## 2.0    INTRODUCTION TO TRANSGENIC RODENT MUTATION MODELS AND ASSAYS

### 2.1    Overview

Induced mutations play a role in carcinogenesis and may be involved in the production of birth defects and other disease conditions. Although it is widely accepted that *in vivo* mutation assays are more relevant to the human condition than *in vitro* assays, our ability to evaluate mutagenesis *in vivo* in a broad range of tissues has historically been quite limited.

The need for effective *in vivo* assays arises out of the complexity of the mutagenic process. Most chemical mutagens and carcinogens, whether natural or synthetic, are not directly mutagenic but are converted to mutagens *in vivo* through the activity of detoxifying enzymes. The active mutagen is frequently a metabolic product, often a minor one, of the detoxification process and may be further metabolised to an inactive and excretable form. The target tissue for toxicity is not necessarily the tissue in which activation occurs; rather, a metabolite may be transported to a target tissue. These complexities, involving uptake, metabolism, transportation between tissues and excretion, are difficult to model *in vitro*.

*In vivo* mutation assays that rely on phenotypic changes in endogenous loci have been developed; however, these assays are limited to particular tissues or developmental stages (Jones, Burkhart-Schultz and Carrano, 1985; Winton, Blount and Ponder, 1988; Aidoo *et al*., 1991). A variety of assays that are based on genotypic selection methods have been developed more recently (Parsons and Heflich, 1997, 1998a, 1998b; McKinzie *et al*., 2001; McKinzie and Parsons, 2002); however, these are generally extremely demanding technically and have shown limited general applicability. *In vivo* assays for chromosomal damage are widely used; however, chromosomal mutation and gene mutation are mechanistically distinct molecular processes, and there are examples in the literature in which clastogenicity does not serve as an adequate surrogate for gene mutation. Cancer bioassays themselves suffer from the need for lifetime exposures of relatively large numbers of animals and for an expensive subsequent analysis.

The development of TGR mutation models has provided the ability to detect, quantify and sequence mutations in a range of somatic and germ cells. The TGR mutation assay is based on transgenic rats and mice that contain multiple copies of chromosomally integrated plasmid and phage shuttle vectors that harbour reporter genes used to detect mutation. Mutagenic events arising in a rodent are scored by recovering the shuttle vector and analysing the phenotype of the reporter gene in a bacterial host. TGR gene mutation assays allow mutations induced in a genetically neutral transgene to be scored in any tissue of the rodent and therefore circumvent many of the existing limitations associated with the study of gene mutation *in vivo*.

The purpose of this review is to summarise the information currently available on TGR assays and suggest the most efficient ways of using the assays based on current knowledge. Specifically, we deal with the use of these assays for testing compounds of unknown carcinogenicity and the use of this information for regulatory purposes. Several previous reviews have appeared in the literature (Provost *et al*., 1993; Ashby and Tinwell, 1994; Dycaico *et al*., 1994; Goldsworthy *et al*., 1994; Gorelick and Thompson, 1994; Gossen, de Leeuw and Vijg, 1994; Mirsalis, Monforte and Winegar, 1994, 1995; Morrison and Ashby, 1994; Shephard, Lutz and Schlatter, 1994; Cunningham and Matthews, 1995; Gorelick, 1995, 1996; Martus *et al*., 1995; Ashby, Gorelick and Shelby, 1997; Vijg *et al*., 1997; Schmezer *et al*., 1998a, 1998b; Dean *et al*., 1999; Nagao, 1999; Schmezer and Eckert, 1999; Suzuki *et al*., 1999a; Nohmi, Suzuki and Masumura, 2000; Stuart and Glickman, 2000; Willems and van Benthem, 2000), although none of these papers provided a detailed

description of the results obtained to date for all the agents tested, as well as a description of the experimental parameters used in those experiments.

This detailed review provides a comprehensive review of the TGR mutation assay literature. In this chapter, we describe TGR mutagenicity models and their use for the analysis of gene and chromosomal mutation. The advantages and disadvantages of TGR assays must be considered in the context of non-transgenic tests for genotoxicity and carcinogenicity; these assays, and their limitations, are detailed in Chapter 3. In order to comprehensively assess the current information available regarding the use of TGR assays, we have developed a database containing transgenic rodent assay information (TRAID). In Chapter 4, we describe the database and summarise the available information as it pertains to the conduct of TGR assays and important parameters of assay performance. The performance of the TGR assay – both in isolation and as part of a battery of *in vitro* and *in vivo* short-term genotoxicity tests – as a mutation test and in predicting carcinogenicity is described in Chapter 5 of this review. Recommended experimental parameters for TGR assays used in product testing are described in Chapter 6. Appendices include a comprehensive description of the experimental TGR data available to date (Appendix A), summary information on the genotoxicity and carcinogenicity of agents that have been evaluated using TGR assays (Appendix B) and experimental data relevant to tissue-specific carcinogens (Appendix C).

## 2.2 Transgenic rodent mutation systems

The first report of a transgenic assay for mutation in mammals was that of Gossen *et al*. (1989), who placed the bacterial *lacZ* gene, encoding β-galactosidase, in a lambda (λ) gt10 vector. Transgenic *lacZ* mice were produced by stable integration of the λgt10 vector into the chromosome of CD2F1 mice. Mutation analysis was carried out by extracting high molecular weight genomic DNA from the tissue of interest, packaging the lambda shuttle vector *in vitro* into lambda phage heads and testing for mutations that arise in the transgene sequences following infection of an appropriate strain of *Escherichia coli*.

A variety of TGR models have subsequently been developed, of which the Muta™-Mouse, the Big Blue® mouse and rat, the *lacZ* plasmid mouse and the *gpt* delta mouse and rat have a sufficient quantity of experimental data associated with them to allow evaluation of their overall performance. These are described below.

### 2.2.1 Muta™Mouse

The *lacZ* transgenic CD2F1 (BALB/C × DBA2) mouse was produced by a micro-injection of λgt10*lacZ* (containing the *lacZ* gene in a single *Eco*RI site of a λgt10 vector; the vector is approximately 47 kilobases [kb] in length, and the *lacZ* transgene is approximately 3 100 base pairs [bp]) into fertilised CD2F1 oocytes (Gossen *et al*., 1989) (Figure 2-1A). Progeny with a variety of copy numbers (3–80) of transgenes was obtained. Among those, strain 40.6, which carries 40 copies of the transgene in a head-to-tail manner at a single site on chromosome 3 (Blakey *et al*., 1995), was maintained as a homozygote and is commercially available as the Muta™Mouse from Covance Research Products (Denver, PA, USA).

To assess mutation, the λgt10*lacZ* shuttle vectors are excised from genomic DNA and packaged into phage heads using an *in vitro* packaging extract (Figure 2-1B). The resultant phages are absorbed onto *E. coli* C (*lacZ⁻*) cells. In initial studies, bacteria were plated onto medium containing X-Gal (a substrate for β-galactosidase that yields a blue product), and blue plaques containing wild-type *lacZ* genes were colorimetrically distinguished from white plaques containing mutant *lacZ⁻* genes (Gossen *et al*., 1991). Subsequently, a simpler and faster selective system was developed in which an *E. coli* C (*galE⁻ lacZ⁻*) host is used for phage infection and mutation selection is carried out on P-Gal medium (Vijg and Douglas, 1996). P-Gal medium is toxic to *galE⁻* strains that express a functional *lacZ* gene; thus, only
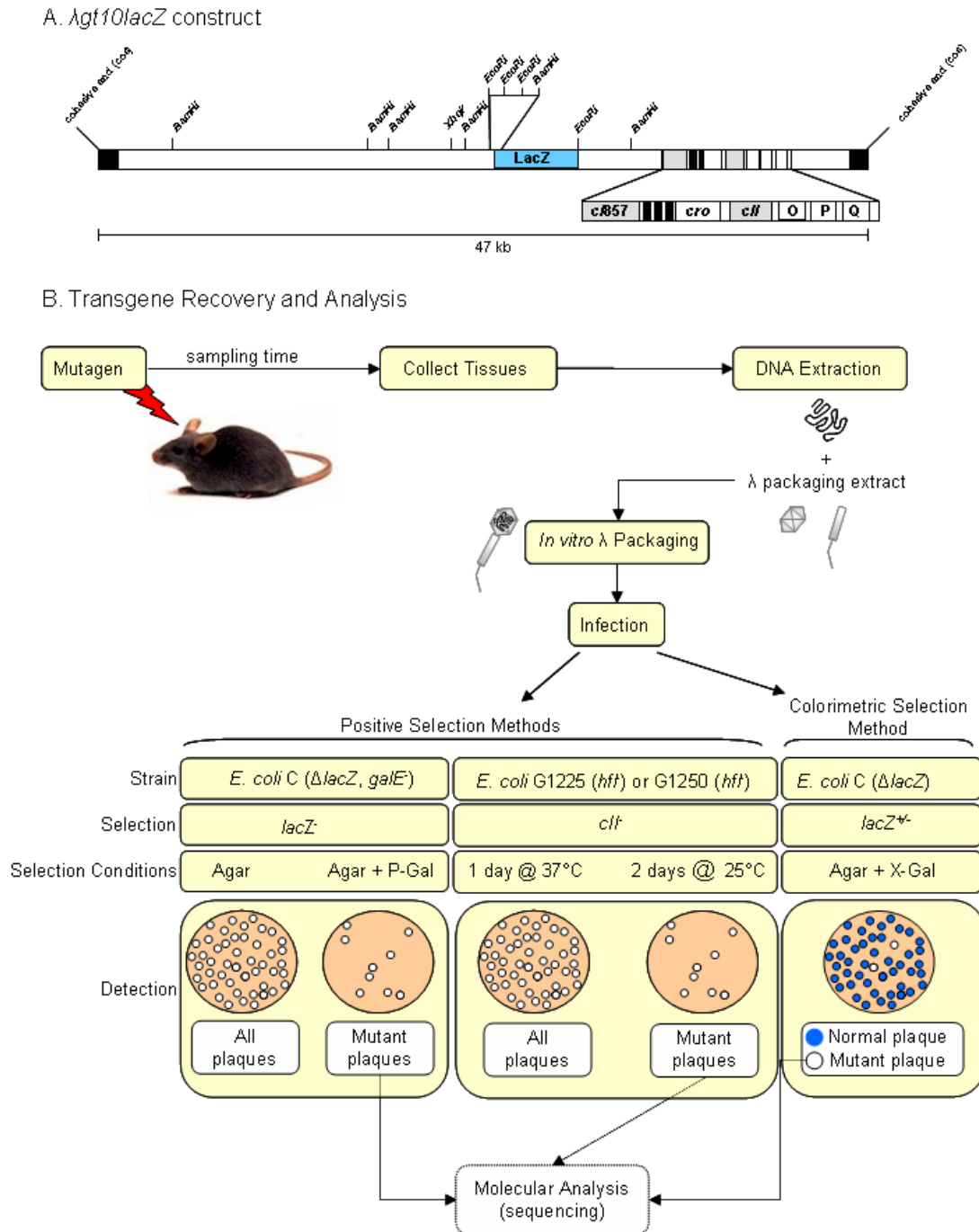
**Figure 2-1.** The Muta™Mouse transgenic mutation assay:
(A) the λgt10*lacZ* construct; (B) transgene recovery and analysis

phages that harbour a mutated *lacZ* will be able to form plaques on P-Gal medium. *LacZ* mutant frequency is determined by calculating the proportion of plaques containing *lacZ* mutations in the phage population, which is estimated on non-selective titre plates.

### 2.2.2   Big Blue®

The Big Blue® mouse and rat transgenic systems are based on the bacterial *lacI* gene. The λLIZα shuttle vector, carrying the bacterial *lacI* gene (1 080 bp) as a mutational target, together with the *lacO* operator sequences and *lacZ* gene (Figure 2-2A), was injected into a fertilised oocyte of C57BL/6 mice to produce transgenic progeny (Kohler *et al*., 1990, 1991a, 1991b). The transgenic C57BL/6 A1 line was also crossed with an animal of the C3H line to produce a transgenic B6C3F1 mouse with the same genetic background as the U.S. National Toxicology Program (NTP) bioassay test strain. The 45.6 kb construct is present in approximately 40 copies per chromosome (Gossen *et al*., 1989), with integration occurring at a single locus on chromosome 4, in a head-to-tail arrangement (Dycaico *et al*., 1994). Both transgenic mouse lines are maintained as a hemizygote for the transgene, and the C57BL/6 mouse is also available as a homozygote. Both C57BL/6 and B6C3F1 transgenic strains are commercially available from Stratagene (La Jolla, CA, USA).

A *lacI* transgenic rat was produced in a Fischer 344 background in the same manner as described above (Dycaico *et al*., 1994). The λLIZα vector is integrated on rat chromosome 4 at 15–20 copies (Stratagene, unpublished data). The Big Blue® Rat, which is sold by Stratagene (La Jolla, CA, USA) exclusively in the homozygous form, contains 30–40 copies of the shuttle vector per genome.

Mutations arising in the rodent genomic DNA are scored in *E. coli* SCS-8 cells (*lacZ*ΔM15) following *in vitro* packaging of the λLIZα phage (Figure 2-2B). White (colourless) plaques will arise from phage bearing wild-type *lacI* (encoding functional Lac repressor) when the SCS-8 host is plated on X-Gal medium. However, mutations in *lacI* will produce a Lac repressor that is unable to bind to the *lac* operator; consequently, α*lacZ* transcription will be derepressed and β-galactosidase will cleave X-Gal, producing a blue plaque. The proportion of blue plaques is a measure of mutant frequency. To date, there has not been an effective positive selection method for *lacI*⁻ mutants developed for the Big Blue® mouse or rat systems.

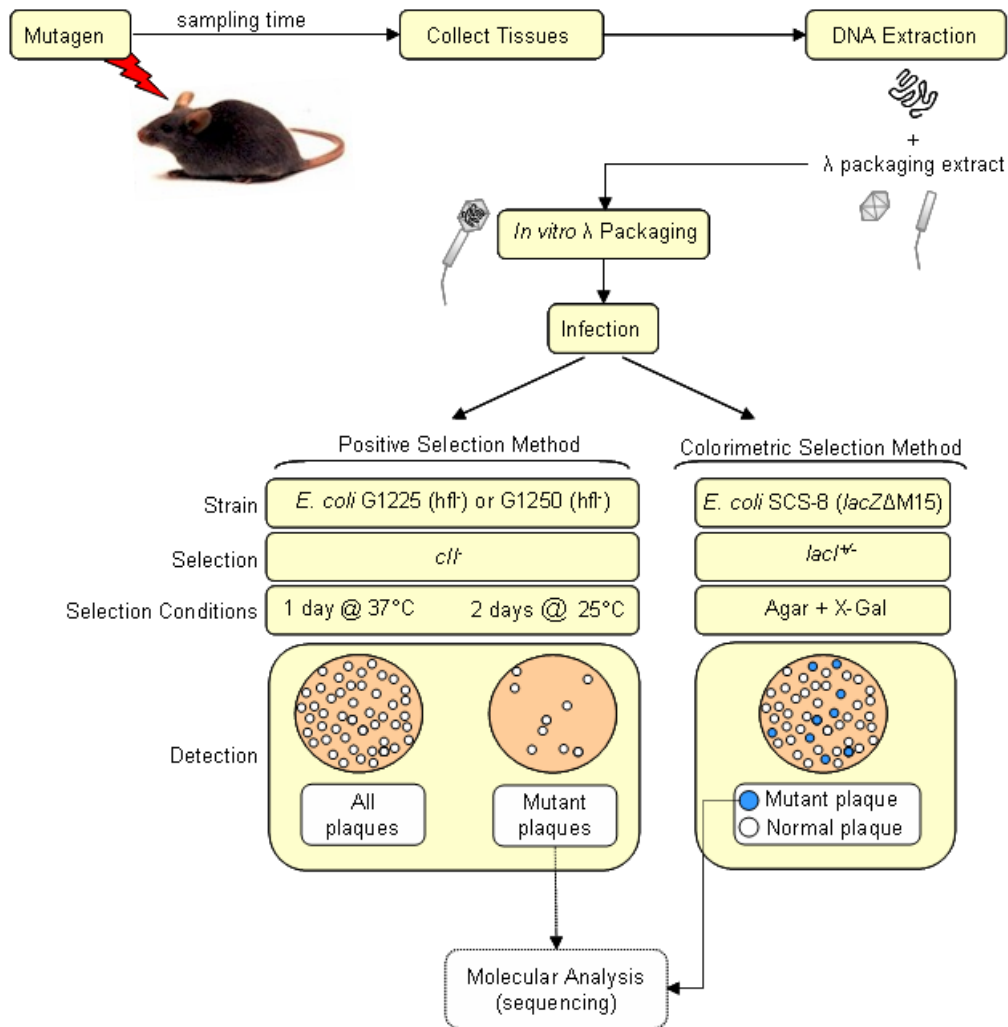### 2.2.3   LacZ *plasmid mouse*

A *lacZ* plasmid mouse has been developed that contains ~20 copies per haploid genome of the pUR288 plasmid (~5 kb harbouring the 3 100 bp *lacZ* gene) integrated into multiple chromosomes of the C57BL/6 mouse (Gossen *et al*., 1995; Martus *et al*., 1995; Vijg *et al*., 1997) (Figure 2-3A). Mouse line 60 contains plasmids integrated on both chromosomes 3 and 4 (Vijg *et al*., 1997). Isolated genomic DNA is digested with *Hin*dIII, which releases single copies of the linearised plasmid from the tandem array. Individual plasmids are then purified by adsorption onto magnetic beads coated with Lac repressor (Gossen *et al*., 1993) (Figure 2-3B). Following elution of the plasmids from the beads, the plasmid DNA is recircularised by T4 DNA ligase. To assess mutation, the recircularised plasmids are electroporated into *E. coli* C (*galE*⁻ *lacZ*⁻), and mutant frequency is determined using the P-Gal positive selection method (Vijg and Douglas, 1996), as described in Section 2.2.1 above.

In principle, the plasmid mouse system differs from the bacteriophage-based models in two significant respects. First, since the plasmid is approximately one-tenth the size of bacteriophages, multicopy concatamers of the plasmids can be isolated from genomic DNA with very high efficiency. This contrasts with phage-based systems, in which very high molecular weight genomic DNA must be isolated in order to obtain intact vectors with high efficiency. Second, a range of deletions arising within the concatamer as well as deletions
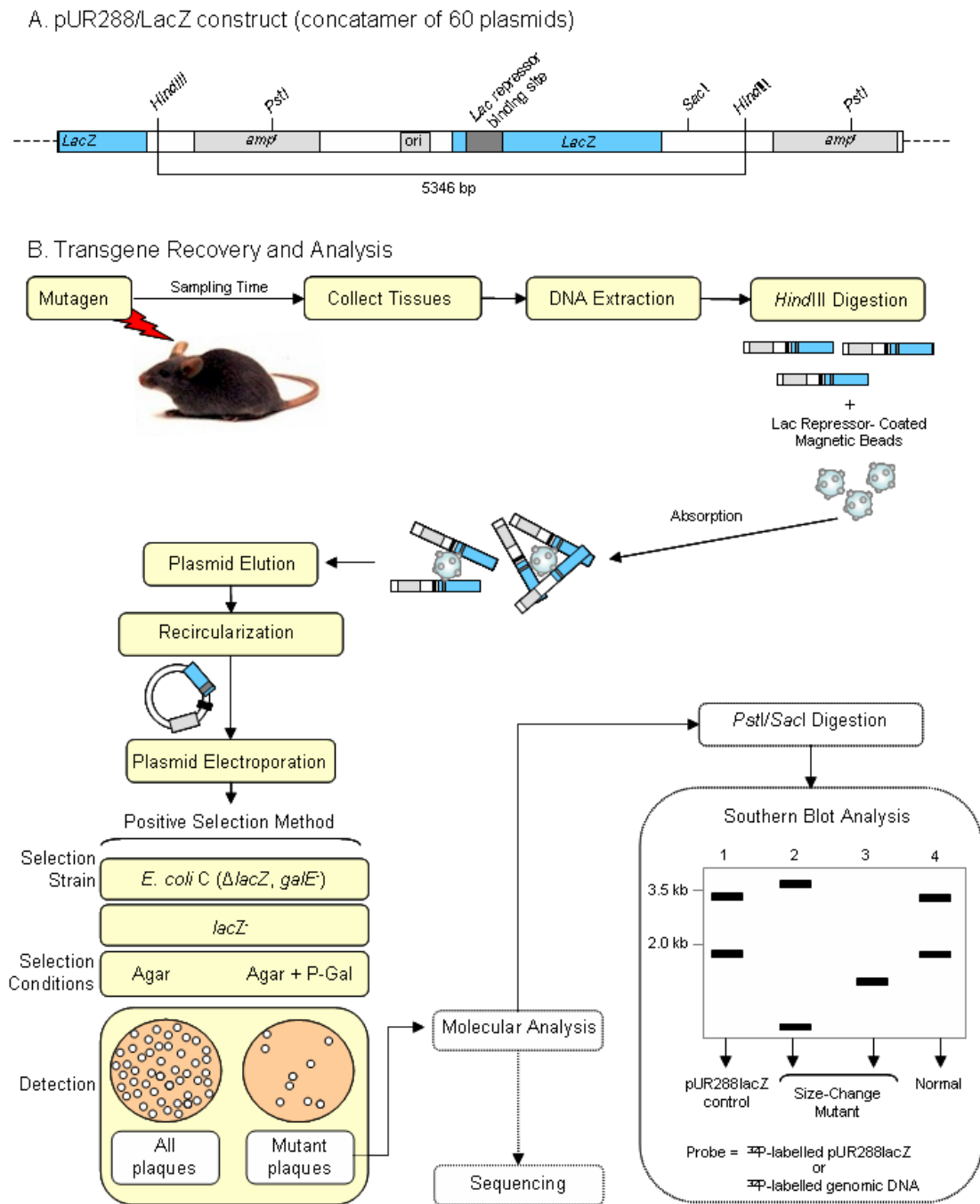
A. λLIZα construct
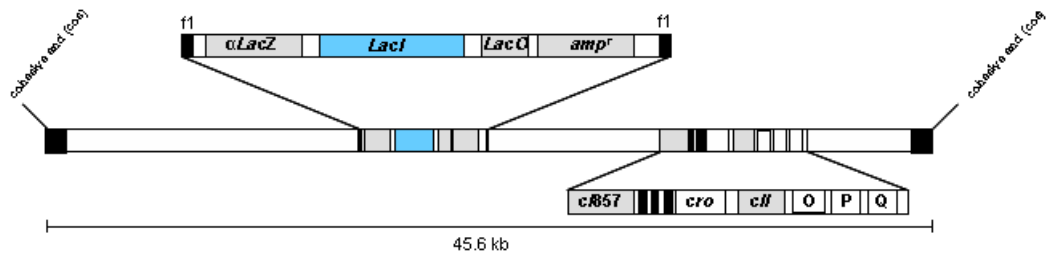


B. Transgene Recovery and Analysis



**Figure 2-2.** The Big Blue® (mouse or rat) transgenic mutation assay:
(A) the λLIZα construct; (B) transgene recovery and analysis

**Figure 2-3.** The *lacZ* plasmid mouse transgenic mutation assay:
(A) the pUR288/*lacZ* plasmid; (B) transgene recovery and analysis

extending from a *lacZ* target gene into 3′-flanking chromosomal sequences can be recovered, detected and characterised (Gossen *et al*., 1995; Martus *et al*., 1995; Dolle *et al*., 1996; Vijg *et al*., 1997). In contrast, bacteriophage-based systems are not efficient for detection of mutants containing deletions, particularly if these deletions extend into or through sequences necessary for phage propagation (see Section 2.3 for further discussion). From a technical point of view, the method of transgene recovery from the genomic DNA (restriction enzyme digestion) contributes modestly to the background spontaneous mutant frequency through a "star" activity associated with *Hin*dIII – that is, cleavage can occur at nucleotide sequences other than the *Hin*dIII restriction enzyme recognition sequence (Dolle *et al*., 1999).

### *2.2.4* **gpt** *delta rodents*

The *gpt* delta mouse was established by microinjection of λEG10 phage DNA (48 kb) into the fertilised eggs of C57BL/6J mice (Nohmi *et al*., 1996) (Figure 2-4A). Phage λEG10 carries about 80 copies of the transgene in a head-to-tail fashion at a single site of chromosome 17 and is maintained as a homozygote (*i.e.* the mouse carries about 160 copies of λEG10 DNA per diploid genome) (Masumura *et al*., 1999b).

More recently, a *gpt* delta rat has been developed in a Sprague-Dawley background (Hayashi *et al*., 2003). The *gpt* delta rat has approximately 10 copies of the λEG10 vector integrated at position 4q24-q31. The transgenic rat is available as a hemizygote only (Hayashi *et al*., 2003).

Mutation in the *gpt* delta mouse and rat can be assessed using 6-thioguanine or Spi⁻ selection, which respond primarily to point mutation and deletion, respectively (Nohmi, Suzuki and Masumura, 2000).

6-Thioguanine selection (Figure 2-4B) uses the 456 bp *gpt* gene of *E. coli* as a reporter gene. The *gpt* gene of *E. coli* encodes guanine phosphoribosyltransferase; phosphorylation of 6-thioguanine is toxic to cells when it is incorporated into DNA. Thus, only cells containing *gpt* mutants can form colonies on plates containing 6-thioguanine. The λEG10 shuttle vector carries a linearised plasmid region flanked by two direct repeat sequences of *loxP*. When *E. coli* strain YG6020 (*gpt⁻*) expressing Cre recombinase is infected with λEG10 rescued from the mice, the plasmid region is efficiently excised from the phage DNA, circularised and propagated as multi-copy-number plasmids carrying the *E. coli gpt* and chloramphenicol aminotransferase (*CAT*) genes. *E. coli* cells harbouring the plasmids carrying mutant *gpt* and *CAT* genes can be positively selected as bacterial colonies arising on plates containing 6-thioguanine and chloramphenicol (Nohmi, Suzuki and Masumura, 2000). The number of rescued phages can be determined by plating the cells on plates containing chloramphenicol alone. The mutant frequency of *gpt* is calculated by dividing the number of colonies arising on plates containing 6-thioguanine and chloramphenicol by the number of colonies arising on plates containing chloramphenicol alone.

Spi⁻ selection (Figure 2-4B) takes advantage of the restricted growth of wild-type λ phages in P2 lysogens (Nohmi *et al*., 1999; Nohmi, Suzuki and Masumura, 2000; Shibata *et al*., 2003). Only mutant λ phages that are deficient in the functions of both the *gam* and *redBA* genes can grow well in P2 lysogens; the Spi⁻ phenotype is exhibited as long as the λ phages carry a *chi* site and the host strain is *recA⁺*. Simultaneous inactivation of both the *gam* and *redBA* genes is usually induced by deletions in the region. The number of rescued phages can be determined using isogenic *E. coli* without prophage P2. The Spi⁻ mutation frequency is calculated by dividing the number of Spi⁻ phages by the number of total rescued phages (Nohmi *et al*., 1999; Nohmi, Suzuki and Masumura, 2000).
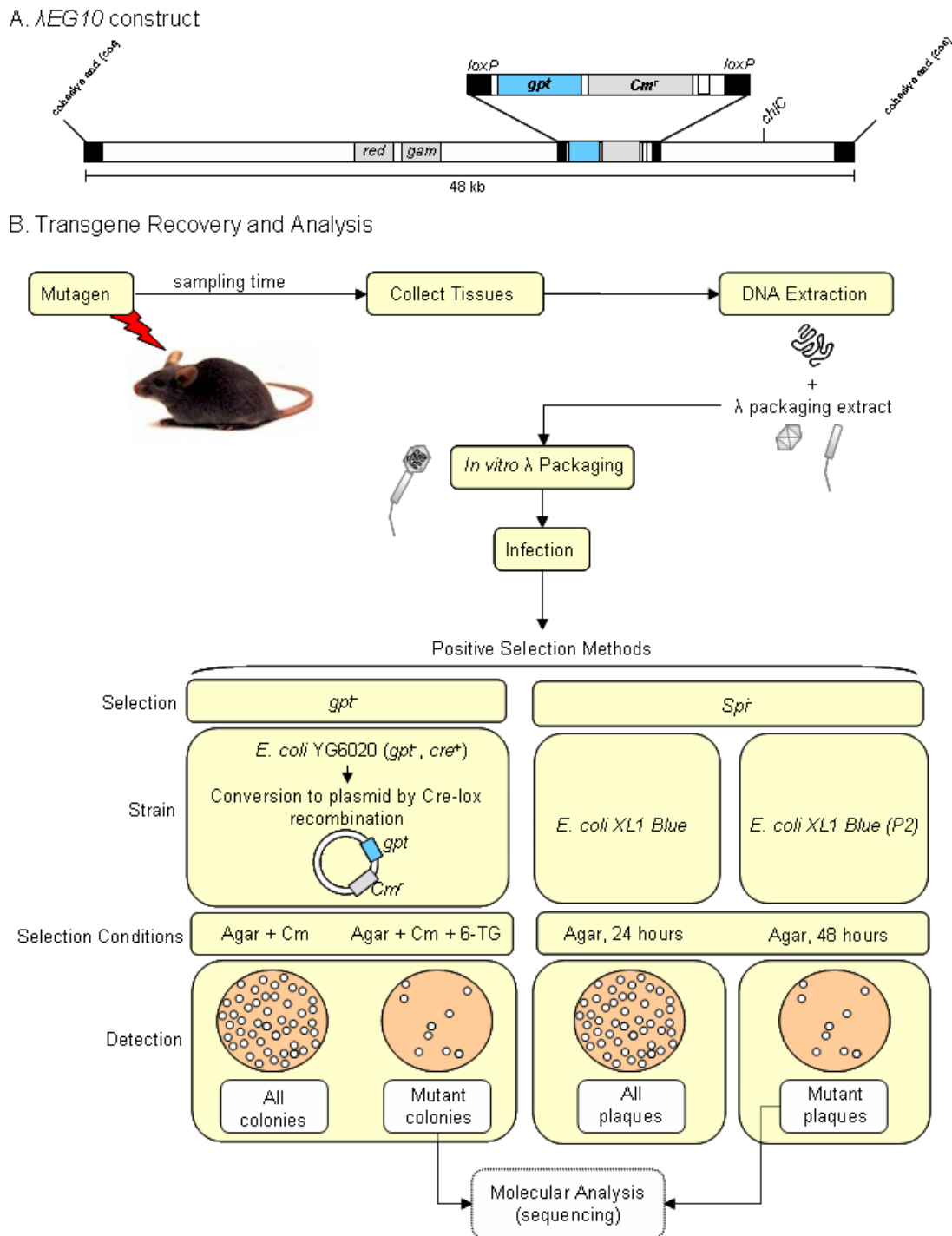
**Figure 2-4.** *gpt* delta mouse transgenic mutation assay:
(A) the λEG10 construct; (B) transgene recovery and analysis

### 2.2.5   Use of the λ cII transgene

The *cII* gene (294 bp) encodes a repressor protein that controls the lysogenic/lytic cycle of λ phage. In *hfl⁻ E. coli*, phages with an active *cII* gene cannot enter a lytic cycle; therefore, only phages with a mutated *cII* gene form plaques. The *cII* selection can be used in systems based on either λgt10*lacZ* (*i.e.* Muta™Mouse) (Figure 2-1B) or λLIZα (*i.e.* Big Blue® mouse or rat) (Figure 2-2B). However, *cII* selection cannot be used in conjunction with the *gpt* delta system, because the λEG10 vector used in the *gpt* delta system has a mutation of *chiC* (Nohmi, Suzuki and Masumura, 2000). Positive selection for *cII* mutants is carried out by adsorbing packaged phages to *E. coli* G1225 (*hfl⁻*), plating the bacteria and monitoring plaque formation after an incubation of 48 hours at 25 °C. Selection is carried out at lower temperature because the phages carry a *cI857* temperature-sensitive mutation, which makes the cI (ts) protein labile at temperatures above 32 °C. Total phage titre is determined by incubating at 37 °C for 1 day (Jakubczak *et al.*, 1996; Nohmi, Suzuki and Masumura, 2000). In principle, the availability of the *cII* gene as a quantifiable mutation target provides the Muta™Mouse and Big Blue® systems with two independent reporter systems. This allows for confirmation of results, evaluation of suspected false positives or negatives and the recognition of jackpot mutations and clonal expansion.

### 2.2.6   Other transgenic systems

Several other transgenic models, including those based on *supF* (Leach *et al.*, 1996a, 1996b), *lacI* (BC-1) (Andrew *et al.*, 1996), *rpsL* (Gondo *et al.*, 1996) and the bacteriophage ΦX174 (Malling and Delongchamp, 2001; Valentine *et al.*, 2004), have been created. However, these models have not been tested sufficiently to allow an evaluation of their performance.

## 2.3   The transgenic rodent mutation experiment

The basic TGR experiment (Figure 2-5) involves treatment of the rodent with a substance over a given period of time via any of several modes of administration that are acceptable in standard toxicological testing. Agents may be administered continuously (*e.g.* through the diet or drinking water) or in discrete doses via injection or gavage; the total period during which an animal is dosed is referred to as the *administration time*. Administration is frequently followed by a period of time, prior to sacrifice, during which the agent is not administered. In the literature, this period has been variously referred to as the sampling time, manifestation time, fixation time or expression time; in this document, this period is referred to as the *sampling time*. After the animal is sacrificed, genomic DNA is isolated from the tissue(s) of interest and purified.

An essential feature of the TGR assay is that mutation detection is achieved *in vitro* following the rescue of reporter gene vectors from the genomic DNA by *in vitro* packaging of the λ shuttle vectors (Gossen *et al.*, 1989; Kohler *et al.*, 1990; Vijg and Douglas, 1996) or excision/religation of integrated plasmids (Gossen and Vijg, 1993; Vijg and Douglas, 1996). There is no minimum acceptable number of plaque-forming units or colony-forming units from an individual packaging reaction: all data are usually aggregated, and mutant frequency is generally evaluated in a sample that contains between $10^5$ and $10^7$ plaque-forming or colony-forming units, as determined by plating on non-selective titre plates. As described above, positive selection methods have been developed to facilitate the detection of mutations in either the *gpt* gene (*gpt* delta mouse, *gpt⁻* phenotype) (Nohmi *et al.*, 1996; Nohmi, Suzuki and Masumura, 2000) or the *lacZ* gene (Muta™Mouse or *lacZ* plasmid mouse) (Gossen and Vijg, 1993; Vijg and Douglas, 1996), whereas *lacI* gene mutations in Big Blue® mouse or rat are routinely detected through colour selection methods. Methodology is also in place to detect point mutations arising in the *cII* gene of the λ phage shuttle vector (Big Blue® mouse
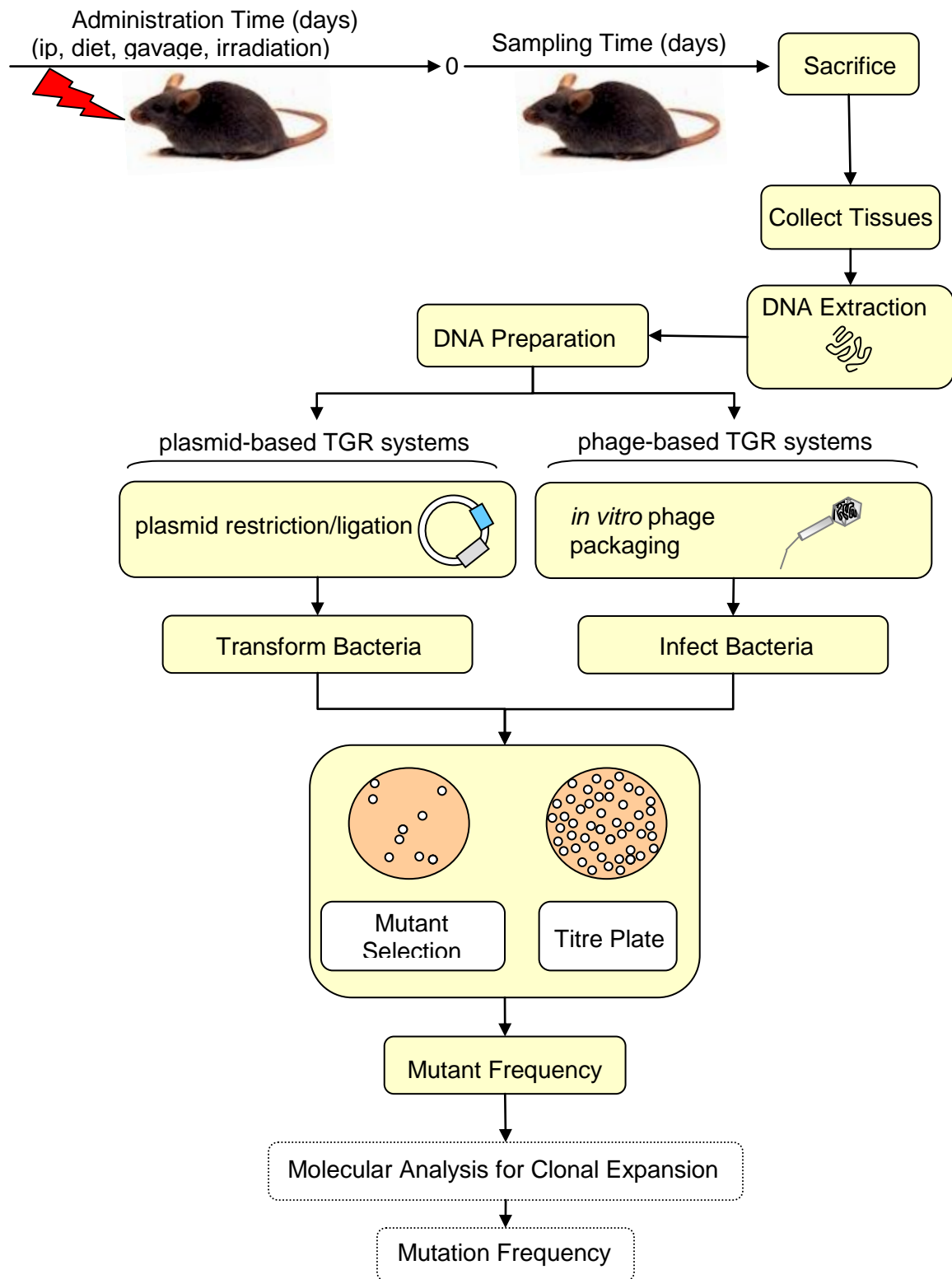
**Figure 2-5.** Transgenic rodent experiment

or rat, Muta™Mouse) (Jakubczak *et al*., 1996; Nohmi, Suzuki and Masumura, 2000) (Figures 2-1B and 2-2B) or deletion mutations in the λ *red* and *gam* genes (*gpt* delta mouse, Spi¯) (Nohmi *et al*., 1999; Nohmi, Suzuki and Masumura, 2000) (Figure 2-4B).

*Mutant frequency* is calculated by dividing the number of plaques/plasmids containing mutations in the transgene by the total number of plaques/plasmids recovered from the same DNA sample. The mutant frequency is generally reported in most TGR mutation studies. On the other hand, *mutation frequency* is determined as the fraction of cells carrying *independent* mutations and requires correction for clonal expansion. When applicable, this is achieved by DNA sequence analysis and subsequent correction of the mutant frequency to account for the presence of jackpot mutations that may arise through the clonal expansion of single mutants during development or cell growth. This molecular analysis is discussed in more detail below.

### 2.3.1   *Molecular analysis of mutations*

One of the advantages of the TGR assays is that the mutant transgenes can be very easily recovered subsequent to selection and sequenced so that the mutation spectrum can be determined. Many of the transgenes used in the current models (*e.g. lacI* [1 080 bp], *gpt* [456 bp] and *cII* [294 bp]) are sufficiently short that they can be easily and rapidly sequenced using existing technology. Sequence analysis of *lacZ* (3 021 bp) mutants is also feasible but is not carried out very frequently owing to the increased cost/complexity associated with sequence analysis of the larger gene target. In general, sequencing of *lacZ* is preceded by genetic complementation analysis that localises the mutation to one of three complementation regions (α, β or ω) in the *lacZ* gene (Douglas *et al*., 1994; Vijg and Douglas, 1996).

When testing drugs or chemicals for regulatory applications, molecular analysis of mutants would not normally be considered necessary, since such analysis would increase the cost and complexity considerably. However, there are situations in which DNA sequence analysis can provide valuable supplementary information. First, DNA sequencing may be useful for providing mechanistic information about the biological mechanisms underlying mutation induction by specific mutagens. The spectrum of mutations is indicative of the mechanism through which an agent induces mutation – that is, the identity of DNA adducts and nature of the DNA repair and replication enzymes that interact with the DNA lesion. In general, such knowledge is obtained by comparing DNA sequence alterations in the transgenes of treated and negative control animals. Proper analysis of mutation spectra may require the sequencing of a large number of mutants, depending upon the molecular mechanism of the mutagen. Second, sequencing data may be useful when high interindividual variation is observed. In these cases, sequencing can be used to assess whether jackpots or clonal expansion events have occurred by identifying the proportion of unique mutants from a particular tissue. Clonal expansion is assumed to have occurred when multiple mutations at the same site in the transgene are recovered from the same tissue of the same animal. Such mutations will most likely have been derived from transgenes in daughter cells of a single mutant cell. Correction of data to account for clonal expansion observed using DNA sequence analysis may result in greater precision in the calculation of mutation frequency (Nishino *et al*., 1996b; Hill *et al*., 2004). Third, knowledge of mutation spectra allows for the comparison of mutagenesis at different loci – for instance, in a transgene as compared with an endogenous locus.

DNA sequence analysis has demonstrated that the overwhelming majority of mutations arising spontaneously or following treatment with mutagens in the Muta™Mouse (*lacZ*), Big Blue® (*lacI*) and *gpt* delta (*gpt*) models are point mutations involving base substitutions or small frameshifts. Some types of mutations may not be readily detectable with the Muta™Mouse (*lacZ*) or the Big Blue® mouse (*lacI*) systems. There has been concern

that large deletions would not be detectable because they will not be recoverable. This would be the case for deletions that extend into the λ vector and inactivate essential phage sequences. In addition, there are size limitations for *in vitro* packaging: λ vectors must have cos sites (segments of single-stranded DNA 12 nucleotide bases long that exist at both ends of the bacteriophage lambda's double-stranded genome) separated by 38–51 kb. Thus, insertions and deletions that produce λ vectors outside this size range will not be recovered using conventional packaging. For instance, Tao, Urlando and Heddle (1993a) found that *lacI* was substantially less mutable than *Dlb-1* by X-rays. As mutant frequencies induced by other mutagens in the *lacI* transgene are not generally lower than in *Dlb-1*, and as X-rays produce a relatively high proportion of deletions as compared with point mutations, the result is consistent with the notion that bacteriophage transgenes will be less sensitive to clastogens (Tao, Urlando and Heddle, 1993a). In principle, some large deletions can be recovered; for example, deletions with one endpoint located in a copy of the reporter transgene (*e.g. lacI* or *lacZ*) and the other endpoint located in a different copy of the transgene should be recoverable and would appear to be much smaller than is actually the case. Whether this actually occurs has not yet been determined.

The *gpt* delta (Spi) and *lacZ* plasmid mouse system are particularly useful for the detection and characterisation of deletion mutations. As described in Section 2.2.4, Spi⁻ selection in the *gpt* delta rodents (Figure 2-4B) is based on the simultaneous inactivation of both the *gam* and *redAB* genes, a molecular event that generally arises when deletions extend into (or through) both genes. Detection of these mutants is limited by packaging constraints; thus, detectable deletions must be less than 10 kb (the largest detected to date is approximately 9.2 kb). Mutants detected by Spi⁻ selection can be easily characterised by DNA sequence analysis (Nohmi and Masumura, 2005).

Deletions in the *lacZ* plasmid mouse may arise internally within the plasmid cluster or may extend into the mouse sequences flanking the transgene cluster. In either instance, the proportion of deletions can be readily detected using Southern hybridisation techniques to characterise mutants following recovery in the *E. coli* host (Figure 2-3B). Size change mutants containing internal deletions will yield smaller fragments when hybridised to plasmid pUR288 sequences. Potentially large genomic rearrangements, including deletions, can be detected by Southern blot hybridisation using non-transgenic genomic mouse DNA as a probe.

### 2.3.2 *Clonal correction*

DNA sequence analysis can facilitate a more accurate assessment of mutation frequencies and/or reduce experimental variance in the event of clonal expansion. Clonal expansion may be of particular concern in rapidly dividing tissues, in tissues stimulated with a mitogen or in situations where the administration and sampling times of the experiment are extremely long. True jackpots or clonal events are, in fact, not very common in the literature. However, there have been a small number of cases in which DNA sequencing has altered the result (*i.e.* changed a positive to a negative) or has increased the statistical significance of a positive result (Shane *et al.*, 1999, 2000c; Singh *et al.*, 2001; Culp *et al.*, 2002). In each case, the administration time was extremely long (*i.e.* between 112 and 180 days). There is some danger in overcorrecting data using DNA sequence analysis. Induced mutations often occur at mutational hotspots; the elimination of all but one mutation at a particular site may theoretically result in the elimination of identical mutations derived from independent mutational events. This correction would reduce the magnitude of the induced response.

**2.4** **Perceived advantages and disadvantages of transgenic rodent mutation assays**

The design of TGR mutation systems over the past 15 years has incorporated certain features deemed desirable for *in vivo* genotoxicity testing. Use of these assays over this period has provided support for a consensus among users regarding the advantages and disadvantages of these assays. These are described below and examined in more detail in Chapter 4 of this document.

### *2.4.1 Advantages*
*2.4.1.1 Numerous tissues for analysis*

The major advantage of TGR mutation systems is that mutation can, in principle, be examined in any rodent tissue from which high molecular weight genomic DNA can be isolated. Mutations are scored in the bacterium subsequent to recovery of the transgene from rodent genomic DNA through either phage packaging or restriction/plasmid re-ligation.

This feature of the TGR assays is attributable to the neutrality of the transgenes (and therefore the neutrality of mutations in the transgenes) in the transgenic rodent. Evidence for the neutrality of mutations has been presented for the *lacI* gene of Big Blue® (Tao, Urlando and Heddle, 1993a), the *lacZ* gene of Muta™Mouse (Cosentino and Heddle, 1996), the *cII* gene of Big Blue® (Swiger *et al*., 1999) and the *gpt* gene of the *gpt* delta mouse (Swiger *et al*., 2001) and has been reviewed recently (Heddle, Martus and Douglas, 2003). Importantly, the genes appear to be transcriptionally inactive, as indicated by heavy methylation of cyto-sine–phosphate–guanine (CpG) sites and an absence of messenger ribonucleic acid (mRNA). A more direct proof of the neutrality of transgene mutations is that in proliferating tissues, mutation frequencies in the transgene tend to reach a maximum several days after an acute mutagenic treatment, and this maximum persists for weeks or months. If the mutation were not neutral, one would expect to see selection at some stage during cellular proliferation.

One potential exception to the observation of genetic neutrality should be noted: in the *lacZ* plasmid mouse, it is possible that some deletion mutations that extend into flanking endogenous genes and can be detected and scored would not be genetically neutral.

*2.4.1.2 Ease of molecular analysis*

The advantages of molecular analysis were discussed in Section 2.3.1. To summarise: 1) DNA sequencing may be useful for providing mechanistic information about the biolog-ical mechanisms underlying mutation induction by specific mutagens; 2) DNA sequencing data may be useful to assess whether jackpots or clonal expansion events are the cause of unusually high interindividual variation; and 3) knowledge of mutation spectra allows for the comparison of mutagenesis at different loci.

*2.4.1.3 Gene mutation assay*

The mutations induced by most mutagens are primarily point mutations that would be detected in the transgenes of transgenic rats and mice. However, the *in vivo* somatic cell genotoxicity tests used most heavily in a regulatory context detect clastogenic/aneugenic events (micronucleus, Section 3.3.1; chromosomal aberrations, Section 3.3.2) or are indicator tests (sister chromatid exchange, Section 3.5.1; unscheduled DNA synthesis, Section 3.5.2). Assays specific for the detection of gene mutations are limited to the mouse spot test (Section 3.4.1) and a series of single locus assays that are limited to specific tissues or developmental stages (Sections 3.4.3.1–3.4.3.3). Thus, in principle, a well-validated TGR test capable of detecting gene mutations in any tissue (Section 2.4.1.1) of animals at any developmental stage (Section 2.4.1.5) would fill a significant existing gap at the level of *in vivo* genotoxicity testing.

*2.4.1.4 Flexible mode of administration*

In TGR assays, the test agent can be administered through any route. This provides the flexibility for a researcher to tailor an experiment to the most appropriate mode of administration; there is no limitation based on a need to distribute the test agent to the target tissue examined using the existing test. For example, unscheduled DNA synthesis in liver and cytogenetics assays in bone marrow or peripheral blood may be unsuitable for evaluation of some substances to which humans are exposed primarily through topical or inhalation exposure. In contrast, TGR assays facilitate testing of "site of contact" mutagens at the site of exposure (Dean *et al*., 1999) and allow for consideration of parameters such as absorption, distribution and tissue-specific metabolism when selecting tissues for analysis.

*2.4.1.5 Relatively few limitations*

Genotoxicity tests often impose testing limitations based on the cell or tissue type or the stage of development. For example, the mouse spot test detects mutations in a subset of cells (the melanoblasts), the *Dlb-1* assay detects mutations in the stem cells of colonic crypts, the *in vivo* micronucleus test is generally carried out using bone marrow or peripheral blood erythrocytes, unscheduled DNA synthesis is typically evaluated in hepatocytes and the rodent dominant lethal assay evaluates male germ cell mutations that result in embryo lethality. In contrast, TGR assays allow the evaluation of mutations that arise in virtually any tissue in animals at all stages of development.

*2.4.1.6 Level of technical ease and reproducibility of results*

TGR assays are composed of an animal handling and treatment component, a DNA isolation component, a vector recovery component and a mutant detection component. There are well-described protocols for each of these steps that allow tests to be carried out by trained technical staff in research or contract laboratories. Although none of the steps require extensive training or expertise, use of the Muta™Mouse or the Big Blue® rodent systems in several laboratories has demonstrated the importance of the quality of the genomic DNA isolation in obtaining high packaging efficiency; optimal results are obtained by individuals with some experience in these manipulations. To date, use of the *gpt* delta rodents and the *lacZ* plasmid mice has generally been limited to a small number of laboratories associated with technical development of the system. The ease of use of these systems by other laboratories cannot yet be evaluated. In some instances, a mutant molecular characterisation component may also be incorporated into the study. This can be carried out in a reasonably well equipped molecular genetics laboratory or can be performed by a DNA sequencing service.

The reproducibility of results using TGR systems within laboratories, and using interlaboratory comparisons, has been reported to be high (Collaborative Study Group for the Transgenic Mouse Mutation Assay, 1996; Willems and van Benthem, 2000). The ease with which molecular characterisation can be achieved helps to identify sources of significant interindividual variation that may arise within an experiment.

*2.4.1.7 Economy of animals*

New toxicological methods must be assessed for the extent to which they address the 3 Rs that are now fundamental principles of toxicity test development: *reduction* in the number of animals used; *refinement* in the procedures to minimise animal suffering or distress; and *replacement* of animal use with alternative tests. TGR mutation models are particularly relevant to *reduction* of animal use, in that they do not use a large number of animals relative to the existing gene mutation assay (*e.g.* the mouse spot test requires ~1 500 animals) or chronic rodent carcinogenicity assays. Moreover, TGR experiments can, in principle, be

combined with other *in vivo* genotoxicity test endpoints, such as the peripheral blood micronucleus assay (S. Itoh *et al*., 1999; T. Itoh *et al*., 2000; Nishikawa *et al*., 2000; Noda *et al*., 2002), the bone marrow micronucleus assay (Manjanatha *et al*., 2004) or even conventional systemic toxicity assays, to further reduce the use of rodents. In addition, TGR assays may, in principle, be used to assess both germ cell and somatic cell mutations, although the administration time and sampling time experimental parameters (see Sections 6.1.2.5 and 6.1.3.1) would have to be very carefully designed in order to allow both types of assays to be carried out adequately.

With regard to *replacement* alternatives, there are a number of *in vitro* mammalian cell lines derived from transgenic rats and mice that are being developed as potential surrogates for the *in vivo* gene mutation model. The *in vitro* models are described in Section 2.4.1.8.

*2.4.1.8 Corresponding transgenic* in vitro *tests*

Although the purpose of this Detailed Review Paper is to review *in vivo* transgenic assays for the detection of gene mutations, it should be noted that there are a number of cell lines that have been derived from these *in vivo* TGR models; these cell lines may be used for *in vitro* mutation assays (Wyborski *et al*., 1995; Suri *et al*., 1996; Erexson, Cunningham and Tindall, 1998; Saranko and Recio, 1998; Erexson, Watson and Tindall, 1999; Ryu *et al*., 1999b; Gonda *et al*., 2001; McDiarmid *et al*., 2001, 2002; Ryu, Kim and Chai, 2002; White *et al*., 2003). The *in vitro* assays use the same mutation detection systems as their corresponding *in vivo* TGR model and therefore may have certain hypothetical advantages over other *in vitro* gene mutation assays (Section 3.2.2), since fewer assumptions will be required in predicting the outcome of, or comparing results with, *in vivo* transgenic mutation tests. Furthermore, some of the cell lines are epithelial in origin. Since most solid tumours arise from such cells (Lieberman and Lebovitz, 1996), another assumption required in extrapolating from *in vitro* to *in vivo* conditions when using fibroblast-based cell lines is reduced. In addition, *in vitro* transgenic models provide a logical platform on which to base follow-up or mechanistic studies relating to *in vivo* mutagenicity results.

Although extensive validation studies against other *in vitro* gene mutation tests have not been conducted, a selection of standard mutagens has been studied with an observed high concordance of results (White *et al*., 2003). As the emphasis continues to shift towards the replacement of *in vivo* animal models, *in vitro* transgenic mutation assays may, in many contexts, provide viable surrogates for *in vivo* gene mutation studies in the corresponding transgenic model.

### 2.4.2 Disadvantages
*2.4.2.1 Limited sensitivity to clastogens*

As described in Section 2.3.1 above, certain deletion or insertion mutations may not be detected in phage-based TGR systems because of packaging constraints. Thus, agents whose genotoxicity arises primarily through clastogenic events may be less likely to be detected in TGR systems as compared with existing clastogenicity assays, such as the *in vivo* bone marrow micronucleus test. The *lacZ* plasmid system, however, is capable of detecting a variety of clastogenic events, including deletions that extend into native chromosomal sequences.

*2.4.2.2 High spontaneous mutant frequency*

The spontaneous mutant frequency in TGR targets appears to be somewhat higher than in endogenous targets and in many tissues appears to increase with the age of the animal (Hill *et al*., 2004). In principle, for a given mutant frequency following treatment, higher

spontaneous mutant frequencies will yield correspondingly smaller fold increases (mutant frequency in treated animals / spontaneous mutant frequency) that may be attributed to the effect of the treatment. Therefore, there has been some concern that the relatively high spontaneous mutant frequency in TGR targets will decrease the sensitivity of the TGR assay relative to assays using endogenous gene targets. However, it has been clearly demonstrated in optimised TGR assay protocols that the sensitivity of transgenes can be enhanced by increasing the administration time. Since mutations in the neutral transgene accumulate linearly with the number of daily administrations given to an animal, longer administration times induce more mutations with time and increase both the induced mutant frequency and the fold increase in treated animals. The sensitivity of TGR assays is discussed in Section 4.8.4.

*2.4.2.3 Cost*

Per unit costs for transgenic animals and experimental consumables are higher than for many other genotoxicity assays. Proper conduct of the assays requires well-trained technical staff. The experimental protocols are not yet amenable to a high degree of automation.