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TRANSGENIC ANIMAL MODELS FOR DETECTION OF IN VIVO MUTATIONS

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ABSTRACT

Transgenic rodent models for measuring mutations provide a tool for assessing tissue-specific mutations following in vivo treatment. These systems are based on the insertion into the rodent genome *Escherichia coli lacI* (lac repressor) or *lacZ* (β-galactosidase) genes that serve as targets for mutations. Following in vivo treatment of animals, genomic DNA is isolated from tissues of interest, and the target gene is screened for mutations using either λ-phage packaging or isolation of the target gene with magnetic affinity capture. In this paper we review the various experimental methods used in the conduct of transgenic mutation assays and discuss critical factors that affect the interpretations of results of these assays.

INTRODUCTION

The ability to measure chemically induced mutations is critical to understanding the production of complex diseases such as birth defects and cancer. Indeed, cancer has been shown to result from a series of mutations in specific oncogenes and tumor suppressor genes (1). Although many in vitro assays have been developed to detect mutations induced by chemicals, these assays have significant limitations. In addition, few in vivo mutation assays are currently available, and none permits measurement of mutations in a wide variety of tissues.

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A revolutionary development in the biological sciences has been the rapid development and deployment of transgenic animal models to study cancer and other diseases (2, 3). A transgenic animal is one in which the genome has been altered in a heritable manner. This alteration is accomplished by introducing foreign DNA sequences into the genome of zygotes or embryos. There are three principal methods for producing transgenic animals: microinjection of DNA into a pronucleus of a zygote, introduction of genetically altered embryonic stem cells into developing embryos, and infection of embryos with retroviruses (4–8). All of the mutation models discussed in this review were produced by microinjecting embryos with recombinant DNA vectors containing a target gene. This method has been most successfully applied to mice because of the relative ease of injecting DNA into the mouse pronucleus; recent studies have demonstrated its feasibility in rats (9).

Transgenic animal models for measuring mutations have provided a major advance in our ability to rapidly assess tissue-specific mutations following chemical treatment. These models are based on the insertion into the genome of specific target genes that can easily be recovered from target tissues and analyzed for mutations. Although several experimental transgenic models for the quantitation of mutation have been reported, the three commercially available systems use a lambda (λ) shuttle vector (10) that carries a *lacI* or *lacZ* target gene. Muta™Mouse, marketed by Hazelton Laboratories (Kensington, MD), contains a *lacZ* transgene in a CD2F1 (BALB/c × DBA/2) mouse (11–14). The Big Blue™ system, marketed by Stratagene (La Jolla, CA), contains a *lacI* target gene and a *lacZ* reporter gene and is available in B6C3F1 and C57Bl/6 mice (15–20), and in F-344 rats (9). The Xenomouse, marketed by Xenometrix (Boulder, CO), contains a *lacZ* target gene in a C57Bl/6 mouse.

In these systems, animals are treated with chemicals, and after a sufficient time to allow fixation of DNA adducts as mutations, genomic DNA is isolated; finally, the target gene is recovered either by exposing the DNA to λ-phage in vitro packaging extracts or by capturing the gene directly using magnetic affinity capture.

A shuttle vector system that uses the bacteriophage ΦX174 inserted as a transgene into a C57Bl/6 mouse has also been developed; this system appears to have a lower background mutant frequency than any of the three commercial systems (21). The lower background frequency may result from this system's being a reverse mutation assay rather than a forward mutation assay. Little additional information is available on this system, and it is not commercially available; it is not discussed further in this review.

Here we describe the three commercially available transgenic mutation systems and the methods used for conducting mutation assays. We focus on different methodologic approaches, how these affect results, and opportunities for improvements in the various assays.

TARGET GENES FOR MUTAGENESIS

All three commercially available transgenic mutation systems use genes that are part of the *lac* operon of *Escherichia coli*, a set of coordinately regulated genes involved in lactose metabolism (22). The *lacI* gene codes for a homotetrameric protein that binds to the *lacO* operator sequence. Binding of the repressor to *lacO* prevents binding of RNA polymerase to *lacP* and transcription of the structural genes *lacZ* (β-galactosidase), *lacY* (permease), and *lacA* (transacetylase). In the absence of an inducer, the *lac* repressor is bound to the operator, and transcription of the *lacZ* gene is blocked, thereby inhibiting β-galactosidase activity. Removal of the repressor allows the polymerase to bind to the promotor, which results in expression of the β-galactosidase gene. β-Galactosidase activity can be measured in *E. coli* by plating on media containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Presence of β-galactosidase activity results in a blue colony or plaque, whereas absence of activity results in a white colony or clear plaque. In the following sections the *lacI* and *lacZ* target genes are discussed in the context of the three different transgenic mutation systems.

The Muta™Mouse system measures mutations within the *lacZ* target gene. Muta™Mouse contains the λgt10LacZ shuttle vector (Figure 1*a*), which is approximately 47 kilobases (kb) long. Both ends of this vector are flanked by *cos* sites, which allow excision and packaging of phage heads. The phage are then used to infect *E. coli* to produce plaques. Screening of plaques is done either colorimetrically (wild type = blue plaque, mutant = clear plaque) or selectively (wild type = no plaques, mutant = plaques).

Xenomouse contains pUR288 (Figure 1*b*), a 5.5-kb plasmid vector flanked by *Hin*dIII restriction sites. The plasmid contains the *lacZ* target gene, the *lacO* sequence (to allow affinity capture with the lacI repressor protein), and the *colE1* and amp^R genes to allow propagation in *E. coli*. In this system, genomic DNA is digested with *Hin*dIII to release the monomeric plasmid sequences. The plasmid is then purified away from genomic DNA by affinity capture and circularized by ligation, then used to electroporate $lacZ^-$, $galE^-$ *E. coli* in the presence of phenyl-β-D-galactoside (P-gal). Mutants are then quantitated by selective growth.

The λLIZ shuttle vector (Figure 1*c*) is used in the Big Blue™ mouse and rat systems. The vector is flanked at each end by *cos* sites, which allow excision and packaging into phage heads. In turn, pLIZ can be excised from the λ-vector as a plasmid. pLIZ contains the *lacI* target gene and the α*lacZ* reporter gene. The α*lacZ* gene codes for the amino portion of β-galactosidase, which complements the *lacZ* gene provided by the *E. coli* host to produce full β-galactosidase activity. In addition the *colE1* and the amp^R genes allow replication and selection of the plasmid in *E. coli*.

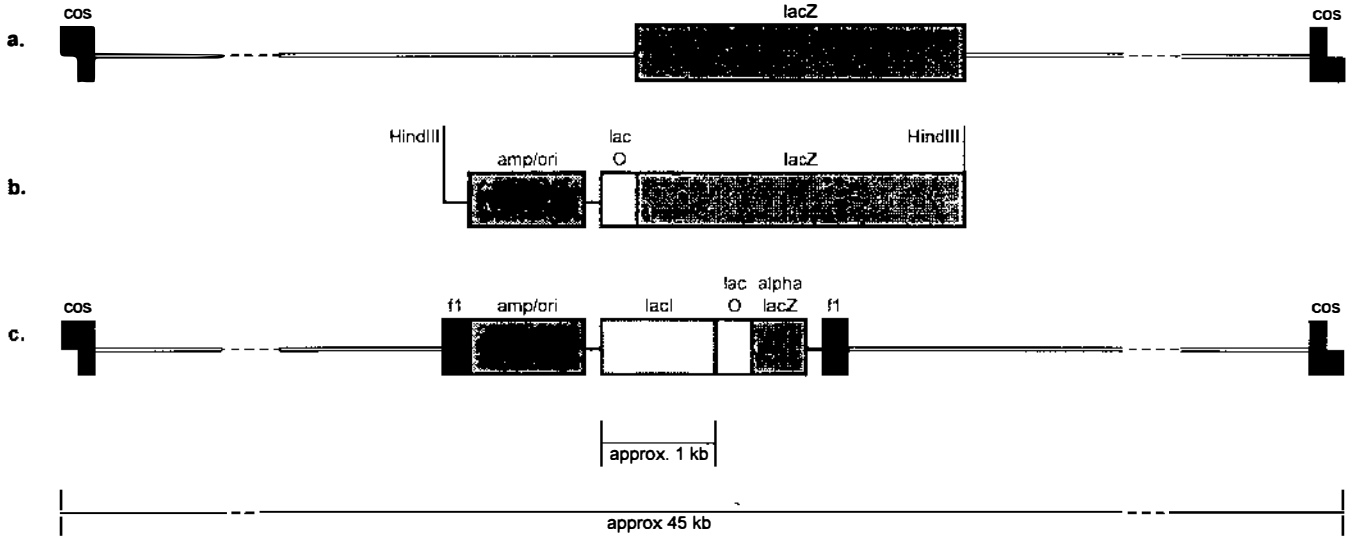

Figure 1 DNA constructs of the three commercially available transgenic mouse models. Diagrams show the elements of a single construct. Constructs are actually present as multiple tandem repeats of 20 or more copies. Double lines represent λ-phage sequences; thick single lines represent plasmid sequences; dashed lines represent discontinuities in the scale. Figures are highly schematic and are only approximately to scale. (*a*) Muta™Mouse contains the 47-kb λgt10LacZ shuttle vector that has the entire *lacZ* target gene and is flanked by *cos* sites, which allow excision and packaging of phage heads. (*b*) Xenomouse contains pUR288, a 5.5-kb plasmid vector flanked by *Hin*dIII restriction sites. The plasmid contains the *lacZ* target gene, the *lacO* sequence to allow affinity capture with the lacI repressor protein, and the *colE1* and *amp*^{R} genes to allow propagation in *E. coli*. (*c*) The lambda/*lacI* shuttle vector of the Big Blue™ mouse. The *cos* sites are separated by ≈ 45 kb. The expanded region represents the portion of the vector that is excised as a phagemid with M13 helper phage. The entire phagemid region is flanked by partial f1 filamentous helper phage origins. The α*lacZ* is flanking the *lacI* target gene). The *colE1* origin is present for plasmid replication, and the ampicillin resistance gene is present for the selection of colonies carrying the plasmid to be used for sequencing the *lacI* target.

Mutations in the *lacI* gene are detected using *lacZ* as a reporter gene. When a functional copy of *lacI* is present, the Lac repressor binds to the *lacO* operator sequence, thereby preventing expression of the α*lacZ* gene. Infection of *E. coli* containing *lacZ* with λLIZ containing wild-type *lacI* results in the production of clear plaques. If a mutation inactivates *lacI*, the repressor will fail to bind to *lacO*, and α*lacZ* will be expressed, the result being a blue plaque after infection of *E. coli*.

The *lacI* gene is the most characterized of all target genes for mutagenesis. To date, more than 30,000 mutants from both prokaryotic and eukaryotic hosts have been sequenced (17, 18, 23–33).

METHODOLOGY

Because transgenic mutation assays have only recently become commercially available, many of the procedures associated with this assay have not been fully optimized. The following sections describe the methods used for each of the major steps of the assay, discuss recent or promising improvements, and present areas where further research is required.

Dosing Regimens and Study Protocols

A number of parameters can affect induction of mutations by chemicals in animals. As in all in vivo studies, toxicity, genotoxicity, or both will be a function of uptake, distribution, detoxification, metabolic activation, DNA repair, DNA replication, and types of DNA adducts formed. These factors in turn may be modified by strain/sex differences, diet, age of animals at time of exposure, dose, duration of exposure, and expression time. It is therefore critical to employ testing protocols that consider all of these variables.

DOSING REGIMEN There has been considerable debate about the appropriate dosing regimen for transgenic mutation models. In vivo genetic toxicology studies have traditionally used short-term dosing regimens. In particular, the in vivo–in vitro unscheduled DNA synthesis assay (34) and the bone marrow chromosome aberration assay (35) have traditionally been conducted by evaluating tissues at several time points after a single dose. An exception is the micronucleus assay, which is optimally conducted using repeat-dose protocols that allow sampling of micronuclei at steady state (36). On the basis of this history, some laboratories have argued that transgenic mutation assays should likewise employ single-dose administration (37). Our laboratory has generally used protocols with administration of several doses, usually over a 1-week period (28, 38). Some laboratories have employed subchronic (28, 38–40) or even chronic (2, 41) dosing regimens.

If human risk assessment, or comparison to rodent bioassay results, is a

desired goal, it makes little sense to administer a single (large) dose of a test chemical to animals. Many chemicals, in addition to producing DNA adducts, may induce cell proliferation. Administering repeated doses of a test chemical therefore mimics more accurately the human situation in that chemicals are being administered during periods in which cell proliferation induced by earlier exposures may be occurring. Indeed, the potent genotoxic agent dimethylnitrosamine (DMN) produces only a modest (2-fold) increase in mutant frequency when administered as a single, large dose (42) but very significant increases when given at lower doses over a period of 5 days or more (28).

Cell proliferation is clearly important for the induction of mutations. In the absence of cell division, chemically induced DNA adducts may be repaired before fixation of mutations. This is most apparent for DMN, where induction of cell proliferation is a prerequisite for DMN-induced mutations. Doses of DMN (2 mg/kg/day) that failed to induce significant hepatotoxicity or cell turnover did not produce mutations; however, increasing the dose to 4 mg/kg/day crossed the toxicity threshold: A significant increase in hepatotoxicity occurs at this dose, and significant elevations in cell replication result (43). At this dose, DMN also produces a significant increase in the mutant frequency (28). Therefore, at doses in which cell proliferation is induced, DMN produces mutations. In the absence of cell proliferation, both DMN and methylmethane sulfonate (MMS) fail to induce hepatic mutations.

Chemically induced mutagenesis should ideally be studied under conditions of subchronic or chronic administration (40), but most laboratories do not have the resources to test many chemicals in 13-, 52-, or 104-week studies. Therefore, a short-term multiple-dose protocol (e.g. a minimum of 3 days up to 2 or 3 weeks) that allows chemical administration in the presence of chemically induced cell turnover seems a reasonable compromise.

SELECTION OF DOSE If one accepts the argument that multiple-dose protocols are preferred for evaluation of mutations, selection of dose levels becomes very important. Our laboratory selects doses for transgenic mutation assays in the same way one would select doses for any multiple-dose toxicity test, such as a 13-week subchronic toxicity study (38). If subchronic toxicity data are unavailable, we conduct a range-finding assay (using nontransgenic mice of the same strain to minimize costs) in which we dose the animals for the number of days they are to be dosed in the definitive study, then observe them for the number of days to be allowed for fixation of mutations (e.g. for a 5-dose/14-day expression time protocol, we would dose for 5 days, then count survivors 14 days after the final dose). The top dose selected is that expected to produce minimal or zero lethality (i.e. about 50% of the lowest dose at which deaths occurred). If subchronic toxicity data are available, we use the highest dose at which animals survived for 13 weeks.

EFFECTS OF FIXATION TIME Fixation time in mutation experiments, also frequently referred to as "expression time," is the period after administration of a single dose, or after the last of multiple doses, and before the animal is sacrificed and tissues are evaluated for mutations. Some expression period is usually required, especially after single-dose administrations, to permit (*a*) uptake and distribution of the chemical, (*b*) metabolic activation to a DNA-reactive form, (*c*) formation of DNA adducts, and (*d*) at least one cell division to "fix" the adduct as a heritable mutation.

Because uptake, distribution, and activation of chemicals are highly dependent on the chemical, the tissue, and the species and sex of the animal, and because differences in cell turnover rates vary widely among tissues, determination of appropriate fixation times is critical to optimizing the detection of mutations in vivo.

In tissues in which mutations occur in stem cells or in slowly dividing cell types, fixation times will generally increase with time or at least will not decline significantly after reaching a plateau. Indeed, this increase has been demonstrated in liver, where mice initiated with diethylnitrosamine (DEN) maintain a very high mutant frequency for up to a year after administration (2). In contrast, when treatments do not produce mutations in stem cells or when mutations are measured in rapidly cycling cells, the mutant frequency (MF) will reach a peak response and then decline with time as cells with mutations go through apoptosis or otherwise disappear from the tissue. For example, treatment of *lacZ* mice with ethylnitrosourea (ENU) produces a gradual increase in MF in the liver, but the MF in the testis and bone marrow peaks after 7 days and then begins to decline (44). A somewhat contradictory study (45) demonstrated that ENU-induced mutations reach a plateau in bone marrow but do not decline with time and that MF continues to rise in liver and male germ cells. A study in skin of both *lacI* and *lacZ* mice demonstrated that a fixation time of at least 7 days was required for detection of DMBA-induced mutations in the skin (46).

Because spermatogonia require a long period for transition to mature spermatozoa, a longer fixation time is clearly called for when evaluating male germ cells. Short fixation times are not adequate to allow the seminiferous tubules to be populated with mutations derived from the stem cells (47).

One concern when mutations are evaluated using a system that requires identification of mutations in bacteria is that DNA adducts formed in vivo may be introduced into *E. coli* as adducted DNA and subsequently converted to a true mutation in vitro. This effect does occur, but it has been shown to be largely dependent on fixation time after treatment, because it relates to in vivo removal (via repair) of mutagenic adducts (48). Such ex vivo mutations can also be identified as "sectored" plaques (discussed below). If the fixation time is long enough to allow repair of DNA adducts, then the incidence of *E.*

coli–derived ex vivo mutations is reduced considerably. Thus, the greatest contribution by host bacteria to the mutant spectrum and frequency will occur in the shortest fixation time. Increased mutant frequency is often observed for longer fixation times (28); this observation suggests that the majority of mutations measured are derived in vivo, and that the contribution by mutations fixed in vitro is relatively small. Nevertheless, care should be taken when following protocols that use very short fixation times (i.e. less than a few days).

OTHER CONSIDERATIONS IN STUDY DESIGN Animal age and strain can have a profound effect on results in all in vivo studies—an effect evident for transgenic mutagenesis models. Studies in our laboratory demonstrated that DMN at 2 mg/kg/day was a potent inducer of hepatic mutations in 3-week-old mice, but no increase was observed in 6-week-old animals at this dose; however, doses of 4 or 6 mg/kg/day yielded significant increases in hepatic mutations in 6-week-old animals (28). Three-week-old mice have poorly developed detoxification mechanisms (49) and elevated basal proliferation rates; this condition could explain the positive response seen at 2 mg/kg/day in these younger animals. Indeed, the background cell proliferation rate in *lacI* mouse tissues declines up to an order of magnitude between weeks 3 and 10 (50).

Selection of a strain of animal is likewise critical when results of mutagenicity testing are to be compared with other toxicology data. The *lacI* systems, which are available in the widely used B6C3F1 mouse and F-344 rat, offer a clear advantage over the *lacZ* system, which is available only in CD2F1 or C57Bl/6 mice. For some chemicals (e.g. DMN, ENU), there appears to be little strain difference in responses (28); however, for 1,3-butadiene (BD), significant strain differences have been reported. BD induced mutations in lungs of CD2F1 *lacZ* mice but not in bone marrow; however, the rate of bioactivation of BD to the monoepoxide and subsequent hydrolysis by epoxide hydrolases in the CD2F1 mouse was 40% lower than the level observed in B6C3F1 mice, and the corresponding level of hemoglobin adducts was lower than expected (51). A similar study conducted in B6C3F1 *lacI* mice demonstrated increases in MF in lung, spleen, and bone marrow (52) that were more consistent with the reported sites of tumor formation.

Preparation of DNA

The method for DNA isolation is in part determined by the type of system used. For λ-phage–based systems, it is critical that the DNA be of high molecular weight (average size 300 kb or greater) to ensure that there is no breakage between *cos* sites (53). For tissue isolation, timing is critical; the faster the samples can be removed from animals and quick frozen in liquid nitrogen, the better are the chances that endogenous nucleases have not digested the genomic DNA. Timing is especially critical in nuclease-rich organs

such as the liver. Traditional protocols for DNA isolation, such as proteinase K/SDS digestion followed by phenol/chloroform extraction and ethanol precipitation may be used. However, samples must be handled gently, especially during the extractions and resuspension of DNA pellets. A recently developed procedure based on large-pore dialysis of digested tissue samples has greatly reduced the number of steps and amount of time required to prepare DNA samples (54). This procedure yields high-concentration, high-molecular-weight DNA without the need for phenol:chloroform extraction or ethanol precipitation.

Plasmid-based systems have the advantage that one need not obtain high-molecular-weight DNA. Since the plasmid is only 5.5 kb long, it is unlikely that any one copy will be sheared by standard isolation methods. Therefore, it is not necessary to take special efforts to prevent shearing of the DNA sample.

Gene Rescue Techniques

LAMBDA PHAGE SYSTEMS Both *lacI* and *lacZ* systems use the λ-phage viral system to provide a vehicle for transfer of the shuttle vectors between eukaryotic and prokaryotic cells. The λ-phage protein extracts recognize and excise the integrated λ-based shuttle vectors at flanking *cos* sequences. These sequences are subsequently encapsidated within λ-phage coat proteins to produce infectious viral particles. Special packaging extracts derived from restriction-negative and β-galactosidase-negative bacteria are used to promote efficient packaging and to limit background β-galactosidase activity. Once packaging is complete, the viral particles are preadsorbed to bacteria, plated on agar, and incubated overnight in the presence of X-gal. Incubation of the viral bacterial mixture produces a lawn of bacteria spotted with viral plaques.

The shuttle-vector packaging reaction involves a simple protocol. Several micrograms of genomic DNA are mixed with Transpack™ or Gigapack™ packaging extracts and incubated for several hours at 30°C. After the reaction, samples are diluted into a phage-stabilizing solution, and aliquots are removed for incubation with bacteria. A fresh stock of host bacteria is grown while the packaging reaction is proceeding, and the stock is adjusted to the proper concentration before mixing with the packaged vector. The concentrations of bacteria and packaging extract vary depending on the type of plating that is to be done. For selective systems, the packaging reactions and bacteria are kept concentrated so that the mixture can be plated at high densities. Dilutions are prepared for plates in which individual plaques need to be screened and counted. During the plating process, the viral/bacterial mix is added to molten agarose and poured over a nutrient-rich agar layer that has been prepoured onto individual plates. Plates are typically incubated overnight and screened the following day.

Successful infection by the phage particles containing the shuttle vector requires the use of special *E. coli* strains, such as SCS-8 (for *lacI* systems) and *E. coli C* (for *lacZ* systems). The methylation pattern of mouse-derived DNA differs from that of bacteria; thus the bacterial host strain must be tolerant of foreign methylation schemes. The SCS-8 strain of *E. coli* is restriction negative in McrA, Mrr, and McrF, which target methylcytosine sequences present in mammalian DNA. To reduce ex vivo, non-mouse-derived mutation, the principal recombination pathway is disabled by a *recA1* mutation. In addition, the correlation of mutation to β-galactosidase activity is ensured by α complementation in which sequences both from the transgene and from the bacterial host are required for activity (55–58).

PLASMID-BASED SYSTEMS An alternative methodology for in vivo mutation analysis has also been demonstrated (59). This system, employed in the Xenomouse system, takes advantage of plasmid vector sequences located internally in the transgenic λ shuttle vectors and flanking *lac* operator and *lacZ* sequences. The plasmid vector sequence is excised by using DNA restriction enzymes instead of depending on λ-phage protein extracts to recognize and excise the integrated vectors. This linear plasmid is then isolated from genomic DNA by magnetic affinity capture. The LacI repressor protein, which strongly and specifically binds to the *lac* operator sequence in the plasmid, is conjugated via antibodies to magnetic beads. The protein/bead conjugate is mixed with the bulk genomic DNA and selectively binds the plasmid DNA, permitting nonspecific genomic DNA to be washed away. The isolated 5.5-kb fragment, which contains the complete pUR288 plasmid sequence, is circularized under dilute ligation conditions, concentrated, and then electroporated into *E. coli* C (*lacZ*) bacteria. Only bacteria transformed by the plasmid are allowed to grow. Use of the affinity capture system and electroporation completely obviates a viral transfer system. The MF is calculated as the number of mutant (clear) colonies divided by the total colony count. This method is potentially 10 to 20 times more efficient than viral transfer systems at rescuing *lac* genes from transgenic mice.

Scoring and Screening Mutants

Determining mutant frequency involves estimating the total number of plaques or colonies plated and identifying all mutant plaques or colonies. The method chosen for screening mutants (i.e. by color or by selection) greatly affects the ease of screening and may subtly alter the spectrum of mutations recovered.

COLOR SCREENING The most common screening procedure involves plating plaques in the presence of X-gal. In the *lacI* system, mutants are blue on a

background of clear wild-type plaques. In *lacZ* systems, mutants are clear on a background of blue wild-type plaques.

Plating conditions It is imperative that laboratories establish standardized procedures for plating and screening, because several factors affect perceived MF. Proper plating density is critical to optimal mutant detection. For the *lacI* system, it is recommended that no more than 15,000 plaques per 625-cm^2 dish be plated. For *lacZ* systems, it is necessary to plate at even lower densities because it is inherently more difficult to detect clear colonies on a blue background. Other factors that can affect sensitivity are X-gal concentration, dryness of plates, and thickness of bottom agar. It is highly recommended that color control mutants be plated routinely to assess sensitivity of the assay. Stratagene has developed a series of *lacI* mutants that have phenotypes ranging from very light blue to dark blue (60).

Mosaic plaque assay An important issue is whether screened mutants are derived from in vivo or ex vivo (i.e. *E. coli*) mutations. Unrepaired DNA adducts present in isolated genomic DNA could result in the fixation of mutations in *E. coli*. Replication-derived mutations in *E. coli* result in mosaic plaques. A mosaic plaque consists of a mixture of wild-type and mutant phage that results in the appearance of a sectored plaque. In this case, one strand of the DNA from the phage contains a DNA adduct and the other does not. Replication-induced mutations at the adducted site would result in two types of DNA: wild-type and mutant. Half of the phage would be mutants if the error occurred during the first round of replication after phage infection. Errors occurring at later rounds of replication would result in progressively smaller fractions of mutant phage. By coring and replating a mosaic plaque, it is possible to determine the fraction of mutant phage.

Whether a plaque is sectored may be difficult to determine using standard plating densities. In the mosaic plaque assay (61), plaques are plated at a very low density: ~2000 plaque-forming units (pfu) per 625-cm^2 dish. Well-isolated primary plaques are then replated to determine the frequency of mosaic plaques. Secondary plaques with a MF greater than 60% are considered in vivo derived, whereas those with MF values below 60% are likely to be ex vivo derived. It is recommended that obvious sectored plaques be tallied separately from nonsectored plaques when one is screening for mutant plaques. When an agent produces a marginally significant positive response, it may be desirable to perform the mosaic plaque assay.

Selectable systems The second method for quantitating *lacI* and *lacZ* mutations makes use of selectable systems that permit high plating densities and minimize the need for multiple platings (61–64). The Muta™Mouse *lacZ*

system uses a positive selection scheme in which phage that contain rescued vectors are used to infect *lac*$^-$,*galE*$^-$ *E. coli*, which are then plated in the presence or absence of phenylgalactoside (P-gal). In the presence of P-gal only *lacZ*$^-$ bacteria will grow and produce plaques. The titer is determined by plating in media without P-gal, which allows both wild-type and mutant *lacZ* *E. coli* to grow. The MF is calculated as the number of mutant plaques divided by the titer.

Selectable systems have also been developed for detecting *lacI* mutants. One example is the minimal-medium lactose system. By altering the host *E. coli* strain, it is possible to obtain lysogenic growth, which results in bacterial colonies rather than plaques. By infecting this host with phage and plating on plates containing lactose as the sole carbon source, it is possible to select for mutants. β-Galactosidase activity resulting from inactivated *lacI* allows utilization of lactose as a carbon source. Therefore, only *lacI* mutants will grow.

To date almost all work has been performed using color selection rather than selectable systems. Therefore, selectable systems will need validation prior to their use for routine testing. One issue is that selectable systems may detect a spectrum of mutations different from that detected by color selection. Another issue is the controversial idea that directed mutagenesis may occur under selective conditions (65), which might confound interpretation of results.

Analysis of Mutations

SIGNIFICANCE OF MUTATIONAL SPECTRA A significant advantage of transgenic rodent mutagenesis assays is that they provide a relatively simple means to analyze mutations at the molecular level. Analysis of mutations can provide mechanistic information about mutagens. The presence of clonally expanded "jackpot" mutations can be confirmed by sequencing several mutants (28). Additionally, in cases where an agent causes only a marginal increase in mutant frequency, a unique spectrum could determine whether an agent is positive or negative.

METHODS OF ANALYSIS Several approaches to the analysis of mutations are possible. In the Big Blue™ system, the pLIZ plasmid can be retrieved using in vivo excision (17). The *lacI* gene can then be sequenced using a series of primers that span the region. Although the *lacZ* gene is three times as long as the *lacI* gene, complementing *E. coli* strains can be used to determine whether a mutation is in the α, β, or ω regions of *lacZ* (JA Gossen, unpublished). The polymerase chain reaction (PCR) (66, 67) can be used with any of the systems to sequence directly from a plaque or colony. Sequencing from PCR products is faster, requires less starting DNA, and is less likely to give sequencing artifacts arising from secondary structures in the template. It is also possible

to detect gross structural alterations by restriction digest analysis of recovered plasmid DNA.

SPONTANEOUS SPECTRA As discussed above, a factor that can confound interpretation of data from transgenic rodent systems is the possibility that a mutation has arisen in *E. coli* rather than in the animal. Comparisons of spontaneous *lacI* mutations in the mouse (18, 48) and in *E. coli* (23) show striking differences. In the mouse system, 75% of C → T transitions occur at CpG dinucleotides. This percentage suggests that they arise from spontaneous deamination of 5-methylcytosine (26, 27). On the other hand no C → T transitions were observed in *E. coli*. In *E. coli*, 67% of mutations are deletions or insertions of a tandem repeat of TGGC; in contrast, only 1.7% of *lacI* mouse mutants involved this sequence.

Sequencing of 89 spontaneous *lacI* mutants shows that approximately 30% of somatic cell and 50% of germ cell mutations are C → T transitions at CpG sites. Overall, 85% are base substitutions and 15% are insertions, deletions, and single-base frameshifts (68).

CHEMICALLY INDUCED MUTATIONS Several mutagenic compounds that are well understood mechanistically have been tested in the *lacI* mouse. Methylnitrosourea (MNU) (61), benzo(a)pyrene (17, 18), DMN (28), and ENU (17) have all shown spectra consistent with known mechanisms of action.

DELETION MUTATIONS One issue yet to be resolved is the ability of the various systems to detect large-scale deletions characteristically induced by agents such as ionizing radiation. Ideally, any system used for genetic toxicology screening would detect both point mutations and large-scale deletions. Lambda-shuttle-vector systems will have an inherent bias against certain deletions because of packaging requirements. High doses of gamma rays were found to induce approximately 20% deletions detectable by restriction digest analysis. These deletions ranged in size from 45 to 249 bp (25) and are much smaller than those found when endogenous genes such as *hprt* or *aprt* are used. The *lacI* locus has been found to be less sensitive than the endogenous *Dlb* locus to X-ray-induced mutations, whereas both loci gave a similar response to the point mutagen ENU (69). The difference in mutagenic response of the two loci may be due to a decreased ability of the *lacI* system to detect large deletions. A variant of the *lacI* system is being developed that is likely to be more sensitive to large deletions. This so-called "polycos" system consists of concatemers of a 3.4-kb monomeric vector that contains the *lacI* gene and is flanked by *cos* sites. Twelve to fifteen copies of the monomer are packaged simultaneously and used to infect *E. coli*. The monomeric plasmid can then be rescued by in vivo excision. Large deletions within or between monomeric

units that still allow packaging of the concatemer should be detectable with this system.

Unpublished data suggest that the plasmid-based *lacZ* system can detect very large deletions. Detection of large deletions is possible because the system does not depend on packaging. Instead, genomic DNA is restricted with an enzyme that normally cuts out the plasmid. The plasmid is then circularized by DNA ligase. Intra-plasmid deletions are detectable as long as the amp^R and origin of replication are present. Intergenic deletions extending into endogenous genomic DNA sequences are also detectable. In the latter case one restriction site is present in the plasmid DNA and the other site in genomic DNA.

Evaluation of Data

No consensus exists about which statistical tests should be used in evaluating data from transgenic mutation assays, and reports of what constitutes a "positive" response often conflict. For example, acetic acid has been reported to produce mutations in skin, with a "significant" 1.7-fold increase in mutant frequency (70); however, the 2.5-fold increase in mutations produced by heptachlor in mouse liver was not considered significant (71).

Several recent publications have addressed the sources of variability in transgenic mutagenesis assays and recommended minimal study designs and appropriate statistical analysis tools (39, 72, 73). One laboratory estimates that at least 5 animals with 200,000 plaques per animal must be analyzed to accurately detect a 2-fold increase in MF, and 10 animals must be evaluated to detect a 50% increase (74); few studies to date have achieved this goal.

SPECIAL APPLICATIONS

Mutagenesis by Nongenotoxic Carcinogens

Our laboratory has been interested in the induction of mutations by nonmutagenic chemicals. While "nonmutagenic mutagenesis" may seem oxymoronic, if one accepts the multistep model of carcinogenesis (1), nongenotoxic carcinogens must increase the number of mutant cells by means other than direct interaction with DNA. Transgenic mutation assays are a unique tool for studying the effects of nongenotoxic chemicals on mutation induction in normal and tumor tissues.

Experiments in our laboratory demonstrated that a burst of cell proliferation induced by nongenotoxic chemicals, in the absence of genetic damage, is unable to induce mutations in the liver. Indeed, carbon tetrachloride and phenobarbital, when administered at doses that produce enormous increases in cell turnover, do not produce an increase in hepatic mutations (50, 75).

Similar results have been reported by other laboratories. The peroxisome proliferator and hepatocarcinogen methylclofenapate failed to induce mutations in *lacI* mice with a single-dose protocol (42). Administration of di(2-ethyl-hexyl)phthalate, heptachlor, or phenobarbital for up to 120 days failed to produce an increase in mutations in *lacI* mice (71, 76).

Relatively short-term bursts of cell proliferation alone do not produce mutations in the liver; however, long-term administration of nongenotoxic cell proliferators could presumably result in clonal expansion of spontaneous mutations, or selective expansion of populations with mutations in specific oncogenes. This expansion may be the principal mechanism of carcinogenesis by nongenotoxic chemicals.

Effects of nongenotoxic chemicals in tissues other than the liver may be more complicated. Recent reports indicate that irritation and ulceration produced in the skin by the "nongenotoxic" chemical acetic acid lead to modest increases in the mutant frequency of *lacZ* mice (70). Similarly, a petroleum distillate that lacked activity in standard genetic toxicity tests produced slight increases in the MF in *lacZ* mice (77). These increases were small ($5.9–6.9 \times 10^{-5}$ compared to $2.5–3.0 \times 10^{-5}$ in acetone controls) and occurred only at doses where ulceration or enhanced cell turnover was evident.

These transgenic systems will allow us to answer fundamental questions about the mechanisms of mutation, but they have also raised questions about the definition of the terms "genotoxic" and "nongenotoxic" (37, 38). MMS induces significant DNA adducts and unscheduled DNA synthesis (UDS) in mouse liver and would therefore clearly be classified as "genotoxic" in the liver (78). Nevertheless, MMS fails to induce *lacI* mutations in B6C3F1 mouse liver, even when administered for up to 21 days (28), and would therefore be classified as "nonmutagenic" in mouse liver. The value of the transgenic mouse assay is its ability to evaluate *relevant* genotoxicity in multiple target tissues (i.e. heritable mutations). In fact, although MMS is "genotoxic" in mouse liver, it does not produce liver tumors. Therefore, DNA adduct or UDS assays may be better predictors of "genotoxicity," but a transgenic mouse model may be a better predictor of mutations and cancer.

Perhaps the best approach to studying mutagenesis by nongenotoxic chemicals is to evaluate mutations in actual tumors. Our laboratory has evaluated the MF in chemically induced liver tumors in *lacI* B6C3F1 mice (2). The *lacI* gene confers no selective growth advantage upon a mammalian cell; therefore, it is useful as a physiologically neutral marker of mutation rates in tumors. We initiated mice with diethylnitrosamine (DEN), then promoted with WY-14,643 (WY) or phenobarbital (PB). Mice receiving DEN+PB or DEN+WY had multiple liver tumors, whereas mice receiving untreated feed after DEN initiation had few tumors. Nontumor tissue from mice treated with DEN yielded approximately 10-fold elevations in the MF, whereas most tumors had

much higher MF than would be predicted from clonal expansion of DEN-induced mutants. These data suggest that nongenotoxic carcinogens such as PB and WY may have little effect on normal hepatocytes but may accelerate mutation rates in initiated cells, thus leading to a rapid progression to tumors.

Animals with Multiple Transgenes

A recent development in the transgenic animal arena has been the mating of different transgenic animal strains. The use of transgenic mutation models provides the means to measure mutations in models carrying other cancer, disease, or regulatory genes. Although this is a very recent development in this field, a few cases of multiple transgene results have been reported. In one study, a mouse containing a gene for over-expression of O^6-alkylguanine-DNA alkyltransferase was crossed with a *lacI* transgene carrier (79). The resulting animals removed O^6-methylguanine adducts more efficiently; using the *lacI* marker gene, the authors demonstrated that fewer mutations were induced by MNU in these animals.

Another promising animal model is a cross between a *lacI* transgenic mouse and a mouse missing one copy of the *p53* tumor suppressor gene (Genpharm International, Mountain View, CA). These mice should be useful for studying the role of *p53* in preventing cell cycling when DNA damage is present, as well as for allowing rapid induction of tumors in mice for the subsequent analysis of *lacI* mutations.

It may prove useful to construct crosses for all major cancer genes, as well as for many human disease genes, so that the contribution of mutation induction in these disease processes can be elucidated.

CONCLUSIONS

Transgenic animal models provide the only system currently available for quantitating chemically induced mutations in a wide range of mammalian tissues in vivo. Combining mutation analysis with the analysis of cell proliferation, mutant sequencing, and other end points in the same animals [e.g. cytogenetic damage (80, 81)] provides a powerful tool for studying mutagenesis in vivo and for elucidating the mechanism of chemical carcinogenicity.

The optimal use of transgenic animals may be in routine toxicology studies such as 30-day, 90-day, or 2-year studies. The B6C3F1 mouse and F-344 rat strains are widely used in general toxicology studies. Since these strains are available as *lacI* transgenic animals, it should be possible to measure many phenomena—general toxicology parameters, clinical chemistry, histopathology, cell proliferation, micronucleus formation, and mutations—in the same animals. In this way, separate in vivo genetic toxicity studies may ultimately

be replaced by routine toxicology tests that include mutation analysis as a routine end point.

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