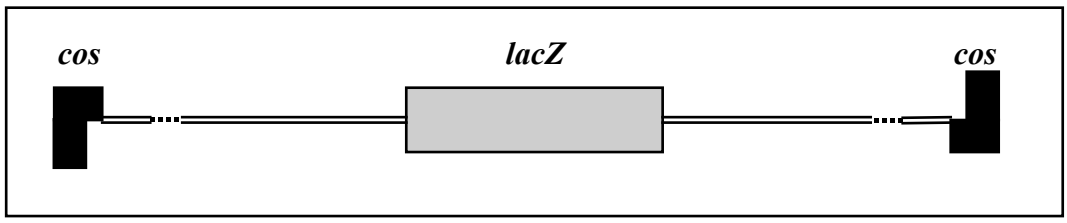

Figure 4: The λgt10-lacZ shuttle vector (Mirsalis et al., 1995)

A selectable system was described by Gossen and Vijg (1993). In this system *E. coli lac$^-$, galE$^-$* is used for phage infection. To determine the recovery the phages are plated on medium containing X-gal; for mutation selection phenyl β-D-galactoside (P-gal) is used. Since β-galactosidase encoded by the *lacZ* gene converts lactose into galactose, normal *lacZ* phages when plated on *E. coli lac$^-$, galE$^-$* in the presence of lactose or lactose derivatives like P-gal will die. They are unable to metabolize the highly toxic UDP galactose, the product of galactose, into UDP glucose. *LacZ* mutated phages will survive since they do not form UDP-galactose.

2.2.1.3 gpt delta transgenic mouse model

A distinct feature of the *gpt*-delta transgenic mouse model by Nohmi *et al.* (1996) is the incorporation of two different positive selection methods in the transgene: the *gpt* gene of *E. coli* for point mutations and/or short deletions and *spi$^-$* selection for (larger) deletions (1 – 10000 bp (Horiguchi *et al.,* 1999). The *gpt*-delta C57Bl/6 mice were obtained after microinjection of C57Bl/6-oocytes obtained after superovulation which resulted in about 80 copies of the vector per chromosome 17. The injected λEG10 shuttle vector (Fig. 5A) is about 48 kb long and composed of the λ2001 vector and a linearilised plasmid. The λ2001 vector carries the *red* and *gam* genes together with a *X*C mutation involved in *spi$^-$* selection. The plasmid possesses the *gpt* gene of *E. coli* and two direct repeat sequences of *loxP* which are recognition sequences for *Cre* recombinase. The coding region of *gpt* is 456 basepairs, which is convenient for the rapid identification of gene mutations by sequencing.

For point mutation or small deletion selection the *E. coli* strain YG6020 (*gpt*$^-$) is used which expresses *Cre* recombinase. Thus when the phage is introduced into strain YG6020 the linearized plasmid is excised, circularized and propagated as a multicopy-number plasmid pYG142 carrying the *gpt* gene. Detection of *gpt* mutations was performed by culture on medium containing 6-thioguanine (6-TG), the packaging efficiency was determined by culture on medium without 6-TG. The ratio between the number of mutants and the packaging efficiency is the mutation frequency for point mutations and/or small deletions.
Spi$^-$ selection (sensitive to P2 interference) requires inactivation of both *red* and *gam* function; mutants are positively identified as *spi*$^-$ plaques in *E. coli* P2 lysogen. For *spi*$^-$ selection rescued phages were infected into *E. coli* XL-1 Blue MRA (P2), poured on λ trypticase plates according to Shimizu *et al.* (1995) and *spi*$^-$ plaques were counted. To enumerate the rescued phages a diluted aliquot was infected into *E. coli* XL-1 Blue MRA. The ratio between these treatments is the mutation frequency for deletions.
The possibility to study two different selection methods one for point mutations and/or short deletions and *spi*$^-$ selection for (larger) deletions is a distinct advantage of this model.

Another transgenic mouse model that uses *gpt* as the reporter gene was developed by Yamada *et al.* (1999). They used the pCGK shuttle vector (Fig. 5B) which contains next to the *E. coli gpt* gene, the kanamycin-resistant gene, an origin of replication and a *cos* region derived from the bacteriophage λ. This vector was linearized with *EcoR1* before micro injection into fertilised eggs from Crj:CD-1 mice, which in turn were implanted into pseudopregnant CD-1 mice. The mice used for mutagenicity tests contain about 50 copies of the vector per haploid genome.

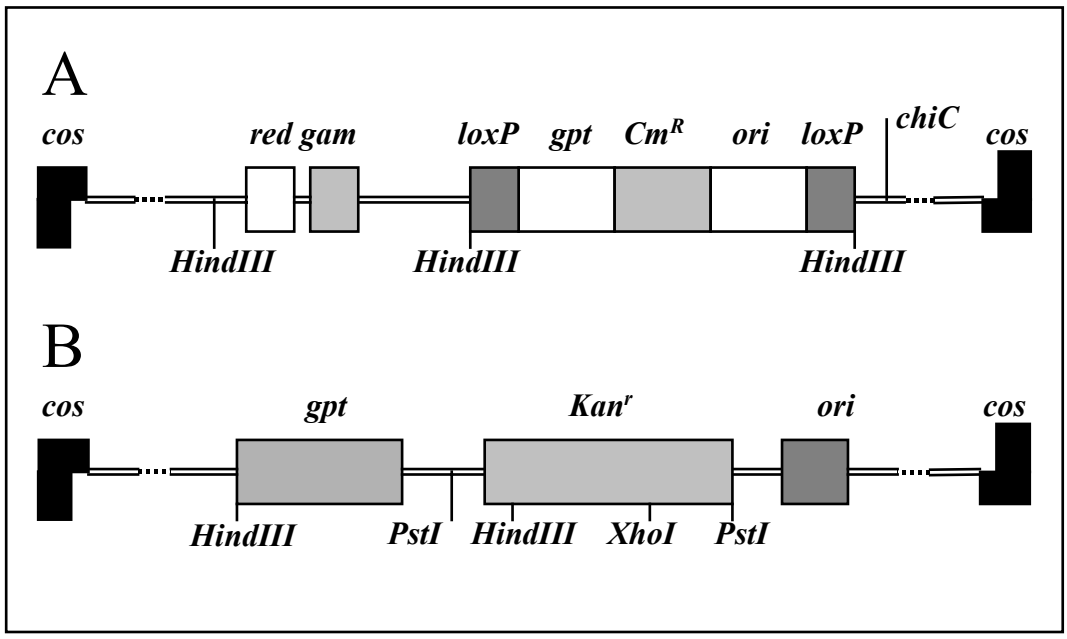

Figure 5: The different vectors used in the gpt transgenic mouse models. (A) The λEG10 shuttle vector used to generate the model developed by Nohmi et al. (1996). B: The pCGK shuttle vector (slightly modified drawn) used by Yamada et al. (1999)

After *in vitro* packaging into phages these were used to infect *gpt*-deficient *E. coli* ZXR15. For the detection of mutants in the *gpt* gene the bacteria were plated on plates with 6-TG; for the determination of the packaging efficiency on plates without 6-TG. The mutant frequency is the ratio

between the total number of mutant plaques for the individual animal and the total number of plaques with rescued shuttle vectors for the individual animal.

An advantage of both transgenic models is that the coding region of *gpt* is only 456 basepairs, which makes it convenient for the rapid identification of gene mutations by sequencing. Next it is a positive selection system which is much more convenient than conventional color selection (Masumura *et al.*, 1999).

2.2.1.4 *phiX174* transgenic mouse model

The *phiX174* model described by Burkhart *et al.* (1993) uses the bacteriophage *phiX174am3cs70,* which has a length of about 5 kb, as a recoverable shuttle vector in transgenic mice. The C57Bl6/J mice are homozygous for the *phiX174am3cs70* shuttle vector and contain about 50 copies in a tandem array per haploid genome. The insert has no apparent effect on the health or breeding capacity of the mice. The reporter gene is *am3* (*amber3*) located in Gene E, which codes in *phiX174* for a lysis inducing protein. The *am3* mutation, a nonsense mutation, renders the mutant phage unable to grow in bacteria that do not carry an amber suppressor such as *E. coli* C (non-permissible host, su^-). It is a reverse mutation model since the model selects on the reversion of the nonsense mutation in *am3*. The mutation in *am3* reverts with a single transition (AT→GC: wild type) or two transversions (AT→TA or AT→CG: both pseudowildtype).

It differs from the earlier mentioned model since the bacteriophages are not preabsorbed by the bacterial host but electroporated into electroporesis competent *E. coli's*. The survival was determined by plating on *E. coli* CQ-2 (sup^+) which contains a suppressor for *am3*, whereas the number of revertants was determined by plating on *E. coli* (sup^-) without the *am3* supressor. The ratio between the number of revertants and the survival is a measure for mutagenicity.

The advantage of this transgenic mouse model is that *phiX174* has been extensively used and therefore provides a useful background of data and experimental design for transition to *in vivo* mutagenicity test. Moreover, since reversion can be accomplished by only three possible base pair substitutions, there is little requirement for sequencing in order to determine the nature of the mutation. The use of vectors that detect only a narrow array of genetic alterations may reveal specific mutagenicity which may go undetected in systems responding to a wide range of genetic alterations (Malling *et al.*, 1998). However, comparing the transgenic *am3* target with the endogenous *hprt* indicated that the potential sensitivity of the *am3* assay is not achieved using the published protocols (Chen *et al.*, 1999).

2.2.1.5 *supF* transgenic mouse model

The *λsupF* transgenic mice model (Leach *et al.*, 1996b) carries 80-100 copies of a λ phage vector *supF* (λsupF; Fig. 6) with the *supF* amber suppressor tRNA gene of *E. coli* along with the c1857 allele of the λ repressor gene as reporter genes. The vector, which is about 48.5 kb long, was microinjected into fertilized oocytes, which in turn were implanted into foster mothers. FISH with *λsupF* DNA probes indicated that the vectors were integrated at a single spot on chromosome 7 (Leach *et al.*, 1996b). The reporter gene *supF* is rather small having a coding region of only 85 bp.

After *in vitro* packaging the phages were infected into *E. coli* PG901 [*Cla lacZ125(am)*] which are without β galactosidase activity due to an amber mutation in the *lacZ* gene which codes for β galactosidase. *E. coli* are cultured on medium containing X-gal and IPTG. Phages with a normal *supF* overcome the amber mutation leading to transcription and translation of β galactosidase which

metabolizes X-gal resulting in blue plaques. Phages with *supF* mutations produce white (colorless) plaques since they cannot suppress the amber mutation in the *lacZ* gene of *E. coli* and consequently do not have β galactosidase activity. The ratio between white and blue plaques is a measure of the mutagenicity.

Both the *supF* gene and transgenic *supF* cell lines have been extensively used and therefore provides a useful background of data and experimental design for transition to *in vivo* mutagenicity test with transgenic *supF* mice. An second advantages of the model is that the coding region of *supF* is only 85 bp, smaller than any other of the reporter genes used, which makes it very convenient for the rapid identification of gene mutations by sequencing.

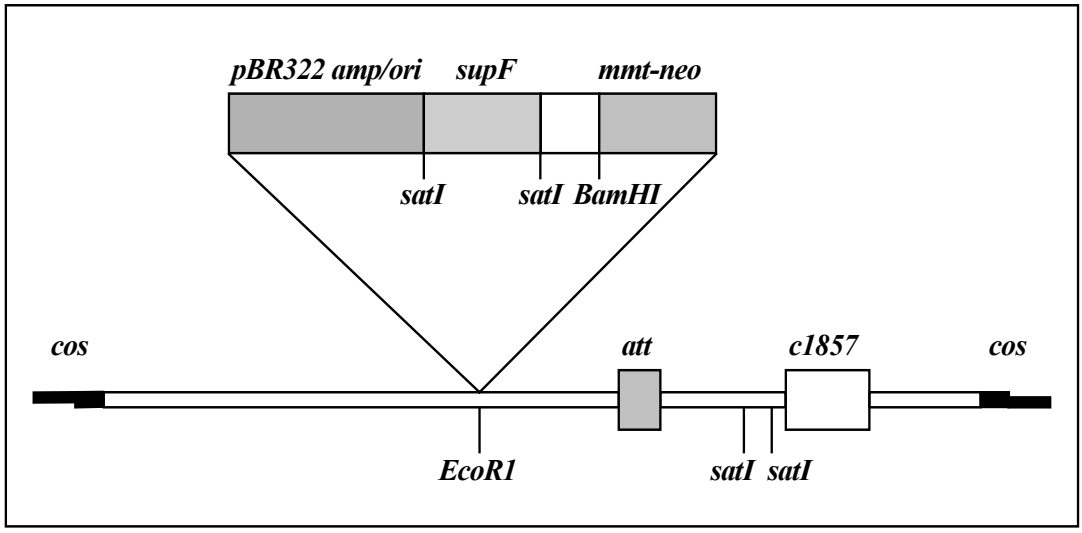

Figure 6: The λsupF shuttle vector (Leach et al., 1996a, 1996b)

2.2.1.6 *cII/cI* transgenic mouse model

Jakubczak *et al.* (1996) described an assay for mutants in the *cII* gene present in bacteriophage λ shuttle vectors (Fig. 3). However, also mutations at the *cI* locus can give rise to plaques under the selective conditions of the assay; consequently the assay is referred to as *cII/cI*. This gene, with a coding region of 294 bp, plays a critical role in the decision between lysis or lysogeny of λ phages following infection of *E. coli*. The gene is present in all transgenic models using bacteriophage λ shuttle vectors. Accumulation of the product of the *cII* gene shortly after infection induces transcription of the *cI* repressor gene leading to lysogeny, and host *Hfl* integrases, which degrade the *cII* protein. Apparently a balance between lysis and lysogeny is maintained. In *Hfl*$^-$ bacteria the *cII* protein is not degraded but accumulated resulting in a continuous lysogeny and will not give rise to plaques. Bacteria with a mutated *cII* or *cI* gene can proceed through a lytic cycle and consequently can form plaques.
To determine the packaging efficiency, the phages were infected into the non-selective *E. coli* G1217 (*hfl*$^+$). Mutants were identified by preabsorbing the phages to *E. coli* G1225 or G1250 (*hfl*$^-$), on which only mutant bacteria form plaques. Since the *cI* gene product appeared to be temperature sensitive, it is also possible to determine the packaging efficiency on *E. coli* G1225 or 1250 (*hfl*$^-$) (Zimmer *et al.*, 1998). Wild type phages will grow lytically in *hfl*$^-$ strains when incubated at 37ºC. This has the advantage that the selection of mutants and the determination of the packaging efficiency are done in the same strain.

CII has a coding region of only 294 bp, which makes also this model convenient for gene mutation identification by sequencing. The positive selection system of the model is much more convenient than conventional color selection and makes it less subjective in scoring mutants. Another advantage of *cII* is that it is a λ phage gene; the *cII* gene can be used as reporter with any λ based mouse or rat mutagenicity assay. In other words any λ based mouse or rat mutagenicity assay has two different reporter genes. By assessment of the mutation frequency of both these genes, false positives/negatives, clonal expansion or Jackpot mutations (Swiger *et al.*, 1999) may be recognized. A final advantage is that *ex vivo* mutations are never a problem because of the immediate commitment to lysogeny/lysis following infection. The decision for lysogeny/lysis is already made before any DNA replication takes place (Watson *et al*., 1998).

2.2.2 Plasmid vector mice

At first instance the assays using a plasmid vectors are identical to that using bacteriophage vectors. However, after the isolation, the genomic DNA is enzymatically cut to release the monomeric plasmid sequences. The plasmid is then purified from the genomic DNA, circulized by ligation and electroporated in *E. coli* deficient for the transgene (fig. 2). Mutants are again quantified by selective growth. An advantage over the bacteriophage vector models is its high rescue efficiency mainly due to the transformation efficiency of the *E. coli* host.

In contrast to the bacteriophage-based models, these plasmid vector based models detect next to point mutations, insertions and small deletions also large deletions. Detection of the latter ones is possible since the system does not depend on packaging. Intra-plasmid deletions or even deletions containing part of the murine genome are detectable as long as the amp^R gene and the origin of replication both present on every copy of the plasmid are available.

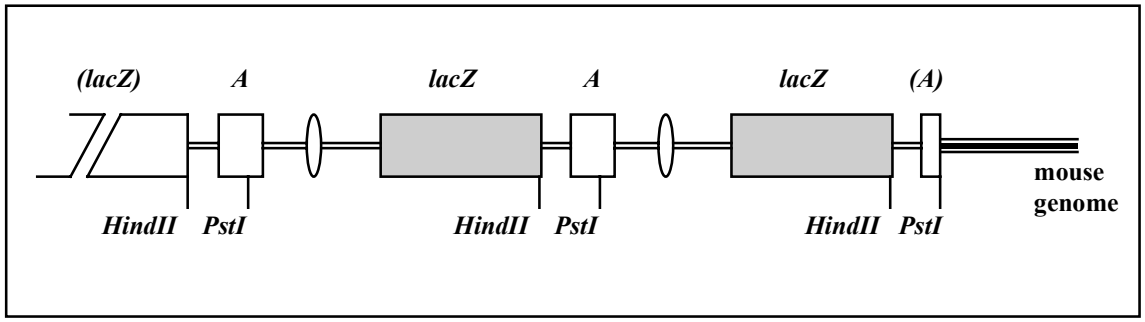

Figure 7: The pUR288 shuttle plasmid (Gossen et al., 1993a)

2.2.2.1 plasmid *lacZ* transgenic mouse model

The *lacZ* plasmid mouse also known as the pUR 288, IM1 or even Xenomouse was made by Gossen *et al.*, (1993b) carries *lacZ* gene of *E. coli* as reporter gene in the pUR288 shuttle plasmid vector (Fig. 7) in C57Bl/6 mice. Approximately 20 copies of the pUR288 plasmid have been integrated head to tail. Integration of the plasmid was observed at different chromosomes, 3, 4, 11 and 12; "line 60" harbors plasmids at chromosomes 3 and 4 (Vijg *et al.*, 1997). The plasmid is about 5 kb long and the *lacZ* reporter gene 3100 bp.

Isolated genomic DNA was digested with *HindIII* or *PstI*, circulated with T4 DNA ligase and electroporated into *E. coli* C (*lacZ*$^-$,galE$^-$). Using the same *galE*$^-$ *E. coli* C strain the mutant selection is identical as those described for the bacteriophage λ *lacZ* model (§ 2.2.1.2). To determine the plasmid

recovery X-gal and for mutation selection P-gal is used. P-gal is metabolized into phenol and galactose by β-galactosidase, the product of *lacZ*. Since galactose in turn is metabolised into the highly toxic UDP-galactose only mutants lacking β-galactosidase will form plaques on this medium.

2.2.2.2 *rpsL* transgenic mouse model

Gondo *et al.* (1996) developed a transgenic mouse model in a background of C57Bl/6J mice with about 350 copies of the *rpsL* gene in a shuttle plasmid. The complete plasmid is only 3000 bp long. An *E. coli* shuttle plasmid pML4 (Fig. 8) carrying next to the *rpsL (strA)* gene the kanamycine-resistant gene, was used. The *rpsL* gene carries an amber mutation and expresses the wild-type phenotype of streptomycin sensitive in *E. coli* harboring *supE* or *supF* mutation. Plasmids with normal *rpsL* expression transform streptomycin-resistant *E. coli* to streptomycin-sensitive ones.

Isolated genomic DNA is digested with *BanII,* circulated with T4 DNA ligase and electroporated into *E. coli* RR1. The RR1 strain is resistant to streptomycin (*rpsL*$^-$) but sensitive to kanamycin. The rescue efficiency was determined by counting the number of plaques after culture on medium containing kanamycin. Mutants were selected by culture on medium containing kanamycin and streptomycin. Since electroporation of *rpsL*$^+$ plasmids make *RR1 E. coli* streptomycin-sensitive, only *E. coli* cells harboring a mutated *rpsL* gene can grow on this medium. The mutation frequency equals the number of mutated *rpsL* bacteria per the total rescued number of bacteria.

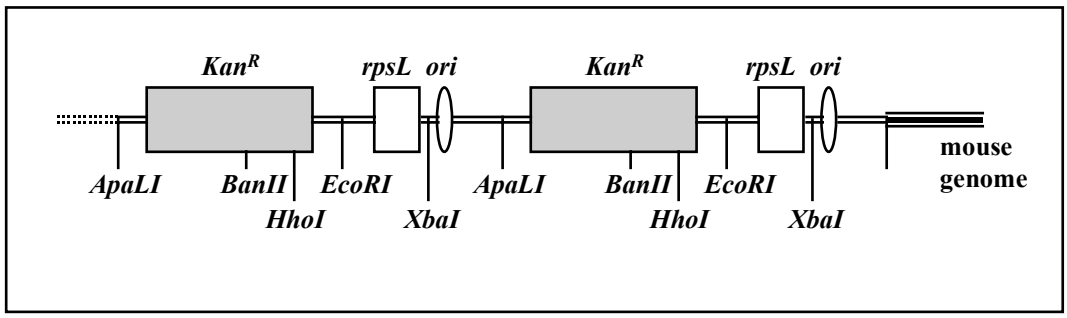

Figure 8: The shuttle plasmid pML4 (Gondo et al., 1996)

Because the reporter gene is only 375 bp long, the *rpsL* transgenic mouse model is very suitable for monitoring mutation spectra. It is possible to sequence the entire reporter gene coding region by setting one pair of primers. Finally it is a positive selection system which is much more convenient than conventional color selection.

Table 3: Overview of mutagenicity test systems in mammal in vivo

model	reporter gene	target size	detection system of mutants	DNA damage detected	remarks
models using endogenous genes					
hprt model	*hprt*	657 bp	6-thioguanine resistance	bps, ins, del	restricted to tissues which can be subcultured
tk model	*tk*	?	trifluorothymidine or 5-bromo-2'-deoxyuridine resistance	bs, ins, del, LOH	restricted to tissues which can be subcultured; heterozygous mice are used; one *tk* allele is knocked out.
aprt model	*aprt*	?	8-azaadenine or 2,6-diamino-purine resistance	bps, ins, del, LOH	restricted to tissues which can be subcultured; heterozygous mice are used; one *aprt* allele is knocked out.
Dlb-1 specific locus assay	*Dlb-1*	?	histochemical detection	bps, ins, del, LOH	restricted to epithelial cells of the small intestine (and colon)
models using transgenes in bacteriophage vectors					
LacZ model	*lacZ*	3100 bp	X-gal resistance phenyl β-D-galactoside resistance	bps, fs, ins, del^{s}	no tissue restriction
LacI model	*LacI*	1080 bp	X-gal resistance	bps, fs, ins, del^{s}	no tissue restriction
gpt-delta model	*gpt red, gam*	456 bp	6-thioguanine resistance *spi*$^{-}$ (sensitive to P2 interference) selection	bps ins, del	no tissue restriction; two selection systems: one for bps and one for del
gpt model	*gpt kan*^{r}	456 bp	6-thioguanine resistance	bps, ins	no tissue restriction
supF model	*supF*	85 bp	X-gal resistance	bps, ins, del^{s}	no tissue restriction
phiX174 model	*am3*	1 bp	selection by *am3* suppression	bp	no tissue restriction; reverse mutation assay
cII/cI model	*cII*	294 bp	selection by *hfl* expression	bps, ins, del^{s}	no tissue restriction; present in all models using λ bacteriophage as vector
models using transgenes in plasmid vectors					
lacZ plasmid model	*lacZ*	3100 bp	phenyl β-D-galacto-side resistance	bps, ins, del	no tissue restriction; detects large deletions
rpsL plasmid model	*rpsL* (*strA*)	375 bp	streptomycine selection	bps, ins, del	no tissue restriction; detects large deletions

Abbreviations
bp, base pair(s); bps, base pair substitution; fs, frame shift; del, deletions; del^{s}, small deletions up to 100 bp; ins, insertions; tran, translocation; LOH, loss of heterozygosity;
X-gal: 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

3. Validation issues

Important aspects of the validation procedure for the novel-gene mutation assays with transgenic mice include: (1) assessment of the reproducibility of the assay through interlaboratory trials, (2) comparison of effects induced in the exogenous transgene with those in endogenous genes, and (3) comparison with *in vitro* and *in vivo* assays currently used to assess the genotoxic or carcinogenic potential of a chemical, with respect to sensitivity, specificity, predictivity, and accuracy.

A number of working groups on various aspects of mutagenicity testing in transgenic animal models have been organised and regularly meetings take place where new developments of gene-mutations assays in transgenic models are discussed. Reports and proceedings of these meetings and working groups have been published. See for example Environ Mol Mutagen 25(3), 1995; Environ Mol Mutagen, 34(2-3), 1999 (Special Issue: Transgenics, Eds Heddle JA, Glickman BW, Nohmi T, van Steeg H); and IARC Scientific Publications no 146, 1999. In addition information on mutation assays with transgenic animal models including a description of the models, data on identification of mutations by DNA sequencing, compounds tested with results and authors are stored in a computer data base (accessible via the InterNet URL: http://darwin.ceh.uvic.ca/bigblue/bigblue.htm).

Most of the novel *in vivo* mutation assays listed in Table 3 suffer from a limited number of data. Only the commercially available Muta^{TM}Mouse (*lacZ*) and Big Blue® (*lacI*) models have been explored by a number of investigators resulting in sufficient knowledge and data for validation. Therefore, the discussion of validation issues in this report is restricted mainly to the two rodent gene mutation assays based on λ shuttle vector systems that are commercially available and have been explored by a number of investigators, i.e., the Muta^{TM}Mouse (*lacZ*) and the Big Blue® (*lacI*) system.

Chapter 3.1 gives a survey of various test characteristics of the Muta^{TM}Mouse (*lacZ*) and the Big Blue® (*lacI*) system known to have influence on the outcome of the tests and playing a role with respect to the differences that are found within and between laboratories.

Chapter 3.2 focuses on the question whether or not the system measures what it is intended to measure. This is elaborated by means of 3 questions, namely: (1) with regard to the origin of the spontaneous and induced mutants, are they from murine or host origin? (2), do the exogenous genes accurately reflect mutations at endogenous loci? And (3), do transgenic and nontransgenic mice respond in a comparable way in terms of metabolism, spontaneous and agent-induced genotoxicity and carcinogenesis. Finally, chapter 3.3 pays attention to the question whether the endpoint studied is predictive of the endpoints of interest, namely, rodent carcinogenicity and heritable effects.

Next to the above mentioned literature sources a number of well-known review articles were used as starting point to discuss the validation issues. The main review articles consulted are those of Gorelick 1995; Heddle and Swiger, 1996; Josephy *et al.*, 1997; Schmezer and Eckert, 1999. In addition, as indicated in the text, a number of more recent, original papers have been included as well.

3.1 Reproducibility within and between laboratories, methodological aspects

3.1.1 Spontaneous mutation frequency, influence of age and tissue specificity

Mutation frequency versus mutation rate[1]

Heddle and Swiger (1996) pay attention to the meaning of the expressions 'mutation frequency' and 'mutation rate'. What we measure is the frequency of mutants, i.e., the number of mutated cells /number of cells examined or the number of mutant colonies or plaques per number of colonies or plaque forming units. From that we infer the mutation rate. There are two mutation rates, the spontaneous rate (mutations per cell division or mutations/unit of time) and the induced rate (mutations/unit dose). Since there are substantial numbers of pre-existing (spontaneous) mutants in mice, it is important to recognise that a doubling of the spontaneous mutation rate/cell division will not lead to a doubling of the mutant frequency. The minimum increase in mutation rate that is needed to double the mutant frequency in one cell division depends on the number of spontaneous mutants per cell division and the number of pre-existing mutants. The rate of mutations per cell division has not been determined *in vivo*. Although there are estimates of the increase in mutant frequency as a function of age, the number of cell divisions over this interval is unknown (Heddle and Swiger, 1996).

Spontaneous mutation frequency

Gorelick (1995) gives an overview of studies aimed at comparing the reproducibility of spontaneous mutation frequencies in untreated transgenic animals within and between laboratories, and the interanimal variability. The data were analysed for sources of variability and it was found that the largest contribution was from the variability between animals. Age is one of the factors underlying interanimal variability. In untreated transgenic mice, the mutant frequency increases very rapidly with age from conception to birth, more slowly from birth to adulthood, and very slowly thereafter (Heddle and Swiger, 1996). The interanimal variability was fairly good reproducible between labs (Gorelick, 1995, Heddle and Swiger, 1996). The influence of age on the spontaneous mutation frequency is an important research issue in various groups, but is not further explored in this paper.

The spontaneous background mutant frequencies in transgenic animals are relatively high compared to the spontaneous background mutant frequencies found in models using endogenous genes. The spontaneous frequency influences the sensitivity of these assays. Induced mutations arise as an absolute increase in mutant frequency, so the lower the spontaneous frequency, the more sensitive the assay is to the same increase. This is apparent in studies in which exogenous and endogenous genes are compared. Thus the high spontaneous background frequency reduces the sensitivity of the transgenic assays. Therefore it is important to optimise the experimental set-up as far as possible to maximise the sensitivity of the assays. See also in section 3.1.2 the paragraph entitled *"Treatment protocol and experimental set-up"*.

Tissue specific background mutation frequencies

Transgenic mouse tissues that have been sampled for determination of the mutation frequency, include liver, lung, nasal mucosa, kidney, urinary bladder, bone marrow, spleen, skin, fore- and glandular stomach, large intestine, colon, brain and heart (Schmezer and Eckhart, 1999). In addition mutation frequencies have been determined in splenic and peripheral blood T-lymphocytes, and in male germ cells (Gorelick, 1995). The spontaneous background mutant frequency in transgenic animals is rather similar in somatic tissue but one order of magnitude higher in intestine and one order lower in germ cells. The significance of the tissue-specific background mutation frequencies is not

[1] MF, mutation frequency, i.e., the number of mutated cells /number of cells examined or the number of mutant colonies or plaques per number of colonies or per number of PFU (= plaque forming units); MR, mutation rate expressed as mutations per cell division, mutations per unit of time or mutations per unit dose

clear. For tissue-specific spontaneous background mutation frequencies see Gorelick (1995), Morrison and Ashby (1994), and de Boer *et al*. (1998). A relation between mitogenic activity and tissue specific mutation frequency is suggested.

3.1.2 Induced mutations, influence of manifestation time and treatment protocol

Manifestation time

Manifestation time is defined as the time required after exposure of the animal before mutants can be detected. The manifestation time is one of the important experimental variables. The minimum time required for expression varies from one tissue to another and may, moreover, be influenced by chemical treatment of the animal. To be valid, comparisons between tissues and treatments must be made after complete expression of the mutations. Factors involved in the length of the manifestation time comprise toxicokinetics, such as uptake and metabolic conversion, DNA repair and replication, and cellular factors such as the turnover time. Cell turnover is considered the most critical factor with regard to the length of the manifestation time. The manifestation time has not been characterised for most tissues. To the extent that measurements are made prior to complete manifestation of the mutations, the mutation frequency may be underestimated substantially. The significance of a negative result is uncertain unless the manifestation time is known (Heddle and Swiger, 1996, Heddle, 1999a, Sun and Heddle, 1999).

Treatment protocol and experimental set-up

Treatment protocols vary considerably between experiments. Variables that affect the outcome of the assay include duration of exposure, way of administering the test substance, and the dose levels selected in relation to the maximum tolerated dose of the chemical in question. Experimental data strongly suggest that chronic treatment protocols are most effective at maximising the sensitivity of the assays (Heddle and Swiger, 1996, Cosentino and Heddle, 1999). This is in concordance with the assumption that mutations in the transgenes seem to be neutral, i.e., that they provide the cell containing them with neither an advantage nor a disadvantage, multiple treatments should thus lead to an additive effect provided the manifestation time is taken into account (Heddle and Swiger, 1996).

Another important point regards the selection of tissues for the assessment of mutagenicity after treatment with a specific chemical. Many chemical carcinogens and mutagens show a tissue specificity, but the relation between target organ specificity for mutagenicity and carcinogenicity has not been explored up to now. Logically, both these target tissues have to be isolated and evaluated next to tissues that are taken routinely such as the liver. Moreover, for validation purposes of mutation tests in transgenic animal models, data from target tissues for carcinogenicity should count more heavily than data from non target tissues. In the validation reviews reported this feature was not always taken into account. It is clear that conflicts may arise when a negative result is found in the target tissue for carcinogenicity but a positive one in the non-targets.

3.1.3 Pooling of tissues and organs and clonal expansion of mutants

In several studies it is reported that the mutation frequency is determined in pooled organ samples because from a single animal not enough genomic DNA could be collected for a reliable determination of the mutation frequency. Krebs and Favor (1997) obtained meaningful results in liver but not in germ cells due to the low amount of genomic DNA extracted which was not packageable in the bacteriophage *lacZ* assay. One prerequisite for pooling of DNA samples is that the number of independent measure-points for non-pooled and pooled tissues in control and exposure groups have to be in the same order, which implies inclusion of extra animals in the experimental set-up. Another

objection against pooling of tissues of different animals within one treatment group is the occurrence of the so-called 'jackpot effect'. Jackpot mutations are mutations which arise from a single mutational event which most likely occurs during development and early growth. Such an event may result in an exceedingly high mutant frequency in all tissues showing the same mutation. Obviously a large jackpot is easily identified among individual animals in control groups. However, identification becomes problematic among animals in treatment groups or when only small jackpots occur because they have arisen later during development. The latter may lead to a false positive conclusion (Heddle and Swiger, 1996).
Recently it has been published that jackpot mutations can be easily identified without sequencing in the *cII* Muta™Mouse or the *cII* Big Blue® mouse, because *cII* and *lacZ* or *lacI* are independent of each other on the same bacteriophage vector (Heddle, 1999b; Swiger *et al.*, 1999). In case a high mutation frequency is found at *lacI*, the same DNA's can be repackaged and cII analysis can be applied. If the high mutation frequency is due to treatment the mutation frequency will be increased in both genes. In case of a jackpot mutation, the mutation frequency in the *lacI* gene is high, whereas that in the *cII* gene is not increased. If the sample is an outlier with respect to mutation frequency at both loci in multiple tissues then the mutator-phenotype may explain such results (Heddle, 1999b; Swiger *et al.*, 1999).

3.1.4 Evaluation of test results and the role of statistical analysis

For assessment of the biological and/or toxicological relevance of an increase in mutation frequencies it is important to consider dose-response relationships and reproducibility, while statistical analysis may be important especially when the levels of induced mutation are low. For discussions on methods to be used for statistical analysis of data obtained in mutation assays see Piegorsch *et al.*, 1997, Delongchamp *et al.*, 1999, Schmezer and Eckert, 1999. In most cases, an increase in mutation frequency of twofold or more over the control value is taken as an indication for a mutagenic effect. However, a twofold increase is not the only criterion for a positive effect. So a 1.3-1.9-fold increase in mutation frequency is reported as a positive result in some experiments after statistical analysis, while in other experiments, increases of twofold and more are not considered to be significant, mainly because of high inter-animal variation.

3.2 Does the system measure what it is intended to measure?

An important validation issue, which raises a number of different questions, is the point whether or not the system measures what it is intended to measure. Three of these questions are discussed below. The first discussion point regards the origin of the spontaneous and induced mutants, the second question discussed below is whether or not the exogenous genes accurately reflect mutations at endogenous loci and the third discussion point regards the question whether transgenic mouse lines respond as nontransgenic mice in terms of metabolism, DNA adduct formation, DNA repair, other genotoxicity endpoints, and spontaneous and agent-induced carcinogenesis.

3.2.1 Are the spontaneous or induced mutants from prokaryotic or murine origin?

A major concern in evaluating mutations with a model that requires bacteria for identification of these mutations is that DNA damage (*e.g.* DNA adducts) formed *in vivo* in the mouse may be introduced into *E. coli* and subsequently converted into a mutation *in vitro*. Theoretically mutations may occur (1) as mouse derived premutagenic DNA adducts, lesions, mismatches that are fixed *in vitro* during

replication in *E. coli*; (2) as artifactual lesions or adducts produced during DNA extraction or processing, and (3) during replication of λ phage within *E. coli*. Although the first effect does occur it is largely dependent on the manifestation time after treatment. If the manifestation time is long enough to allow repair of DNA damage in the mouse then the incidence of *E. coli* derived mutations is reduced significantly (Mirsalis *et al.,* 1994). Postmitotic cells like those of brain of transgenic mice treated with ENU were investigated at several times after treatment. An increased mutation frequency was only observed several days after treatment. If the mutations had originated in *E. coli* than an increase whould have been expected immediately after treatment when the DNA damage levels are highest (Gossen and Vijg, 1993; Dollé *et al.,* 1999). Moreover, *lacZ* mutation spectra from these cells isolated from the brain showed a pattern different from spectra obtained from ENU-treated *E. coli* cells (Gossen and Vijg, 1993). Dollé *et al*. (1999) confirmed the possibility that most mutants rescued from the mouse had really originated in the bacterial host by comparing the *lacZ* mutant frequency in plasmids obtained from the mouse with those in plasmids derived from *E. coli*. These authors found that although *E. coli* derived mutations may contribute up to 20% to the mouse spectrum, most of the mutations seem to be derived from the same precursor, suggesting a much lower contribution. Indeed they reported mutations in *E. coli* which could not be identified among those found in the mouse suggesting that the mutations detected arose in *E. coli* during the growth period necessary for preparing the plasmids and probably represent a jackpot effect. Moreover, only mutations occurring in the *E. coli* host during the first round of replication have a chance of detection. A replication-derived mutation would likely be present on only one strand of the transgene and could give rise to mosaic mutant plaques or colonies. A mosaic plaque or colony consists of a mixture of mutants and wild type cells resulting in a sectored appearance.
Hill *et al.* examined to what degree the Big Blue® assay generates mutations that occur outside of the mouse by investigation of plaque morphology. Generally four different types of mutant plaque morphologies are observed in the standard Big Blue® assay, i.e., circular (i.e. the plaque circumference is at least 50% blue), pinpoint (a dot of blue colour peripherally located in a wild type plaque), sectored and noncircular plaques. The circular mutant plaques are analysed in the Big Blue® as murine-derived events.
The most direct evidence for the murine derived origin of circular mutants was the similarity in the types of mutations found in jackpot and nonjackpot mutations of circular mutant plaques. In addition, about half of the spontaneous mutations in the *lacI* transgene were transitions and transversions at CpG dinucleotides, a mammalian-specific feature. The mutation pattern observed at *lacI* is consistent with AT mutation pressure operating in a GC rich DNA and approaches that reported for observed germline human factor IX mutations. Furthermore, the spontaneous mutation pattern of circular Big Blue® mutants differed significantly from that of an endogenous *lacI* gene in *E. coli*. Pinpoint mutants, which a priori were not expected to be mouse-derived, have a mutation pattern consistent with the mutation pattern of an endogenous *E. coli lacI* gene. Moreover, analysis of induced mutagenesis studies revealed mutation frequencies and patterns for the Big Blue® circular mutants which were comparable to endogenous mouse genes. In reconstruction experiments, blue plaques derived from a superinfection with wild type and mutant phage produced approximately 50% blue and 50% clear plaques on replating. This phenomenon was not seen when plaques derived from mouse were replated in the Big Blue® assay. Hill *et al.* concluded that several lines of evidence indicated that the circular mutants were derived primarily from the mouse (Hill *et al.*, 1999).

3.2.2 Comparability of transgenes and endogenous mammalian genes as mutational targets

An assumption in the development and the use of transgenic assays is that mutations at these loci accurately reflect mutations at endogenous loci. However, the transgenic targets differ in several ways from the endogenous loci. First, the sequences and location in the genome differ between transgenes

and endogenous loci. Secondly, the prokaryotic DNA is heavily methylated, is non-transcribed and is embedded in bacteriophage DNA. Third, the transgenes are usually present in multiple tandem copies (Cosentino and Heddle, 1999). The relevance of the transgenic assay results for mutations that normally occur in the cellular genome, can be demonstrated by comparison between these loci if possible in the same tissues. While the exogenous reporter genes can be measured in every tissue of the transgenic rodents as long as sufficient amounts of DNA can be collected, only a few endogenous genes and tissues are suitable for measuring mutations in animals *in vivo* (see chapter 2). Animal models suitable for measurement of mutations at endogenous genes are listed in Table 3 and described in section 2 and comprise the *Dlb-1*, the *hprt*, the *aprt* and the *tk* models. Table 4 summarises the experiments in which mutation induction in endogenous genes is compared with that in exogenous reporter genes.

***Big Blue*® *assay* (see Table 4).**
Tao *et al.* showed that increases in spontaneous and ethylnitrosourea (ENU)-induced mutant frequencies were similar at the *Dlb-1* locus and the *lacl* transgene, whereas X-ray induced increases in mutations were detected at the *Dlb-1* locus but not in the transgene (Tao *et al.*, 1993 cited in Hill *et al.*, 1999). The *Dlb-1* locus has not been cloned so it is unknown whether *lacI* and *Dlb-1* loci are differentially sensitive to different types of DNA damage (substitutions at AT base pairs and large deletions, respectively). Walker *et al.* compared mutation frequencies and mutational spectra in the *lacI* transgene with the same in the endogenous *hprt* gene in splenic lymphocytes. ENU-induced mutant frequencies and mutation patterns appeared to be similar in the *lacI* transgene and the endogenous *hprt* gene in splenic T-lymphocytes (Walker *et al.*, 1994 cited in Gorelick, 1995 and Hill, 1999). However the ~10-fold higher background mutation frequencies in the *lacI* gene made it a somewhat less sensitive target for detecting increases in mutation frequencies. Skopek *et al.* examined mutation induction by ENU and B[a]P in endogenous and exogenous genes in splenocytes in *lacI* mice. The mutation induction by ENU was similar in both loci, while B[a]P produced many more *lacI* mutations than *hprt* mutations (Skopek *et al.*, 1995, 1996 cited in Cosentino and Heddle, 1999). Casciano *et al.* (1999) compared the mutational response and spectra of the *hprt* and *lacI* transgene in Big Blue® F344 rats after treatment with the point mutagens 7,12 dimethylbenzanthracene (DMBA), and N-hydroxy-2-acetylaminofluorene (N-OH-2-AAF) and the clastogen thiotepa. These chemicals target the mammary gland, liver and blood cells, respectively. *LacI* mutation frequency and type were evaluated in these tissues as well as in splenocytes, while *hprt* mutation and frequency were measured in splenocytes. The results with DMBA and N-OH-2-AAF indicated that, although the mutant frequencies varied among genes and tissues (no further details given), the types of mutations induced by these point mutagens were similar in target and surrogate tissue and consistent with the DNA damage produced by the agents. The frequency of thiotepa-induced mutants was 2.8 fold lower (see publication of Chen *et al.*, 1998b) in the *lacI* gene than in the *hprt* gene, although the *hprt* gene recovered a fraction of large deletions not found among the *lacI* gene (Casciano *et al.*, 1999, Chen *et al.*, 1998b; Manjanatha *et al.*, 1998; Mittelstaedt *et al.*, 1998). Walker *et al.* (1999) compared the mutagenicity of the indirect-acting agent, cyclophosphamide (CP) for *hprt* and *lacI* mutations in splenic lymphocytes in transgenic Big Blue® mice treated once ip with 0, 25, or 100 mg CP/kg and necropsied 6 weeks later. There was a significant dose-related increase in *hprt* mutation frequencies; however, the mutation frequencies in *lacI* of lymphocytes from the same CP-treated animals were not significantly different from that in control animals. *Hprt* mutational spectra data in CP-treated transgenic and nontransgenic mice were different from those of control mice, whereas the spectra of mutations in *lacI* of lymphocytes from Big Blue® mice were not significantly changed after CP treatment. These data indicate that CP-induced mutations were detectable in the *hprt* gene but not in the *lacI* transgene of splenic lymphocytes, that are a non-target tissue for CP-induced cancer in mice

(Walker *et al.*, 1999; see also Gorelick *et al.* 1999, section 3.2.3, who examined *lacI* mutation induction in target and non-target tissues of CP-treated *lacI* mice).

***Muta^{TM}Mouse* (Table 4).**

Van Delft *et al.* (1998) compared alkylation-induced mutagenesis *in vivo* in (a) *lacZ* and *hprt* in spleen cells, and (b) *lacZ* and *Dlb-I* in small intestine from lambda *lacZ*$^{+/0}$/Dlb-I$^{a/b}$ mice. Induction of mutations by ethyl- and methylnitrosourea (ENU, MNU) and ethyl methanesulphonate (EMS) was investigated at 7 weeks after a single i.p. dose of each of these chemicals. In the small intestine, treatment with various dosages of ENU (10-150 mg/kg) resulted in a linear dose-response in both *lacZ* and *Dlb-I*. MNU (30 mg/kg) was also mutagenic in *lacZ* and *Dlb-I*, while EMS (250 mg/kg) did not significantly induce mutations in either gene. In spleen, ENU gave a linear dose-related response in both *lacZ* and *hprt*, MNU induced mutations in both *lacZ* and *hprt*, and EMS was only positive for *lacZ*. In most cases, the induction factor (ratio treated over controls) for mutations in *lacZ* was lower than that for *hprt* and *Dlb-1*, presumably due to a higher background in *lacZ* and/or a lower mutability of *lacZ* (van Delft *et al.*, 1998). Cosentino and Heddle (1999a) compared the mutation induction in the *lacZ* transgene of the Muta^{TM}Mouse and the endogenous *Dlb-1* gene in the epithelium of the small intestine.

The chemicals examined, i.e., MNU, EMS, MMS, B(a)P, MMC and BrdU, were selected according to the different DNA alterations they create; the presumptive repair pathways involved and whether the compound is a direct acting or a metabolically activated agent. The chemicals were administered once by gavage and mutations were quantified two weeks after treatment. Only treatment with B(a)P, MMS and MNU resulted in a dose-related increase in mutation frequencies for both loci. All chemicals tested induced similar mutant frequencies at the *Dlb-1* locus and at the *lacZ* transgene. The acute treatments generally produced only modest increases in mutant frequencies at both loci. The higher background frequency observed at the *lacZ* transgene reduces the ability of the transgenic assay to detect the same absolute increase in mutation frequencies. Despite the different spontaneous mutation frequencies, the induced mutation frequencies at the *Dlb-1* locus and *lacZ* transgene were not significantly different and were nearly identical after treatment with B(a)P, BrdU, EMS, MNU, MMC and MMS. Although each mutagen produces a distinct spectrum of mutations, resulting from the specificity of DNA binding and the type of DNA repair involved, all of the agents induced comparable increases in mutant frequencies at both loci (Cosentino and Heddle 1999a).

The above experiments regard acute studies in which test animals were treated with relatively high, often toxic doses of genotoxic carcinogens. Only a few studies are available in which endogenous genes and exogenous genes are compared in the same animal using chronic repeated dosing protocols. Cosentino and Heddle reported on an extension of the endogenous/transgenic locus comparisons to chronic exposures. The similarities between the endogenous and transgenic loci that were generally striking after acute treatments, were by no means so evident after chronic exposure. Under these conditions the endogenous loci (*Dlb-1* and *hprt*) showed much less mutations than expected in comparison with the transgenes (*lacI*, *lacZ*), based on the experience with acute exposures to B(a)P or ENU (Cosentino and Heddle, 1999b).

From the experiments summarised above it is concluded that, despite differences in mutation properties of the various model mutagens, the responses of the exogenous loci (*lacI*, *lacZ* transgene) and the endogenous loci (*Dlb-1*, *hprt*) in animals *in vivo* were generally fully comparable upon acute dosing when expressed in terms of increases in mutation frequencies measured in the same tissues. Differences in mutation induction between endogenous and exogenous loci were found for *hprt* in

Table 4 Comparison of mutation induction in endogenous and exogenous reporter genes in Big Blue® (lacI) mice/rats or in Muta™Mouse (lacZ) mice[2]

	Chemicals	Small intestinal epithelium			Splenocytes/splenic lymphocytes			Additional remarks
		Dlb-1	*lacI*	*lacZ*	*Hprt*	*lacI*	*lacZ*	
Tao *et al.*, 1993 cited in Hill *et al.*, 1999	ENU, ip X-rays	+ +	+ -		nt +	nt +		*LacI* mice 1 x ip
Walker *et al.*, 1994 cited in Hill *et al.*, 1999	ENU				+	+		*LacI* mice Mutation spectrum similar in endo- and exogenous locus
Skopek *et al.*, 1995 cited in Cosentino and Heddle, 1999a	ENU				+	+		*LacI* mice 1 – 3 x i.p.
Skopek *et al.*, 1996 cited in Cosentino and Heddle, 1999a	B[a]P				+	++		*LacI* mice
Chen *et al.*, 1998b Casciano *et al.*, 1999	thiothepa				++	+		*lacI* transgenic rats (Big Blue® F344 rats).
Manjanatha *et al.*, 1998 Casciano *et al.*, 1999	DMBA				++	+		*lacI* transgenic rats (Big Blue® F344 rats).
Van Delft *et al.*, 1998	ENU MNU EMS	+, dr + -		+, dr + -	+, dr + -		+, dr + +	$\lambda lacZ^{+/0}/Dlb\text{-}1^{a/b}$ mice Treatment: 1 x ip Manifestation time: 7 weeks
Cosentino and Heddle, 1999a	MNU BrdU EMS MMS B[a]P MMC	++, dr +, dr +, ss +, dr +, dr +, ss		++, dr + ±, ns +, dr +, dr ±, ns				F_1 (Muta™Mouse x SWR)mice. Treatment: 1 x po. Manifestation time: 2 weeks
Walker *et al.*, 1999	CP				+	-		*LacI* mice Treatment: 1 x ip Manifestation time: 6 weeks

spleen with EMS and cyclophosphamide, as well as for *Dlb-1* in intestinal epithelium with X-rays (Table 4). These results can only partly be attributed to the lower sensitivity of the exogenous genes compared to the endogenous genes as a consequence of the generally lower control (background) mutation frequencies found in endogenous than in the exogenous reporter genes. However, the general concordance between the increases in mutation frequencies in exogenous and endogenous genes indicates that the λ *lacZ* and *lacI* transgenic mice are a promising model for testing the potential mutagenicity of chemicals *in vivo*.

[2] Studies were not evaluated. The test outcomes in the table represent the conclusion as given in the papers, i.e., –, no treatment-related increase; ±, outcome inconclusive; +, treatment-related increase; ++, increase in MF more pronounced relative to other MFs in the same series of experiments. Abbreviations; nt, not tested; dr, dose-related, ss, statistically significant, ns, not statistically significant.

3.2.3 Comparability of transgenic and nontransgenic mice in terms of toxicokinetics and dynamics

Several lines of evidence indicate that the transgenic mouse responds as expected for a non-transgenic mouse with a similar genetic background in terms of chromosome damage, DNA adducts, or UDS as well as gene mutation (Gorelick, 1995). Also comparison of mutagenicity results obtained in transgenic rodents with agent-induced carcinogenic effects in rodents point to similar responses in transgenic and non-transgenic rodents (Gorelick, 1995; Gorelick *et al.*,1999; Schmezer and Eckert, 1999).

Gorelick *et al.* (1999) reported that CP-induced mutations were detectable in *lacI* transgenic mice in target tissues for CP tumorigenesis (lung, urinary bladder) but not in tissues not susceptible to tumour formation by CP in B6C3F1 mice (kidney, bone marrow, splenic T-lymphocytes). DNA sequencing revealed that the spectra of mutations in *lacI* from lung and urinary bladder were significantly changed after high-dose CP treatment, with a significant increase in the frequency of AT → TA transversions found in both tissues and a significantly elevated frequency of deletions in the lungs. Conversely, in vehicle-treated mice, the two predominant classes of *lacI* mutations recovered in lung and urinary bladder were GC → AT transitions at CpG sites and GC → TA transversions. These CP exposures were also genotoxic as measured by the significant induction of micronuclei in peripheral blood 48 hr after exposure (Gorelick *et al.*,1999).

Schmezer and Eckert summarised experimental data from mutagenicity studies in which the Muta™Mouse (*lacZ*) or the Big Blue® (*lacI*) mouse/rat system were used. The review summarises the results obtained with 51 chemical compounds and three types of radiation (Schmezer and Eckert, 1999). For each of the chemicals the route of administration, the dose levels applied, the manifestation time, the tissue(s) examined, and the results, i.e., the authors conclusion with respect to the outcome of the test, i.e., positive, negative or inconclusive with the mutation ratio ($MF_{test}/MF_{control}$) is given for each of the tissues examined for a total of circa 120 experiments. The listing includes one study in which the transgenic Fischer 344 rat was used, the other studies were performed with the mouse strains.

In about half of the experiments the test substance was administered by ip injection, in about 30% orally (cells/tissues examined, liver), in 6 out of 120 exposure was by inhalation (cells/tissues examined: lung, spleen, liver, bone marrow), and in 11 out of 120 the test substance was applied dermally (cells/tissues examined: skin, bone marrow).

Of the more than 50 agents tested in the transgenic assays, 35 have been evaluated in the *IARC Monographs* series: six were classified in Group 1, 12 in Group 2A and 14 in Group 2B. The organs and tissues that have been sampled for mutations include liver, lung, nasal mucosa, kidney, urinary bladder, bone marrow, spleen, skin, fore- and glandular stomach, large intestine, colon, brain and heart.

Schmezer and Eckert conclude that several examples demonstrate the suitability of these assays for tissue-specific mechanistic studies of mutagenicity *in vivo*: *ortho*-anisidine, a potent urinary bladder carcinogen in mice and rats gave negative results in standard assays for genotoxicity in rodents, whereas evaluation of the mutagenicity of this compound to the Big Blue® mouse bladder showed weak but consistent evidence of a positive response. The chemotherapeutic anti-oestrogen tamoxifen is a potent carcinogen in rat liver but shows no activity in most standard assays for genotoxicity *in vitro*; however, it was clearly identified as a hepatic mutagen in *lacI* Fischer 344 rats. The species-specific liver carcinogen aflatoxin B, induces hepatic tumours in humans and rats but is not tumorigenic in mouse liver. In the *lacI* system hepatic mutagenesis has been shown in rats but not in mice (Schmezer and Eckert, 1999). *N*-Nitrosodimethylamine is a well-known hepatic carcinogen in

rats after oral administration, but hepatic tumours occur only rarely after long-term exposure by inhalation, although a high incidence of nasal cavity tumours was induced. This route-dependent shift in carcinogenesis correlates well with the mutagenicity of the compound *in vivo*, as a strong, dose-dependent increase in mutation frequency is seen in the nasal mucosa of Muta^{TM}Mouse exposed by inhalation (Schmezer *et al.*, 1997 cited in Schmezer and Eckert, 1999). The direct-acting alkylating agents *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and β-propiolactone, known to act at the site of administration, induce tumours in the stomach of rodents after oral administration; the *lacZ* system was used to demonstrate the gastric specificity of these genotoxic carcinogens (Schmezer and Eckert, 1999).

While some of the compounds were studied by only one investigator, a few chemicals such as *N*-ethyl-*N*-nitrosourea (ENU), *N*-nitrosodimethylamine (NDMA), *N*-methyl-*N*-nitrosourea (MNU), cyclophosphamide, 7,12-dimethylbenz[a]anthracene (7,12-DMBA), and benzo[a]pyrene (B(a)P) were analysed by several groups. Table 5 summarises the results obtained with these chemicals in independent experiments per tissue/cell examined separately for Muta^{TM}Mouse and Big Blue® system.

Although the experimental conditions varied between the different studies, the results generally appeared reasonably well reproducible between independently conducted experiments. Results obtained in Muta^{TM}Mouse and Big Blue® system were fully comparable except for the results obtained with NDMA. Exposure to NDMA resulted in increased mutation frequencies in the liver irrespective of the way of administering the test substance, i.e., the oral (p.o.), the intraperitoneal (i.p.) or the inhalation route. However, mutagenicity in kidney and lung tissues was seen in Big Blue®, but not in Muta^{TM} Mouse, despite the comparable dose levels used in both assays, and the only marginal differences in exposure and manifestation time between the two experiments.

The review by Schmezer and Eckert demonstrates the suitability of these mutagenicity assays with transgenes *in vivo* for tissue-specific mechanistic studies of mutagenicity *in vivo*, and route and species specificity. The review does not pay attention to evaluation of experimental set-up and test results of the carcinogenicity studies, while data on mutagenic properties are restricted to that obtained in the *lacZ* or *lacI* transgenes.

The literature search did not reveal data comparing type and frequency of spontaneous occurring tumours in ageing transgenic and nontransgenic rodents.

Table 5 Mutagenic results obtained in lacI and lacZ transgenic mice with chemicals tested by more than one laboratory with, after the colon, the number of experiments in which the tissues were examined[3]

	Big Blue® mouse (BB)				Muta™ mouse (MM)			
	po	ip	inh	derm	po	Inhalation	ip	derm
NDMA, IARC classification 2A (BB, 8 experiments, MM, 5 experiments)								
Kidneys		+,1[a]					-,1[b]	
Liver	+,2	+,6			+,2	+,1	+,2	
Spleen							+,1	
Bone marrow		-,1						
Testes		-,1						
Lungs	-,1	+,1[a]				-,1	-,1[b]	
Nasal mucosa					-,1	+,1		
Forestomach	-,1							
Urinary bladder	-,1	-,1						
7,12-DMBA, no IARC classification given (BB, 1 experiment, MM, 2 experiments)								
Bone marrow								+,1
Skin				+,1				+,2
Cyclophosphamide, IARC classification 1 (BB, 1 experiment, MM, 2 experiments)								
Bone marrow							+,2	
Spleen		+,1						
B(a)P, IARC classification 2A, (BB, 2 experiments, MM, 2 experiments)								
Spleen							+,2	
Liver	+,1	+,1						
MNU, IARC classification 2A (BB, 3 experiments)								
Spleen		+,2						
Liver	+,1	+,2						
Brain		+,1						
Lungs	-,1	+,1						
Male germ cells		+,1						
Forestomach	-,1							
Kidneys	-,1							
ENU, IARC classification (BB, 8 experiments, MM, 13 experiments)								
Liver		+,1					+,6; (+),1; -,1	
Bone marrow		+,1					+,6	
Testes							+,1	
Spleen		+,2					+,1	
Male germ cells		+,3					+,2	
Lymphocytes (spleen)		+,2						
Lungs							+,2	
Brain							-,2	
Seminiferous tubules, vas deferens, small intestine, kidneys, urinary bladder, heart							+,1	

[a] treatment 1 mg/kg bw, 5d, manifestation time 7 d Suzuki *et al.*, 1996b

[b] treatment 1 mg/kg bw, 4 d, manifestation time 14 d Suzuki *et al.*, 1997

[3] Data taken over from Schmezer and Eckert, 1999, +, positive; (+), inconclusive; -, comparable with control

3.3 Predictivity of the novel gene mutation assays for carcinogenicity and for heritable effects

3.3.1 Validation assessment: mutagen/carcinogen correlations

Gorelick analysed the predictivity of the two *lac* assays for carcinogenicity with 21 chemicals tested in *lacI* and 12 in *lacZ*. These chemicals were selected because data from a rodent carcinogenicity study as well as the results from the Salmonella / microsome test were available for comparison with the transgenic mouse mutation data. Moreover the chemicals included in this validation had been tested in one of the *lac* assays in a tissue where tumours develop. The chemicals were subdivided into four categories namely: mutagenic and nonmutagenic carcinogens, and mutagenic and nonmutagenic noncarcinogens. Mutagenicity was defined solely by the response in the Salmonella / microsome test. Table 6 summarises the transgenic mouse assay results according to rodent carcinogenicity and Ames test results.

Table 6 Summary of transgenic mouse mutation assay results according to rodent carcinogenicity and Salmonella/microsome test results with number of chemicals showing the combination of results as indicated[A]

	Carcinogen[B]		Noncarcinogen	
Transgenic	SAL+[C]	SAL-	SAL+	SAL-
Big Blue® (*lacI*) n = 21				
+	10[C]	2	0	0
-	2	4	1	2
Muta™Mouse (*lacZ*) n = 12				
+	9	1	0	0
-	0	1	0	1

[A] Tables 6 and 7 have been taken over from Gorelick (1995)
[B] Carcinogenic in the mouse.
[C] SAL, Salmonella/microsome test.

Gorelick emphasised that the validation is hampered by a number of deficits, i.e.,

- Some of the experimental data were only available in abstract form and were not from peer-reviewed literature.
- In many cases the study could not be evaluated because no data of individual mice were available.
- The choice of chemicals was not balanced with respect to chemical and physical classes.
- It is not clear from all reports whether the mutation assays were conducted with the optimal study design.
- The way of administering the test substance was not always the same in the carcinogenicity and the transgenic mutagenicity assay.

Table 7 shows the positive and negative predictivity of the transgenic mouse assays for predicting rodent carcinogenicity. Table 8 gives a survey of the terminology used to describe screening tests and screening programmes. In the *lacI* assay the specificity and the positive predictivity were 100% for this set of chemicals. The sensitivity and the negative predictivity, however, were 67% and 33%

respectively. It is possible that the sensitivity and negative predictivity could be improved by using standardised protocols and optimised study designs. The overall accuracy (concordance) for this set of chemicals was 71% (see Table 7).

Table 7 Characteristics of the transgenic mouse mutation assays for predicting rodent carcinogenicity [A] ([A], see Table 6)

Characteristic	Response	
	Big Blue® (*lacI*), n = 21	Muta™Mouse (*lacZ*), n = 12
Sensitivity[4]	67% (12/18)	91% (10/11)
Specificity[5]	100% (3/3)	100% (1/1)
+ Predictivity[6]	100% (12/12)	100% (10/10)
- Predictivity[7]	33% (3/9)	50% (1/2)
Overall accuracy[8]	71% (15/21)	92% (11/12)
Overall accuracy for the Salmonella/microsome test for this set of chemicals[9]	67% (14/21)	83% (10/12)

Table 8 Terms used to describe screening tests and screening programmes (taken over from IARC, 1999)

Test outcome	Carcinogen		
	Yes	No	Total
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	N = a + b + c + d
Term	Definition		
Sensitivity	a/(a + c)		
Specificity	d/(b + d)		
Positive predictivity (predictive value)	a/(a + b)		
Negative predictivity	d/(c + d)		
Accuracy (concordance)	(a + d)/N		

4 Percentage of carcinogens with a positive result in the transgenic mutation assay
5 Percentage of noncarcinogens with a negative result in the transgenic mutation assay.
6 Percentage of positive results in the transgenic mutation assay that are carcinogens.
7 Percentage of negative results in the transgenic mutation assay that are noncarcinogens.
8 Percentage of chemicals tested for which the transgenic mutation results agree with the carcinogenicity results.
9 Percentage of chemicals tested for which the Salmonella/microme test results agree with the carcinogenicity results.

The *lacZ* system showed 100% specificity and positive predictivity for carcinogenicity with 91% sensitivity and 50% negative predictivity for the set of 12 chemicals listed. The overall accuracy was 92%. Gorelick concluded that based on this preliminary analysis, a positive result in *lacI* or *lacZ* is predictive of carcinogenicity, but a negative result is not wholly predictive. The negative predictivity of the assays require further investigation (Gorelick, 1995).

Remarks and conclusion
The article of Gorelick (1995) summarised above is the most recent publication on validation of results obtained in transgenic mouse mutation assays for predicting carcinogenicity. Taken together the results of the *lacI* and *lacZ* tests, the results of this study bring out that the transgenic mouse mutation assays show a very good positive predictivity (22/22) and specificity (4/4) of 100% each, while the sensitivity (22/29) and the overall accuracy (26/33) are 75% and 79% respectively. But the negative predictivity (4/11) is only 36%. In this context it should be realised that the validation of Gorelick does not discriminate between genotoxic and non-genotoxic carcinogens and does not attempt to answer a number of important questions such as regarding the possible relationship between the results obtained in the transgenic mutation assay and other genotoxic and carcinogenic properties of the chemicals, also in relation to the presumed mode of action. Assuming that the Salmonella positive carcinogens represent genotoxic carcinogens, the chemicals were divided in two groups namely, 1) genotoxic carcinogens and 2) non-genotoxic carcinogens and non carcinogens. Each of the two groups was subdivided according to the result of the transgenic mouse mutation assays, i.e., positive or negative. The sensitivity, specificity, positive predictivity, negative predictivity, and the accuracy for predicting genotoxic carcinogenicity was 90%, 75%, 86%, 82%, and 85% respectively. In other terms, the negative predictivity is increasing from 4/11 (36%) to 9/11 (82%).
As pointed out by Gorelick the set-up of the validation study suffered from several shortcomings, e.g., with respect to the evaluation of the underlying studies and the selection of the chemicals (see also the description of this study hereabove).

The results of the validation study of Gorelick showed an excellent positive predictivity of the bacteriophage λ *lacZ* and *lacI* assays for carcinogenicity, but the negative predictivity was rather low. It is not clear to what extent the results of the study are influenced by the selection of the chemicals and the limited set-up of the study. Moreover, the study of Gorelick leaves unanswered a number of other questions, e.g., with regard to the target organ specificity for mutagenicity in the transgenic animals in relation to the target organ specificity for carcinogenicity and questions regarding the possible relationship between genotoxicity and carcinogenicity profile in relation to the mode of action.

3.3.2 Heritable effects in rodents and results obtained in *lacI*/*lacZ* mutagenicity tests

The available data do not allow any conclusion with respect to the predictive value of germ cell mutagenicity assays in transgenic animals for heritable effects in humans. Ashby *et al.* (1997), Shelby and Tindall (1997), and van Delft *et al.* (1997) have compared the results obtained in germ cell mutagenicity tests in transgenic animals with those in the most commonly used non-transgenic germ cell mutagenicity assays, namely, the dominant lethal, heritable translocation and specific locus tests for the model mutagens ENU, iPMS and MMS. Comparison of these results is however very complex among others due to differences in type of genetic damage measured in the different assays, and the characteristic exposure and experimental requirements intrinsic to each of these assays.
The literature search did not reveal experiments in which germ cell mutagenicity was measured in exogenous and endogenous genes in the same animal.

4 Discussion

The novel *in vivo* gene mutation assays are promising new tools to determine the mutagenic potential of chemicals. The results so far show a good reproducibility. The assays can be used as a multi-endpoint system; other *in vivo* genotoxic endpoints, e.g., micronuclei, chromosomal aberrations or sister chromatid exchanges in bone marrow or blood cells, or unscheduled DNA synthesis in liver cells can be evaluated simultaneously in the same animals. Since (sub)chronic treatment of the animals rather improves its sensitivity (Tao and Heddle, 1994), these assays can be easily incorporated in, for instance, a subacute 28-day or even a subchronic 90-day toxicity test.

At present, the novel *in vivo* gene mutation assays are already used to assess the putative mutagenic capacity of chemicals. Assays with transgenic animals are predominantly performed with the commercially available bacteriophage λ-based Muta^{TM}Mouse (*lacI*) and Big Blue® mouse (*lacZ*). In a few cases these models have even been used for legislation of chemicals. However, because they are not yet fully validated and because an OECD guideline does not (yet) exist, they can only be used for confirmation and verification of results obtained with other genotoxicity tests or when all other tests give rise to an overall equivocal conclusion. One has to be very careful with data from assays with transgenics particularly when the results point to a non-mutagenic potential of the test compound, since the predictive power for carcinogenicity has not been sufficiently evaluated and the negative predictivity was rather low.

Although the results obtained so far with the novel *in vivo* gene mutation models are well matched, the transgenic models are preferable to assays that use endogenous genes. The models using endogenous genes suffer from a tissue restriction. Only those tissues where the genes are expressed and which allow subculture *in vitro*, can be investigated. Another disadvantage is that selection pressure against cells carrying mutations in the endogenous reporter genes may exist and influence the mutant frequencies. However, the possibility to detect loss of heterozygosity (LOH) is a big advantage of the *aprt* and *tk* models. LOH was found in 65% of the spontaneous *tk* mouse lymphocyte mutants (Dobrovolsky *et al.*, 1999a) and in 69% of spontaneous *aprt* mutants (Wijnhoven *et al.*, 1998). After ENU treatment also 35% of the *tk* and 10% of the *aprt* mutants contained LOH. Apparently LOH is a common mutational event in heterozygous autosomal genes *in vivo* (Dobrovolsky *et al.*, 1999b).

In contrast to the assays using endogenous genes, the transgenic models theoretically do not possess such a tissue restriction. The transgene is transmitted by the germ cells and thus therefore present in all cells. Consequently, the mutation frequency can be determined in every tissue, i.e., in somatic cells and in germ cells. There may be, however, a practical tissue restriction. Some tissues or organs are so small that it is difficult to isolate enough DNA to perform a reliable assay. Pooling of DNA samples may only then be a solution if enough animals are exposed so that the same number of measure-points will be obtained as for a tissue without pooling.

An objection against the use of transgenic animals in mutagenicity tests performed for legislation could be, although unlikely, that these animals differ from normal wild type mice, e.g., in absorption, metabolism, and distribution, suggesting that the results might be unreliable or even irrelevant. However, Heddle's group nicely demonstrated the neutrality of mutations in the *lacI* or *lacZ* genes (Tao *et al.*, 1993; Tao and Heddle, 1994; Cosentino and Heddle, 1996); the mutations do not give selective advantage or disadvantage to cells containing them. Transgenic mice responded as expected for non-transgenic mice with a similar genetic background.

The transgenic models can be divided into bacteriophage-based and plasmid-based models using either a bacteriophage vector or a plasmid vector. At present, the bacteriophage λ-based models are

commonly used. They are suitable for the detection of point mutations, insertions, and small deletions but do not allow detection of large deletions or large insertions. The multiple copies of the shuttle vector are separated from the murine genome by *cos* sites. These *cos*-sites together with a certain length of the vector are essential for excision and packaging into phage heads. Consequently, if the *cos*-sites are deleted, packaging is not possible and the deletion will be missed. Next, *in vivo* packaging requires a λ vector that is minimally 42 kb and maximally 52 kb long. Large intra-vector deletions or insertions (>5 kb) result in vectors which are too short or too long to package and these deletions will be lost too. The plasmid models do not have this problem. Detection of large deletions is possible since the system does not depend on packaging. Deletions are detectable as long as the amp^R gene and the origin of replication, present on every copy of the vector, are available. Next to intra-plasmid deletions also large deletions involving the flanking region of the murine genome can be recovered. In such cases a fragment will include a mouse sequence from the breakpoint in the flanking region up to the nearest restriction site (*HindIII*). Dollé *et al.* (1999) demonstrated the importance of the possibility to detect large deletions. Various chemicals are known to induce large deletions next to point mutations, small insertions, and small deletions. For example, exposure to X-rays resulted in both types of DNA damages (Gossen *et al.*, 1995), whereas ENU predominantly induced point mutations (Dollé *et al.* 1999). A considerable proportion of the spontaneous mutants appeared to be large deletions (Dollé *et al.* 1999). Because large deletions can be detected, plasmid vector models should be considered preferable to bacteriophage vector models.

The *lacZ* plasmid model, and probably also the other plasmid model, and the models using endogenous genes *aprt* and *tk* can detect chromosomal aberrations (Dollé *et al*., 1996; Wijnhoven *et al*., 1998; Dobrovolsky *et al*., 1999a). These chromosomal aberrations, large deletions and LOH, would have been detected in a classical bone marrow chromosome aberration or micronucleus assay accepted that bone marrow exposure occurred. However, further investigations have to show in how far these intra-chromatid size mutations and LOH occurring in the plasmid models as well as the *aprt* and *tk* models in only one chromosome are representative for the scala of aberrations which can be detected in the classical *in vivo* tests for clastogenicity. For legislation purposes this may have great implications; positive *in vitro* results whether these are gene mutations or chromosomal aberrations may then be confirmed with only one *in vivo* assay.

Another advantage of the novel *in vivo* gene mutation models is, that mutation spectra can be determined. Particularly the models with small reporter genes like *supF* (85 bp), *cII* (294 bp), *rpsL* (375 bp), or *phiX174* are very convenient for the rapid identification of gene mutations by sequencing. However, although DNA sequencing may be useful for mechanistic studies, for legislation purposes sequencing is less important. Genotoxicity testing is carried out for hazard identification. DNA sequencing may only be helpful when an induction of the mutation frequency is to be expected, but only a slightly increased mutation frequency is actually found as compared to the one found in control animals. In that specific case, a mutation spectrum in treated animals different from the endogenous spectrum points to a genotoxic capacity of the tested compound.

In vivo gene mutation assays with transgenic animals suffer from a high spontaneous background mutant value. This may be rather problematic since low increases in mutation frequencies may be lost in the background frequency, diminishing the sensitivity of the assay. De Boer *et al.* (1999) demonstrated that in Big Blue® mice (*lacI*) transitions at GC basepairs predominate and that these most frequently occur at CpG sites, which was also found for other transgenic models. There are two possible explanations for this high spontaneous mutant frequency. The first may be that cytosines within these sequences of non-transcribed genes are often heavily methylated. Methylated cytosines are prone to spontaneous deamination resulting in conversion of 5-meC to thymine. Thymine is not repaired by glycosylases leading to GT mismatches and finally to GC→AT transitions. The *lacI* gene

possesses 95 of these CpG sites whereas the murine *hprt* gene only has 10 CpG sites, which are only weakly methylated because the *hprt* gene is a transcribed gene. To study the effect of CpG content on the frequency and type of spontaneous mutation, Skopek *et al.* (1998) developed transgenic animals with an analogue of the bacterial *lacI* target gene (*mrkII*), which contains a reduced number of CpG sequences (95 in *lacI* versus only 13 in *mrkII*). Substantial reduction of the number of CpG sequences in the *lacI* transgene did not significantly reduce the rate of spontaneous mutation or alter the contribution of CpG-related events (32% in *lacI* versus 23% in *mrkII*.). This suggests that other factors are also operating to establish frequency and composition of spontaneous mutations in transgenic targets. The second explanation may be difference in repair. The transcribed genes, e.g., *hprt,* are repaired much more efficiently than the non-transcribed (*lacI* or *lacZ*).

Because in transgenic mice the mutation frequency is determined in a bacterial transgene normally not present in the mouse genome, it is often suggested that these transgenic mouse models are not suitable models for mutagenicity testing. To test the relevance of the transgenic mutation model the mutation frequencies of the transgenes can be compared to those of the endogenous genes in transgenic mice. There are several differences between the bacterial transgenes and the endogenous genes, which may influence the outcome of this comparison. The transgene is not expressed and consequently not subject to preferential repair; there are multiple copies of the transgene per mouse cell as compared to single copies of native mammalian genes; the transgene is highly methylated, and finally the differences in mutant selection may provide different filters for the recovery of phenotypic mutations (Walker *et al.*, 1996). Moreover, it is important to remember that a given reporter gene will have a different target size for every mutagen or class of mutation (Skopek, 1995). Despite these differences the results with the *lacI* and *lacZ* models show a similar pattern as those obtained with the *Dlb-1* model (Tao and Heddle, 1994; Zhang *et al.*, 1996a, 1996b; van Delft *et al.*, 1998; Cosentino and Heddle, 1999). That the number of mutants observed differ by a factor 10, is easily explained by the fact that each mutant colony represents one mutable locus, whereas each villus is supplied by about 10 stem cells explaining the factor 10. The only difference in results so far was obtained with X-rays due to the fact that the bacteriophage λ-based *lacI* and *lacZ* models do not detect large deletions. Induced mutation frequencies in these transgenic models appeared also rather similar to those obtained with the endogenous *hprt* gene (Skopek 1995; Walker *et al.*, 1996; Lynch *et al.*, 1998; Chen *et al.*, 1998b, Mittelstaedt *et al.*, 1998; Manjanatha *et al.*, 1998). Differences between the *lacI* and *hprt* genes in the kinetics of mutant induction, in the frequencies of induced mutants, and in the sensitivity of mutant detection could be explained at least partially by the properties of both genes (Chen *et al.*, 1998b, Mittelstaedt *et al.*, 1998; Manjanatha *et al.*, 1998). However, due to the higher spontaneous frequencies in transgenic mice, the relative increase over the background is generally lower for the transgenes as compared to the endogenous *hprt* gene. From these data it is evident that the results obtained with transgenes are to a large extent comparable with those of endogenous genes. However, this comparison may be distorted in some degree since these assays were performed predominantly with *N*-ethyl-*N*-nitrosourea or other clear mutagenic compounds.

At present validation of the commercially available bacteriophage λ-based transgenic mouse models is in progress. The most recent publication on the predictive value of the novel gene mutation tests for carcinogenicity shows that the Big Blue® and Muta™Mouse systems perform well with regard to positive predictivity, specificity, sensitivity, and overall accuracy (Gorelick, 1995). Although many of the data were obtained with non-optimised protocols, the sensitivity in the Big Blue® and Muta™Mouse systems was 67 and 91% respectively, which is a better result than the sensitivity found in the classical *in vitro* and *in vivo* gene mutation assays. Moreover a specificity and a positive predictivity of even 100% was reported for both models. A major problem is the poor negative predictivity of 33% in the Big Blue® and 50% in the Muta™Mouse model. From these results it is concluded that a positive result in a gene mutation test with bacteriophage λ-based *lacI* or *lacZ* mice

almost certainly indicates that the compound is carcinogenic. Without additional information, however, a negative result does not automatically point to non-carcinogenicity. In view of the very small data base used to examine the predictivity of the Big Blue® and Muta™Mouse systems for carcinogenicity, it is recommended to extend the study on the predictivity of the novel gene mutation tests for carcinogenicity by using the latest mutagenicity and carcinogenicity data also in relation to new insights and taking into account the available data on the mode of action of several of the carcinogens.

A protocol for the performance of the discussed novel *in vivo* gene mutation assays is not yet available. A number of working groups and meetings has been organised to discuss the novel gene mutation assays. As assays with transgenics are assumed to be the most promising, the discussion is predominantly focused on the use of transgenic animals and properly speaking only on the commercially available bacteriophage λ-based Muta™Mouse and Big Blue® mice. During the International Workshop on Genotoxicity Test Procedures, held in Washington in March 1999, it was concluded that consensus has to be reached first on some important issues: e.g., number of animals/groups, number of treatments, way of administering the test substance, manifestation times, tissues to be sampled, statistics, and so on. The number of treatments and with that the manifestation times were by far the most discussed items. One to 5 treatments, followed by a manifestation time of several weeks, were generally accepted as not suitable. There is a preference for treatment periods of 28 days or when necessary even longer but not exceeding 180 days since then neoplastic lesions may occur. The neutrality of mutations in the *lacI* or *lacZ* genes, indicated by a constant mutant frequency after cessation of exposure and an additive response after weekly exposure, was demonstrated (Tao *et al.*, 1993; Tao and Heddle, 1994; Cosentino and Heddle, 1996). The additive response gives support to the suggestion that chronic exposure would increase the sensitivity of the assay. Evidence supporting this suggestion is accumulating rapidly. For example, multiple treatments with mitomycine C resulted in a 2-fold increase in *lacZ* mutations in the bone marrow, whereas a single acute dose did not induce an increase in *lacZ* mutations at all (Suzuki *et al.*, 1993).

5 Novel gene mutation assays: implications for regulatory bodies

The novel *in vivo* gene mutation assays are promising new assays for mechanistic and fundamental studies on the occurrence of gene mutations. This conclusion is above all based on the large amount of data obtained with the commercially available Muta^{TM}Mouse (*lacZ*) and Big Blue® mouse or rat (*lacI*) but given the large similarities in theory and design, the conclusion probably also applies to the other less well known models. In general, these models show a good positive but a low negative predictivity for carcinogenicity. However, it is not clear to what extent these results are influenced by the selection of the chemicals and the limited set up of the validation studies. Further these models show a good reproducibility. A negative point is the relatively high background mutation rate. Immediately, the question arises which of these different assays meets best the requirements essential for legislation purposes. At first sight many will prefer the bacteriophage based-models and particularly the commercially available Muta^{TM}Mouse (*lacZ*) and Big Blue® mouse or rat (*lacI*). The advantages of these models are obvious: the mutation frequencies can be determined in every tissue or cell type, the results are reproducible, and the models have already been extensively used and therefore provide a useful background of data and experimental design. However, they suffer from a major drawback; large deletions can not be measured. Next, the large size of the phage and the rather high number of copies per haploid genome may give rise to problems, since it requires large amounts of packaging extracts to collect a sufficiently high number of reporter genes. The models using a plasmid vector may be a better choice; with these models large deletions can be detected. However, the plasmid models are not (yet) extensively used and consequently there are not many experimental data. A major point against both plasmid- and bacteriophage-based models is that they use exogenous reporter genes, which are heavily methylated and not transcribed. These features may influence chemical mutagenicity. Up to now, however, no major influence has been shown in studies aimed at comparing chemical mutagenicity in assays using exogenous and endogenous reporter genes. The models using an endogenous gene as reporter gene do not have the latter disadvantages. Moreover, they detect the broadest spectrum of mutations, next to point mutations, frameshifts, insertions and (large) deletions, also loss of heterozygosity (*aprt* and *tk*), and they are more sensitive than the ones using exogenous reporter genes as a consequence of the lower background mutation rate. However, they demonstrate an important tissue restriction, as application of these assays is restricted to those tissues which express the reporter gene and/or which can be subcultured *in vitro*. Secondly, the selective marker is only present once or twice in contrast to the transgenic models with multiple copies of the transgene.

Apparently, it is very difficult to choose between these models. In view of the complexity of the various pathways that can lead to mutations, the wide range of different mutational target genes and the fact that the animal model remains a surrogate for the human situation, it is unlikely that the perfect model harbouring all the advantages of the above mentioned models will ever be developed. On the basis of the above mentioned features of the various models a preference exists for the transgenic models, whereas plasmid vector models are considered preferable to bacteriophage vector models since the former may detect large deletions in addition to point mutations. However, the appropriate model has to be chosen for each test compound on a case by case basis. To make a reliable choice, all known toxicological data have to be taken into account, e.g., which type of DNA damage occurs in *in vitro* genotoxicity tests and which are the target tissues in (sub-) chronic studies. For legislation purposes a properly standardised (OECD) guideline has to be developed. This guideline should give clear directives with regard to, among others, the duration of the manifestation time, the number and level of test concentrations as well as the route of exposure. Moreover the guideline should give the possibility to determine for each test compound on a case by case basis

which tissues or cell types have to be evaluated taking into account all known toxicological data. A repeated dose protocol (28 days or longer) has to be strongly recommended. Repeated dosing of a test compound more accurately mimics the human situation in that the chemicals are present during periods in which cell proliferation induced by earlier exposures may be occurring. Next, the incorporation of these novel gene mutation tests into other toxicological tests (e.g. sub-acute 28-day toxicology test) should be recommended in order to decrease the use of experimental animals. Ideally, this genotoxic endpoint should be seen as an additional endpoint in standard toxicology tests. Meanwhile, strategies for genotoxicity testing have to be adjusted. In those cases where a tiered system is used, e.g., as for the legislation of new chemicals, *in vitro* positive results have to be confirmed by *in vivo* tests. At present, any positive *in vitro* assay, independently of whether it demonstrates an increase in chromosomal aberrations or in gene mutations, is always followed by an *in vivo* cytogenetic bone marrow/peripheral blood cell test and/or an *in vivo* unscheduled DNA synthesis test, although none of these tests detect gene mutations. With the novel *in vivo* gene mutation tests it is possible to confirm putative positive results from *in vitro* gene mutation tests with a proper follow-up assay. Since with the *in vivo* assays using transgenes, the induction of gene mutations can be determined in any tissue, it is also possible to investigate the induction of DNA damage in germ cells, which is an EU consideration in case of positive *in vivo* results. Adjustment can even go further. If further testing with plasmid models or models using endogenous reporter genes shows that these models detect also clastogens, *in vivo* testing even may be restricted to a single test. In those cases where a battery approach is used, the *in vivo* gene mutation test has to be added to the strategy. Whether there will only be an addition to or whether there will be a replacement of the cytogenetic bone marrow or peripheral blood cell assay, which is mostly part of such a battery, depends on the outcome of the above mentioned further testing on clastogenicity.
Although these novel assays are not yet fully validated and a test guideline is not yet available, the bacteriophage λ-based *lacI* and *lacZ* models may already be used for legislation. However, the assays should not be used on a routine base and not be carried out without a clear purpose like confirmation and verification of results obtained in other genotoxicity tests. Moreover, in view of the uncertainties involved with regard to the negative predictivity for carcinogenicity, one has to be very careful with a negative response in these assays.

The results of these novel gene mutation assays are so promising and the importance for legislation is so great that further investigations of the existing models and the development of new models should be recommended.

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