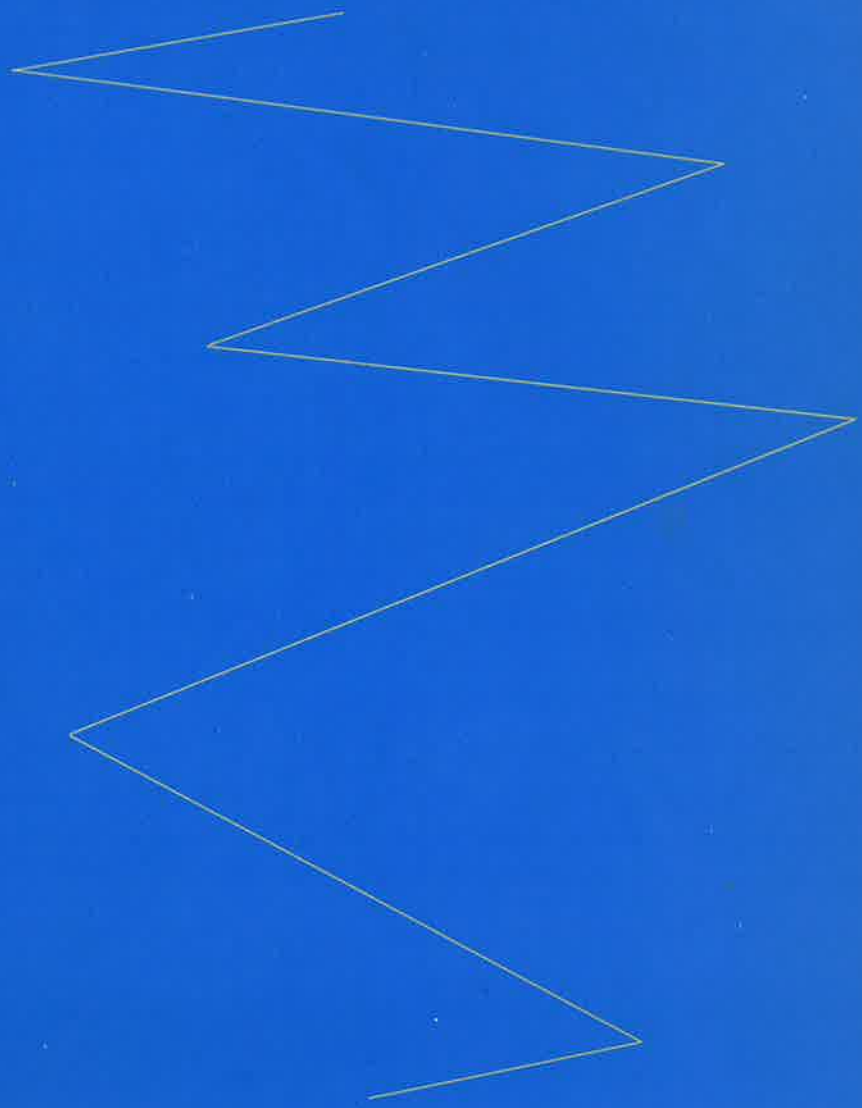
Development of Transgenic Mouse Models for Studying Gene Mutations in Vivo

Jan A. Gossen



I 2200

DEVELOPMENT OF TRANSGENIC MOUSE MODELS FOR STUDYING GENE MUTATIONS IN VIVO

This thesis will also be available as a publication of Ingeny b.v.

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Gossen, Jan Albert

Development of transgenic mouse models for studying gene mutations in vivo /
Jan Albert Gossen. - [S.l. : s.n.] (Thesis Publishers, Amsterdam). -Ill.
Thesis Leiden. -With ref. - With summary in Dutch.
ISBN 90-9006034-0
NUGI 743
Subject headings: DNA / toxicologie

DEVELOPMENT OF TRANSGENIC MOUSE MODELS FOR STUDYING GENE MUTATIONS IN VIVO

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het college van dekanen
te verdedigen op woensdag 7 april 1993
te klokke 14.15 uur

door

JAN ALBERT GOSSEN

geboren te Ruinerwold in 1961

THESIS PUBLISHERS
AMSTERDAM 1993

Promotiecommissie:

Promotor:	**Prof. Dr. Ir. P.H.M. Lohman**
Co-promotor:	**Dr. J. Vijg, Ingeny B.V., Leiden en Harvard Medical School, Boston, U.S.A.**
Referent:	**Dr. F. Berends, Medisch Biologisch Laboratorium TNO**
Overige leden:	**Prof. Dr. H. A. de Boer** **Prof. Dr. Ir. A.A. van Zeeland** **Prof. Dr. C.J.M. Melief**

The research presented in this thesis was conducted at the TNO Institute for Experimental Gerontology, Rijswijk and at Ingeny B.V., Leiden. Publication of this thesis was financially supported by Ingeny B.V..

Aan mijn ouders
Voor Ditta en Rens-Jan

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1 has been published in a condensed form as a review article in Trends in Genetics (1993), 9, 27-31.

1.1 Introduction

Gene mutations have been implicated in the etiology of cancer, developmental anomalies, genetic diseases and aging, and their formation is considered as the first step in the long chain of events leading to these physiological endpoints. Alterations in the genetic information encoded in the DNA may arise as a consequence of error-prone DNA replication and repair, or as the result of either endogenous DNA-damaging substances or exposure to exogenous factors, e.g chemical mutagens or ionizing/UV irradiation. In order to be able to directly correlate mutations with their ultimate physiological endpoints, knowledge on the nature and the relative frequency of mutations in somatic and germ cells is essential.

In the past years, many different techniques have been developed in order to obtain a more fundamental insight in the chain of events that ultimately lead to DNA mutations. Most of the methods developed to date, however, are limited to the *in vitro* situation and do not allow comparative analysis of mutations in various organs and tissues in an intact organism. The main difficulty in studying mutagenesis in genes of higher organisms is the lack of techniques to identify and isolate mutated genes with a high efficiency.

The mammalian gene that has been used most extensively as a target for both *in vivo* and *in vitro* mutagenesis studies, is the gene coding for hypoxanthine phosphoribosyl transferase (HPRT). The so called HPRT assay, however, can only be applied to detect mutations in a limited number of cell types and does, therefore, not allow comparative analysis of gene mutations in all organs and tissues.

Transgenic animal technology development in the early 1980's, to allow the introduction of foreign DNA into the germline of mammals by microinjection of fertilized oocytes, has found widespread application in molecular biology, for example, for studying different aspect of development, tumorigenesis, the immmune response, etc. In addition, this technique created new possibilities for developing systems to study

gene mutations *in vivo*.

In this thesis, the development and application of transgenic mouse models for studying spontaneous and/or induced mutations *in vivo* are described. As the mutational target gene the bacterial LacZ gene was chosen. In order to recover the LacZ gene with high efficiency from transgenic mouse DNA, the gene was cloned into a bacteriophage lambda shuttle vector. This construct was introduced into the germline of mice, and a number of transgenic mouse strains with different copy numbers of the shuttle vector were obtained. This vector can be rescued from genomic mouse DNA through packaging into empty phage particles; the resulting phages can be propagated on E.coli bacteria to yield "plaques". Since bacteria are used that do not produce the LacZ gene product, i.e. ß-galactosidase, mutations in the LacZ gene that destroy its expression or the activity of this enzyme can be scored: because the chromogenic substrate 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal) is added to the growth medium, phages containing a non-mutant LacZ gene give rise to a blue plaque and phages containing a mutated LacZ gene give rise to colourless plaque. Mutation frequencies in a specific organ or tissue are determined as the ratio of colourless versus blue plaques. A variety of mutational events can be detected by this system, including point mutations, frameshifts and small deletions and insertions. Deletions and insertions larger than about 7 kb go undetected since efficient rescue of bacteriophage lambda vectors is restricted to a minimum and maximum size of the vector. Two transgenic mouse strains, harbouring different copy numbers of the LacZ transgene at different chromosomal positions, were investigated for spontaneous and chemical-induced mutations in various organs and tissues. A number of spontaneous and chemical-induced LacZ mutants were analyzed by DNA sequencing. In addition, an alternative, plasmid-based, approach for rescuing integrated LacZ transgenes from transgenic mouse DNA was developed.

1.2 Methods for predicting the mutagenic/carcinogenic potential of chemicals

In view of the alleged causal relationship between the mutagenic action of chemicals and their carcinogenicity, *in vitro* test systems have been developed for the assessment of the mutagenic potential of compounds. The *in vitro* assay most extensively used for testing the mutagenicity of chemicals is the Ames' Salmonella test (Ames et al., 1973; Maron and Ames, 1983). This assay is based on the use of a set of histidine-requiring strains of the bacterium *Salmonella typhimurium*. It is called a reverse-mutation assay because the assay strains are mutated in one of the genes of the histidine biosynthetic pathway and, consequently, cannot synthesize histidine, an essential amino acid. An additional mutation is required to revert the cells to histidine independence. One of the limitations of using prokaryotic assays is that bacteria lack many of the metabolic enzyme pathways present in mammalian cells, some of which may actually convert compounds into mutagenic agents. Mammalian metabolism can be imitated *in vitro* by adding rodent liver homogenate (S9) to the assay. The enzymes in this homogenate may activate compounds that would otherwise be non-mutagenic.

It has now been shown that the Ames test has a sensitivity (percentage of established carcinogens according to long-term rodent carcinogenicity assays that were identified as mutagens) of about 54% and a specificity (percentage of non-carcinogens identified as non-mutagens) of 70% (Zeiger et al., 1986). Similar results were obtained by Tennant et al. (1987) who reported that the salmonella assay would only identify about 45% of the established carcinogens. Although the Ames test has been reevaluated using more stringent criteria, which resulted in an increased specificity, the total number of mutagenic carcinogens detected by the assay was also significantly reduced (Prival and Dunkel, 1989). In addition, inclusion of three other short-term *in vitro* assays (the mouse micro-nucleus assay,

chromosome aberrations and sister chromatid exchanges), did not significantly improve the performance of in vitro assays for predicting rodent carcinogenicity (Tennant et al, 1987; Haseman et al., 1988).

To test the potential carcinogenic effect of a chemical in vivo, the long-term rodent carcinogenicity assay now serves as legislated standard for assessing carcinogenic risk, and the specific locus test serves as the standard for the assessment of heritable damage. In the long-term carcinogenicity assay, rodents are treated acutely or subchronically with a chemical and the animals are monitored during their life-time for the occurrence of tumours. Large studies have shown that this assay has a low specificity but a high sensitivity; all definite human carcinogens tested were positive (Ennever et al., 1987). Long-term rodent carcinogenicity studies, however, are expensive, time-consuming (3 to 4 years), require high numbers of experimental animals and are often conducted at high, near toxic doses, which does not reflect the low dose ranges to which humans are exposed. Attempts to extend these investigations to lower dose ranges, however, are limited since these are even more time-consuming and expensive because of the still larger number of animals that would be required.

Recently, Ames and Gold suggested that treating rodents with near toxic doses of a test chemical for long periods of time may cause chronic mitogenesis (Ames and Gold, 1990a and 1990b), which may account for the high number of chemicals positive in the long-term rodent carcinogenicity assay (50% of all chemicals tested sofar; Abelson, 1990 and 1992). This view has been contested (Hay, 1991; Weinstein, 1991), but a definite conclusion cannot be reached since data on rodent carcinogenicity at low dose levels are scarce, and comparison with in vivo mutagenesis data is virtually impossible because of lack of data (except for the HPRT and HLA-A locus).

In order to study the mutagenic effects of potential mutagenic agents in germ cells, the mouse specific locus test was developed, by Russell (1951). This method is the most

efficient one to screen for transmitted germ-cell mutations, and it has provided important information on factors affecting the mutation rate in germ cells. The method involves mating homozygous wild type mice, treated or controls, to untreated tester stock mice, homozygously recessive at seven marker loci. Six of those loci control coat pigmentation colour or pattern and the seventh locus controls the size of the external ear. The offspring is subsequently analyzed for phenotypic changes due to a mutation in one, or more, of the seven marker loci. In the newer biochemical specific locus test, mutagenic effects are determined by assaying changes in the electrophoretic pattern in selected, accessible gene products. The advantage of this biochemical test is that mutagenic effects in 33 different loci are determined, increasing the potential sensitivity of the test as well as reducing the numbers of animals needed. The advantages of these assays are that methods to screen for mutations are simple and fast and, since the animals are alive, presumed mutations can be subjected to genetic analysis. The general disadvantages of these assays, however, are that they require thousands of animals and treatments are usually performed using high doses of a potential mutagen.

In summary, it has become clear that there is a serious need of other systems for the assessment of the mutagenicity of chemicals, which yield results that are more relevant for the mammalian (human) situation than the present assays, and/or are less demanding with respect to costs, time or the number of animals. Preferably, these systems should detect mutations in genes present in the mammalian genome and - if possible - should be applicable in <u>in vivo</u> experiments.

1.3 Mutation detection in endogenous mammalian genes

The direct analysis of somatic DNA mutations in different organs and tissues, requires the development of highly sensitive techniques since these mutations occur at very low frequency. Due to the lack of such systems, mutation detection in naturally present genes in mammalian cells has thusfar been mainly restricted to those genes which, when mutated, give rise to a selectable or recognizable phenotype of a cell or are involved in genetic disease. The types of mutations which are most amenable to molecular analysis are small mutations such as base substitutions (transitions and transversions) and deletions and insertions of up to 100 kb. During the past years different techniques have been developed to rapidly analyze such mutations, e.g, Pulsed Field Electrophoresis (large deletions/insertions), Southern blot analysis (small deletions/insertions), Denaturing Gradient Gel Electrophoresis (point mutations), DNA sequence analysis (point mutations), etc. These methods have been applied for the analysis of mutations in a number of mammalian genes. Those genes which have been studied most extensively are discussed below.

1.3.1 The HPRT gene

By far the most information about *in vivo* somatic mutations comes from studies of the HPRT locus (reviewed by Thacker, 1985; Stout and Caskey 1988). The HPRT gene is an X-linked gene that upon inactivation confers resistance to cytotoxic purine analogues, such as 6-thioguanine. Because of this X-linkage only one allele needs to be mutated to obtain this effect, both in males (one X chromosome) and in females (one active X chromosome). Because selection of the mutants demands culturing of the cells in selective media, studying mutations at the HPRT locus can be performed only on cells that are capable to proliferate, such as T- and B-lymphocytes isolated e.g. from blood of humans or rodents (Albertini et

al., 1982; Morley et al.,1983), cell lines of different sources, primary human fibroblasts and primary fibroblastoid and epithelioid cells from various organs and tissues of rodents (Hakoda et al., 1989).

The frequency of mutations at the HPRT locus has been studied predominantly in T-lymphocytes isolated from the blood of human individuals. For example, mutation frequencies have been analyzed in apparently healthy individuals (Trainor et al., 1984; Albertini et al., 1985; Henderson et al., 1986), in individuals with defects in DNA-damage processing (Papadopoulo et al., 1990), individuals exposed to ionizing radiation (Messing and Bradley, 1985; Hakoda et al., 1988) or cytotoxic drugs (Dempsey et al., 1984), or individuals exposed to environmental or industrial chemicals (Tompa and Sapi, 1989). In general, spontaneous mutation frequencies in apparently healthy individuals were found to range from about 0.6 x 10^{-6} in newborns to about 16 x 10^{-6} in aged individuals (Carrano, 1989). The mutation frequencies observed, however, were characterized by a large inter-individual variation which may in part be due to experimental factors (Featherstone et al., 1988) or due to donor variations such as age, sex and life style (Cole et al., 1988).

Several methods have been developed to investigate the nature of mutations at the HPRT locus. Due to the large size of the HPRT gene (the genomic sequence spans about 34 kb and 44 kb in mice and humans, respectively) point mutations are relatively difficult to analyze. Simpson et al. (1988) and Vrieling et al. (1988), however, developed a method based on the use of the polymerase chain reaction (PCR) to amplify the HPRT cDNA from HPRT mutant cell lines. PCR technology was invented by Mullis (Mullis and Falloona, 1987) and first described in 1985 by Saiki et al.; it is based on the use of two oligonucleotide primers flanking a region of interest. Through successive steps of DNA denaturation, primer annealing and extension using a thermo-stable DNA polymerase, a particular sequence can be amplified more than a million-fold. Upon cloning of the amplified mutant HPRT sequence into a

sequencing vector, point mutations in the HPRT-coding sequence can be analyzed by nucleotide sequencing. Obviously, this method can only be applied to analyze those mutant cells in which the HPRT gene, despite a mutation which inactivates the HPRT, still produces HPRT mRNA. Large scale alterations can be detected by means of Southern blot analysis using HPRT specific probes (Alvi and Williams, 1992; O'Neill et al., 1990). More recently, it has been shown that deletions of exons in the human and hamster HPRT gene can be determined using a so called multiplex PCR method, in which all exon sequences are simultaneously amplified using a set of HPRT-specific primers (Gibbs et al., 1990; Rossiter et al., 1991). In general, it has been found that in human T-lymphocytes about 15 to 57% of the spontaneous mutants analyzed contain large deletions (Bradley et al., 1987; Albertini et al., 1985; Turner et al., 1985). Interestingly, it has been shown that the spectrum of mutations in newborns appeared to be quite different. McGinnes et al. (1989) observed large deletions in 81% of the mutants. By using T-cell receptor rearrangements as a marker they showed that a considerable part of these mutations had been independently arisen in pre-thymic stem cells, which is in contrast with the mostly post-thymic T-lymphocyte mutants in adults. These results indicate that different mechanisms are responsible for the mutations observed at the HPRT locus in T-lymphocytes isolated from blood of individuals of different age. It has also been shown that the mutation frequency at this locus increases significantly with age. Studies in which the mutational spectra are compared between mutant T-lymphocytes isolated from young and old individuals may, therefore, provide greater insight in the mechanism(s) that underlie the age-dependent increase of mutations at this locus.

In addition to the analysis of spontaneous mutations at the HPRT locus, this system also has the potential to investigate induced mutations. Lymphocytes, for example, obtained from donors exposed *in vivo* to environmental carcinogens can be analyzed and compared to controls.

Disadvantages of the HPRT system for predicting the mutagenicity of compounds, however, is that the analysis is time-consuming and it does not allow comparative analysis of mutation frequencies in multiple organs and tissues.

1.3.2 The APRT locus

A system comparable with the HPRT system is the adenine phosphoribosyl transferase gene (APRT) in Chinese hamster cells. Because this gene is autosomal, in vivo studies can not be performed. Hemizygous cell lines, however, are available that allow selection of cells containing a mutant APRT gene in medium containing 8-azaadenine. The APRT gene is a relatively small gene of about 2.5 kb (Nalbantoglu et al., 1986). Although the small size facilitates the analysis of mutations at the DNA sequence level, it may affect the types and distribution of mutations recovered. Indeed, the predominant type of mutations observed at the APRT locus are basepair substitutions (Meuth, 1990). Large deletions at this locus do occur but at a low frequency and are characterized by their distinctive directionality (Nalbantoglu et al., 1987). All deletions analyzed thusfar, ranging in size up to 170 kb, have one breakpoint within the gene and a second breakpoint upstream. No deletions have been reported downstream of the APRT gene. These results suggest the presence of an essential gene or structure in the 3' region which would be lethal when deleted in the hemizygous strains used in all studies.

In contrast to the data obtained by Meuth (1990), deletion type mutations at the APRT locus were not found among 90 mutants analyzed by de Jong et al. (1988). Instead, a predominance for G:C -> A:T transitions was observed, which probably reflects cytosine deamination, a commonly encountered spontaneous DNA lesion (Ehrlich et al., 1981 and 1990). The differences observed in mutational spectra at the APRT locus between the two laboratories underline the difficulties in working with selectable loci. Similar observations have been

encountered in studies employing the selectable xanthine/guanine phosphoribosyl transferase (gpt) gene in CHO cell lines (Romak et al., 1989; Tindall and Stankowski, 1989), and are both likely to be due to differences in cell growth conditions.

In addition to its use in studying forward mutations, the APRT gene has also been applied in studying reverse mutations by introducing a defined point mutation into the wild-type sequence. An advantage of such an approach is that specific mutations, e.g. caused by chemicals, can be analyzed which circumvents the necessity of DNA sequencing. A system using transgenic human cell lines, each containing a different mutant mouse APRT gene, was described by Schaff et al. (1990). Treatment of the cells with mutagens known to induce point mutations, revealed the utility of such cell lines in studying site-specific reversion mutations. However, due to the fact that in this system only specific point mutations can be detected at a single site, the applicability for studying spontaneous or induced mutations is limited.

1.3.3 The glycophorin A locus

Glycophorin A (GPA) is a cell-surface protein of human erythrocytes which occurs in two forms, M and N; it is the product of codominantly expressed alleles on chromosome 4 (Langlois et al., 1985). In the GPA expression-loss assay, pairs of fluorescently tagged monoclonal antibodies specific for the M and N allelic forms of GPA are used to label erythrocytes from heterozygous MN donors. By using flow cytometry and sorting, cells that have lost or gained one of the forms can be detected. The method offers the advantage that it is fast and applicable to the *in vivo* situation. It has been successfully applied in studies on the long-term health effects of human exposure to ionizing radiation (Langlois et al., 1987). This method, however, suffers from the drawback that characterization of the mutants is not possible and mutant cells can therefore not be proven as being

due to gene mutation.

1.3.4 The HLA-A locus

The HLA genes encode multiallelic cell-surface antigens. Mutations at the HLA-A locus in human blood lymphocytes can be analyzed in individuals heterozygous for HLA-A2 or HLA-A3. Lymphocytes containing a mutation in one of these genes can be selected *in vitro* by immunoselection using monoclonal antibodies (Morley et al., 1990). Although this method is technically demanding, it permits quantitation and molecular analysis of both gene and chromosomal mutations (Turner et al., 1988; Morley et al., 1990). In general, spontaneous mutation frequencies in blood lymphocytes of human individuals ranged from 7×10^{-6} in newborns to 65.3×10^{-6} in elderly individuals (Grist et al., 1992). Analysis of HLA-A gene mutations indicated that the predominant types observed were point mutations (60-70%; Morley et al., 1990; Grist et al., 1992). An important finding obtained with the HLA-A assay was that about 1/3 of the mutants was due to mitotic recombination. The high frequency with which this type of mutation occurred at the HLA locus and the absence of such mutations at the HPRT locus (only one gene copy present), may explain the overall higher spontaneous mutation frequency at the HLA locus. Similar to the results obtained with the HPRT assay, the frequency of mutational events at the HLA-A locus such as point mutations and mitotic recombination events increased significantly with age (Grist et al., 1992).

1.3.5 Dlb-1 gene

Other elegant procedures to detect somatic mutations are based on in situ histochemistry. Winton et al. (1988) described the development of a mutation-induced marker in mice heterozygous at the Dlb-1 locus which determines the expression of binding sites for lectin in intestinal epithelium. A similar method was described by Griffiths et al.

(1988) based on X-linked histochemistry. These *in situ* methods for demonstrating somatic mutations are valuable since they allow to determine mutation frequency at a specific locus in different cell types (provided they express the gene).

1.3.6 Oncogenes and tumour-suppressor genes

In somatic cells dominant oncogenes are generated by the induction of point mutations in "normal" genes (proto-oncogenes), or by gene rearrangements. Examples of this are activation of the ras oncogene by point mutations (Fox et al., 1990), the chromosomal rearrangements involved in chronic myelogenous leukaemia (Rowley, 1973) and translocations involved in the generation of the bcl-abl oncogene (Croce, 1987). More recently, mutations which inactivate tumour-suppressor genes, like the p53 and retinoblastoma gene, have been analyzed (Levine, 1990; Hollstein et al., 1991). Mutational analysis of tumour-suppressor genes is of special interest since mutations in these genes have been found in a large variety of cancers. The mutational spectra observed in the p53 tumour-suppressor gene indicate that the majority of mutations occurred in evolutionary highly conserved regions and the type of mutation varied considerably among different cancer types. In colon tumours, for example, the predominant type of mutations are G:C->A:T transitions (68%), all occurring at CpG dinucleotides. These findings are consistent with the endogenous mutational mechanism due to deamination of 5-methylcytosine at CpG dinucleotides in the mammalian genome (Ehrlich and Wangh, 1981; Ehrlich et al., 1990). Analysis of mutant p53 genes isolated from lung, bladder and liver cancers, however, indicated that 5-methylcytosine deamination is not a major source of mutations in these cancers. Recent data on the p53 mutational spectra in human hepatocellular carcinomas from China (Hsu et al., 1991) and southern Africa (Bressac et al., 1991) indicated that all mutations were transversions occurring at a single site. Since these tumours were from individuals known to be exposed to aflatoxin B_1 and

hepatitis B, respectively, these results may indicate that carcinogens leave specific fingerprints. Upon the availability of a large data base of p53 mutations, analysis of these mutations may provide a tool for diagnosis or prognosis of various cancers in individuals exposed to (environmental) carcinogens.

1.3.7 Genes involved in heritable genetic disorders

Because of their etiological importance, many germline mutations are presently being analyzed in genes involved in heritable diseases. This refers to the low-density lipoprotein receptor (Hobbs et al., 1991), factor VIII (Youssoufian et al., 1988), factor IX (Koeberl et al., 1990), glucose-6-phosphate dehydrogenase (G-6-PD) (Vulliami et al, 1992), dystrophin (Chamberlain et al., 1988), glucocerebrosidase (Beutler, 1992) and cystic fibrosis transmembrane regulator (CFTR; Tsui, 1992). Large data bases on germline mutations in these genes are now becoming available and these indicate that each locus is characterized by its own mutational spectrum. Germline mutations in the factor VIII gene, for example, are despite its large size (about 186,000 bp) characterized by a high incidence (95%) of single basepair mutations. Mutations in the CFTR gene on the contrary are characterized by a high incidence of deletions, observed in 66% of the mutants. This variation may reflect individual characteristics of the genes, such as length, the presence of repetitive sequences, local configuration and/or transcriptional activity.

A major source of germline mutations are G:C->A:T transitions occurring within CpG dinucleotides. Cooper and Kraczak (1990) analyzed the mutational spectrum of single basepair substitutions among genes involved in human genetic disease and found that this type of mutation was present in 31.7% of the mutant genes. These results suggest that, like in cancers, 5-methylcytosine deamination is a major mechanism for spontaneous mutagenesis in germ cells.

1.4 PCR-based methods for the analysis of somatic mutations

One of the most promising new methods for analyzing mutations occurring at very low frequency in complex tissues, such as somatic mutations, is based on the use of PCR. A number of methods using PCR have been developed and are described below.

A procedure based on the use of denaturing gradient gel electrophoresis (DGGE) to separate mutant and non-mutant PCR-amplified target sequences was proposed by Vijg and Uitterlinden (1987; see also Lohman et al., 1987). Mutation detection in this system is based on the fact that the electrophoretic mobility of a double-stranded DNA molecule in a polyacrylamide gel at a particular concentration of denaturants is highly dependent on its sequence content. The sensitivity of this system is such that short double-stranded DNA molecules (up to 1 kb) that differ at only one position can be separated, since they will migrate to different positions in a denaturing polyacrylamide gel. Thilly et al. demonstrated that it is possible to separate in denaturing gradient gels PCR-amplified mutant HPRT sequences obtained from uncloned populations of human B-lymphocytes treated with high doses of different mutagens (Thilly et al., 1990; Cariello et al., 1990; Keohavong et al; 1991). Application of this method for scoring mutations occurring at low-frequency, however, has not been established yet. Despite the use of high-fidelity polymerases such as Vent, Sequenase or T7, and alterations in the experimental conditions (Keohavong and Thilly, 1989; Cariello et al., 1991 and 1991a), the PCR amplification step was found to introduce too many misincorporations, thereby overshadowing spontaneously arisen mutations. The sensitivity of these methods was found to be such that only mutants present at a frequency of 10^{-3} or greater could be detected (Cariello et al., 1991), which restricts the potential application of the method to the detection of hot-spots for mutation (Keohavong and Thilly, 1992). Still, DGGE can be used to analyze spontaneous mutants

provided a pre-selection is introduced, as was shown by Skopek et al. (1992). They first selected cells containing a mutated HPRT gene by culturing them in the presence of 6-thioguanine. A large set of mutant cells, all containing different mutations, was then analyzed by DGGE. Both spontaneous and ethylnitrosourea-induced mutations occurring at a frequency of 5 - 75 x 10^{-6} in splenic T cells of mice could be detected.

A highly sensitive method for analyzing single basepair (bp) changes within the Taq I enzyme recognition site by means of a restriction-fragment length polymorphism/polymerase chain reaction (RFLP/PCR) approach was described by Sandy et al. (1992). This method allowed the detection of as few of 5 mutant copies among 10^8 wild-types and it can in principle be applied to the detection of spontaneous or induced mutations in disease-related genes in humans (Chiocca et al., 1992). Despite its high sensitivity and the fact that specific gene sequences can be analyzed without the selection of phenotypically altered cells, this method has its limitations, the most important of which is that only those mutations present within a (Taq I) restriction enzyme site are detectable.

Jeffreys et al. (1990) developed a method to specifically amplify and characterize in vivo deletion mutations at the D1S8 minisatellite locus in human genomic DNA isolated from blood and sperm. This method is based on the amplification of the D1S8 locus using specific primers followed by separation of the amplified DNA fragments on a agarose gel. All fragments smaller than the wild type band are eluted from the gel and are again amplified using PCR. In this way deletion type mutation occurring at very low frequency could be detected. All other types of mutations, however, go undetected in this system.

All these approaches allow to monitor mutations directly in tissue DNA. However, each method has its own drawbacks since either only a limited number of mutation types can be detected or only a limited number of tissues can be analyzed, which demonstrates the need to develop additional methods. In

general, however, the PCR technique can now routinely be applied to characterize mutations occurring at a frequency of >0.1%. This includes mutations occurring at so called "hot spots" and germline mutations when each body cell contains a mutant gene copy. Application of the PCR technology for the detection of somatic mutations, however, requires dramatic improvements in the experimental conditions, such as, the use of new DNA polymerases characterized by much lower error rates. Once such improvements have been achieved, the PCR technique could become a valuable assay for the detection of somatic mutations.

1.5 The use of shuttle vectors for studying gene mutations

In order to be able to study spontaneous or induced mutations in all cells of the body of an experimental animal, a system is required in which, starting with genomic DNA from various organs and tissues, mutant genes can be rescued and subsequently selected for among non-mutant genes. In addition, such a system should be able to detect mutations occurring at very low frequency, i.e. permit the detection of one mutant gene copy among 100,000 non-mutants. The systems described in 1.3, which are based on the use of selectable genes, all have severe disadvantages, which limit their applicability. In general, they are restricted to very special cell types (e.g. GPA, HLA, Blb-1) or require cells that can be cultured in vitro (e.g. HPRT, APRT). Sometimes the selection is technically demanding and/or laborious, in others the characterization of mutations is not (yet) possible.

One approach that has been taken to circumvent these problems is based on the use of so-called shuttle vectors. Initially, the design was to construct vectors carrying a well studied reporter gene that can be rapidly isolated and analyzed at the sequence level (Calos and Dubridge, 1989). Shuttle vectors can be introduced into mammalian cells and subsequently retrieved in ("shuttled" to) bacteria for the discrimination between mutated and non-mutated target genes. During the past years different types of shuttle vectors, with different mutational target genes, have been used for studying gene mutations in in vitro assays. The most commonly used vectors were: (1) SV40-based vectors, which replicate autonomously in the nucleus of mammalian cells (Lebkowski et al., 1986; Macgregor et al., 1987); (2) vectors based on Epstein-Barr virus (EBV), which replicate as a plasmid in the nucleus, but under cellular control (DuBridge et al., 1987; Eckert et al., 1988); (3) retroviral vectors, which integrate in the mammalian genome (Ashman, 1989; Itehata et al., 1989); and (4) bacteriophage lambda vectors which also integrate in the mammalian genome (Glazer et al., 1986).

The most commonly used target genes in mutation studies are genes encoding bacterial reporter enzymes, e.g. Lac repressor (LacI), galactokinase (galK), xanthine/guanine phosphoribosyl transferase (gpt), suppressor tRNA (supF), the α fragment of ß-galactosidase (LacZα) or ß-galactosidase (LacZ). All these genes have been sequenced and have a relatively small size (<3000 bp) which facilitates the analysis of mutations by sequencing. Shuttle vector systems have been applied in a wide variety of studies, such as those aimed at investigating either spontaneous or induced mutations. Specific approaches using shuttle vectors included: (1) comparative studies on mutagenesis in both repair deficient and proficient cell lines (Moriwaki et al., 1991); (2) determination of mutation spectra in shuttle vectors containing specific lesions (Yang et al., 1987; Klein et al., 1990);, (3) studying the fidelity of mammalian DNA polymerases (Hauser et al., 1988; Thomas et al., 1991); and (4) studying spontaneous or mutagen-induced recombination events (Hellgren and Lambert, 1989; Mudgett and Taylor, 1990).

Initially, most of the shuttle vectors described were extrachromosomally located in the nucleus. Results obtained, however, indicated that such vectors were characterized by high spontaneous mutation frequencies, in some cases up to 1% (Calos et al., 1983; Ashman and Davidson, 1984). Analysis of individual reporter genes revealed that mutations were due to deletions, duplications, point mutations and insertions of cellular DNA (Razzaque et al., 1983). It was suggested that these mutations were the result of damage incurred during transfection and transport of the vector to the nucleus (Calos et al., 1983). These findings led to the search for alternative methods to introduce the shuttle vector into cells. However, although several new methods were tried, each variation seemed to have its own inherent difficulties which finally resulted in the use of shuttle vectors which integrate in the genome. A major advantage of this approach is that shuttle vectors which integrate in the genome more closely resemble the natural environment of mammalian endogenous

genes. Moreover, in view of the much lower spontaneous mutation frequency in integrated marker genes, more sensitive systems were obtained (Glazer et al., 1986; Goring et al., 1987; Tindall and Stankowsky, 1989).

Glazer et al. (1986) described the use of integrated bacteriophage lambda shuttle vectors, provided with the supF mutational target gene, for studying UV-induced mutagenesis in mouse L cells. In this system, cell lines were used containing up to 100 copies of the marker gene, which contributes to the sensitivity. The vectors could be rescued by exposing the genomic DNA to a bacterial packaging extract, which in theory should allow the rescue of about 7000 vectors/μg genomic DNA/integrated copy. The results obtained, however, indicated that rescue of these vectors was highly inefficient ($\pm$ 5 pfu/μg genomic DNA/copy). The results described in this thesis provide evidence that bacteriophage lambda shuttle vectors integrated in chromosomal mammalian DNA are highly methylated, which may lead to host-restriction during *in vitro* packaging or after retrieval into *E.coli*. Host-restriction is the consequence of the phenomenon that eukaryotic DNA is modified; this modified DNA, for example DNA containing 5-methylcytosine, is recognized and degraded when introduced in *E.coli*. Two *E.coli* host-restriction systems had been characterized at that time: MCR-A and MCR-B (Raleigh et al., 1988). However, application of *E.coli* host strains mutated in MCR-A and MCR-B did not result in improved rescue efficiencies; this result was indicative of the presence of additional host restriction systems. By using the host-restriction negative strain *E.coli* C, high rescue efficiencies could be obtained (Gossen and Vijg, 1988; this thesis). Similar results were later obtained by Kohler et al. (1990), who also demonstrated increased rescue efficiencies upon the use of host restriction negative *E.coli* strains.

1.6 Transgenic mice

An important step forward in the direction of a mammalian *in vivo* mutagenicity assay was made when the shuttle vector approach was combined with the possibility to generate transgenic mice. The first transgenic mice were produced by microinjection of SV40 DNA into the blastocoel cavity of early embryos (Jaenisch and Mintz, 1974) and, subsequently, by exposing early embryos to infectious retroviruses (Jeanisch, 1976). However, the majority of transgenic animals produced during the last ten years were generated by direct microinjection of recombinant-DNA constructs into pronuclei of fertilized eggs (Fig. 1; Gordon et al., 1980). This method now allows a nearly infinite variety of different DNA molecules to be introduced into fertilized eggs of e.g. mice, rats, rabbits, cattle and sheep (for a review, see Pursel et al., 1989). Microinjection has the advantage that there is no constraint on the size or sequence of the DNA that is to be introduced. DNA fragments are microinjected at the one-cell stage and they usually integrate in the genome at one site, presumably randomly, prior to the first cell division, such that all cells of the resulting embryo carry the foreign DNA. The efficiency with which transgenic mice can be produced averages about 25%, when linear DNA molecules are microinjected (Palmiter and Brinster, 1986). Transgenic mice obtained can be bred to transmit the foreign DNA to half of their progeny. These heterozygous animals are then bred in order to obtain a homozygous line. Characterization of homozygous transgenic animals can be performed using Southern blot analysis (Southern, 1978) or by means of in situ hybridisation. The latter technique, in principle, also allows to determine at which chromosome the transgene(s) have been integrated. When more than one copy has been integrated, which is generally observed with microinjected DNA, the multiple copies are always arranged in head-to-tail arrays which may contain up to hundreds of copies. This type of arrangement is presumed to be the consequence of homologous recombination

between injected molecules (Brinster et al, 1981). This was recently confirmed in an experiment in which three partially overlapping genomic fragments of the human serum albumin gene were injected into mouse oocytes (Pieper et al., 1992). The results obtained showed that 20 out of 27 transgenic mice contained functional genes, which must have resulted from homologous recombination between the three overlapping fragments before integration in the mouse genome occurred.

The microinjection technique for generating transgenic animals has now found widespread application in molecular biology. Examples are its use in studying gene expression, the action of oncogenes, insertional mutagenesis and the construction of transgenic mice as models for human genetic diseases (for reviews, see Camper, 1987; Compere et al, 1988; Hanahan, 1989; Conelly et al, 1989). More recently, an improved technique for generating transgenic animals has been developed, termed gene-targeting. This technique is based on homologous recombination between DNA sequences residing in the chromosome and the newly introduced DNA sequences. It has the potential to modify any gene, in a defined manner, in any species from which functional embryonic stem cells can be obtained (Capecchi, 1989). The procedure is performed in vitro, and it allows the selection of transgenic stem cells with the desired properties before the next step is made. Thus far, however, functional embryonic stem cells could only be obtained from a single mouse strain, 129/Sv. The use of other embryonic stem cells originating either from different mouse strains or other species, e.g. the rat, have not been reported yet. The applicability of the gene-targeting technique for generating mice containing specific mutations has now been well established. It overcomes many of the limitations of the microinjection technique, such as variable expression of introduced genes due to integration in transcriptionally active or inactive genomic regions, or integration of the microinjected DNA fragments in a functional gene (insertional mutagenesis).

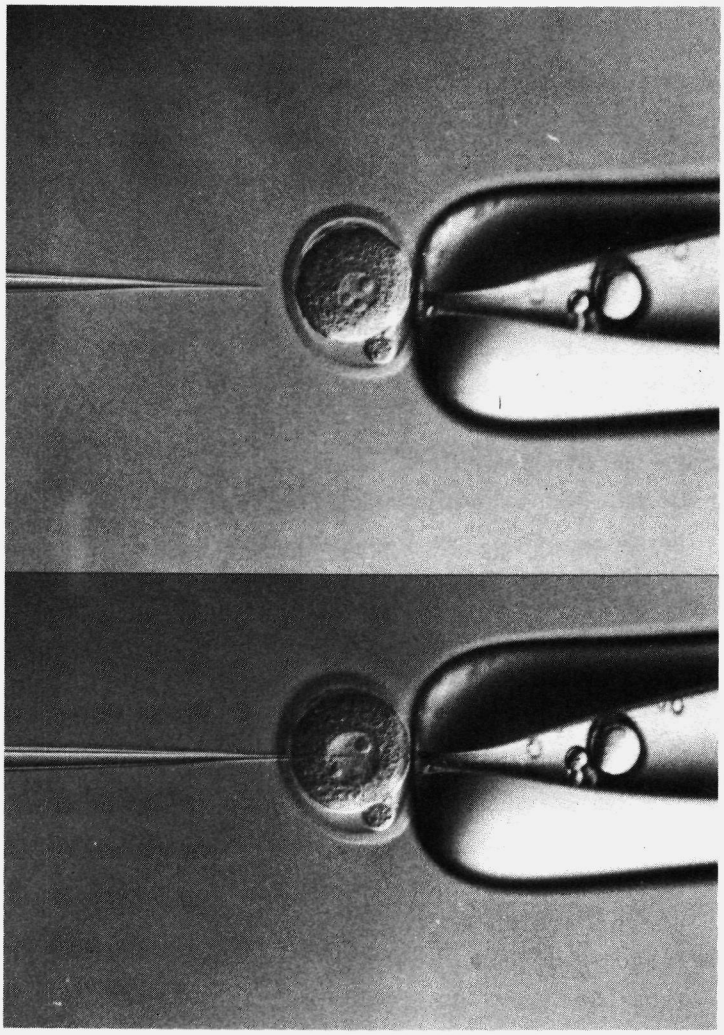


Figure 1. Microinjection of foreign DNA into mouse oocytes.

1.7 Transgenic mouse models for studying the mutagenic/ carcinogenic effects of chemicals

Transgenic animal technology has been applied in developing improved mutagenicity/carcinogenicty testing systems (for a review see Cordaro, 1989). The approaches used include germline transmission of oncogenes to increase the sensitivity for chemical-induced tumour formation. The transgenic mice described are characterized by high levels of expression of e.g. the myc oncogene (Stewart et al., 1984) and pim-1 oncogene (Breuer et al., 1989). Studies in which pim-1 transgenic mice were treated with ethylnitrosourea (ENU) indicate that such a transgenic mouse model can be used as sensitive system for studying the carcinogenicity of chemicals. However, since these oncogenes are involved in tumour development in only a limited number of tissues, only mutagens acting on these tissues will be detected as a carcinogen, which limits the applicability of these models as a short-term carcinogenicity assay.

Transgenic mice carrying an inactivated p53 tumour-supressor gene (along with two wild type copies) have also been generated (Lavigeur et al., 1989). These transgenic mice were characterized by a higher risk of developing various forms of cancer. More recently, transgenic mice have been generated in which both alleles of the p53 tumour-suppressor gene were inactivated by means of gene targeting techniques (Donehower et al., 1992). These p53-deficient mice were found to be highly prone to spontaneous development of a variety of cancers by 6 months of age. This increased susceptibility could make them a valuable tool for studying compounds suspected to be carcinogenic in humans.

A second approach involves the use of the bacterial reporter gene LacZ to detect somatic DNA recombination in transgenic mice (Matsuoka et al., 1991). In this system the LacZ gene lacking its promoter is placed in the opposite orientation relative to the murine actin promoter, a house-keeping promoter which directs expression in a large number of

cell types. Rearrangements which result in relocating the LacZ gene in the correct transcriptional orientation, e.g due to recombination events, can be detected by staining cells with X-gal, a chromogenic substrate of ß-galactosidase (see 1.1).

The third approach is based on the use of shuttle vectors, harbouring a bacterial reporter gene, to study gene mutations <u>in vivo</u> in a more general way. An overview of the presently available transgenic mouse mutation models is shown in Fig. 2, and are described separately.

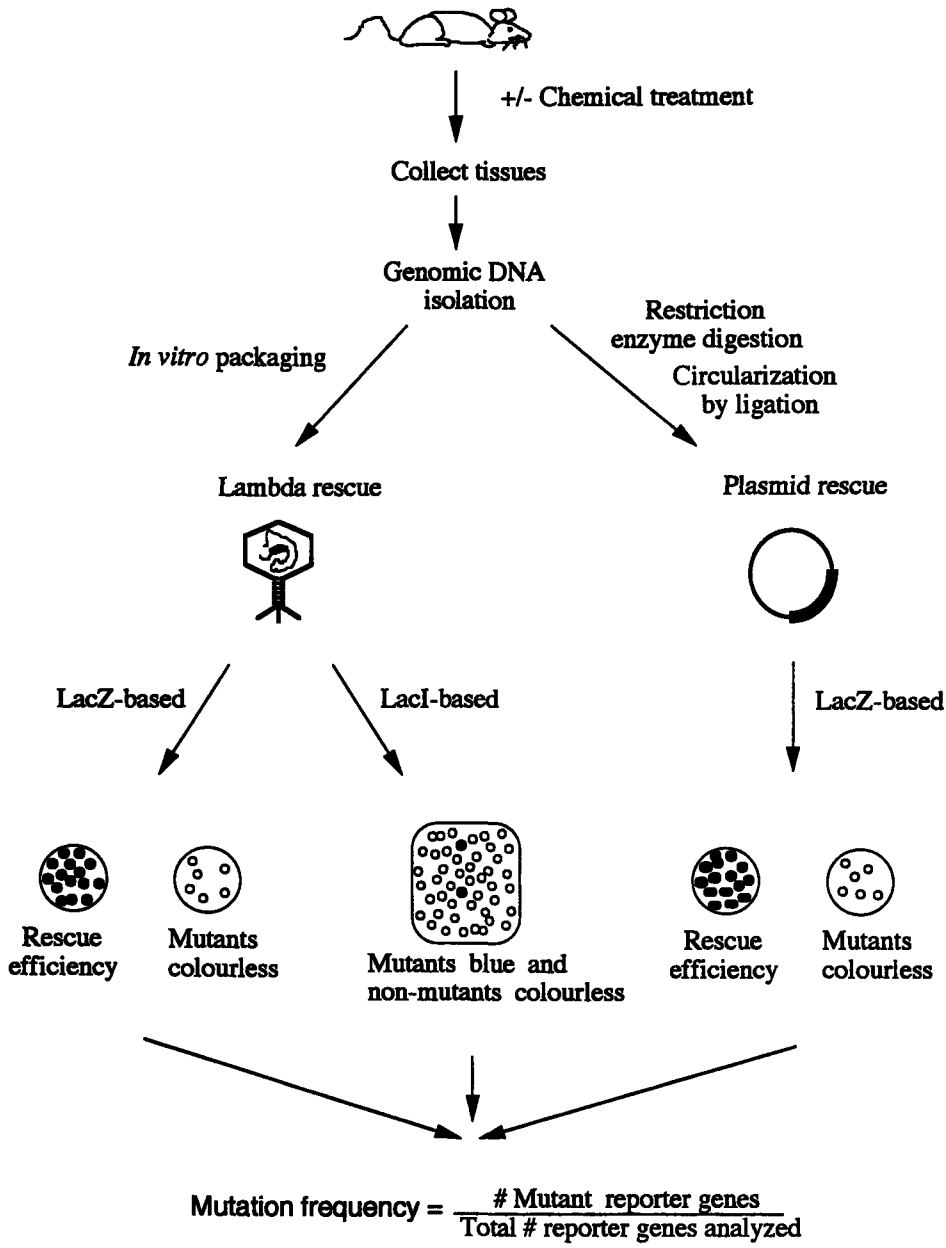


Figure 2. Overview of transgenic mouse mutation models.

1.7.1 Bacteriophage lambda-based transgenic mouse models

A transgenic mouse model based on the use of a bacteriophage lambda shuttle vector containing the bacterial LacZ gene as a target for mutagenesis was described by Gossen et al. (1989; this thesis). This transgenic mouse model was constructed by micro-injection of about 150 copies of the bacteriophage lambda gt10-LacZ shuttle vector into fertilized CD2 mouse oocytes. Transgenic mice which develop from such micro-injection experiments usually carry multiple copies of the foreign DNA in a head-to-tail arrangement at a single site in the genome. Rescue of these vectors is performed by exposing total genomic DNA, isolated from different organs or tissues, to an E.coli *in vitro* packaging extract. During the process, the terminase enzyme present in the packaging extracts recognizes and cuts the lambda cos-sites, resulting in the formation of single lambda molecules each of which is packaged into an empty phage-head. Phages are then plated on a host-restriction negative, $LacZ^-$ *E.coli* strain in the presence of the chromogenic substrate X-Gal for selection of mutant and non-mutant LacZ phages. Mutation frequencies for a particular organ or tissue are determined as the ratio between colourless (mutated) and blue (non-mutated) plaques (for a schematic representation of the assay system, see Fig.2). On the basis of the same principle other systems have been described (Kohler et al., 1989 and 1991; Fig.2). One of these models was not based on LacZ as the mutational target gene but on LacI. The LacI gene encodes the Lac repressor protein which, by binding to the operator sequence in front of the LacZ gene, is able to negatively regulate LacZ expression. Consequently, infection of $LacZ^+$ E.coli host cells with phages containing a non-mutant LacI gene will give rise to colourless plaques, and phages containing a mutant LacI gene will give rise to blue plaques. Since the visual scoring of a few blue plaques among a large number of colourless plaques is much easier then scoring a few colourless plaques among a large number of blue plaques, the LacI system offers a distinct advantage compared

to a LacZ-based system. This advantage, however, has been overtaken by the development of a selective system for isolating mutant LacZ phage. This system is based on the use of a GalE⁻ E.coli host which is highly sensitive for galactose (Gossen and Vijg, 1992b). Since the ß-galactosidase enzyme encoded by the LacZ gene converts lactose into galactose, non-mutant LacZ phages are unable to propagate when plated on this strain in the presence of lactose or lactose analogues such as phenyl-ß-D-galactoside: the GalE⁻ E.coli host cells lyse before the LacZ phages have time to multiply and infect neighbouring cells. This selective system allows to plate at least 300,000 phages on a single small petri-dish (Gossen and Vijg, 1992b), and it overcomes the laborious task of analyzing large numbers of plaques to accurately determine mutation frequencies.

1.7.2 Plasmid-based transgenic mouse models

In addition to lambda-based models, more recently a transgenic mouse model for mutagenesis studies based on the efficient rescue of plasmid vectors from genomic DNA, has been generated (this thesis). In this approach, rescue of LacZ-containing plasmid vectors is based on the binding of the Lac repressor protein to the operator sequence located in front of the LacZ gene. LacZ plasmid sequences are released from the genomic DNA by excision with a restriction enzyme, and given the opportunity to bind to the Lac repressor protein which is conjugated to a magnetic support particle. The use of a magnetic particle concentrator then allows to specifically recover LacZ plasmid sequences. After circularization, the concentrated plasmids are transferred into a bacterial host by means of electroporation. Using the same GalE⁻ E.coli C strain as described for the bacteriophage lambda-based system, also this system allows to select for colonies harbouring mutant vectors (Fig. 2; Gossen et al., 1992a).

Plasmid vectors offer the distinct advantage of having a size which is only 1/10 of a bacteriophage lambda vector; the average size of a LacZ-containing plasmid is 5 kb and that of

a bacteriophage lambda vector 50 kb. Due to the large size of the latter and the fact that some of the bacteriophage lambda-based transgenic mice carry up to 80 copies at a single genomic site (4 x 10^6 bp), high molecular weight DNA must be isolated in order to rescue intact vectors with reasonable efficiencies. In general, however, the average size of genomic DNA isolated from organs or tissues is about 200-300 kb, indicating that multiple double strand breaks will be present within the lambda concatemer. Because of the relatively small size of plasmid vectors, the presence of double strand breaks every 200-300 kb will not interfere with plasmid rescue. A second advantage of plasmid rescue is that, owing to the high capacity of LacI repressor magnetic beads, large amounts of plasmid can be purified from restriction-enzyme digested genomic DNA in a single step. Although bacteriophage lambda vectors also can be purified from genomic DNA, using field inversion gel electrophoresis of genomic DNA digested with a restriction enzyme which cuts outside the lambda concatemer (Gossen et al., 1989), the inclusion of this complex procedure is time-consuming.

1.8 Other applications of bacteriophage lambda transgenic mice

In addition to its use in detecting somatic or germ cell mutations, the transgenic mice described in this thesis can be applied for a number of other purposes. These transgenic mice carry multiple copies of the bacteriophage lambda vector and some of the strains analyzed carry up to 80 copies (about 4 x 10^6 bp) at a single site in the genome. Such large amounts of foreign DNA can easily be detected by means of in situ hybridization techniques using, for example, biotin-labelled bacteriophage lambda probes.

In principle, this allows to use somatic cells of transgenic mice as marker cells in transplantation studies, to follow the transport of these cells in acceptor animals. Such an application has been described by Huppes et al. (1992). In this study, foetal liver cells isolated from bacteriophage lambda transgenic mice were injected into "twitcher" mice to analyze the migration pattern of the injected cells. Through a combination of in situ hybridisation and immunochemistry techniques it was shown that injected transgenic donor cells could be found throughout the brain of the "twitcher" mouse, which underlines the applicability of transgenic mouse cells as marker cells in transplantation studies.

A second application of bacteriophage lambda transgenic mice is their use in studying aneuploidy. Aneuploidy represents a cytogenetic event in which a cell or an organism has an abnormal number of chromosomes. The role of aneuploidy in prenatal and postnatal abnormalities and also in carcinogenesis has been established in a number of studies (Evans et al., 1985). A number of test systems has been developed during the past years, most of which are based on cytogenetic assays, for example, via the analysis of non-disjunction in interphase nuclei after in situ hybridization with human chromosome-specific repetitive DNA sequences as probes. Such probes, however, are not available for mice. Natarajan et al. (1990) recently described a substitute, i.e.

the use of bacteriophage lambda transgenic mice for aneuploidy testing. Since the homozygous transgenic mice used in these studies contain the bacteriophage lambda concatemer in both chromosomes, in situ hybridization of normal body cells with a biotin-labelled lambda probe will reveal two hybridizing spots. Those body cells in which 0, 1, 3 or more spots are detected are considered aneuploid.

1.9 The LacZ mutational target gene: its sequence and molecular analysis

The transgenic mice that stood central in the studies described in this thesis, all contain the LacZ gene as the target for mutation induction. The E.coli LacZ gene is part of the Lac operon and has a size of 3087 bp (the complete sequence is shown in Fig. 3). It encodes the enzyme ß-galactosidase, a tetrameric protein with four identical subunits. Although the mechanism of action of the ß-galactosidase enzyme has not been fully elucidated, the active region of the protein has been shown to be near amino acid residues Met-502 and Glu-461 (Fowler and Smith, 1983).

In order to analyse mutations within the LacZ structural gene, DNA sequence analysis can be performed. However, the LacZ gene, together with the regulatory sequences, has a considerable length. This latter target size contributes to the suitability of the gene for mutagenicity studies, but is not very favourable for the characterization of the mutants at the DNA sequence level. A pre-screening of the mutants as to the region where the sequence alteration has occurred, however, has been developed (this thesis). The method, termed α, ß, Ω complementation, is based on the fact that the ß-galactosidase protein is divided into three regions: the α, ß and Ω region. Polypeptide chains corresponding to these regions have been isolated, and it has been shown that these enzymatically inactive chains are able to complement each other to result in the formation of an active ß-galactosidase protein (Ullman et al., 1967; Villarejo et al., 1972). The complementation assay performed by transferring a mutant LacZ gene to 3 different E.coli strains harbouring partial LacZ deletions: for example DH5α (ß + Ω donor) to identify LacZ mutants containing a mutation in the LacZ-α region, W4680 (α + Ω donor) to identify LacZ mutants containing a mutation in the LacZ-ß region and Hfr 30 x 9000 (α + ß donor) to identify LacZ mutants containing a mutation in the LacZ-Ω region, respectively. The nature of mutations in LacZ mutants can

subsequently be analyzed by means of DNA sequence analysis using primers specific for each region.

Figure 3. DNA sequence of the LacZ gene

```
      -160       -150       -140       -130       -120       -110       -100        -90        -80
TCATGCAGCT CGATTCATTA GGCACGACAG GTTTCCCGAC TGGAAAGCGG GCAGTGAGCG CAACGCAATT AATGTGAGTT AGCTCACTCA

       -70        -60        -50        -40        -40        -30        -20        -10          0
TTAGGCACCC CAGGCTTTAC ACATTTATGC TTCCGGCTCG TATAATGTGT GGAATTGTGA GCGGATAACA ATTTCACACA GGAAACAGCT

        10         20         30         40         50         60         70         80         90
ATGACCATGA TTACGGATTC ACTGGAATTC CTGGGGATGC CCGTCGTTTT ACAACGTCGT GACTGGGAAA ACCCTGGCGT TACCCAACTT

       100        110        120        130        140        150        160        170        180
AATCGCCTTG CAGCACATCC CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA GGCCCGCACC GATCGCCCTT CCCAACAGTT GCGCAGCCTG

       190        200        210        220        230        240        250        260        270
AATGGCGAAT GGCGCTTTGC CTGGTTTCCG GCACCAGAAG CGGTGCCGGA AAGCTGGCTG GAGTGCGATC TTCCTGAGGC CGATACTGTC

       280        290        300        310        320        330        340        350        360
GTCGTCCCCT CAAACTGGCA GATGCACGGT TACGATGCGC CCATCTACAC CAACGTGACC TATCCCATTA CGGTCAATCC GCCGTTTGTT

       370        380        390        400        410        420        430        440        450
CCCACGGAGA ATCCGACGGG TTGTTACTCG CTCACATTTA ATGTTGATGA AAGCTGGCTA CAGGAAGGCC AGACGCGAAT TATTTTTGAT

       460        470        480        490        500        510        520        530        540
GGCGTTAACT CGGCGTTTCA TCTGTGGTGC AACGGGCGCT GGGTCGGTTA CGGCCAGGAC AGTCGTTTGC CGTCTGAATT TGACCTGAGC

       550        560        570        580        590        600        610        620        630
GCATTTTTAC GCGCCGGAGA AAACCGCCTC GCGGTGATGG TGCTGCTCTG GAGTGACGGC AGTTATCTGG AAGATCAGGA TATGTGGCGG

       640        650        660        670        680        690        700        710        720
ATGAGCGGCA TTTTCCGTGA CGTCTCGTTG CTGCATAAAC CGACTACACA AATCAGCGAT TTCCATGTTG CCACTCGCTT TAATGATGAT

       730        740        750        760        770        780        790        800        810
TTCAGCCGCG CTGTACTGGA GGCTGAAGTT CAGATGTGCG GCGAGTTGCG TGACTACCTA CGGGTAACAG TTTCTTTATG GCAGGGTGAA

       820        830        840        850        860        870        880        890        900
ACGCAGGTCG CCAGCGGCAC CGCGCCTTTC GGCGGTGAAA TTATCGATGA GCGTGGTGGT TATGCCGATC GCGTCACACT ACGTCTGAAC

       910        920        930        940        950        960        970        980        990
GTCGAAAACC CGAAACTGTG GAGCGCCGAA ATCCCGAATC TCTATCGTGC GGTGGTTGAA CTGCACACCG CCGACGGCAC GCTGATTGAA

      1000       1010       1020       1030       1040       1050       1060       1070       1080
GCAGAAGCCT GCGATGTCGG TTTCCGCGAG GTGCGGATTG AAAATGGTCT GCTGCTGCTG AACGGCAAGC CGTTGCTGAT TCGAGGCGTT

      1090       1100       1110       1120       1130       1140       1150       1160       1170
AACCGTCACG AGCATCATCC TCTGCATGGT CAGGTCATGG ATGAGCAGAC GATGGTGCAG GATATCCTGC TGATGAAGCA GAACAACTTT
```

1180 1190 1200 1210 1220 1230 1240 1250 1260
AACGCCGTGC GCTGTTCGCA TTATCCGAAC CATCCGCTGT GGTACACGCT GTGCGACCGC TACGGCCTGT ATGTGGTGGA TGAAGCCAAT

1270 1280 1290 1300 1310 1320 1330 1340 1350
ATTGAAACCC ACGGCATGGT GCCAATGAAT CGTCTGACCG ATGATCCGCG CTGGCTACCG GCGATGAGCG AACGCGTAAC GCGAATGGTG

1360 1370 1380 1390 1400 1410 1420 1430 1440
CAGCGCGATC GTAATCACCC GAGTGTGATC ATCTGGTCGC TGGGGAATGA ATCAGGCCAC GGCGCTAATC ACGACGCGCT GTATCGCTGG

1450 1460 1470 1480 1490 1500 1510 1520 1530
ATCAAATCTG TCGATCCTTC CCGCCCGGTG CAGTATGAAG GCGGCGGAGC CGACACCACG GCCACCGATA TTATTTGCCC GATGTACGCG

1540 1550 1560 1570 1580 1590 1600 1610 1620
CGCGTGGATG AAGACCAGCC CTTCCCGGCT GTGCCGAAAT GGTCCATCAA AAAATGGCTT TCGCTACCTG GAGAGACGCG CCCGCTGATC

1630 1640 1650 1660 1670 1680 1690 1700 1710
CTTTGCGAAT ACGCCCACGC GATGGGTAAC AGTCTTGGCG GTTTCGCTAA ATACTGGCAG GCGTTTCGTC AGTATCCCCG TTTACAGGGC

1720 1730 1740 1750 1760 1770 1780 1790 1800
GGCTTCGTCT GGGACTGGGT GGATCAGTCG CTGATTAAAT ATGATGAAAA CGGCAACCCG TGGTCGGCTT ACGGCGGTGA TTTTGGCGAT

1810 1820 1830 1840 1850 1860 1870 1880 1890
ACGCCGAACG ATCGCCAGTT CTGTATGAAC GGTCTGGTCT TTGCCGACCG CACGCCGCAT CCAGCGCTGA CGGAAGCAAA ACACCAGCAG

1900 1910 1920 1930 1940 1950 1960 1970 1980
CAGTTTTTCC AGTTCCGTTT ATCCGGGCAA ACCATCGAAG TGACCAGCGA ATACCTGTTC CGTCATAGCG ATAACGAGCT CCTGCACTGG

1990 2000 2010 2020 2030 2040 2050 2060 2070
ATGGTGGCGC TGGATGGTAA GCCGCTGGCA AGCGGTGAAG TGCCTCTGGA TGTCGCTCCA CAAGGTAAAC AGTTGATTGA ACTGCCTGAA

2080 2090 2100 2110 2120 2130 2140 2150 2160
CTACCGCAGC CGGAGAGCGC CGGGCAACTC TGGCTCACAG TACGCGTAGT GCAACCGAAC GCGACCGCAT GGTCAGAAGC CGGGCACATC

2170 2180 2190 2200 2210 2220 2230 2240 2250
AGCGCCTGGC AGCAGTGGCG TCTGGCGGAA AACCTCAGTG TGACGCTCCC CGCCGCGTCC CACGCCATCC CGCATCTGAC CACCAGCGAA

2260 2270 2280 2290 2300 2310 2320 2330 2340
ATGGATTTTT GCATCGAGCT GGGTAATAAG CGTTGGCAAT TTAACCGCCA GTCAGGCTTT CTTTCACAGA TGTGGATTGG CGATAAAAAA

2350 2360 2370 2380 2390 2400 2410 2420 2430
CAACTGCTGA CGCCGCTGCG CGATCAGTTC ACCCGTGCAC CGCTGGATAA CGACATTGGC GTAAGTGAAG CGACCCGCAT TGACCCTAAC

2440 2450 2460 2470 2480 2490 2500 2510 2520
GCCTGGGTCG AACGCTGGAA GGCGGCGGGC CATTACCAGG CCGAAGCAGC GTTGTTGCAG TGCACGGCAG ATACACTTGC TGATGCGGTG

2530 2540 2450 2560 2570 2580 2590 2600 2610
CTGATTACGA CCGCTCACGC GTGGCAGCAT CAGGGGAAAA CCTTATTTAT CAGCCGGAAA ACCTACCGGA TTGATGGTAG TGGTCAAATG

2620 2630 2640 2650 2660 2670 2680 2690 2700
GCGATTACCG TTGATGTTGA AGTGGCGAGC GATACACCGC ATCCGGCGCG GATTGGCCTG AACTGCCAGC TGGCGCAGGT AGCAGAGCGG

2710 2720 2730 2740 2750 2760 2770 2780 2790
GTAAACTGGC TCGGATTAGG GCCGCAAGAA AACTATCCCG ACCGCCTTAC TGCCGCCTGT TTTGACCGCT GGGATCTGCC ATTGTCAGAC

2800 2810 2820 2830 2840 2850 2860 2870 2880
ATGTATACCC CGTACGTCTT CCCGAGCGAA AACGGTCTGC GCTGCGGGAC GCGCGAATTG AATTATGGCC CACACCAGTG GCGCGGCGAC

2890 2900 2910 2920 2930 2940 2950 2960 2970
TTCCAGTTCA ACATCAGCCG CTACAGTCAA CAGCAACTGA TGGAAACCAG CCATCGCCAT CTGCTGCACG CGGAAGAAGG CACATGGCTG

2980 2990 3000 3010 3020 3030 3040 3050 3060
AATATCGACG GTTTCCATAT GGGGATTGGT GGCGACGACT CCTGGAGCCC GTCAGTATCG GCGGAATTAC AGCTGAGCGC CGGTCGCTAC

3070 3080 3090 3100 3110 3120 3130 3140 3150
CATTACCAGT TGGTCTGGTG TCAAAAATAA TAATAACCGG GCAGGCCATG TCTGCCCGTA TTTCGCGTAA GGAAATCCAT TATGTACTAT

3058
TTGAATTC

Underlined are the Lac operator (TGTGTGGAATTGTGAGCGGATAACAATT TCACACA), the start codon (ATG) and stop codons $(TAA)_3$. The LacZα region is located from position 1 to 450, the LacZß region from position 451 to approximately 1750 and the LacZΩ region from approximately position 1751 to 3086.

1.10 Outline of this thesis

At the outset of the investigations described in this thesis (1986/87), the technology for generating transgenic mice by microinjecting fertilized mouse oocytes was still rather young, and transgenic mice harbouring a target gene for mutagenicity studies did not exist yet. It was decided to use a bacteriophage lambda vector containing the LacZ gene as a target for mutagenesis. This type of vector was selected because of the possibility of retrieval via packaging in empty phage heads and propagation in E.coli. The LacZ gene was chosen as mutational target gene since easy and well-tried methods for the detection of the intact gene product, i.e. active ß-galactosidase, were available. After microinjection of this vector into fertilized mouse eggs a number of transgenic mice was obtained, a few of which were chosen for further breeding to obtain homozygous strains. In this stage little was known about the possibilities to rescue integrated bacterial shuttle vectors with reasonable efficiencies from transgenic mice. Indeed, the first results obtained were not encouraging. Our observation that bacteriophage lambda sequences introduced into the mouse genome become heavily methylated and the fact that E.coli K12 bacterial hosts are able to recognize and degrade methylated DNA (a phenomenon termed host restriction) offered a possible explanation for the poor results. This insight finally led us to use an E.coli C host strain. This strain appeared to lack the ability to restrict foreign DNA (Wood, 1972), and the use of such a host-restriction negative strain eventually also allowed the rescue of integrated shuttle vectors from mouse genomic DNA with high efficiency (Gossen et al., 1988).

From there on it became possible to evaluate transgenic animals as tools in mutagenicity studies, by analyzing phage rescued from various organs of control and mutagen-treated mice. Furthermore, different strains were compared to analyze the influence of different chromosomal positions of the mutational target gene on mutation induction. Also, a number

of mutants was analyzed at the DNA sequence level to establish the changes introduced. Since the use of bacteriophage lambda vectors, as well as their packaging and retrieval method, was found to have its limitations, the possibility to develop an alternative approach was considered and the use of a plasmid vector transferred to the mouse germline was brought into practice. Finally, selective systems were developed to improve the scoring of LacZ mutants.

In Chapter 2 the development of the lambda-gt10LacZ transgenic mice and their application in studying spontaneous and ENU-induced mutations are described. In addition, some of the mutant LacZ genes isolated from brain DNA of ENU-treated mice were analyzed at the DNA sequence level to determine the nature of ENU-induced mutations.

Chapter 3 describes studies on the mutagenicity of ENU, procarbazine and chlorambucil in a number of organs and tissues of lambda-gt10LacZ transgenic mice injected with these compounds. The results showed dose dependent and organ and tissue specific mutation frequencies. Amplification of mutant LacZ genes by PCR, followed by restriction enzyme digestion and separation on agarose gels showed that the predominant type of mutations detected were point mutations and/or small deletions/insertions (<25 bp).

Chapter 4 describes the analysis of a transgenic mouse strain, termed strain 35.5, in which the lambda-gt10LacZ concatemer was integrated at the X-chromosome. Both male and female mice of this strain were found to be characterized by a 25-100 times higher spontaneous mutation frequency in liver and brain, compared to transgenic mice harbouring the LacZ gene at autosomal positions. In addition to the high spontaneous mutation frequencies in somatic tissues, deletion-type mutations were found to occur with high frequency in the 3′ region of the lambda-gt10LacZ concatemer, in germ cells.

Chapter 5 describes the DNA-sequence analysis of LacZ mutants isolated from liver and brain of mice from strain 35.5. The results showed that the mutations analyzed were tissue-specific and, compared to mutant LacZ genes isolated

from other transgenic mouse strains with different integration sites, dependent on the chromosomal position of the LacZ transgene.

Chapter 6 describes the development of a second system for rescuing LacZ genes from transgenic mice. For this study transgenic mice harbouring the LacZ gene as part of a plasmid shuttle vector were used. The plasmid rescue system is based on the high affinity of the LacI repressor protein for the Lac operator in front of the LacZ gene. By conjugating the LacI repressor protein to magnetic beads, Lac-operator containing sequences could be rescued with high efficiency from transgenic mouse genomic DNA.

1.11 References

Abelson, P.H. (1990) Testing for carcinogens with rodents, Science (Editorial) 249, 1357.

Abelson, P.H. (1992) Exaggerated carcinogenicity of chemicals, Science (Editorial) 256, 1609.

Albertini, R.J., K.L. Castle and W.R. Borcherding (1982) T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood, Proc. Natl Acad. Sci. (U.S.A.), 79, 6617-6621.

Albertini, R.J. (1985) Somatic gene mutations in vivo as indicated by the 6-thioguanine-resistant T-lymphocytes in human blood, Mutation Res. 150, 411-422.

Alvi, N.K., and Williams, G.M. (1992) Restriction fragment pattern analysis of HPRT mutations induced in rat epithelial cells by alkylating and arylating agents, Mutation Res. 265, 283-295.

Ames, B.N., F.D. Lee, W.E. Durston (1973) An improved bacterial test system for the detection and classification of mutagens and carcinogens, Proc. Natl. Acad. Sci. (U.S.A.) 70, 782-786.

Ames, B.N., Magaw, R., Gold, L.S. (1987) Ranking possible carcinogenic hazards, Science 236, 271-280.

Ames, B.N. & Gold, L.S. (1990) Chemical carcinogenesis: too many rodent carcinogens, Proc. Natl. Acad. Sci. USA 87, 7772-7776.

Ames, B.N. & Gold, L.S. (1990) Too many rodent carcinogens: Mitogenesis increases mutagenesis, Science (Perspective) 249, 970-971.

Ashman, C.R. & Davidson, R.L. (1984) High spontaneous mutation frequency in shuttle vector sequences recovered from mammalian cellular DNA, Proc. Natl. Acad. Sci. USA 81, 5170-5174.

Ashman, C.R. (1989) Retroviral shuttle vectors as a tool for the study of mutational specificity (base substitution/ deletion/mutational hotspot), Mutat. Res. 220, 143-149.

Beutler, E. (1992) Gaucher disease: New molecular approaches to diagnosis and treatment, Science 256, 794-799.

Bressac, B., Kew, M., Wands, J. & Ozturk, M., (1991) Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa, Nature 350, 429-431.

Breuer, M., Siebos, R., Verbeek, S., van Lohuizen, M., Wientjes, E. & Berns, A. (1989) Very high frequency of lymphoma induction by a chemical carcinogen in pim-1 transgenic mice, Nature 340, 61-63.

Brinster, R.L., Chen, H.Y., Trumbauer, M.E., Senear, A.W., Warren, R., Palmiter, R.D. (1981) Somatic expression of herpes thymidine kinase in mice following microinjection of a fusion gene in eggs, Cell 27, 223-231.

de Boer, J.G. and Glickman, B.W. (1991) Mutational analysis of the structure of the adenine phosphoribosyltransferase enzyme of Chinese hamster, J. Mol. Biol. 221, 163-74.

Calos, M.P., Lebkowski, J.S. and Botchan, M.R. (1983) High mutation frequency in DNA transfected into mammalian cells, Proc. Natl. Acad. Sci. (U.S.A.) 80, 3015-3019.

Camper, S.A. (1987) Research application of transgenic mice, BioTechniques 7638-650.

Cariello, N.F., Keohavong, P., Kat, A.G., Thilly, W. (1990) Molecular analysis of complex human cell populations: mutational spectra of MNNG and ICR-191, Mutation Res. 231, 165-176.

Cariello, N.F., Swenberg, J.A., Skopek, T.R. (1991) Fidelity of Thermococcus litoralis DNA polymerase (Vent™) in PCR determined by denaturing gradient gel electrophoresis, Nucleic Acids Res. 19, 4193-4198.

Cariello, N.F., Swenberg, J.A., De Belles, A., Skopek, T.R. (1991a) Analysis of mutations using PCR and denaturing gradient gel electrophoresis, Environ. Molec. Mutagen. 18, 249-245.

Capecchi, M.R. (1989) Altering the genome by homologous recombination, Science 244, 1288-1296.

Carrano, A.V. (1989) Summary of the workshop on mammalian in vivo somatic mutation, Genome 31, 458-459.

Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Ngyuen, P.N., Caskey, C.T. (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification, Nucleic Acids Res. 16, 11141-11156.

Chiocca, S.M., Sandy, M.S., Cerutti, P.A. (1992) Genotypic analysis of N-ethyl-N-nitrosourea-induced mutations by Taq I restriction fragment length polymorphism/ polymerase chain reaction in the c-H-ras1 gene, Proc. Natl. Acad. Sci. USA 89, 5531-5535.

Cole, J., Green, M.H.L., James, S.E., Henderson, L., Cole, H. (1988) A further assessment of factors influencing measurements of thioguanine-resistant mutant frequency in circulating T-lymphocytes, Mutation Res. 204, 493-507.

Compere, S.J., Baldacci, P., Jaenisch, R. (1988) Oncogenes in transgenic mice, Bioch. Bioph. Acta 948, 129-149.

Connelly, C.S., Fahl, W.E., Iannaccone, P.M. (1989) The role of transgenic animals in the analysis of various biological aspects of normal and pathologic states, Exp. Cell Res. 183, 257-276.

Cooper, D.N. & Krawczak, M. (1990) The mutational spectrum of of single base-pair substitutions causing human genetic disease: patterns and predictions, Hum. Genet. 85, 55-74.

Cordaro, J.C. (1989) Transgenic mice as future tools in risk assessment, Risk Analysis 9, 157-168.

Croce, C.M. (1987) Role of chromosome translocations in human neoplasms, Cell 49, 155-156

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J. & Bradley, A., (1992) Mice deficient for the p53 are developmentally normal but susceptible to spontaneous tumours, Nature 356, 215-221.

DuBridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H., and Calos, M.P. (1987) Analysis of mutation in human cells by using an Epstein-Barr virus shuttle vector system, Mol. Cell. Biol. 7, 379-387.

Dubridge, R.B. & Calos, M.P. (1988) Recombinant shuttle vectors for the study of mutation in mammalian cells, Mutagenesis 3, 1-9.

Eckert, K.A., Ingle, C.A., Klinedinst, D.K., and Drinkwater, N.R. (1988) Molecular analysis of mutations induced in human cells by N-ethyl-N-nitrosourea, Carcinogenesis 1, 50-56.

Ehling, U.H., Charles, D.J., Favor, J., Graw, J., Kratoch-Vilova, J., Neuhauser-Klaus, A., Pretsch, W. (1985) Induction of gene mutations in mice: the multiple endpoint approach, Mutation Res. 150, 393-401.

Ehrlich, M. & Wangh, R.Y. (1981) 5-Methylcytosine in eukaryotic DNA, Science 212, 1350-1357.

Ehrlich, M., Zhang, X.Y. & Inamdar, N.M. (1990) Spontaneous deamination of cytosine and 5-methylcytosine residues in DNA and replacement of 5-methylcytosine residues with cytosine residues, Mutation. Res. 238, 277-286.

Ennever, F.K., Noonan, T.J., Rosenkranz, H.S. (1987) The predictivity of animal bioassays and short-term genotoxicity tests for carcinogenicity and non-carcinogenicity to humans, Mutagenesis 2, 73-78.

Evans, H.G. (1985) Neoplasia and cytogenetic abnormalities in: Aneuploidy, Etiology and Mechanisms, Plenum, New York, 165-178.

Featherstone, T., P.D. Marshall, E.J. Evans (1987) Problems and pitfalls in assessing human T-lymphocyte mutant frequencies, Mutation Res. 179, 215-230.

Fowler, A.V. & Zabin, I. (1977) The amino acid sequence of ß-galactosidase of Escherichia coli, Proc. Natl. Acad. Sci. USA. 74, 1507-1510.

Fowler, A.V. & Smith, P.J. (1983) The active site regions of LacZ and ebg ß-galactosidase are homologous, J. Biol. Chem. 258, 10204-10207

Fox, T.R., Schumann, A.M., Watanabe, P.G., Yano, B.L., Maher, V.M., McCormick, J.J. (1990) Mutational analysis of the H-ras oncogene in spontaneous C57Bl/6 x C3H/He mouse liver tumors, and tumors induced with genotoxic and nongenotoxic hepatocarcinogenesis, Cancer Res. 50, 4014-4019.

Gibbs, R.A., Nguyen, P.N., Edwards, A., Civitello, A.B., Caskey, C.T., (1990) Multiplex DNA deletion detection and exon sequencing of the hypoxanthine phosphoribosyl transferase gene in Lesch-Nyhan families, Genomics 7, 235-244.

Glazer, P. M., Sarker, S. N. and Summers, W. C. (1986) Detection and analysis of UV-induced mutations in mammalian cell DNA using a lambda phage shuttle vector Proc. Natl. Acad. Sci. USA, 83, 1041-1044.

Gordon, J.W. and Ruddle, F.H. (1981) Integration and stable germline transmission of genes injected into mouse pronuclei, Science 214, 1244-1246.

Goring, D.R., Gupta., Dubow, M.S., (1987) Analysis of spontaneous mutations in a chromosomally located HSV-1 thymidine kinase (TK) gene in a human cell line, Somat. Cell. Molec. Genet. 13, 47-56.

Gossen, J.A. and J. Vijg (1988) E.coli C: a convenient host strain for cloning highly methylated DNA, Nucleic Acids Res. 16, 9343.

Gossen, J.A., W.J.F. de Leeuw, C.H.T. Tan, P.H.M. Lohman, F. Berends, D.L. Knook, E.C. Zwarthoff and J. Vijg. (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying gene mutations in vivo. Proc. Natl. Acad. Sci. (U.S.A.) 86, 7971-7975.

Gossen, J.A. and J. Vijg (1990) Transgenic mice as a model to study gene mutations: Application as a short-term mutagenicity assay, Prog. in Clin. and Biol. Res. Part A: Basic Mechanisms 340A, 347-354.

Gossen, J.A., W.J.F. de Leeuw, A. Verwest, P.H.M. Lohman and J. Vijg (1991). High somatic mutation frequencies in a LacZ transgene integrated on the mouse X-chromosome, Mutation Res. 250, 423-429.

Gossen, J.A., Molijn, A.C., Douglas, G.R., Vijg, J. (1992a) Application of galactose-sensitive E.coli strains as selective hosts for LacZ$^-$ plasmids, Nucleic Acids Res. 20, 3254.

Gossen, J.A., de Leeuw, W.J.F., Molijn, A.C., Vijg, J. (1992b) A selective system for LacZ$^-$ phage using a galactose-sensitive E.coli host, BioTechniques, In press.

Gossen, J.A., W.J.F. de Leeuw, and Vijg, J. (1992c) DNA sequence analysis of spontaneous mutations at a LacZ transgene integrated on the mouse X-chromosome, Mutagenesis, In press.

Gossen, J.A., W.J.F. de Leeuw, A.C. Molijn, Vijg, J. (1992d) Plasmid rescue from transgenic mouse DNA using LacI repressor protein conjugated to magnetic beads, BioTechniques, In press.

Gossen, J.A. & Vijg, J.A. (1993) Transgenic mice as model systems for studying gene mutations in vivo, Trends in Genetics, 9, 27-31.

Griffiths, D.F.R., Davies, S.J., Williams, D., Williams, G.T. & Williams, E.D. (1988) Demonstration of somatic mutation and colonic crypt clonality by X-linked enzyme histochemistry, Nature 333, 461-463.

Grist, S.A., McCarron, Kutlaca, A., Turner, D.R., and Morley, A.A., (1992) In vivo human somatic mutation: frequency and spectrum with age, Mutation Res. 266, 189-196.

Hakoda, M., Akiyama, M., Kuoizuma, S., Awa, A.A., Yamakido, M., and Otake, M. (1988) Increased somatic cell mutant frequency in atomic bomb survivors, Mutation Res. 179, 215-230.

Hanahan, D. (1989) Transgenic mice as probes into complex systems, Science 246, 1265-1275.

Harris, C.C. (1990) Chemical and physical carcinogenesis: advances and perspectives for the 1990s, Cancer Res. (suppl) 51, 5023-5044.

Harris, A.L. (1991) Telling changes of base, Nature 350, 377-378.

Haseman, J.K., Margolin, B.H., Shelby, M.D., Zeiger, E., Tennant, R,W. (1988) Do short-term tests predict rodent carcinogenicity? Science (Technical comments) 241, 1232-1233.

Hauser, J., Levine, A.S., and Dixon, K. (1988) Fidelity of DNA synthesis in a mammalian in vitro replication system, Mol Cell. Biol. 8, 3267-3271.

Hay, A. (1991) Carcinogenesis: Testing times for the tests, Nature 350, 555-556.

Hellgren, D. & Lambert, B. (1989) Mechanisms for recombination between stably integrated vector sequences in CHO cells, Mutation Res. 215, 197-204.

Hobbs, H.H., Russel, D.W., Brown, M.S., Goldstein, J.L. (1991) The LDL receptor locus in familial hypercholesterolemia: Mutational analysis of a membrane protein, Annu. Rev. Genet. 24, 133-170.

Hollstein, M., Sidranski, D., Vogelstein, B., Harris, C.C. (1991) p53 mutations in human cancers, Science 253, 49-53.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., & Harris, C.C. (1991) Mutational hotspot in the p53 gene in human hepatocellular carcinomas, Nature 350, 427-428.

Hupkes, W., de Groot, C.J.A., Ostendorf, R.H., Bauman, J.G.J., Gossen, J.A., Smit, V., Vijg, J. and Dijkstra, C.D. (1992) Detection of migrated allogeneic oligodendrocytes throughout the central nervous system of the galactocerebrosidase-deficient twitcher mouse. J. Neurocytology 21, 129-136.

Janatipour, M., Trainor, K.J., Kutlaca, R., Bennet, G., Hay, J., Turner, D.R., and Morley, A.A. (1988) Mutations in human lymphocytes studied by an HLA selection system, Mutation Res. 189, 221-226.

Jaenisch, R., and Mintz, B. (1974) Simian virus 40 sequences in DNA of healthy adult mice derived from preimplantation blastocytes injected with viral DNA, Proc. Natl. Acad. Sci. USA 71, 1250-1254.

Jaenisch, R. (1976) Germline integration and Mendelian transmission of the exogenous Moloney leukemia virus, Proc. Natl. Acad. Sci USA 73, 1260-1264.

Jaenisch, R. (1988) Transgenic animals, Science 240, 1468-1472.

Jeffreys, A.j., Neuman, R., Wilson, V. (1990) Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis, Cell 60, 473-485.

De Jong, P.J., Grosowski, A.J., Glickman, B. (1988) Spectrum of spontaneous mutation at the APRT locus of Chinese hamster ovary cells: an analysis at the DNA sequence level, Proc. Natl. Acad. Sci. USA 85, 3499-3503.

Keohavong, P. and W.G. Thilly (1989) Fidelity of DNA polymerases in DNA amplification, Proc. Natl. Acad. Sci. USA 86, 9253-9257.

Keohavong, P., Liu, V.F., and Thilly, W. (1991) Analysis of point mutations induced by ultraviolet light in human cells, Mutation Res. 249, 147-159.

Keohavong, P., & Thilly, W.G. (1992) Mutational spectrometry: A general approach for hot-spot point mutations in selectable genes, Proc. Natl. Acad. Sci. USA 89, 4623-4627.

Klei, J.C., Bleeker, M.J., Lugterink, J.T., van Dijk, W.J., van den Elst, H., van den Marel, G.A., van Boom, J.H., Westra, J.G., Berns, A.J.M., and Kriek, E. (1990) Use of shuttle vectors to study the molecular processing of defined carcinogen-induced DNA damage: mutagenicity of single O^4-ethylthymine adducts in Hela cells, Mutation Res. 18, 4131-4137.

Koeberl, D.D., Bottema, C.D.K., Ketterling, R.P., Bridge, P.J., Lillicrap, D.P., Sommer, S.S. (1990) Mutations causing hemophilia B: Direct estimate of the underlying rates of spontaneous germ-line transitions, transversions, and deletions in a human gene, Am. J. Hun. Genet. 47, 202-217.

Kohler, S.W., G.S. Provost, P.L. Kretz, M.J. Dycaico, J.A.Sorge, J.M. Short (1990) Development of a short-term in vivo mutagenesis assay: the effects of methylation on the recovery of a lambda shuttle vector from transgenic mice, Nucleic Acids Res. 18, 3007-3013.

Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, Putman, D.L., and Short J.M. (1991) Spectra of spontaneous and mutagen-induced mutations in the LacI gene in transgenic mice, Proc. Natl. Acad. Sci. USA 88, 7958-7962.

Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, Putman, D.L., and Short J.M. (1991) Analysis of spontaneous and induced mutations in transgenic mice using a lambda ZAP/LacI shuttle vector, Environ. Molec. Mutag. 18, 316-321.

Langlois, R.G., Bigbee, W.L., and Jensen, R.H. (1985) Measurements of the frequency of human erythrocytes with gene expression loss phenotypes at the glycophorin A locus, Hum. Genet. 74, 353-362.

Langlois, R.G., Bigbee, W.L., Kyoizuma, S., Nakamura, N., Bean, M.A., Akiyama, M., and Jensen, R.H. (1997) Evidence for increased somatic cell mutations at the glycophorin A locus in atomic bomb survivors, Science 236, 445-448.

Lavineur, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., Bernstein, A. (1989) High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene, Molec. Cell. Biol. 9, 3982-3991.

Lebkowski, J.S., Miller, J.H., and Calos, M. (1985) Determination of DNA sequence changes induced by ethyl methanesulfonate in human cells, using a shuttle vector system, Molec. Cell. Biol. 6, 1838-1842.

Levine, A.J. (1990) Tumor suppressor genes, BioEssays 12, 60-66.

Lohman, P.H.M., J. Vijg, A.G. Uitterlinden, P. Slagboom, J.A. Gossen and F. Berends (1987) DNA methods for detecting and analyzing mutations in vivo, Mutation Res. 181, 227-234.

MacGregor, G.R., James, M.R., Arlett, C.F., Burke, J.F. (1987) Analysis of mutations occurring during replication of a SV40 shuttle vector in mammalian cells, Mutat. Res. 183, 273-278.

Malling, H.V. & Burkhart, J.G. (1989) Use of phi X174 as a shuttle vector for the study of in vivo mammalian mutagenesis, Mutat. Res. 212, 11-21.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maron, D.M. & Ames, B.N. (1983) Revised methods for the Salmonella mutagenicity test, Mutation Res. 113, 172-215.

Matsuoka, M., Nagawa, F., Okazaki, K., Kingsbury,L., Yoshida, K., Müller, U., Larue, D.T., Winer, J.A., Sakano., H. (1991) Detection of somatic DNA recombination in the transgenic mouse brain, Science 254, 81-86.

McGinnes, M.J., Nicklas, J.A., Albertini, R.J. (1989) Molecular analysis of in vivo HPRT mutations in human T-lymphocytes; IV. Studies in newborns, Environ. Mol. Mutagen. 14, 229-237.

Messing, K., and Bradley, W.E.C. (1985) In vivo mutant frequency rises among breast cancer patients after exposure to high doses of gamma-radiation, Mutation Res. 152, 107-112.

Meuth, M. (1990) The structure of mutation in mammalian cells, Biochem. Biophys. Acta. 1032, 1-17.

Moriwaka, S., Yagi, T., Imamura, S., Takebe, H. (1991) Analysis of plasmid propagated in mouse 6-methylguanine-DNA methyltransferase-deficient cells in comparison with proficient cells, Cancer Res. 51, 6219-6223.

Morley, A.A., K.J. Trainor, R.S. Seshadri and R.G. Ryall (1983) Measurement of in vivo mutations in human lymphocytes, Nature London, 302, 155-156.

Morley, A.A., Grist, S.A., Turner, D.R., Kutlaca, A., and Bennet, G. (1990) Molecular nature of in vivo mutations in human cells at the autosomal HLA-A locus, Cancer Res. 50, 4584-4587.

Mudgett, J.S. & Taylor, W.D. (1990) Recombination between irradiated vector DNA and chromosomal DNA in African green monkey kidney cells, Molec. Cell. Biol. 10, 37-46.

Mullis, K. and Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction, Methods Enzymol. 155, 335.

Myhr, B.C. (1991) Validation studies with Mutamouse: A transgenic mouse model for detecting mutations in ivo, Environ. Molec. Mutag. 18, 308-315.

Myhr, B.C., Gossen, J.A., Lohman, P.H.M., Brusick, D, Vijg, J. (1992) Investigation of in vivo mutagenesis at the LacZ transgene in Mutamouse, submitted.

Nalbantoglu, J., Phear, G.A., Meuth, M. (1986) Nucleotide sequence of the hamster adenine phosphoribosyl transferase (aprt) gene, Nucleic Acids Res. 14, 1914.

Nalbantoglu, J., Phear, G.A., Meuth, M. (1987) DNA sequence analysis of spontaneous mutations at the APRT locus of hamster cells, Molec. Cell. Biol. 7, 1445-1449.

Natarajan, A.T., Vlasblom, S.E., Manca, A., Lohman, P.H.M., Gossen., Vijg, J., Beerman, F., Hummler, E., Hansman, I. (1990) Transgenic mouse - an in vivo system for detection of aneugens, Prog. in Clin. and Biol. Res. Part B: Metabolism, Testing methods and Chromosomes 340B.

O'Neill, J.P., Hunter, T.C., Sullivan, L.M., Nicklas, J.A., and Albertini, R.J. (1990) Southern blot analysis of human T-lymphocyte mutants induced in vitro by gamma-irradiation, Mutation Res. 240, 341-347.

Palmiter, R.D. and Brinster, R.L. (1986) Germline transformation of mice, Ann. Review Genet. 20, 465-499.

Papadopoulo, D., Guillouf, C., Mohrenweiser, H., Moustacchi, E. (1990) Hypomutability in Fanconi anemia cells is associated with increased deletion at the HPRT locus, Proc. Natl. Acad. Sci. USA 87, 8383-8387.

Pieper, F.R., de Wit, I.C.M., Pronk, A.C.J., Kooiman, P.M., Strijker, R., Krimpenfort, P.J.A., Nuyens, J.H., de Boer, H.A. (1992) Efficient generation of functional transgenes by homologous recombination in murine zygotes, Nucleic Acids Res. 20, 1259-1264.

Prival, M.J. & Dunkel, V.C. (1989) Reevaluation of the mutagenicity and carcinogenicity of chemicals identified as "false positives" in the Salmonella typhimurium mutagenicity assay, Environ. Molec. Mutagen. 13, 1-24.

Pursel, V.G., Pinkert, C.A., Miller, K.F., Bolt, D.J., Campbell, R.G., Palmiter, R.D., Brinster, R.L. & Hammer, R.E. (1987) Genetic engineering of livestock, Science 244,1281-1288.

Raleigh, E.A., Murray, N.E., Revel., Blumenthal, R.M., Westaway, D., Reith, A.D., Rigby, P.W.J., Elhai, J., Hanahan, D. (1988) McrA and McrB restriction phenotypes of some E.coli strains and implications for gene cloning, Nucleic Acids Res. 16, 1563-1575.

Razzaque, A., Mizusawa, H., Seidman, M.M. (1983) Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells, Proc. Natl. Acad. Sci. USA 80, 3010-3014.

Romak, S., Leong, P., Sockett, H., Seidman, M.M. (1989) DNA base sequence changes induced by ultraviolet light mutagenesis of a gene on a chromosome in chinese hamster ovary cells, J.Mol. Biol. 209, 195-204.

Rossi, A.M., Thyssen, J.C.P., Tates, A.D., Vrieling, H., Nataraja, A.T., Lohman, P.H.M., van Zeeland, A.A. (1990) Mutations affecting splicing in man are detected more frequently in somatic than in germ cells, Mutation Res. 244, 353-357.

Rossiter, B.J.F., Fuscoe, J.C., Muzny, D.N., Fox, M., Caskey, T. (1991) The Chinese hamster HPRT gene: Restriction map, sequence analysis and multiplex PCR deletion screening, Genomics 9, 247-256.

Runnebaum, A., Nagarajan, M., Bowman, M., Soto, D., Sukumar, S. (1991) Mutations in p53 as potential molecular markers for human breast cancer, Proc. Natl. Acad. Sci. USA 88, 10657-10661.

Russell, W.L. (1951) X-ray-induced mutations in mice. Cold Spring Harbor Symp. Quant. Biol. 16, 327-356.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G., Erlich, H.A., Arnheim, N. (1985) Enzymatic amplified ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science 230, 1350-1354.

Sandy, M.S., Chiocca, S.M., Cerutti, P.A. (1992) Genotypic analysis of mutations in Taq I restriction recognition sites by restriction fragment length polymorphism/ polymerase chain reaction, Proc. Natl. Acad. Sci. USA 89, 890-894.

Shaff, D.A., Jarret, R.A., Dlouhy, S.R., Ponniah, S., Stockelman, M., Stambrook, P.J., Tishfield, J.A. (1990) Mouse transgenes in human cells detect specific base substitutions, Proc. Natl. Acad. Sci. USA 87, 8675-8679.

Simpson, D., Crosby, R.M., Skopek, T.R. (1988) A method for specific cloning and sequencing of human HPRT cDNA for mutation analysis, Biochem. Biophis. Res. Commun. 151, 487-492.

Stewart, T.A., Pattengale, P.K., Leder, P. (1986) Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MMTV/myc fusion genes, Cell 38, 627-637.

Stout, J.T. & Caskey, C.T. (1985) HPRT: Gene structure, expression and mutation, Ann. Rev. Genet. 19, 127-148.

Tennant, R.W. B. H. Margolin, M.D. Shelby, E. Zeiger, J.K. Haseman, J. Spalding, W. Caspary, M. Resnick, S. Stasiewicz, B. Anderson, R. Minor (1987) Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays, Science 236, 933-941.

Thacker, J. (1985) The molecular nature of mutations in cultured cells: A review, Mutation Res., 150, 431-442.

Thomas, D.C., Roberts, J.D., Sabatino, R.D., Myers, T.W., Tan, C.K., Downey, K.M., So, A.G., Bambara, R.A., and Kunkel, T.A. (1991) Fidelity of mammalian DNA replication and replicative DNA polymerases, Biochemistry 30, 11751-11759.

Thilly, W.G., V.F. Lui, B.J. Brown, N.F. Cariello, A.G. Kat and P. Keohavong (1989) Direct measurement of mutational spectra in humans, Genome 31, 590-593.

Tindall, K.R. & Stankowski, L.F. (1989) Molecular analysis of spontaneous mutations at the gpt locus in Chinese hamster ovary (AS52) cells, Mutation Res. 220, 241-253.

Tompa, A., and Sapi, E. (1989) Detection of 6-thioguanine resistance in human peripheral blood lymphocytes (PBL) of industrial workers and lung cancer patients, Mutation Res. 210, 345-351.

Trainer, K.J., Wigmore, D.J., Chrysostomou, A., Dempsey, J.L. Seshadri, R., and Morley, A.A. (1984) Mutation frequency in human lymphocytes increases with age, Mech. Age. Develop. 27, 83-86.

Tsui, L. (1992) The spectrum of cystic fibrosis mutations, Trends in Genetics 8, 392-398.

Turner, D.R., A.A. Morley, M. Haliandros, R. Kutlaca, B.J., Sanderson (1985) In vivo somatic mutations in human lymphocytes frequently result from major gene alterations, Nature London 315, 343-345.

Turner, D.R., Grist, S.A., Janatipour, M., and Morley, A.A. (1988) Mutations in human lymphocytes commonly involve gene duplication and resemble those seen in cancer cells, Proc. Natl. Acad. Sci. USA. 85, 3189-3192.

Ullman, A., Jacob, F., Monod, J. (1967) Characterization of in vitro complementation of a peptide corresponding to an Operator-proximal segment of the ß-galactosidase structural gene of Escherichia coli, J. Molec. Biol. 24, 339-343.

Villarejo, M., Zamenhof, P.J., Zabin, I. (1972) ß-galactosidase and in vivo α-complementation, J. Biol. Chem. 247, 2212-2216.

Vijg, J. and A.G. Uitterlinden (1987) A search for DNA alterations in the aging mammalian genome: an experimental strategy, Mech. Ageing Dev. 41, 47-63.

Vrieling, H., Simons, J.W.I.M., van Zeeland, A.A. (1988) Nucleotide sequence determination of point mutations at the mouse HPRT locus using in vitro amplification of HPRT mRNA sequences, Mutation Res. 198, 107-113.

Vulliami, T., Mason, P., Luzzatto, L. (1992) The molecular basis of glucose-6-phosphate dehydrogenase deficiency, TIG 8, 138-143.

Weinstein, B. (1990) Mitogenesis is only one factor in carcinogenesis, Science 251, 387-388.

Winton, D.J., Blount, M.A. & Ponder, B.A.J. (1988) A clonal marker induced by mutation in mouse intestinal epithelium, Nature 333, 463-466.

Yang, J.L., Maher, V.M., and McCormick, J.J. (1987) Kinds of mutations formed when a shuttle vector containing adducts of benzo-a-pyrene-7,8-diol-9,10-epoxide replicates in COS7 cells, Molec. Cell. Biol. 7, 1267-1270.

Young, S.S. (1988) Do short-term tests predict rodent carcinogenicity? Science 241, 1232-1233.

Youssoufian, H., Antonarakis, S.E., Bell, W., Griffin, A.M., Kazazian, H.H. (1988) Nonsense and missense mutations in hemophilia A: estimate of the relative mutation rate at CG dinucleotides, Am. J. Hum. Genet. 42, 718-725.

CHAPTER 2

EFFICIENT RESCUE OF INTEGRATED SHUTTLE VECTORS FROM TRANSGENIC MICE: A MODEL FOR STUDYING MUTATIONS IN VIVO

Jan A. Gossen, Wiljo J.F. de Leeuw, Cecilia H.T. Tan, Ellen C. Zwarthoff, Frits Berends, Paul H.M. Lohman, Dick L. Knook, and Jan Vijg.

Published in the Proceedings of the National Academy of Science USA, (1989) 86, 7971-7975.

Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying mutations *in vivo*

(carcinogenesis/aging/short-term mutagenicity assay/*N*-ethyl-*N*-nitrosourea/transition mutations)

JAN A. GOSSEN*†, WILJO J. F. DE LEEUW*, CECILIA H. T. TAN*, ELLEN C. ZWARTHOFF‡, FRITS BERENDS†, PAUL H. M. LOHMAN§, DICK L. KNOOK*, AND JAN VIJG*¶

*TNO Institute for Experimental Gerontology, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands; †TNO Medical Biological Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands; ‡Department of Pathology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; and §Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7971–7975, October 1989
Genetics

Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying mutations *in vivo*

(carcinogenesis/aging/short-term mutagenicity assay/*N*-ethyl-*N*-nitrosourea/transition mutations)

JAN A. GOSSEN*†, WILJO J. F. DE LEEUW*, CECILIA H. T. TAN*, ELLEN C. ZWARTHOFF‡, FRITS BERENDS†, PAUL H. M. LOHMAN§, DICK L. KNOOK*, AND JAN VIJG*¶

*TNO Institute for Experimental Gerontology, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands; †TNO Medical Biological Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands; ‡Department of Pathology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; and §Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

Communicated by Richard B. Setlow, July 17, 1989 (received for review January 23, 1989)

ABSTRACT **To study gene mutations in different organs and tissues of an experimental animal, we produced transgenic mice harboring bacteriophage λ shuttle vectors integrated in the genome in a head-to-tail arrangement. As a target for mutagenesis, the selectable bacterial *lacZ* gene was cloned in the vector. The integrated vectors were rescued from total genomic DNA with high efficiency by *in vitro* packaging and propagation of the phages in a LacZ⁻ strain of *Escherichia coli* C. The background mutation frequencies in brain and liver DNA appeared to be low, as was indicated by the absence of colorless plaques among 138,816 and 168,160 phage isolated from brain and liver DNA, respectively. Treatment of adult female transgenic mice with *N*-ethyl-*N*-nitrosourea resulted in a dose-dependent increase of the frequency of mutated vectors isolated from brain DNA, up to 7.4×10^{-5} at 250 mg of the alkylating agent per kilogram of body weight. At this dose, in liver DNA of the same mice, mutation frequencies were $\approx 3 \times 10^{-5}$. DNA sequence analysis of four mutant vectors isolated from brain DNA indicated predominantly G·C → A·T transitions. These results demonstrate the value of this transgenic mouse model in studying gene mutations *in vivo*. In addition to its use in fundamental research, the system could be used as a sensitive, organ-specific, short-term mutagenicity assay.**

In recent years methods have been developed to study mutagenesis in mammalian cells (1). Most of these methods are limited to the *in vitro* situation and do therefore not allow comparative studies on mutation induction in various organs and tissues in the intact organism. The main difficulty in studying mutagenesis in endogenous genes of higher organisms is the lack of techniques to identify and isolate mutated genes with high efficiency. A mammalian gene that has been used extensively as a target for both *in vivo* and *in vitro* mutagenesis studies is the gene coding for hypoxanthine phosphoribosyltransferase (HPRT). Cells mutated in this gene can be selected on the basis of their resistance to 6-thioguanine. The results obtained with this method suggest that the spontaneous mutation frequency in human T lymphocytes is about 10^{-5} (2). However, the HPRT assay is laborious, is limited to one or a few cell types, and may not always reflect mutation frequency in the tissue of origin (3).

A promising category of short-term mutagenicity assays is based on the use of so-called shuttle vectors (see ref. 4 for a review). These are vectors containing a marker gene that can be introduced into mammalian cells and then retrieved ("shuttled") to bacteria in which selection takes place between mutated and nonmutated marker genes. Most of the shuttle vectors described are extrachromosomally located in the nucleus (4), although shuttle vectors that integrate in the genome also have been used (5). The latter situation has the advantage that it more closely resembles the natural environment of mammalian endogenous genes, but it has not been used on a large scale because rescue of vectors integrated in the genome was very inefficient (5).

Recently, Gossen and Vijg (6) described the use of *Escherichia coli* C as a convenient host strain for the highly efficient rescue of vectors from mammalian DNA. Here we demonstrate that the induction of gene mutations in different organs and tissues *in vivo* can be studied through a combination of shuttle vector with transgenic animal technology. We produced transgenic mice harboring in every cell of their bodies multiple copies of a bacteriophage λ vector, integrated in the genome in a head-to-tail arrangement. The shuttle vector carries the bacterial *lacZ* gene as a target for mutagenesis, allowing selection of mutated and nonmutated vectors upon retrieval to LacZ⁻ bacteria.

The applicability of these transgenic mice as an *in vivo* model for studying mutation induction was tested by treating transgenic animals with various doses of *N*-ethyl-*N*-nitrosourea (EtNU) and then determinating the mutant frequency in DNA from brain and liver. In addition, the nature of mutants isolated from brain DNA was investigated by DNA sequence analysis.

MATERIALS AND METHODS

Shuttle-Vector Construction. The complete bacterial *lacZ* gene was obtained as a 3.9-kilobase (kb) *Dra* I fragment from plasmid pMC1511 (a gift from B. Enger, TNO Medical Biological Laboratory). *Eco*RI linkers were attached and the fragment was cloned in the single *Eco*RI site of λgt10 (Promega) (Fig. 1).

Production of Transgenic Mice. Transgenic mice were produced as described by Hogan *et al.* (7). Approximately 150 copies of the monomeric λgt10LacZ vector were injected into the male pronucleus of fertilized eggs of (BALB/c × DBA/2)CD2 F_1 mice, which were then implanted into pseudopregnant BCBA mice. Positive offspring were identified by Southern blot analysis of *Eco*RI-digested tail DNA with λgt10LacZ as a probe.

DNA Isolations and Hybridization Analysis. Freshly isolated tissue was homogenized and incubated overnight at 65°C in 3 volumes of 100 mM EDTA/50 mM Tris·HCl, pH 7.5/100 mM NaCl/1% SDS containing proteinase K (Boehringer) at 200 μg/ml. After mixing with 0.2 volume of 8 M potassium acetate the solution was kept at 4°C for 30 min and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DMSO, dimethyl sulfoxide; EtNU, *N*-ethyl-*N*-nitrosourea; FIGE, field-inversion gel electrophoresis; HPRT, hypoxanthine phosphoribosyltransferase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

¶To whom reprint requests should be addressed.

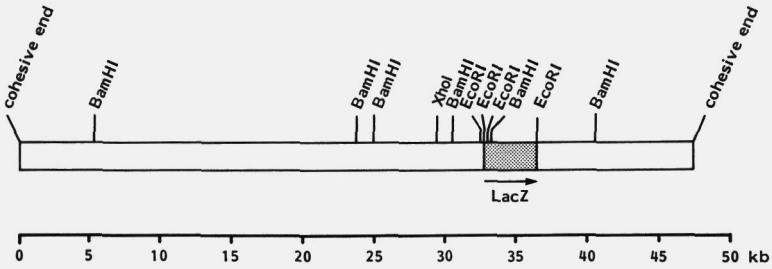


FIG. 1. The λgt10LacZ shuttle vector. Indicated are the recognition sites for the restriction enzymes *Bam*HI, *Eco*RI, and *Xho* I and the bacterial *lacZ* gene [3126 base pair (bp)] that provides the target for mutagenesis. The vector lacks a recognition site for the restriction enzyme *Xba* I.

subsequently extracted with 1 volume of chloroform. DNA was ethanol-precipitated and solubilized in 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA. All restriction enzyme digestions were performed under conditions recommended by the supplier (BRL). For Southern analysis, fragments were fractionated by agarose gel electrophoresis and subsequently transferred to GeneScreen*Plus* nylon filters (NEN). DNA dot blot analysis was performed using a microsample manifold (Bio-Rad) as described by the manufacturer. Hybridization analysis was performed in the presence of 7% (wt/vol) SDS. The probes were ^{32}P-tagged by oligonucleotide labeling (BRL) to a specific activity of 5×10^8 cpm/μg of DNA.

Purification of λgt10LacZ from Genomic DNA. High molecular weight DNA (≈100 μg) was digested with the restriction enzyme *Xba* I. Preparative field-inversion gel electrophoresis (FIGE) was performed in a vertical 1.5% agarose gel (Hoefer) in 1× TBE (89 mM Tris·HCl, pH 8.0/89 mM boric acid/1 mM EDTA) at 250 V for 4 hr at 14°C. The forward pulse time was 0.6 sec, the reverse pulse time 0.2 sec, and the ramp factor 0. A small slice of the gel was stained in a solution containing ethidium bromide at 0.1 μg/ml. The position of the λgt10LacZ concatamers in the high molecular weight area of the gel (above 50 kb and well separated from the other mouse genomic *Xba* I fragments) was established once by Southern blot analysis with the vector as a probe. On the basis of this information, in all subsequent experiments the λgt10LacZ concatamers were recovered from this part of the gel by electroelution for 16 hr at 125 V in 0.1× TBE (Bio-trap, Schleicher & Schuell). DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.6) and 2 volumes of ethanol and subsequently solubilized in 4 μl of 10 mM Tris·HCl, pH 8.0/0.1 mM EDTA.

Treatment of Transgenic Mice with EtNU. Female transgenic animals of strain 20.2 (3–6 months old) were treated with 10, 100, or 250 mg of EtNU (Sigma) per kilogram of body weight. EtNU was dissolved in dimethyl sulfoxide (DMSO) and immediately injected intraperitoneally (50 μl per animal). Animals were sacrificed 1 or 7 days after treatment.

***In vitro* Packaging and Phage Analysis.** *In vitro* packaging of purified λgt10LacZ concatamers was performed with Gigapack Gold extracts under conditions recommended by the manufacturer (Stratagene). Viable phage particles were propagated with an *E. coli* C strain (*E. coli* C LacZ$^-$) lacking the entire *lacZ* gene. The latter was found to be necessary to prevent homologous recombination between the *E. coli* C *lacZ* gene and the λgt10LacZ vectors isolated from EtNU-treated mice (unpublished results). The LacZ$^-$ *E. coli* C strain was obtained by γ irradiation of wild-type *E. coli* C (obtained from Phabagen Collection, Department of Molecular Cell Biology, University of Utrecht, The Netherlands). Wild-type *E. coli* C, incubated overnight at 37°C, was plated on 9-mm Petri dishes containing LB medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 10 g of NaCl per liter) in 1.5% agar (Difco) with isopropyl β-D-thiogalactoside (135 μg/ml; BRL) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal, 450 μg/ml; BRL). The plates were irradiated with 25 Gy, incubated overnight at 37°C, and analyzed for the presence of colorless colonies, i.e., absence of β-galactosidase (LacZ) activity. Colorless colonies were subsequently analyzed by Southern blot analysis for the absence of the *lacZ* gene (results not shown). Phage analysis was performed by plating about 20,000 phage with *E. coli* C LacZ$^-$ per 241 × 24-mm Petri dish, in the presence of isopropyl β-D-thiogalactoside (135 μg/ml), X-Gal (450 μg/ml), 10 mM $MgSO_4$ (Merck), and 0.2% maltose (Merck). After overnight incubation at 39°C, the plates were analyzed by eye for β-galactosidase activity. As a control, phage from colorless plaques were always replated. To analyze for the presence of large sequence changes in the *lacZ* gene, λ DNA from colorless plaques was isolated, digested with *Eco*RI, *Dra* I, *Hae* III, *Msp* I, *Sau*3A1, or *Hae* II, size-fractionated in an agarose gel, and subjected to Southern blot analysis using the complete *lacZ* gene as a probe. The reproducibility of this protocol was determined by the isolation and plating of vectors from the same DNA samples repeatedly in independent experiments.

DNA Sequencing. Mutant *lacZ* genes were subcloned into the *Eco*RI site of M13mp18 or M13mp19. Sequencing was performed according to Sanger *et al.* (8) as described in the M13 sequencing system manual (Pharmacia), using [α-[^{35}S]thio]dATP (Amersham) and four different oligonucleotide primers representing sequences occurring at about 600-bp intervals throughout the *lacZ* gene.

RESULTS

λgt10LacZ Transgenic Mice. The bacteriophage λ vector transferred to the mouse germ line comprised the complete *E. coli lacZ* gene (Fig. 1), which encodes the readily detectable enzyme β-galactosidase when expressed. From microinjection experiments with this λgt10LacZ vector, 23 transgenic animals were obtained, according to hybridization analysis of tail DNA. Four mice with different copy numbers of integrated vectors were selected to be bred into strains. Dot blot analysis showed that the number of vector copies that were integrated per haploid genome of transgenic mice 20.2, 34.1, 35.5, and 40.6 varied between 3 and 80 (Fig. 2).

Southern analysis of liver DNA digested with *Dra* I, *Dra* I/*Bcl* I, or *Bam*HI, with the whole vector as a probe, revealed identical restriction patterns for each transgenic animal (Fig. 3*A*). In addition, DNAs isolated from various other organs and from animals from different generations were analyzed, and all revealed identical restriction patterns of the vector (results not shown). This indicated that (*i*) no detectable rearrangements (>50 bp) had taken place during the integration process or during transmission to the progeny and (*ii*) the

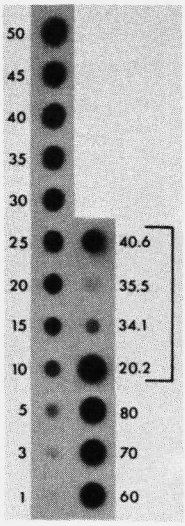


FIG. 2. Dot blot analysis of the number of copies of the λgt10LacZ vector integrated in the genome of mice. High molecular weight DNA was isolated from liver and aliquots (5 μg) were applied to a GeneScreen*Plus* filter (bracketed area). As a standard, tail DNA from a nontransgenic animal was mixed with λ DNA in proportions varying from approximately 1 to 80 copies per cell. Hybridization analysis was performed using the λgt10LacZ vector as a probe. The number of copies that are integrated per haploid genome is approximately 80 in transgenic mouse strain 20.2, 8 in strain 34.1, 3 in strain 35.5, and 35 in strain 40.6.

microinjected λ monomers were integrated in a head-to-tail arrangement in the genome. The latter was also indicated by the presence of unique hybridizing fragments, containing the λ *cos* site, after *Dra* I, *Dra* I/*Bcl* I, or *Bam*HI digestion and Southern blot analysis using a 1150-bp *Dra* I 3′ λ probe (Fig. 3*B*).

Vector Retrieval. Head-to-tail integration of three or more vectors as indicated by the results described above, without the λ cohesive ends being damaged, is a prerequisite for rescue of the vector from genomic DNA by *in vitro* packaging. Upon the linking of two intact cohesive ends, one *cos* site is formed. During packaging, the terminase enzyme present in the *in vitro* packaging extracts recognizes and cuts the *cos* sites, whereafter the excised λ DNA molecule is packaged into empty pre-heads. Treatment of chromosomal DNA of transgenic mouse 20.2.12 with the packaging extract indeed resulted in 47-kb vector monomers (results not shown), indicative of the formation of intact *cos* sites upon microinjection of λgt10LacZ monomers in fertilized mouse eggs. However, rescue of the vector by *in vitro* packaging with *E. coli* K-12 as a host was very inefficient. The most likely reason for this low efficiency appeared to be the hypermethylated state of the vectors, a phenomenon previously described for retroviral sequences introduced into the genome of mice (11), which could lead to host restriction during *in vitro* packaging or after retrieval to bacteria. Southern anal-

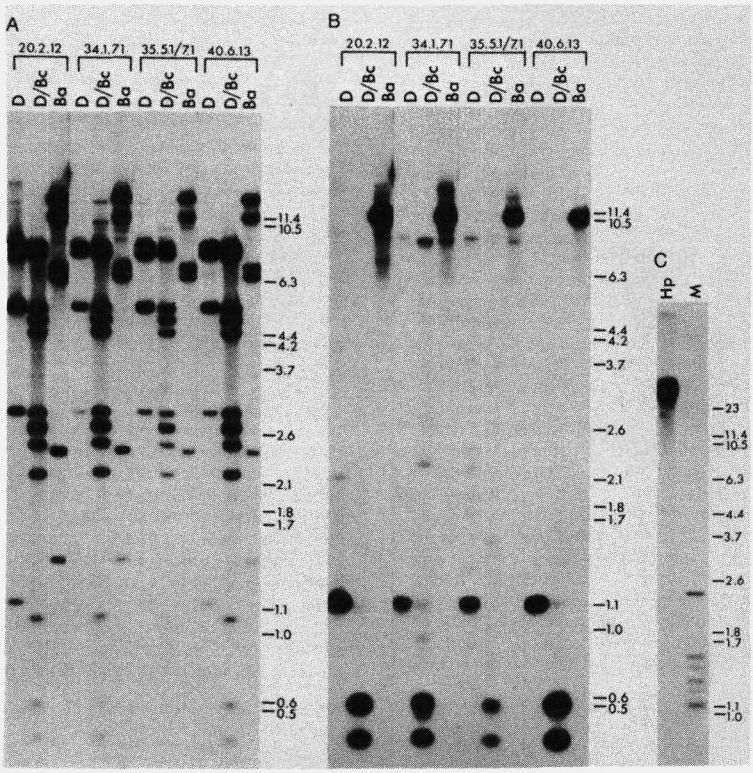


FIG. 3. (*A* and *B*) Southern analysis of *Dra* I- (lanes D), *Dra* I/*Bcl* I- (lanes D/Bc), and *Bam*HI- (lanes Ba) digested DNA from the liver of transgenic mice of strains 20.2.12, 34.1.71, 35.5.1/7.1, and 40.6.13 with λgt10LacZ as a probe (*A*) or with a 1150-bp *Dra* I 3′ λ probe (*B*). The autoradiograph shown in *B* was obtained by rehybridizing the filter that had been used to obtain the autoradiograph in *A*. The weakly hybridizing bands, visible in both autoradiographs, are either due to rearrangements near the 3′ and 5′ sites of integration (9) or due to multiple integrations in a single chromosome separated by several short mouse DNA sequences (10). (*C*) Southern analysis of *Hpa* II- (lane Hp) and *Msp* I- (lane M) digested liver DNA of transgenic mouse 35.5.7., with λgt10LacZ as a probe, to determine the methylation status of the integrated λgt10LacZ vector. Size markers (kb) are shown at the right of each autoradiograph.

ysis of liver DNA of transgenic mouse 35.5.7, after digestion with the isoschizomers *Hpa* II and *Msp* I, indicated that in the integrated vectors all CCGG sites were completely methylated (Fig. 3*C*). The same result was obtained with genomic DNAs isolated from brain and testis of this and from other transgenic mouse strains (results not shown).

Highly methylated vectors can be rescued with high efficiency by using *E. coli* C-derived packaging extracts and propagation of the phage with *E. coli* C (6). The maximum number of vectors that could be rescued from total genomic DNA of transgenic mouse 20.2.12 with the standard amount of commercially available packaging extract was about 9000 plaque-forming units per microgram of DNA (6). The amount of DNA that can be added to such *in vitro* packaging extract, however, is limited to only a few micrograms. One extract will therefore not allow the rescue of the large number of vectors necessary for mutation analysis.

To greatly increase the number of vectors that could be rescued efficiently from total genomic mouse DNA, the λgt10LacZ vector was purified from genomic DNA by preparative FIGE of *Xba* I-digested genomic DNA. Since the vector lacks a recognition site for this enzyme, *Xba* I generates large restriction fragments containing concatamers of the 47-kb vector. According to this principle, vector-containing fragments were separated from the other mouse genomic *Xba* I fragments (having a mean length of only about 5 kb) by FIGE, eluted from the gel, and packaged *in vitro*. In this way up to 1.4×10^6 vectors could be rescued in a single packaging experiment from 100 μg of *Xba* I-digested liver DNA of transgenic mouse 20.2.12.

Mutation Frequencies. By applying the purification step described above, the background mutation frequency was determined in vectors isolated from genomic brain and liver DNA. The phage were analyzed after propagation in *E. coli* C LacZ$^-$ in the presence of the chromogenic β-galactosidase indicator X-Gal. Mutants were then scored on the basis of absence of β-galactosidase activity (colorless plaques). The results indicated the absence of mutant vectors among 138,816 vectors isolated from brain DNA and 168,160 vectors isolated from liver DNA of a control transgenic mouse (Table 1). Similar results were obtained when vectors were rescued from liver and brain of mice treated with DMSO only (Table 1) or with mice from transgenic mouse strain 40.6 (results not shown). These results indicate a low background mutation frequency in these organs, which is an important prerequisite for studying induced mutagenesis.

To demonstrate the applicability of this system for testing the mutagenicity of chemicals *in vivo*, adult female transgenic mice of strain 20.2 were treated with 10, 100, or 250 mg of EtNU per kilogram of body weight. Again, DNA from brain and liver was assayed for mutations. To discriminate between mutations that arose in the mice and those that could occur (after packaging) in the bacteria, two animals treated with EtNU (100 mg/kg) were sacrificed 1 day after treatment. Vectors isolated from organs of these mice should have still contained, at this short time after treatment, EtNU-induced DNA lesions (12) that could give rise to mutations in the bacteria. All other animals were sacrificed 7 days after treatment.

The results obtained for the brains indicated a dose-dependent increase, up to 7.1×10^{-5}, of the mutation frequency in vectors isolated 7 days after treatment (Table 1). The mutation frequencies observed in vectors isolated from liver also indicated an increase of the mutation frequency; however, at an EtNU dose of 250 mg/kg, lower values were observed (Table 1). No increase in the mutation frequency was observed in brain and liver of animals sacrificed 1 day after treatment (Table 1), indicating that induced mutations detected in this system most likely originate in the mouse and not in the bacteria.

Table 1. Mutation frequencies in the λgt10LacZ shuttle vector rescued from brain and liver of control and EtNU-treated transgenic mice

Organ	Mouse*	EtNU, mg/kg	No. of phage analyzed	No. of mutants	Mutation frequency × 10^5
Brain	1a	0	138,816	0	ND
	2b	0	81,792	0	ND
	3c	100	92,608	0	ND
	4c	100	80,960	0	ND
	5d	10	198,208	3	1.5
	6d	100	167,296	5	3.0
	7d	100	204,032	7	3.4
	8d	250	197,312	13	6.7
	9d	250	67,832	5	7.4
Liver	1a	0	168,160	0	ND
	2b	0	148,464	1	0.7
	3c	100	151,232	0	ND
	4c	100	146,336	1	0.7
	5d	10	121,632	0	ND
	6d	100	141,088	5	3.5
	7d	100	95,808	3	3.1
	8d	250	137,760	3	2.1
	9d	250	141,488	2	1.4

ND, not detectable.
*Each entry represents data on a single animal that was untreated (mouse 1a), treated with DMSO only (2b), sacrified 1 day after treatment (3c and 4c), or sacrificed 7 days after treatment (5d–9d).

Mutant Characterization. A total of 49 mutant phage were isolated from brains and livers of animals treated with EtNU. Because of the large size of the target gene (3126 bp), determination of the kind of mutations induced by EtNU was initially performed by restriction enzyme analysis. Southern blot analysis of λ DNAs digested with *Eco*RI, *Dra* I, *Hae* III, *Msp* I, *Sau*3A1, or *Hae* II, with the *lacZ* gene as a probe, revealed no detectable size changes. Therefore, the mutants represent base-pair substitutions or small deletions or insertions. This was confirmed by DNA sequence analysis of four mutant *lacZ* genes isolated from brain DNA, indicating G·C → A·T transitions in all cases, resulting in the formation of a stop codon in the 5′ part of the *lacZ* gene (Table 2).

DISCUSSION

Study of either spontaneous or induced mutations in various organs and tissues of experimental animals has been precluded by the difficulty in rescuing and identifying mutated mammalian genes with high efficiency. The results obtained with the transgenic mouse model presented in this article demonstrate that it is possible to quantitate and characterize such mutations in genomic DNA by using an integrated bacteriophage λ vector in combination with *E. coli* C as a host strain.

Transfer of the shuttle vector to the mouse genome by microinjection of fertilized eggs resulted in its intact integration, without detectable deletions or rearrangements. In

Table 2. DNA sequence analysis of mutant *lacZ* genes isolated from brain DNA of EtNU-treated mice

Mutant	EtNU, mg/kg	Sequence alteration	Position	Target sequence
1	10	C·G → T·A	505	CGGCTAGGA
2	100	G·C → A·T	203	GCCTAGTTT
3	100	G·C → A·T	900	CTGTTGAGC
4	100	C·G → T·A	1072	GATTTGAGC

For each *lacZ* mutant a 9-bp region is listed, in which the mutated base is underlined.

addition, rescue of the shuttle vector from genomic DNA, isolated from different organs and tissues of (untreated) transgenic mice indicated a very low background mutation frequency, which is an important prerequisite for studying induced mutagenesis at low doses of a mutagen. The observed low background mutation frequency indicates a low spontaneous mutation frequency *in vivo*, which is in agreement with results described by others using the HPRT assay with human T cells *in vivo* (2) or using integrated bacteriophage λ vectors in cell lines (5). However, in contrast to the transgenic mouse system, these systems do not allow the determination of spontaneous mutation frequencies in different organs and tissues, for example, in relation to tumor susceptibility and aging (13). In addition, if integration of the microinjected vectors occurs at random, the influence of genomic site on the mutation frequency can be investigated by using different transgenic mouse strains. Preliminary results indicate that the spontaneous mutation frequency in transgenic mouse strain 35.5 is much higher (about 10×10^{-5}) than in the other three strains, for which mutants could not be detected among ≈150,000 plaques. This indicates that the particular site of integration in strain 35.5 is more sensitive for mutagenesis.

To test the ability of the system to detect induced mutations in various organs and tissues, transgenic mice were treated with various doses of EtNU. The results indicated for liver and brain a dose-dependent increase of the frequency of colorless plaques, up to 7.4×10^{-5} for the brain. The sensitivity of the system is illustrated by the fact that colorless plaques were obtained even at the lowest EtNU dose (10 mg/kg). At the higher doses of EtNU (100 and 250 mg/kg), the interanimal variation in induced mutation frequency is very small (Table 1), which underlines the applicability of this system to detect organ-specific mutation induction. However, with intraperitoneally injected compounds such as EtNU, differences between organs can be due to a number of factors, such as transport via the lymph of blood, uptake by the various organs and tissues, or toxicity to surrounding organs after administration of high doses of a mutagen. Such toxicity could be the reason for the observed decrease of the mutation frequency in liver between 100 and 250 mg of EtNU per kilogram of body weight.

Determination of the exact nature of EtNU-induced mutations by DNA sequence analysis showed that the predominant mutation observed was a G·C → A·T transition. This is in agreement with results of previous studies on the mutagenicity of EtNU, both in bacteria and in mammalian cells (14, 15). However, it has been suggested that base-pair substitutions are the result of miscoding during DNA replication as a consequence of the presence of O^6-ethylguanine, the main adduct induced by EtNU. Since in our case the mutant vectors were isolated from postmitotic brain tissue of adult transgenic mice, DNA replication was probably not involved in the induction of base-pair substitutions. Instead, they may have resulted from an error-prone DNA-repair process. Interestingly, DNA sequence analysis also revealed that the mutated base (either guanine or cytosine) was always flanked on the 5′ side by a thymine and on the 3′ side by a guanine (Table 2). This observation suggests that at particular sites, neighboring bases influence either the susceptibility to DNA damage or the repairability of an EtNU-induced lesion. Characterization of a large number of mutants may provide clues as to how mutations are induced in brain or other postmitotic organs. In general, induction of DNA damage, repair, mutagenesis, and carcinogenesis can now be studied in one animal model system. This may provide insight into the chain of events that leads from DNA damage and DNA repair via mutations to biological end points such as aging and carcinogenesis.

In summary, we anticipate that the λgt10LacZ transgenic mice described in this paper will be useful as an *in vivo* model for studying spontaneous mutation frequencies and the relation of DNA damage and repair to mutagenesis, carcinogenesis, and aging in different organs and tissues. A major application of the system could be in the field of genetic toxicology testing; its capacity to demonstrate mutagenicity of carcinogenic compounds could make it a suitable short-term mutagenicity assay, in particular with respect to organ specificity. As such, the system might fulfill a longstanding deficiency in *in vivo* mutagenicity systems (16) and could be used in addition to methods presently available in genetic toxicology testing (e.g., the Ames test, tests for chromosome aberrations or sister chromatid exchanges, and the mouse lymphoma test), which have only a limited sensitivity and specificity even when a combination of assays is used (17).

We thank Drs. R. Brown, P. van der Putte, and A. G. Uitterlinden for helpful discussions during this study, Drs. R. B. Setlow and G. R. Douglas for critically reading the manuscript, and Mr. E. J. van der Reyden and Mr. A. A. Glaudemans for preparation of the photographs. Part of this work was supported by the Stuurgroep Onderzoek op het terrein van de Ouder wordende Mens (SOOM). This work is the subject of a patent application.

1. Lehman, A. R. (1985) *Mutat. Res.* **150**, 61–67.
2. Albertini, R. J., O'Neill, J. P., Nicklas, J. A., Heintz, N. H. & Kelleher, P. C. (1985) *Nature (London)* **316**, 369–371.
3. Featherstone, T., Marshall, P. D. & Evans, H. J. (1987) *Mutat. Res.* **179**, 215–230.
4. DuBridge, R. B. & Calos, M. P. (1988) *Mutagenesis* **3**, 1–9.
5. Glazer, P. M., Sarkar, S. N. & Summers, W. C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1041–1044.
6. Gossen, J. A. & Vijg, J. (1988) *Nucleic Acids Res.* **16**, 9343.
7. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
8. Sanger, E., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **80**, 5463–5467.
9. Palmiter, R. D. & Brinster, R. L. (1986) *Annu. Rev. Genet.* **20**, 465–499.
10. Michalova, K., Bucchini, D., Ripoche, M., Pictet, P. & Jami, J. (1988) *Hum. Genet.* **80**, 247–252.
11. Jahner, D. & Jaenisch, R. (1985) *Mol. Cell. Biol.* **5**, 2212–2220.
12. Frei, F. V., Swenson, D. H., Warren, W. & Lawley, P. D. (1978) *Biochem. J.* **174**, 1031–1044.
13. Vijg, J. & Uitterlinden, A. G. (1987) *Mech. Ageing Dev.* **41**, 47–63.
14. Burns, P. A., Gordon, A. J. E., Kunsman, K. & Glickman, B. W. (1988) *Cancer Res.* **48**, 4455–4458.
15. Drinkwater, N. R. & Klinedinst, D. R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3402–3406.
16. Minister of National Health and Welfare and Minister of the Environment (1988) *Environ. Mutagenesis* **11**, 261–304.
17. Tennant, R. W., Margolin, B. H., Shelby, M. D., Zeiger, E., Haseman, J. K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B. & Minor, R. (1987) *Science* **236**, 933–944.

CHAPTER 3

INVESTIGATION OF IN VIVO MUTAGENESIS AT THE LACZ TRANSGENE LOCUS IN MUTAMOUSE

Brian C. Myhr, Jan A. Gossen, Alfred J.W. Hoorn, David J. Brusick, Paul H.M. Lohman, and Jan Vijg.

3.1 Introduction

The use of transgenic mice to detect mutations in a recoverable gene, stably integrated within the mouse genome, has been described recently (Gossen et al., 1989; Kohler et al., 1990; Kohler et al., 1991; Myhr and Brusick, 1991; Myhr, 1991). Of particular interest to this laboratory is the λgt10-lacZ model initially described by Gossen et al. (1989). Several mouse strains with different transgene copy numbers and placement within the genome were constructed. The bacterial lacZ gene, cloned within the lambda vector, serves as the target for detecting mutagenesis. This gene is rescued with reasonably high efficiency from the mouse DNA by *in vitro* packaging and propagation of the phage on E. coli C $lacZ^-$. One of the mouse strains (20.2) was used to demonstrate the induction of lacZ mutations in brain and liver tissues of female animals exposed to N-ethyl-N-nitrosourea (ENU).

By allowing an investigator to assess induced mutations in any tissue, transgenic mouse models clearly have potential to significantly alter the ways in which genotoxicity and risk assessment are investigated. To explore this potential, transgenic mouse strain 40.6 (Gossen et al., 1989) was acquired by Hazleton Research Products, Inc., Denver, PA, to establish a consistent source of animals. These animals, designated Muta™Mouse, were investigated for responses to known mutagenic chemicals.

The purpose of this paper is to describe the results obtained in several tissues after exposures of animals to different mutagens. These results provide insight regarding the experimental parameters that will be important to the quantitation of *in vivo* mutagenesis in future studies. In addition, a practical assay method is described for the efficient recovery of lacZ genes from mouse DNA and the measurement of the frequency of $lacZ^-$ genes.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals used in this study were of reagent grade or better. N-ethyl-N-nitrosourea (ENU, CAS No. 759-73-9), chlorambucil (CHL, CAS No. 305-03-3), cyclophosphamide monohydrate (CP, CAS No. 6055-19-2), and acrylamide (AA, CAS No. 79-06-1) were purchased from Sigma Chemical Co., St. Louis, MO. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG, CAS No. 70-25-7) and 9,10-dimethylbenz(a)anthracene (DMBA, CAS No. 57-97-6) were obtained from Aldrich Chemical Co., Milwaukee, WI. Procarbazine hydrochloride (PRO, CAS No. 366-70-1) was kindly provided by the U.S. National Toxicology Program chemical repository.

3.2.2 Transgenic mice

The construction and characterization of the lacZ transgenic mouse, Muta™Mouse, was described by Gossen et al. (1989). Briefly, the mice were produced by microinjection of fertilized eggs of (BALB/c x DBA/2) mice using monomeric copies of λgt10lacZ DNA. After the identification of transgenic offspring and subsequent breeding to homozygosity, several mouse strains containing different transgene copy numbers and chromosomal locations were obtained. Strain 40.6 contained at least 35 copies at one autosomal site per haploid genome and was selected for these studies. The mice, homozygous for the lacZ transgene, were bred as an SPF colony at the TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands, and were used for the i.p. dosing experiments. The same animal colony was acquired by Hazleton Research Products, Inc., Denver, PA, to provide a commercial source of transgenic mice designated as Muta™Mouse (CD_2-LacZ80/HazfBR); animals from this source were used for the skin-painting experiments. All ani-

mals were grouped randomly in polycarbonate cages with hardwood chips and kept on a 12-hour light/dark cycle. Rodent chow and water were provided at liberty.

3.2.3 Animal Dosing and Tissue Collection

Chemical dosing solutions were prepared fresh just before use. ENU was dissolved in dimethylsulfoxide (DMSO). Phosphate-buffered saline (PBS) was the solvent for CP, AA and PRO. CHL was dissolved in 70% ethanol in PBS and then diluted with PBS to give an ethanol concentration of 7% by volume; a fine, milky suspension formed at a CHL concentration of 0.75 mg/ml. For the skin painting, MNNG and DMBA were dissolved in Analar grade acetone (Aldrich).

The i.p. dosing regimen was either a single injection (acute) or injections of equal amounts on 5 successive days (subchronic). The dosing volumes were based on individual animal weights and were approximately 0.1 ml/animal for ENU in DMSO, 0.2 ml/animal for the chemicals in PBS, and 0.9 ml/animal for CHL in 7% ethanol/PBS. Only male animals 8-10 weeks of age were used (typical weight of 25 gm). Animals were sacrificed by cervical dislocation at the indicated times after the last treatment. The desired organs were immediately dissected and frozen in liquid nitrogen. Bone marrow from the tibia and femur of both hind legs was flushed into a tube by injecting PBS into the marrow from a 25G needle fitted to a syringe. The collected marrow was centrifuged, the supernatant discarded, and the marrow cell pellet frozen in liquid nitrogen. After freezing, the tissues were stored at -70°C until DNA was extracted for analysis.

For the skin painting studies, the lower dorsal areas of 9-10 week-old animals were shaved with electric clippers 2 days prior to dosing. Any animals with nicked skin were excluded from treatment. Each animal was dosed with 0.2 ml of acetone (controls) or test chemical solution in acetone, using a micropipet to deliver incremental amounts as evenly as possible over approximately 10 cm^2 of shaved skin. After

dosing, the animals were housed individually. The animals were sacrificed by CO_2 inhalation 7 days later. The dosed skin areas were excised and frozen immediately in liquid nitrogen, then stored at -70°C until analyzed.

3.2.4 DNA Isolation

Genomic DNA was isolated from frozen tissues with only slight modifications for each tissue type. Liver cell nuclei were prepared by gentle homogenization of approximately one-half the liver in 10 ml ice-cold TMS-100 (50 mM Tris.HCl, pH 7.6, 3 mM Mg-acetate, 250 mM sucrose, 0.2% Triton X-100) in a Potter-Elvehjem-type homogenizer fitted with a Teflon pestle. Cell debris was removed by filtering the homogenate through a double layer of cheese-cloth. Cell nuclei were collected by centrifugation, washed twice with 10 ml aliquots of ice-cold TMS-100, then resuspended in 10 ml of lysis buffer (10 mM Tris.HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA).

One testis was similarly homogenized per 10 ml ice-cold lysis buffer. A bone marrow pellet was thawed and resuspended in a small volume of ice-cold lysis buffer, then further diluted to 5 ml. Frozen skin (approximately 400 mg) was simply minced with scissors and suspended in 10 ml lysis buffer.

Tissue digestion was subsequently performed in lysis buffer supplemented with 0.9% sodium dodecylsulfate and 900 µg/ml proteinase K (Boehringer). This mixture was incubated for 2-3 hours at 50°C in a shaking waterbath or overnight at 37°C. The tissue digests were carefully but thoroughly mixed with an equal volume of equilibrated phenol:chloroform (1:1) solution, pH 8 (Sambrook et al., 1989; or product 0883, Amresco Inc., Solon, OH). For bone marrow, it is important to mix for 10-20 minutes in tubes slowly rotated end-to-end on a wheel mixer. The aqueous phase was separated by centrifugation (1500 xg for 15 min), transferred to a clean tube, mixed with one-fifth volume of 8 M potassium acetate, then extracted with one volume of chloroform. After phase separation at 2500 xg for 30 minutes at 4°C, the upper aqueous phase was transferred

to a clean polypropylene tube. The DNA was precipitated by adding 2 to 2.5 volumes of ice-cold ethanol. The collected DNA was washed with cold ethanol and dissolved in TE-4 buffer (10 mM Tris.HCl, pH 8, 4 mM EDTA). The DNA solution was stored at 4°C, and the concentration was determined by absorbance at 260 nm, using an absorbance of 1.0 as equivalent to 50 μg/ml. High molecular weight was confirmed by electrophoresis of each DNA sample in 0.7% agarose.

3.2.5 Transgene Recovery

The λgt10lacZ sequences were recovered from the mouse genomic DNA by *in vitro* packaging reactions with Gigapack II Gold extracts (Stratagene, La Jolla, CA). Approximately 5 to 7.5 μg of genomic DNA in 5 μl were added to each reaction mixture. After incubation at 37°C for 3 hours, the reaction was terminated by the addition of 0.5-1.0 ml SM buffer (50 mM Tris.HCl, pH 7.5, 10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin) and stored at 4°C. Viable lambda phage particles were titered with E. coli C ($lacZ^-$) (Gossen and Vijg, 1988; Gossen et al., 1989). Several packaging reactions were used to obtain a large number of phage per mouse DNA sample. Typical packaging efficiencies were 10,000 to 25,000 pfu/μg DNA.

3.2.6 Mutant Frequency Determination

A phage titer expected to produce 2000 to 4000 pfu was adsorbed to 2.5 x 10^8 E. coli C ($lacZ^-$), then plated in 9 ml of 0.75% agarose in Luria-Bertani (LB) medium containing 10 mM $MgSO_4$, 0.2% maltose, and 0.45 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal, Boehringer). This mixture was poured onto 150 mm culture plates already containing 15 ml of bottom agar (1.5% agar in LB). After incubation overnight at 37°C, the blue plaques were enumerated by counting one-tenth areas on several representative plates and multiplying by 10 and the

total number of plates. The blue plaques represented the total population of $lacZ^+$ genes analyzed. Clear or light blue plaques represented presumptive $lacZ^-$ mutant genes. All of these plaques were picked, resuspended in SM buffer, and spotted onto X-gal/LB/agar plates to check for phenotypic stability after overnight incubation at 37°C. These phage isolates were scored as mutant if colorless or light blue plaques were obtained; an all blue response indicated no mutation.

The mutant frequency (MF) for a mouse DNA sample was defined as the ratio of confirmed mutant plaques to the total blue plaques analyzed. The results from several packaging reactions and phage platings were combined for the analysis of each DNA sample.

The phage plating procedure evolved during this study so that the above description holds for the skin painting and tissue negative control data. The i.p. dosing studies used 1000 to 20,000 phage in large 245 x 245 mm plates, and isopropyl β-D-thiogalactoside (135 μg/ml) was included in the agarose medium. This inducing agent was found to be unnecessary for color development; also the smaller 150 mm culture plates are much less costly and more convenient for handling and the scoring of mutants. More recently, the bottom agar has been eliminated; now a 15 ml volume of phage/bacteria/X-gal/LB agarose is poured per 150 mm plate, and the X-gal concentration has been reduced to 0.35 mg/ml.

3.2.7 DNA Sequencing

Mutant lacZ genes were isolated from λgt10lacZ vectors by EcoRI digestion and subsequent cloning into the M13mp18 sequencing vector. After isolation of single-stranded template DNA, sequencing reactions were performed using Taq polymerase, dideoxy nucleotides (Applied Biosystems, Ramsey, NJ) and 10 lacZ primers, located at approximately 300 bp intervals along the lacZ gene. Samples were analyzed using an automated DNA sequencer (Applied Biosystems).

3.2.8 Screening for Deletion Mutants

To screen for the presence of deletions (or insertions) larger than 15 bp, mutant lacZ genes were amplified by means of PCR directly from mutant λgt10lacZ phage, using the λgt10 specific primers, LD3 and LD4, located near the unique EcoRI site. Reactions were performed in a 50 μl volume containing 2 mM dATP, dGTP, dCTP, and dTTP (Pharmacia), 0.3 μg of primers, LD3 and LD4, 1 unit of Taq polymerase (BRL), and reaction buffer [50 mM KCl/10 mM Tris.HCl, pH 8.3/0.25 mM $MgCl_2$/20 μg/ml bovine serum albumin]. The samples were subjected to repeated cycles of 2 min at 95°C, 2 min at 50°C, and 4 min at 72°C, by using a Bio-med thermocycler. PCR products were subsequently digested with the restriction enzymes, BglI and RsaI, which generated eight fragments of 586, 557, 505, 479, 360, 342, 312 and 187 bp in size. The samples were analyzed by agarose gel electrophoresis using 3% Nusieve agarose (FMC). As controls, non-mutant phage and 2 mutant phage containing deletions of 21 and 1021 bp were used.

3.3 Results

3.3.1 Mutant Phage Detection

The efficiency of visual detection of a few mutant lacZ phage plaques among a large number of blue plaques, produced by $lacZ^+$ phage, was investigated. A constant titer of $lacZ^-$ phage was mixed with increasing numbers of $lacZ^+$ phage, adsorbed to the bacteria, and poured with X-gal/agarose into 150 mm culture plates. Several experiments were performed in which the $lacZ^-$ titer varied from 8 to 70 pfu per plate and the wildtype $lacZ^+$ titer was increased from 0 to 3700 pfu per plate. The results obtained (data not shown), indicate that the apparent titer of $lacZ^-$ phage decreased as the plating density of $lacZ^+$ phage was increased. Considerable variability was obtained between experiments, but on average, the visual detection of $lacZ^-$ plaques was 70% efficient at 1500 $lacZ^+$ plaques/plate and 25% at 3700 plaques/plate. Experiments performed with and without a bottom layer of LB/agar medium gave essentially the same results. A plating density of 1500 pfu/plate was considered practical for the analysis of $lacZ^-$ mutations in phage prepared from MutaMouse DNA samples.

In the absence of bottom agar, the visual clarity of the agarose medium containing the plaques is considerably improved, allowing rapid visual detection of the clear or light blue plaques. The results show (data not shown) no loss in frequency of $lacZ^-$ plaques caused by the elimination of the bottom agar layer. The total pfu/plate was somewhat reduced, however, to approximately 80% of pfu in plates containing bottom agar. In either case, the percentage of $lacZ^-$ plaques appearing as light blue increased from 10% to 100% as the density of $lacZ^+$ plaques was increased from 500 to 3000 pfu/plate. These light blue plaques were picked and confirmed as clear plaques when spotted onto X-gal/agarose plates.

Table 1. Frequency of LacZ⁻ genes in tissues from control animals

Tissue	Animal	LacZ⁺ Plaques Scored	LacZ⁻ Mutants	Average MF x10⁶
Skin	1	1,059,759	29	27.4
	2	1,013,281	28	27.6
	3	1,062,595	37	34.8
	4	1,092,815	32	29.3
	5	1,156,650	24	20.7
	6 [a]	1,204,389	28	23.2
	7 [a]	1,050,676	34	32.4
				MF ± S.D. = (27.9 ± 4.9) x 10^{-6}
bone marrow	1	1,049,068	29	27.6
	2	1,161,165	43	37.0
	3	1,011,951	47	46.4
	4	1,078,767	18	16.7
	5	1,023,630	23	22.5
	6	857,044	102	119.0
	7	926,204	12	13.0
				MF ± S.D. = (40 ± 37) x 10^{-6}
Liver	1	689,286	14	20.3
	2	643,523	28	43.5
	3	864,963	29	33.5
	4	474,636	10	21.1
	5	613,132	21	34.3
				MF ± S.D. (31 ± 10) x 10^{-6}
Testis	1	290,339	1	3.4
	2	255,875	2	7.8
	3	77,184	1	13.0
	4	70,160	1	14.0
				MF ± S.D. = (7 ± 5) x 10^{-6}

Male animals, 8-10 weeks
[a] Animals exposed to 0.2 ml acetone per 10 cm^2 skin area.

3.3.2 Spontaneous Mutant Frequencies

Data were collected on the frequency of lacZ⁻ mutations in male, control animals, 8-10 weeks of age. Skin, bone marrow, and liver tissues from different animals were analyzed (Table 1). The largest data base was obtained for skin, in which each

of 7 animals was analyzed to approximately 1 x 10^6 lacZ genes. The average mutant frequency (MF) of lacZ$^-$ genes in skin was 27.9 x 10^{-6}, and the observed variation between animals was small (standard deviation of 4.9 x 10^{-6}).

Very similar average MF values were obtained for bone marrow and liver. The sample sizes and/or numbers of animals analyzed were smaller than for skin, so less is known about animal-to-animal variability. The liver MF appeared to vary little between animals, whereas a wide range of MF values was apparently found among the bone marrows of different animals (7 to 116 x 10^{-6}). In testes, the spontaneous MF remained uniformly low among the 4 animals (7 x 10^{-6}) even though low numbers of plaques were analyzed.

3.3.3 Mutagenesis with ENU

The lacZ$^-$ mutant frequencies induced by i.p. treatments with ENU are shown in Table 2. Both an acute and subchronic (5 day) dosing regimen were compared, and several tissues were harvested 3, 7, and 10 days after the last treatment. The high dose of 250 mg ENU/kg did not cause deaths or visible toxicity, but later studies on peripheral blood indicated this dose caused considerable depression of bone marrow (T. Suzuki, personal communication).

After treatment with ENU, dose-related increases in MF were observed in bone marrow, liver, and testes, which were the only tissues analyzed from these animals. The largest response occurred in the bone marrow, where subchronic dosing at 50 mg/kg x 5 induced a MF of 1016 x 10^{-6}, representing a 40-fold increase over the control bone marrow MF of 25 x 10^{-6}. Subchronic dosing was much more effective in causing bone marrow mutations relative to an equivalent total dose given once. Thus, 250 mg ENU/kg induced a MF of 293 x 10^{-6} at 10 days after treatment compared to the MF of 1016 x 10^{-6} obtained for the subchronic 50 mg ENU/kg x 5 treatment. Also, the harvest time was clearly an important variable in determining the

magnitude of the induced MF. With subchronic dosing, the bone marrow MF clearly showed a progressive increase in MF as the harvest time was increased from 3 days to 10 days (data not shown). Longer harvest times were not investigated. With acute dosing at 250 mg ENU/kg, the MF also increased with the harvest time (data not shown). However, for the less toxic acute dose of 100 mg ENU/kg, a sharp peak in MF appeared to occur at 7 days. Both animals showed this response (12 $lacZ^-$/10,528 $lacZ^+$ and 11 $lacZ^-$/26,848 $lacZ^+$).

The liver mutation response to subchronic dosing with ENU was about one-fifth the bone marrow, and essentially equivalent results were obtained for both methods of dosing (Table 2). The sampling sizes were too small at these lower MF values to clearly resolve the effect of harvest time.

Table 2. Induction of LacZ⁻ mutants by intraperitoneal dosing with ENU

Treatment Group	Harvest Time (Days)	bone marrow			liver			testis		
		$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6	$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6	$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6
Control[a]	-	237,850	6	25	121,024	1	8	546,214	3	6
SUBCHRONIC:										
20 mg/kg	3	46,368	5	108	113,840	16	141	115,464	2	17
50 mg/kg	3	39,600	15	379	75,840	5	66	73,920	4	54
20 mg/kg	7	30,656	4	130	98,640	12	122	267,239	10	37
50 mg/kg	7	96,128	60	624	105,040	24	228	124,800	7	56
20 mg/kg[b]	10	36,800	20	523	45,440	4	88	155,632	2	13
50 mg/kg	10	46,240	47	1016	108,240	22	203	136,928	3	22
ACUTE:										
100 mg/kg	3	266,099	16	60	132,400	13	98	326,712	3	9
250 mg/kg	3	127,448	11	86	278,160	16	58	121,355	5	41
100 mg/kg	7	37,376	23	615	95,120	12	126	240,288[a]	4	17
250 mg/kg	7	214,032[a]	35	164	99,136	6	61	40,064	7	175
100 mg/kg	10	125,948	11	87	91,680	3	33	122,014	5	41
250 mg/kg	10	157,152	46	293	81,840	20	244	183,280	3	16

Control animals received 0.1 ml DMSO i.p.
Plaque data are pooled from two animals, except as noted: [a]Three animals, [b]One animal.

The high dose of ENU appeared to induce a MF that increased with harvest time, whereas the low dose may have yielded an earlier response that was returning to the background MF at 10 days.

The testes responses were small and barely detectable for the sampling sizes ($lacZ^+$ plaques) scored. An isolated, large increase in MF (175 x 10^{-6}) was obtained for an acute treatment with 250 mg ENU/kg, testes harvested at 7 days, which represented a 32-fold increase over background (Table 2). However the calculated MF could easily drop as the sample size is increased above 40,064 $lacZ^+$ plaques. Fluctuations in MF for the different harvest times probably represented sampling effects rather than any discernible effect of time after treatment. If all treatment groups are pooled, a total of 55 $lacZ^-$ mutants among 1,907,696 $lacZ^+$ plaques is obtained, giving a MF of 29 x 10^{-6} or a 5-fold increase over the spontaneous MF for testes.

3.3.4 Mutagenesis with CHL

The mutagenic effects of single, i.p. doses of CHL were investigated. As shown in Table 3, a dose of 10 mg/kg induced small increases in MF in bone marrow, liver and testes. Harvest times of 3 to 21 days were used, but no trend in response was observable with the small sample sizes used. If data from all harvest times are combined, approximately one million plaques were analyzed for each tissue, and the mutant frequencies were 60 x 10^{-6}, 62 x 10^{-6}, and 81 x 10^{-6} for bone marrow, liver, and testes, respectively. Interestingly, one animal appeared to be a high responder for liver and testes, but not for bone marrow. This animal was observed at 14 days after treatment and yielded a MF of 523 x 10^{-6} in the liver (10 $lacZ^-$/19,120 $lacZ^+$) and a MF of 743 x 10^{-6} in the testes (55 $lacZ^-$/74,000 $lacZ^+$).

The high dose of 25 mg CHL/kg was selected from a prestudy with CD-1 mice, all of which survived this dose. Unex-

pectedly, exactly one-half of the exposed MutaMice died. The mutagenic effects of this highly toxic dose in three analyzed tissues were essentially equivalent to those obtained at 10 mg CHL/kg.

3.3.5 Other chemical mutagens

Three additional chemical mutagens, PRO, CP and ACR, were screened for mutagenic activity in bone marrow, using the subchronic i.p. dosing regimen, followed by harvest times of 3,7 and 10 days (Table 4). One high, but nonlethal, dose level was used for each chemical. PRO at 200 mg/kg x 5 was mutagenic as observed earlier for ENU, but the time course of the response was reversed. Thus, instead of an increasing MF with longer harvest times, the response was maximal at 3 days and declined thereafter. CP at 100 mg/kg x 5 was considerably less mutagenic, and the time course seemed to parallel that of PRO; at 10 days, however, mutagenesis was not detectable. ACR at 50 mg/kg x 5 induced a weak response that remained constant over the 3-10 day harvest period. The average response was a MF of 75 x 10^{-6} (29 $lacZ^-$/388,400 $lacZ^+$), which was 5 times the concurrent control MF and 2 times the average historical MF for bone marrow (Table 1).

Table 3. Induction of LacZ⁻ mutants by acute intraperitoneal dosing with Chlorambucil

Treatment Group	Harvest Time (Days)	bone marrow			liver			testis		
		$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6	$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6	$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6
Control	- -	156,080[a]	5	32	93,120	2	22	147,344	2	14
10 mg/kg	3	103,696	11	106	79,008	5	63	128,304	3	23
10 mg/kg	7	162,880	9	55	161,760	4	25	119,588	8	67
10 mg/kg	10	123,936	7	56	70,448	3	43	109,792	7	64
10 mg/kg	14	123,360	7	57	73,680	16	217	180,160	57	316
10 mg/kg	21	199,824	7	35	97,800	5	51	131,600	7	53
25 mg/kg[a]	3	112,560	8	71	114,224	2	18	254,512	6	24
25 mg/kg	7	173,760	10	58	89,408	6	67	172,640	2	12
25 mg/kg	10	117,760	8	68	107,768	8	74	75,360	5	66

Control animals received 0.9 ml 7% ethanol in PBS i.p.

Plaque data are pooled from two animals, except as noted: [a]Three animals

[a] i.p. LD50 dose

Table 4. Induction of LacZ⁻ mutants in bone marrow by subchronic intraperitoneal dosing with chemical mutagens

Treatment Group	Harvest Time (Days)	bone marrow		
		$LacZ^+$ Plaques Scored	$LacZ^-$ Mutants	Average MF x 10^6
Control	- - -	194,080	3	15
Procarbazine 200 mg/kg x 5	3	95,120	130	1367
	7	116,560	90	772
	10	112,704	58	515
Cyclophosphamide 100 mg/kg x 5	3	125,744	12	95
	7	97,600	8	82
	10	161,520	3	19
Acrylamide 50 mg/kg x 5	3	129,040	8	62
	7	124,240	11	89
	10	135,120	10	74

Control animal received 0.2 ml PBS i.p.
Plaque data are pooled from two animals.

3.3.6 Skin mutation studies

The possible induction of mutations in skin by the topical application of mutagenic chemical was investigated with several dose levels of both MNNG and DMBA. The sample size analyzed per animal was increased to 0.5 to 1 x 10^6 lacZ genes to reduce the statistical fluctuation in the MF calculation. Seven days after a single application of either MNNG or DMBA, dose-related increases in MF were clearly observable (Table 5).

MNNG at 100 μg per animal was not detectably mutagenic, but an average 5-fold increase in MF was induced by the 300 μg treatment. An increase in dose to 600 μg MNNG/animal had no further effect. Two-fold variations in MF occurred between the two animals at each dose level. With DMBA, which requires metabolic activation to react with DNA, treatment with 4 μg/animal was not mutagenic, but the 10 μg dose induced a 2-

fold increase in MF. A maximum average MF of 101 x 10^{-6} was obtained at 40 μg DMBA/animal, and no further increase in MF was obtained by increasing the dose to 100 μg. A large variation in response between the two animals exposed to 40 μg DMBA was noted; one animal barely responded, while the other exhibited a high MF of 166 x 10^{-6}.

Table 5. Induction of LacZ⁻ mutants in skin by single topical applications of chemical mutagens

Treatment Group	Animal	skin		
		LacZ⁺ Plaques Scored	LacZ⁻ Mutants	Average MF x 10^6
Control	1	1,050,676	34	28
	2	1,204,389	28	
MNNG				
100 μg	1	1,012,735	23	23
300 μg	1	488,452	102	146
	2	539,330	48	
600 μg	1	764,365	69	150
	2	1,591,917	285	
DMBA				
4 μg	1	514,290	14	27
	2	427,816	11	
10 μg	1	1,009,656	49	55
	2	1,006,832	62	
40 μg	1	522,684	19	101
	2	519,484	86	
100 μg	1	1,334,291	133	83
	2	938,083	55	

Control animals received 0.2 ml acetone on approximately 10 cm^2 skin area.
Harvest time = 7 days

3.3.7 LacZ⁻ mutant sequencing

Phage isolated from six, independent, colorless plaques were sequenced in the lacZ gene in order to demonstrate the

existence of mutations and provide some information on the nature of the molecular changes. The phage were selected from bone marrow samples exposed to PRO because the very high mutant frequency would make it likely that the selected phage would represent induced mutations rather than spontaneous ones.

The results of the sequence analysis are shown in Table 6. All of the six phage isolates did, in fact, show mutations, and two of the isolates (C11-9 and C10-39) contained two mutations. All of the 8 mutations were single base pair changes, divided into 3 transitions and 5 transversions. Cytosine was not represented as a target, yet in all cases, cytosine was either adjacent to the mutated base or adjacent to the target base-pair of d.s. DNA. The positions of the mutations were distributed over the length of the lacZ gene; two of the positions were adjacent.

Table 6. DNA sequence analysis of LacZ⁻ mutants from bone marrow exposed to procarbazine

Mutant Identity	LacZ Position	LacZ Target Sequence	LacZ Sequence Alteration
C11-3	233	GAAAGCTGG	G → A
C11-5	587	CTGCTCTGG	T → G
C11-9	1076	CGAGGCGTT	G → C
	2804	CCGTACGTC	A → T
C10-39	2123	GTGCAACCG	A → T
	2803	CCCGTACGT	T → C
C10-21	2384	CCGCTGGAT	T → C
C10-41	2702	CGGGTAAAC	T → A

Mutants obtained from bone marrow harvested 7 days after treatment with procarbazine, 200 mg/kg x 5.

In the course of other experiments not described here, one male animal was found to exhibit an extremely high MF of approximately 8000 x 10^{-6} in the analyzed tissues of bone

marrow, liver, skin and testes. (This animal was, in fact, given a single i.p. injection of mitomycin C, 1 mg/kg, but no other similarly treated animal yielded a MF even one one-hundredth this value). In order to test the hypothesis that this animal was the offspring from a parental germ cell mutation, the lacZ genes from a mutant plaque from each of the four tissues were sequenced. The results (Table 7) showed that all mutant LacZ genes contained a T:A -> A:T transversion mutation at position 873 in the LacZ gene, resulting in the formation of a stopcodon.

Table 7. DNA sequence analysis of LacZ⁻ mutants from the tissues of a presumptive mutant animal

Tissue	LacZ Position	LacZ Target Sequence	LacZ Sequence Alteration
Bone marrow	873	GTTATGCCG	T → A
Liver	873	GTTATGCCG	T → A
Skin	873	GTTATGCCG	T → A
Testes	873	GTTATGCCG	T → A

Phage from one lacZ⁻ plaque selected per tissue type were sequenced. Animal #3156, male, 9 weeks old.

3.3.8 Screening for deletion mutants

In addition to base-pair changes, the lacZ mutation assay system should be able to detect base deletions up to the size of the lacZ gene itself. The existence and general frequency of deletions greater than about 15 base pairs was investigated by amplification of the lacZ genes from mutant plaques, followed by agarose gel analysis of 8 fragments produced by double digestion with BglI and RsaI. A collection of 94 mutant phage isolates was screened, which represented a variety of tissues from both
control and mutagen-treated animals. Only one deletion mutant of approximately 350 bp was found among the 94 mutant isola-

tes. The lacZ mutation position was approximately 2300 to 2650. This deletion may have been spontaneous or induced because the origin was skin DNA that showed a mutant frequency of only 36 x 10^{-6} after a single, topical exposure to 20 mg of acetic acid.

3.4 Discussion

The results of this preliminary survey of in vivo mutagenesis, utilizing the lacZ transgenic MutaMouse and a number of well-known chemical mutagens, were very encouraging toward the development of a practical and quantitative assay of mutagenesis in mouse tissues. The method of analysis described herein for the frequency of $lacZ^-$ mutants is robust and easily transferred among laboratories. A number of changes have been introduced since the original description (Gossen et al., 1989) in order to reduce the cost of labor and materials. For example, the field-inversion gel electrophoresis step for the enrichment of transgene sequences in the genomic DNA was eliminated as being unnecessary for the efficient packaging of λgt10lacZ sequences into phage particles. Digestion of genomic DNA with XbaI can be helpful in improving the packaging efficiency of some DNA preparations, but even this step is usually not worth the additional effort and cost. Elimination of the bottom agar in the bacterial cultures allowed further economies and improved the ease of visual detection of mutant plaques. A number of other changes had the combined effect of routinely yielding ≥ 150,000 pfu per packaging reaction with a minimum of effort in preparing DNA samples. The described method is still by no means optimized, and current experiments indicate that yields of 1,000,000 pfu per packaging reaction and more efficient scoring will be realized in future studies.

To date, we are not aware of any operational fault (other than not bothering to confirm suspicious plaques) that will lead to an erroneously high $lacZ^-$ mutant frequency. On the other hand, a loss in apparent MF does occur with an increasing density of blue, $lacZ^+$ plaques on the bacterial lawn.

Much of the MF data in this report was collected prior to the realization of this density effect, when plaque densities of 3000 or more per area of a 150 mm plate were used. Thus, it should not be surprising that future studies with these chemicals will yield 2- to 3-fold larger MF values for the same treatments, simply from the use of improved scoring methods.

Aside from method development, the purpose of this study was to determine whether or not MutaMouse was responsive to a variety of chemical mutagens and if so, to obtain information on how to proceed with the quantitation of the responses. The results obtained with seven different chemicals showed that all of these known mutagens could be detected as mutagenic in one or more tissues with at least a 50-fold range in magnitude of induced MF values. The calculated MF values in this study are not important, as they will surely change (upward) as the techniques improve. What is important is the demonstration that the method of dosing, dose level of each chemical, choice of tissue, choice of harvest time, etc. are all important experimental variables in studying in vivo mutation.

The dependence of MF on harvest time will be particularly important to study in the future quantitation of mutagenic responses in each tissue. The dynamics of mutant induction and the effects of cell replication on the MF are not known for any tissue. In this study, the MF induced in bone marrow by toxic treatments with ENU was still rapidly increasing at 10 days after treatment. Presumably, this response would decrease at longer times as the mutant progeny moved out into the circulatory system. If stem cells were mutated, sudden and temporary bursts in MF might subsequently occur as the mutant progeny first expand and then move out. For the less toxic, single treatment with 100 mg ENU/kg, a sharp maximum in MF occurred at 7 days. And finally, the PRO and CP treatments induced maximum MFs in bone marrow at 3 days. For any given chemical, the higher toxicity associated with higher doses may cause a delay in the appearance of the maximum MF, so that measurements obtained at a constant harvest time could appear

to yield a saturated or inverse dose-response. Skin mutagenesis by MNNG and DMBA appeared to saturate at the high dose levels, for example, but longer harvest times might have revealed higher MFs.

Careful inspection of the MF data in this study and simple considerations of statistical fluctuations in MF values as a function of population size (using Poisson distributions, which may or may not be appropriate) reveal the need for a large amount of additional work in order to quantitate mutagenic responses that are less than 20-fold over spontaneous frequencies. For example, a 5-fold apparent increase in MF would require a lacZ gene population (sample size) of 270,000 in both control and treated tissues in order to be substantiated with 95% probability. Exponentially larger populations are required for the resolution of smaller responses. These considerations don't even take into account the variability between animals regarding spontaneous MF, reaction to a given dose of chemical, and response time for each tissue. Thus, in order to obtain test results that can be duplicated in other laboratories or compared with other in vivo or in vitro mutation assay systems, it is necessary to resolve the issues of sample size, number of animals per dose condition, and the use of several harvest times for each tissue type.

This laboratory is taking the approach of defining the distribution of spontaneous mutant frequencies among MutaMouse animals for several types of tissues. Approximately 10^6 lacZ genes are analyzed per tissue as a practical attempt to minimize statistical fluctuations. The results in Table 1 represent progress toward this goal and already are indicating some conclusions. Skin and liver tissues have essentially the same spontaneous MF (28×10^{-6}) and are showing minimal variation between animals. In contrast, the bone marrow MF may be quite variable. A 15-fold variation between animals was observed, although this range may decrease as the analysis proceeds with larger sample sizes. This result indicates more effort will be necessary to quantitate mutagenic events in bone marrow than in liver or skin. Testicular tissue appears to exhibit a low

spontaneous MF (7×10^6), but confirmation by additional data is still needed. A similar observation has been made at the lacI locus in transgenic C57BL/6 mice, although no data on population sizes was provided (Kohler et al., 1991).

Acknowledgements

We especially thank the following persons for their excellent technical skills and long hours in the laboratory: Laura Custer, Wiljo J.F. de Leeuw, Arjen Bakker, Kelley Intehar and Hoda Khouri. These studies were supported by research and development funds from Hazleton Washington, Inc.

3.5 References

Gossen, J.A., W.J.F. de Leeuw, C.H.T. Tan, E.C. Zwarthoff, F. Berends, P.H.M. Lohman, D.L. Knook and J. Vijg (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations in vivo, Proc. Natl. Acad. Sci. (U.S.A.), 86, 7971-7975.

Gossen, J.A. and Vijg, J. (1988) E.coli C: a convenient host strain for cloning highly methylated DNA, Nucleic Acids Res. 16, 9343

Kohler, S.W., G.S. Provost, P.L. Kretz, M.J. Dycaico, J.A.Sorge and J.M. Short (1990) Development of a short-term, in vivo mutagenesis assay: the effects of methylation on the recovery of a lambda phage shuttle vector from transgenic mice, Nucleic Acids Res., 18, 3007-3013.

Kohler, S.W., G.S. Provost, A. Fieck, P.L. Kretz, W.O. Bullock, J.A. Sorge, D.L. Putman and J.M. Short (1991) Spectra of spontaneous and mutagen-induced mutations in the lacI gene in transgenic mice, Proc. Natl. Acad. Sci. (U.S.A.), 88, 7958-7962.

Myhr, B., and D. Brusick (1991) A transgenic mouse model for genetic toxicology studies, Lab Animal, 20, 31-35.

Myhr, B. (1991) Validation studies with Muta™Mouse - A transgenic mouse model for detecting mutations in vivo, Environ. Molec. Mutagen, 18, 308-315.

Sambrook, J., E.R. Fritsch and T. Maniatis (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 2nd Ed., Appendix B.

CHAPTER 4

HIGH SOMATIC MUTATION FREQUENCIES IN A LACZ TRANSGENE INTEGRATED ON THE MOUSE X-CHROMOSOME

Jan A. Gossen, Wiljo J.F. de Leeuw, Aart Verwest, Paul H.M. Lohman, and Jan Vijg.

Published in Mutation Research (1991) 250, 423-429.

Mutation Research, 250 (1991) 423–429

ADONIS 002751079100200X

MUT 02508

High somatic mutation frequencies in a LacZ transgene integrated on the mouse X-chromosome

Jan A. Gossen, Wiljo J.F. de Leeuw, Aart Verwest, Paul H.M. Lohman and Jan Vijg

Medscand INGENY, P.O. Box 685, 2300 AR Leiden, TNO Institute of Ageing and Vascular Research, and Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, 2333 AL Leiden (The Netherlands)

(Accepted 5 April 1991)

Keywords: Transgenic mice; Spontaneous mutations; LacZ shuttle vector; Mutational 'hot spot'; Mouse X-chromosome

Summary

To study spontaneous and induced mutagenesis in vivo we recently constructed a series of transgenic mice harboring different numbers of bacteriophage lambda shuttle vectors, provided with a LacZ mutational target gene, integrated in their genome. The transgenic mice enabled analysis of spontaneous and induced mutation frequencies in postmitotic tissues like liver and brain. The obtained data indicated spontaneous mutation frequencies in the order of 10^{-5}–10^{-6}. Here we report a 25–100 times higher spontaneous mutation frequency in liver and brain DNA of mice from strain 35.5, with the lambda-gt10LacZ concatemer integrated on the X-chromosome. These results indicate the presence of a mutational 'hot spot' in the mammalian somatic genome in vivo.

A fundamental understanding of spontaneous mutagenesis in higher organisms is important for obtaining insight into the ultimate cause of complex phenomena such as cancer and aging (Bishop, 1991; Vijg, 1990) and may provide a better rationale for risk assessment of potential genotoxicants. Studying spontaneous mutagenesis in mammalian cells in vivo requires sensitive systems, which allow to comparatively analyze spectra of mutations occurring at low frequency in different organs and tissues of an experimental animal. Presently, by far the most information about in vivo somatic mutations comes from studies of the HPRT locus in lymphocytes (reviewed by Thacker, 1985; Stout and Caskey, 1988). This method is based on 6-thioguanine selection of HPRT-deficient T-lymphocyte cells from peripheral blood (Albertini et al., 1982; Morley et al., 1983). Spontaneous mutation frequencies were found to be in the order of 1–10 per million cells (Albertini et al., 1985; Turner et al., 1985). Recently a similar method has been developed for cloning B-lymphocytes with mutations in the HPRT gene (Hakoda et al., 1989). In principle these systems can be used to obtain data sets on mutation spectra of the HPRT gene in cells that can be readily isolated and clonally assessed in vitro. Disadvantages of these systems are that their application is time-consuming, only a limited number of cell types can be analyzed and the observed mutations may not always reflect muta-

Correspondence: Dr. J.A. Gossen, Medscand INGENY, P.O. Box 685, 2300 AR Leiden (The Netherlands).

tion frequencies in the tissue of origin (Featherstone et al., 1989).

In order to directly analyze different organs and tissues for the presence of low-frequency DNA mutations other systems have been developed. A procedure based on the use of denaturing gradient gel electrophoresis (DGGE) to separate mutant from non-mutant PCR-amplified target sequences was proposed by Vijg and Uitterlinden (1987) and Lohman et al. (1987). Recently, it has been demonstrated that it is possible in denaturing gradient gels to separate heteroduplexed mutant HPRT sequences obtained from uncloned, complex populations of human B-lymphocytes treated with high doses of different mutagens, and cultured in the presence of 6-thioguanine (Thilly et al., 1990). Application of this method for studying mutations occurring at low frequency, however, has not been established yet. Despite the use of high-fidelity polymerases such as T3 and T7 and alterations in the experimental conditions (Keohavong et al., 1989), the PCR amplification step was found to introduce too many misincorporations to detect spontaneous mutation frequencies in the order of 10^{-5} (J.A. Gossen, unpublished results). Jeffreys et al. (1990) developed a method to specifically amplify and characterize in vivo deletion mutations at the MS minisatellite locus in human genomic DNA isolated from blood and sperm. This is the first method that allows one to monitor (deletion-type) mutations directly in tissue DNA; however, point mutations and other mutations in less unstable loci go undetected in this method, which demonstrates the need to develop additional methods. As yet, such methods are not available and therefore mutation analysis in various organs and tissues of humans is not yet possible.

Transgenic animal technology has recently been applied in developing improved mutagenesis testing systems. We have recently constructed several transgenic mouse strains harboring multiple copies of a bacteriophage lambda shuttle vector, provided with the LacZ gene as a target for mutagenesis (lambda-gt10LacZ), integrated in a head-to-tail arrangement in the genome (Gossen et al., 1989). It was demonstrated that efficient rescue of the shuttle vectors is possible by in vitro packaging using *E. coli* C as a host strain (Gossen and Vijg, 1988). The phages can subsequently be analyzed for the absence of β-galactosidase activity by plating on *E. coli* C host cells in the presence of IPTG and X-Gal. In addition, this system allows easy identification of mutations in the LacZ gene by means of DNA sequencing.

Here we present data on spontaneous mutation frequencies in liver and brain DNA of mice from 3 different transgenic mouse strains. Assuming random integration of the lambda-gt10LacZ concatemer in the mouse genome (Palmiter and Brinster, 1986), this system allows determination of spontaneous mutation frequencies in different organs and tissues, in relation to the site of integration in different transgenic mouse strains. The results obtained indicate that in at least one strain, 35.5, the lambda-gt10LacZ concatemer is integrated in a mutational 'hot spot'.

Materials and methods

Lambda-gt10LacZ transgenic mice

Transgenic mice were produced by micro-injection of approximately 150 copies of the monomeric lambda-gt10LacZ vector into the male pronucleus of fertilized eggs of (Balb/c × DBA/2)CD2 F_1 mice, which were then implanted into pseudopregnant BCBA recipients. Transgenic offspring were identified by Southern-blot analysis of *Eco*RI-digested tail DNA using lambda-gt10LacZ as a probe (see also Gossen et al., 1989).

DNA isolations

Freshly isolated brain tissue was homogenized, incubated overnight at 37°C in 3 volumes of 10 mM Tris–HCl, pH 7.5/150 mM NaCl/20 mM EDTA/1% SDS, containing 200 μg/ml proteinase K (Boehringer) and extracted with one volume of phenol–chloroform (1:1) and one volume of chloroform. Genomic DNA was ethanol-precipitated and solubilized in 10 mM Tris–HCl, pH 7.5/0.1 mM EDTA.

Freshly isolated liver tissue was homogenized in 50 mM Tris–HCl, pH 7.5/3 mM MgAc/0.25 M sucrose/0.2% Triton X-100 at 4°C and the nuclei were pelleted by centrifugation (2500 rpm, 10 min at 4°C). Nuclei were resuspended in 10 mM Tris–HCl, pH 7.5/150 mM NaCl/20 mM

EDTA/1% SDS, containing 200 μg/ml proteinase K and incubated at 37°C for 2 h. After extractions with one volume of phenol–chloroform (1:1) and one volume of chloroform, genomic DNA was ethanol-precipitated and solubilized in 10 mM Tris–HCl, pH 7.5/0.1 mM EDTA.

Tail tissue was minced and incubated overnight at 65°C in 100 mM EDTA/50 mM Tris–HCl, pH 7.5/100 mM NaCl/1% SDS, containing 200 μg/ml proteinase K. After mixing with 0.2 volume 8 M potassium acetate the solution was kept at 4°C for 30 min and extracted with one volume of chloroform. Genomic DNA was ethanol-precipitated and solubilized in 10 mM Tris–HCl, pH 7.5/0.1 mM EDTA.

Southern-blot analysis

Tail DNA, digested with the restriction enzyme *Dra*I, was size-fractionated on a 1% agarose gel and subsequently alkaline-transferred to Hybond-N^+ nylon membranes (Amersham). Hybridization analysis was performed overnight in 0.5 M $NaHPO_4$ pH 7.2/1 mM EDTA/ 7% (wt/vol) SDS at 65°C. As a probe, a *Dra*I fragment of 1.1 kb located at the 3′ end of the lambda-gt10LacZ vector was used. This fragment was radiolabeled by the random prime method (BRL).

Determination of spontaneous mutation frequencies

Rescue of lambda-gt10LacZ vectors from genomic DNA was performed by in vitro packaging (Giga Pack Gold, Stratagene), either directly from 5–10 μl total genomic DNA (1 μg/μl), or after pre-purification of the lambda-gt10LacZ concatemer from genomic DNA by preparative field-inversion gel electrophoresis of *Xba*I-digested DNA (Gossen et al., 1989). Phage analysis was performed by plating about 15 000 phage with *E. coli* C $LacZ^-$ per 241 × 241 mm Petri dish, in the presence of isopropyl β-D-thiogalactoside (135 μg/ml; BRL), X-gal (450 μg/ml; molecular probes), 10 mM $MgSO_4$ (Merck) and 0.2% maltose (Merck). Mutation frequencies were determined in liver and brain DNA, isolated from male and female transgenic mice, as the ratio between colorless (LacZ gene mutated) and blue (LacZ gene non-mutated) plaques.

Amplification of mutant LacZ genes

Mutant LacZ genes were amplified in a 50-μl reaction volume containing mutant lambda-gt10LacZ phages, 2 mM dATP, dGTP, dCTP, and dTTP (Pharmacia), 0.3 μg of primers LD3 and LD4 (located near the unique *Eco*RI site of lambda-gt10 vector), 1 unit Taq polymerase (BRL) in 1 × reaction buffer (50 mM KCl/10 mM Tris–HCl, pH 8.3/0.25 mM $MgCl_2$/20 μg/ml BSA). The samples were subjected to repeated cycles of 2 min at 95°C, 2 min 60°C, and 4 min 72°C, by using a Bio-med thermocycler.

DNA sequencing

Amplified mutant LacZ genes were subcloned into the M13mp18 sequencing vector. DNA sequencing was performed according to the M13 sequencing manual (Pharmacia), using the T7 DNA polymerase, [α-[^{35}S]thio]dATP and a set of 10 LacZ oligonucleotide primers located at 300-bp intervals throughout the LacZ gene.

Results and discussion

In order to compare spontaneous mutation frequencies at 3 different loci, lambda-gt10LacZ shuttle vectors were recovered from liver and brain DNA of mice from 3 transgenic mouse strains: 20.2 (80 copies per haploid genome), 35.5 (15 copies per haploid genome) and 40.6 (40 copies per haploid genome).

The results obtained with transgenic mouse strains 20.2 and 40.6 indicate spontaneous mutation frequencies in liver and brain DNA of between 1×10^{-6} and 1×10^{-5} (Table 1). Such mutation frequencies are in the same range as the spontaneous mutation frequencies found in animal and human T-lymphocytes using the HPRT clonal assay (Horn et al., 1984; Albertini et al., 1985; Turner et al., 1985). Interestingly, considerably higher spontaneous mutation frequencies were observed in mice from strain 35.5, with mean values ranging from about 7.9×10^{-5} for liver DNA and 9.2×10^{-5} for brain DNA. A significantly higher spontaneous mutation frequency was observed in brain DNA of homozygous animals as compared to hemizygotes (see below).

To investigate the nature of the increased

TABLE 1

SPONTANEOUS MUTATION FREQUENCIES IN lambda-gt10LacZ RESCUED FROM LIVER AND BRAIN DNA OF 3 DIFFERENT TRANSGENIC MOUSE STRAINS

Strain	Organ (number of animals analyzed)	Number of plaques analyzed	Number of mutants	Mutation frequency ($\times 10^{-5}$)
20.2	liver (3)	656883	1	0.1
	brain (3)	521356	0	nd
40.6	liver (4)	258562	1	0.7
	brain (4)	1022000	1	0.1
35.5 [a]	liver (17)	758936	62	8.1 ± 6.1
	brain (19)	598938	29	5.1 ± 2.4
35.5 [b]	liver (5)	288136	21	7.3 ± 3.5
	brain (9)	405568	63	15.5 ± 8.8

[a] Hemizygote; [b] homozygote; nd, not detectable.
For brain DNA, isolated from hemi- or homo-zygous mice of strain 35.5, the spontaneous mutation frequencies were significantly different ($p < 0.05$), as determined by Student's t-test.

spontaneous mutation frequency in the lambda-gt10LacZ concatemer in this specific transgenic mouse strain, knowledge concerning its chromosomal position is essential. Indeed, the primary and/or higher-order structure of the lambda-gt10LacZ concatemer and/or its flanking sequences may well be the underlying cause of the increased susceptibility to mutagenesis. The finding that after crossings between hemizygous mice the female progeny were homozygous only, and that after crossings between hemizygous male mice and non-transgenic female mice the lambda-gt10LacZ concatemer was transmitted to the daughters only, indicated that the lambda-gt10LacZ concatemer in strain 35.5 is located on the X-chromosome.

Southern-blot analysis of tail DNA of mice, obtained from crossings between hemizygous male (X^zY) and female (X^zX) 35.5 mice and from crossings between hemizygous 35.5 male (X^zY) and female mice not possessing the 35.5 lambda-gt10LacZ concatemer (XX) revealed rearrangements of 3′ flanking regions of the lambda-gt10LacZ concatemer (Fig. 1; Table 2). In addition to a strong hybridizing 1.2-kb head-to-tail fragment containing the lambda cos-site, 12 other fragments are detected by this probe (Fig. 1). These fragments are due to extensive rearrangements near the 3′ site of integration, the occurrence of which during integration is typical for transgenesis (Palmiter and Brinster, 1986). It should be noticed that after these integration-associated rearrangements have taken place, the lambda-gt10LacZ concatemer and its 3′ and 5′ surrounding regions should be stable as has been observed for all other transgenic mouse strains except the 35.5 strain.

Among 96 mice of strain 35.5 analyzed, 9 out of 57 females (15.8%) and only 1 out of 39 males (3%) contained deletions and/or duplications in the 3′ flanking region (Table 2; Fig. 1, indicated by arrows), 2 female animals were found to contain both a deletion and a duplication. In contrast to the instability observed in the 3′ flanking region, deletions and/or duplications were not detected in the 5′ flanking region. In addition, although all other lambda-gt10LacZ transgenic mouse strains were found to contain lambda parts in the regions flanking the concatemer, instabilities were never observed.

The high frequency of deletions and/or duplications observed in female mice indicates deletion and duplication events of 3′ regions occurring preferentially during male meiosis. The meiotic origin of the rearrangements is indicated by our observation that the same deletions and duplications were also present in liver and brain DNA of the same mice (results not shown). Fur-

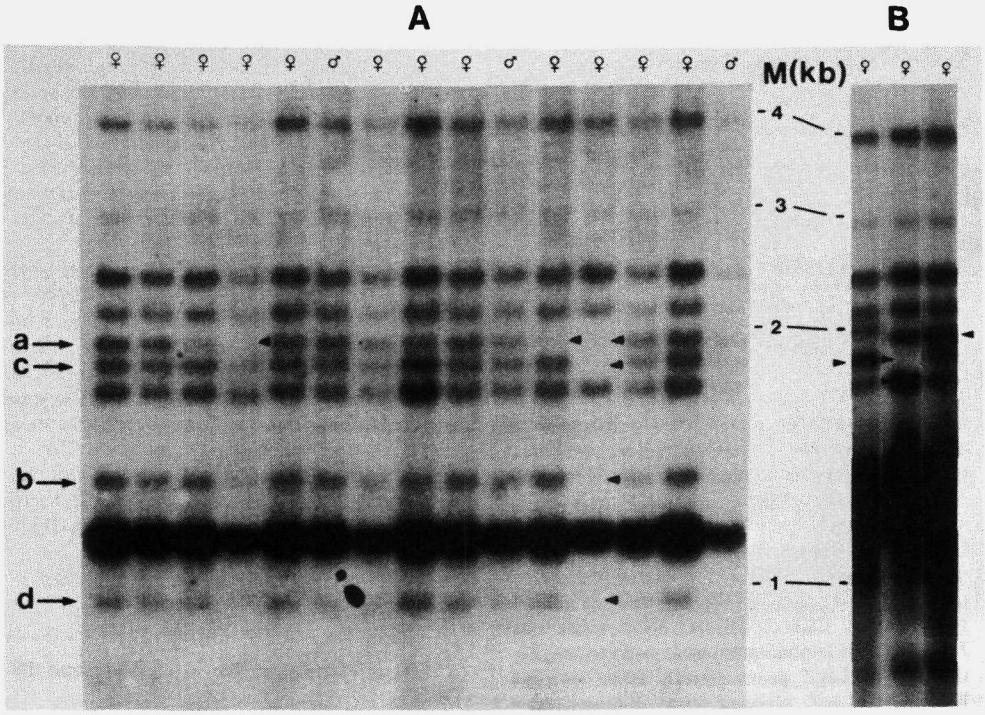


Fig. 1. Southern-blot analysis of tail DNA of mice obtained from crossings between hemizygous 35.5 male and female (A) and from crossings between 35.5 male and female mice not possessing the 35.5 lambda-gt10LacZ vector (B). Deletions/duplications are detected by arrows (a, b, c and d).

TABLE 2

TRANSMISSION OF 3′-FLANKING SEQUENCES IN STRAIN 35.5

Parents	Offspring	Number of mice with deletions/duplications
A) $X^zX \times X^zY$	female 41	6
	male 39	1
B) $X^zY \times XX$	female 16	3
	Total female 57	9 (15.8%)
	male 39	1 (3%)

Transmission of 3′ flanking sequences of the lambda-gt10LacZ concatemer in strain 35.5 was analyzed by crossing (A) hemizygous male and female mice of strain 35.5 (lambda-gt10LacZ concatemer integrated on the X-chromosome indicated by the symbol Z) and (B) hemizygous males of strain 35.5 with females not possessing the 35.5 lambda concatemer on the X-chromosome.

thermore, lambda-containing parts of the 3′ flanking regions were found to undergo deletion with different frequency; the relative frequencies with which fragments, indicated as a, b, c and d in Fig. 1, were deleted are 8.5%, 3.5%, 1.7% and 0.8%, respectively. In this regard it is tempting to speculate that the deletion frequency reflects the increasing distance 3′ from the lambda-gt10LacZ concatemer into the pseudoautosomal region of the X-chromosome (Rouyer et al., 1986). This region of the X-chromosome is characterized by increasing recombination frequencies towards the telomere. If recombination events between X- and Y-chromosomes during meiosis are involved, occasionally 3′ lambda sequences should be present on the Y-chromosome. This, however, has not been observed among 30 male mice analyzed,

TABLE 3

SEQUENCE ANALYSIS OF SPONTANEOUS LacZ MUTANTS ISOLATED FROM LIVER DNA OF MICE OF STRAIN 35.5

Mutant	Sequence alteration [a]	Position	Target sequence
L-1	T:A → A:T (TV)	1242	TGTAAGTGG
L-2	G:C → A:T (TS)	1676	TACTAGCAG
L-3	C:G → A:T (TV)	1270	AACCAACGG
L-4	A:T → T:A (TV)	1648	GGGTTACAG
L-5	G:C → T:A (TV)	1721	GTCTTGGAC

[a] TS indicates transition and TV indicates transversion. For each LacZ mutant a 9-bp region is listed, in which the mutated base is underlined.

which suggests that part of the deletions and duplications of the 3′ flanking region could be the result of intrachromosomal rearrangements during premeiotic stages.

To analyze the kind of mutations present in LacZ genes isolated from liver and brain DNA, 90 mutants were analyzed for the presence of large structural changes (> 30 bp) by means of PCR analysis. Lambda primers, located near the *Eco*RI site of lambda-gt10, were used to amplify the mutant LacZ genes directly from colorless lambda-gt10LacZ plaques. Gel electrophoretic analysis of the PCR products indicated no large structural changes, with the exception of 2 mutants, which contained deletions up to 1100 bp. DNA sequence analysis of 5 mutants indicated that single base substitutions, resulting either in amino acid substitutions or in the formation of a stop codon, were predominant (Table 3).

It is well documented that the majority of base-pair mutations (68%) appear to be replication-dependent (Cooper and Krawczak, 1990). It is therefore likely that the high spontaneous mutation frequency in strain 35.5 reflects misincorporations opposite apurinic sites by DNA polymerases (Loeb, 1989) for example as a consequence of differences in chromatin structure or the asynchronous replication of active and inactive X-chromosomes during cell replication (Schmidt and Migeon, 1990). The latter could be tested by crossing the 35.5 strain with Searle's translocation mice [t(X,16)] and comparing mutation frequencies in females where the lambda-gt10LacZ concatemer is on the normal or on the rearranged X-chromosome. If the lambda-gt10LacZ concatemer is indeed preferentially present on the active X-chromosome, this may account for the observed difference in spontaneous mutation frequency between hemi- and homo-zygote brain DNAs (Table 1) and would explain the absence of differences in mutation frequencies between hemizygous male and female mice.

Our results suggest that the high spontaneous mutation frequency in liver and brain DNA of strain 35.5 is due to the location of the lambda-gt10LacZ concatemer at a highly unstable chromosomal region, making it more susceptible to both germinal and somatic mutagenesis. The large differences in spontaneous mutation frequencies between 35.5 and the other 2 strains may therefore indicate the presence of a mutational 'hot spot' in the somatic genome, which has hitherto only been found for some minisatellite loci (Kelly et al., 1989; Jeffreys et al., 1990).

Acknowledgements

This is Publication No. 4 of Medscand INGENY.

We thank A.G. Uitterlinden for critical comments during the course of this study and Mr. A.A. Glaudemans for preparation of the photograph. Part of this study was performed at the TNO Institute for Experimental Gerontology, Rijswijk (The Netherlands).

References

Albertini, R.J., K.L. Castle and W.R. Borcherding (1982) T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood, Proc. Natl. Acad. Sci. (U.S.A.), 79, 6617–6621.

Albertini, R.J., J.P. O'Neill, J.A. Nicklas, N.H. Heintz and P.C. Kelleher (1985) Alterations of the HPRT gene in human in vivo derived 6-thioguanine-resistant T-lymphocytes, Nature (London), 316, 369–371.

Bishop, J.M. (1991) Molecular themes in oncogenesis, Cell, 64, 235–248.

Cooper, D.N., and M. Krawzcak (1990) The mutational spectrum of single base-pair substitutions causing human genetic disease: patterns and predictions, Hum. Genet., 85, 55–74.

Featherstone, T., P.D. Marshall and E.J. Evans (1987) Problems and pitfalls in assessing human T-lymphocyte mutant frequencies, Mutation Res., 179, 215–230.

Horn, P.L., M.S. Turker, C.E. Ogburn, C.M. Disteche and G.M. Martin (1984) A cloning assay for 6-thioguanine resistance provides evidence against certain mutational theories of aging, J. Cell. Physiol., 121, 309–315.

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

Gossen, J.A., W.J.F. de Leeuw, C.H.T. Tan, P.H.M. Lohman, F. Berends, D.L. Knook, E.C. Zwarthof and J. Vijg (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying gene mutations in vivo, Proc. Natl. Acad. Sci. (U.S.A.), 86, 7971–7975.

Hakoda, M., Y. Hirai, S. Kusonoki and M. Akiyama (1989) Cloning of in vivo-derived thioguanine-resistant human B-cells, Mutation Res., 210, 26–34.

Loeb, L.A. (1989) Endogenous carcinogenesis: molecular oncology into the twenty-first century, Cancer Res., 49, 5489–5496.

Lohman, P.H.M., J. Vijg, A.G. Uitterlinden, P. Slagboom, J.A. Gossen and F. Berends (1987) DNA methods for detecting and analyzing mutations in vivo, Mutation Res., 181, 227–234.

Jeffreys, A.J., R. Neuman and V. Wilson (1990) Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis, Cell, 60, 473–485.

Kelly, R., G. Bulfield, A. Collick, M. Gibbs and A.J. Jeffreys (1989) Characterization of a highly unstable mouse minisatellite locus: evidence for somatic mutation during early development, Genomics, 5, 844–856.

Keohavong, P., and W.G. Thilly (1989) Fidelity of DNA polymerases in DNA amplification, Proc. Natl. Acad. Sci. (U.S.A.), 86, 9253–9257.

Morley, A.A., K.J. Trainor, R.S. Seshadri and R.G. Ryall (1983) Measurement of in vivo mutations in human lymphocytes, Nature (London), 302, 155–156.

Palmiter, R.D., and R.L. Brinster (1986) Germline transformation of mice, Annu. Rev. Genet., 20, 465–499.

Rouyer, F., M. Simmler, C. Johnsson, G. Vergnaud, H.J. Cooke and J. Weissenbach (1986) A gradient of sex linkage in the pseudoautosomal region of the human sex chromosomes, Nature (London), 319, 291–295.

Schmidt, M., and B.R. Migeon (1990) Asynchronous replication loci on human active and inactive X-chromosomes, Proc. Natl. Acad. Sci. (U.S.A.), 887, 3685–3689.

Stout, J.T., and C.T. Caskey (1985) HPRT: structure, expression and mutation, Annu. Rev. Genet., 19, 127–148.

Thacker, J. (1985) The molecular nature of mutations in cultured cells: a review, Mutation Res., 150, 431–442.

Thilly, W.G., V.F. Lui, B.J. Brown, N.F. Cariello, A.G. Kat and P. Keohavong (1989) Direct measurement of mutational spectra in humans, Genome, 31, 590–593.

Turner, D.R., A.A. Morley, M. Haliandros, R. Kutlaca and B.J. Sanderson (1985) In vivo somatic mutations in human lymphocytes frequently result from major gene alterations, Nature (London), 315, 343–345.

Vijg, J. (1990) DNA sequence changes in aging: how frequent, how important?, Aging, 2, 105–123.

Vijg, J., and A.G. Uitterlinden (1987) A search for DNA alterations in the aging mammalian genome: an experimental strategy, Mech. Ageing Dev., 41, 47–63.

CHAPTER 5

DNA SEQUENCE ANALYSIS OF SPONTANEOUS MUTATIONS AT A LACZ TRANSGENE INTEGRATED ON THE MOUSE X-CHROMOSOME

Jan A. Gossen, Wiljo J.F. de Leeuw, Arjen Q. Bakker and Jan Vijg

Accepted for publication in Mutagenesis

5.1 Abstract

Transgenic mice with integrated shuttle vectors containing the LacZ mutational target gene, were used to study spontaneous mutational events in vivo. The transgenic mouse strain used carries the LacZ transgene on the X-chromosome and was previously found to be characterized by an about 25-fold higher spontaneous mutation frequency in liver and brain compared to at least three other transgenic mouse strains. To determine the nature of in vivo spontaneous mutational events in this mouse strain, 35 mutant LacZ genes isolated from liver and brain were analyzed at the DNA sequence level. The results obtained indicate that single basepair changes were predominant in both liver and brain. However, in liver the majority of mutations were transitions whereas in brain predominantly transversions were observed. Six mutants appeared to contain multiple dispersed mutations, separated by as much as 44 bp. Mutations were generally located within a 500 bp region encoding the active site of the ß-galactosidase enzyme.

5.2 Introduction

Spontaneous mutations in vivo are thought to arise by a number of mechanisms, including errors made during replication or repair synthesis of DNA. A major source of spontaneous mutations is thought to be spontaneous deamination of 5-methylcytosine (Ehrlich et al., 1981, 1990) and recent evidence of mutation at CpG sites in the low-density lipoprotein receptor and the p53 tumour suppressor gene support this notion (Rideout et al., 1990). In general, deamination of 5-methylcytosine most frequently occurs at CpG sites and accounts for about 30% of known point mutations leading to genetic diseases or cancer (Cooper & Krawzcak, 1990). Although it is not possible to distinguish between spontaneous mutations and mutations caused by low level ("natural") exposure to mutagens, evidence has recently been obtained that specific DNA-damaging agents leave characteristic mutational spectra. Studies of the mutational spectrum in mutant p53 tumour suppressor genes, isola-

tional spectrum in mutant p53 tumour suppressor genes, isolated from lung (Jones et al, 1992), skin (Brash et al., 1991) and hepatocellular (Bressac et al., 1991) cancers of people exposed to cigarette smoke, UV light and aflatoxin B1, respectively, showed that chemicals leave specific "fingerprints"; using in vitro assays the type of mutations observed had previously been shown to be specific for these carcinogens.

In order to further investigate the mechanisms involved in spontaneous or induced mutagenesis in vivo, transgenic mice carrying bacterial reporter genes have recently been developed (Gossen et al., 1989; Kohler et al., 1991). Application of these models allows to analyze mutational events occurring at very low frequency in all organs and tissues and to rapidly characterize significant numbers of mutants at the DNA sequence level.

We have previously reported high spontaneous mutation frequencies in a LacZ transgene integrated at the mouse X-chromosome (strain 35.5; Gossen et al., 1991). Compared to other transgenic mouse strains, in which the LacZ transgene is integrated at different autosomal chromosomal positions, mutation frequencies in liver and brain were approximately 25 times higher. These results indicated the presence of a mutational "hot spot" in the mammalian somatic genome in vivo. To further investigate the high spontaneous mutation frequency in transgenic mouse strain 35.5, we have now cloned and sequenced 35 LacZ mutants isolated from liver and brain. Here we show that spontaneously occurring mutations at the LacZ transgene are tissue specific and comprise a variety of events, including single base-pair substitutions, frameshifts and deletions.

5.3 Materials and Methods

5.3.1 Lambda-gt10LacZ transgenic mice

Transgenic mice were produced by micro-injection of approximately 150 copies of the monomeric lambda-gt10LacZ vector into the male pronucleus of fertilized eggs of (Balb/c x DBA/2)CD2 F1 mice, which were then implanted into pseudopregnant BCBA recipients. Transgenic offspring were identified by Southern-blot analysis of EcoRI-digested tail DNA using lambda-gt10LacZ as a probe (Gossen et al., 1989). A number of transgenic mouse strains with different integration sites of the lambda-gt10LacZ vector were obtained. All of these strains were characterized by low spontaneous mutation frequencies, with the exception of strain 35.5 with the lambda-gt10LacZ shuttle vector integrated on the X-chromosome. This strain is used in the present study.

5.3.2 DNA isolations

DNA isolations were performed essentially as described by Gossen et al. (1990). Briefly, freshly isolated brain tissue was homogenized, incubated overnight at 37 °C in 3 volumes of 10 mM Tris.HCl, pH 7.5/150 mM NaCl/20 mM EDTA/1% SDS, containing 200 µg/ml proteinase K (Boehringer) and extracted with one volume of phenol-chloroform (1:1) and one volume of chloroform. Genomic DNA was ethanol precipitated and solubilized in 10 mM Tris.HCl, pH 7.5/0.1 mM EDTA.

Freshly isolated liver tissue was homogenized in 50 mM Tris.HCl, pH 7.5/3 mM MgAc/0.25 M sucrose/0.2% Triton-X-100 at 4°C and the nuclei were pelleted by centrifugation (2500 rpm, 10 min at 4°C). Nuclei were resuspended in 10 mM Tris.HCl, pH 7.5/150 mM NaCl/20 mM EDTA/1% SDS, containing 200 µg/ml proteinase K and incubated at 37°C for 2 h. After extractions with one volume of phenol-chloroform (1:1) and one volume of

chloroform, genomic DNA was ethanol-precipitated and solubilized in 10 mM Tris.HCl, pH 7.5/0.1 mM EDTA.

5.3.3 LacZ mutant collection

The 35 LacZ mutants analyzed in this study were randomly chosen from a collection of mutants isolated from livers and brains of 15 different animals of strain 35.5. Rescue of lambda-gt10LacZ vectors from genomic DNA was performed by in vitro packaging (Giga Pack Gold, Stratagene), either directly from 5-10 μl total genomic DNA (1μg/μl), or after pre-purification of the lambda-gt10LacZ concatemer from genomic DNA by preparative field-inversion gel electrophoresis of XbaI digested DNA (Gossen et al., 1989). Phage analysis was performed by plating about 15,000 phage with E.coli C LacZ$^-$ per 241 x 241 mm Petri dish, in the presence of isopropyl ß-D- thiogalactoside (135 μg/ml; BRL), X-gal (450 μg/ml; Molecular Probes), 10 mM $MgSO_4$ (Merck) and 0.2% maltose (Merck). Mutant LacZ phage were identified on basis of absence of ß-galactosidase activity. Mutant phage were always replated with E.coli C LacZ$^-$ to confirm mutant phenotype. As a control, about 100,000 phage isolated from transgenic mouse liver DNA of strain 40.6 were plated and analyzed for mutant LacZ phage. The spontaneous mutation frequency in this control genomic DNA was previously determined to be in the order of 10^{-5}-10^{-6} (Gossen et al., 1989).

5.3.4 α, ß, Ω LacZ complementation assay.

To test if mutant LacZ genes contain mutations in the region of the LacZ gene encoding either the α, ß or Ω part of the ß-galactosidase protein, a number of mutant phage were replated on 3 different E.coli strains: DH5α (ß + Ω donor) to identify phage containing mutations in the LacZ-α region, W4680 (α + Ω donor; Cook and Lederberg, 1962) to identify

phage containing mutations in the LacZ-ß region and Hfr 30 x 9000 (α + ß donor; Newton et al., 1965) to identify phage containing mutations in the LacZ-Ω region, respectively. Phage infection and plating were performed as described under 5.3.3.

Strains W4680 and Hfr 30 x 9000 were kindly provided by Dr B. Bachman of the E.coli Genetic Stock Centre (Department of Biology, Yale University, P.O. Box 6666, New Haven, CT 06511-7444, USA). Strain DH5α was obtained from Gibco BRL.

5.3.5 Amplification of mutant LacZ genes

Mutant LacZ genes were amplified in a 50 µl-reaction volume containing mutant lambda-gt10LacZ phages, 2 mM dATP, dGTP, dCTP, and dTTP (Pharmacia), 0.3 µg of primers LD3 and LD4 (located near the unique EcoR I site of lambda-gt10 vector), 1 unit Taq polymerase (BRL) in 1 x reaction buffer [50 mM KCl/10 mM Tris.HCl, pH 8.3/ 0.25 mM $MgCl_2$/ 20 µg/ml BSA]. The samples were subjected to repeated cycles of 2 min at 95 °C, 2 min 60 °C, and 4 min 72 °C, by using a Bio-med thermocycler.

5.3.6 DNA sequencing

Amplified mutant LacZ genes were subcloned into the M13mp18 sequencing vector. Based on the results obtained in the α, ß, Ω LacZ complementation assay, the α, ß or Ω region of the LacZ gene was sequenced. DNA sequencing was performed according to the M13 sequencing manual (Pharmacia), using the T7 DNA polymerase, [α-[^{35}S]thio]dATP and a set of 10 LacZ oligonucleotide primers located at 300 bp intervals throughout the LacZ gene.

5.4 RESULTS

5.4.1 Isolation and characterization of mutant LacZ genes

A total of 36 mutant LacZ genes was isolated from liver and brain of 15 (6 hemi- and 9 homozygous) mice from strain 35.5. All mutants analyzed in this study were characterized by a reduced or complete absence of ß-galactosidase activity compared to non-mutant LacZ phage. The latter was tested by re-plating both mutant and non-mutant phage with E.coli C ($LacZ^-$) host cells in the presence of the chromogenic substrate X-gal. Subsequently, mutant phage were tested in a α, ß, Ω LacZ complementation assay to determine the region of the LacZ gene in which mutations were present. The results obtained (Table 1) indicate that most LacZ phage mutants are characterized by absence of ß complementation when plated on E.coli strain W4680. Those mutants characterized by absence of ß and Ω complementation contained a -1 frameshift or single basepair mutation resulting in the formation of a stopcodon within the LacZ-ß region.

As presented in Table 2, a variety of mutational events were observed among the mutant LacZ genes. The most frequent mutational events were single base pair changes, observed in 15 of the 26 mutants. The remaining mutations were -1/+1 frameshifts and deletions.

Table 1. Analysis of LacZ mutants analyzed in the α, ß, Ω complementation assay

mutant no.	DH5α (ß+,w+)	W4680 (α+,w+)	Hfr 3000x90 (α+,ß+)	expected region of mutation*	region of mutation*
L-2	+/-	-	+	ß	ß
L-3	+/-	-	-	ß	ß
L-8	-	-	+	α-ß	ß
L-9	-	-	+	α-ß	ß
L-10	+	-	+	ß	ß
L-11	+/-	-	-	ß	ß
L-12	+/-	-	+	ß	ß
L-13	+/-	-	-	ß	ß
L-16	+/-	-	-	ß	ß
B-1	+/-	-	+	ß	ß
B-6	+/-	-	-	ß	ß
B-10	+/-	-	+	ß	ß
B-11	+/-	-	+	ß	ß
B-12	+/-	-	+	ß	ß
B-13	+/-	-	+	ß	ß
B-15	+/-	+	-	Ω	Ω
B-16	+/-	-	+	ß	ß
B-17	-	-	-	α	α
B-18	+	+/-	+	ß	ß
B-19	+	+	-	Ω	Ω

\+ = ß-galactosidase activity
\- = no ß-galactosidase activity
+/- = reduced ß-galactosidase activity
* The LacZ-α,LacZ-ß and LacZ-Ω regions are located from positions 1 to about 450, 451 to about 1750 and 1751 to about 3086, respectively.

Table 2. Summary of spontaneous mutations at the LacZ transgene isolated from liver and brain of mice from strain 35.5

	liver	brain
# LacZ genes analyzed*	18	17
# animals analyzed	9	11
Transitions		
G:C -> A:T	9	4
A:T -> G:C	3	1
Transversions		
G:C ->T:A	2	6
G:C ->C:G	0	0
A:T ->C:G	0	0
A:T ->T:A	2	4
Frameshifts		
-1	4	2
-2	0	0
Deletions	1	3
Insertions	0	1
multiple mutations	3	3

* includes data from Gossen et al. (1991)

5.4.2 Base substitutions

The location and type of base substitution events are shown in Tables 3 and 4. In liver the most predominant type of mutations were transitions, with G:C -> A:T being predominant. The mutational pattern observed in brain was significantly different from liver, with G:C -> A:T and A:T -> T:A transversions being most common ($p=0.03$; as determined by Fisher's Exact test) and G:C -> A:T transitions least common ($p=0.03$).

The majority of mutants analyzed contained single basepair substitutions resulting in the formation of a missense or stop codon. However, in 6 mutants multiple mutations were observed. Of these mutants, mutant L-15, contained two single basepair substitutions

Table 3. Spontaneous transition mutations at the LacZ transgene in liver and brain of mice from strain 35.5

Animal no.	Mutant no.	Sequence alteration	Target sequence	Position	Amino acid alteration
11	L-2	C:G -> T:A	AACC<u>C</u>ACGG	1270	his->asp
12	L-3	G:C -> A:T	TACT<u>G</u>GCAG	1676	trp->stop
108	L-7	T:A -> C:G	TCCA<u>T</u>AAAA	1577	ile->thr
155	L-9	T:A -> C:G	CGTCT<u>G</u>GGA	1720	trp->arg
23	L-10	C:G -> T:A	CTTT<u>C</u>GCTA	1592	ser->leu
83	L-12	C:G -> T:A	TGGT<u>C</u>GCTG	1388	ser->leu
119	L-14	T:A -> C:G	GCTG<u>T</u>GCCG	1562	val->ala
105	L-16	C:G -> T:A	GAAG<u>C</u>AGAA	1159	gln->stop
137	L-17	G:C -> A:T	ACAG<u>G</u>GCGG	1708	gly->ser
105	B-9	C:G -> T:A	CACG<u>C</u>GATG	1640	ala->val
	B-16	A:T -> G:C	GCCG<u>A</u>CGGC	974	asp->gly
119	B-18	G:C -> A:T	GTGC<u>G</u>CTGT	1181	arg->his
155	B-19	C:G -> T:A	CTTC<u>C</u>AGTT	2884	glu->stop

Table 4. Spontaneous transversion mutations at the LacZ transgene in liver and brain of mice from strain 35.5

Animal no.	Mutant no.	Sequence alteration	Target sequence	Position	Amino acid alteration
12	L-4	A:T -> T:A	GGGT<u>A</u>ACAG	1648	asn->tyr
12	L-5	G:C -> T:A	GTCT<u>G</u>GGAC	1721	trp->leu
12	L-6	T:A -> A:T	TGTA<u>T</u>GTGG	1242	tyr->stop
12	B-1	C:G -> A:T	CCCA<u>C</u>GCGA	1638	his->gln
17	B-2	C:G -> A:T	TGCC<u>C</u>GATG	1520	pro->gln
80	B-7	C:G -> A:T	TGAG<u>C</u>GAAC	1329	ser->arg
30	B-10	A:T -> T:A	ATTG<u>A</u>AACC	1265	glu->val
30	B-11	A:T -> T:A	ATTG<u>A</u>AACC	1265	glu->val
30	B-12	A:T -> T:A	ATTG<u>A</u>AACC	1265	glu->val
155	B-13	C:G -> A:T	GGAG<u>C</u>CGAC	1490	ala->asp
97	B-15	T:A -> A:T	ACTA<u>T</u>CCCG	2736	tyr->stop

5.4.3 Frameshifts and deletions

The location and sequence alteration of frameshift and deletion mutations are shown in Table 5. All frameshift mutations involved -1 basepair deletions and a single basepair insertion. Two deletions were observed, one 21 basepair inframe deletion in mutant L-8 located between a 4 basepair CGCT direct repeat, and one frameshift in mutant B-16 between a 4 basepair GCCG inverted repeat. One larger deletion, of 1046 basepairs, was found in mutants B-4 and B-5.

Table 5. Spontaneous frameshift and deletion mutations at the LacZ transgene in liver and brain of mice from strain 35.5.

Animal no.	Mutant no.	Sequence alteration	Target sequence	Position
11	L-1	del G:C	CGAT.TACG	1524
155	L-8	del 21 bp	CTTT.CGCT	1592-1613
137	L-18	del C:G	GCTA.CGGC	1318
45	B-3	del A:T	TAAC.GTCT	1651
155	B-14	ins A:T	CCCCTTTTC	114/115/116

5.4.4 Multiple mutations

In total six mutants were characterized by the presence of two mutations (Table 6). Mutant L-15 was found to contain a tandem base substitution, consisting of a C:G -> T:A transition and a G:C -> T:A transversion. Mutants L-11, L-13, and B-6 contained a basepair substitution and a -1 frameshift mutation. The nucleotides affected were in tandem (mutants L-11 and L-13) or 44 basepairs apart (mutant B-6). Mutants B-4 and B-5 contained a basepair substitution and a 1046 basepair deletion separated by 6 basepairs. Mutants L-11 and L-13 and mutants B-4 and B-5 were isolated from the same animal in independent packaging experiments.

Table 6. Multiple dispersed mutations at the LacZ transgene in liver and brain of mice from strain 35.5.

Animal no.	Mutant no.	Sequence alteration	Target sequence	Position	Amino acid alteration
83	L-11	G:C -> A:T	TTGCGAATA	1627	glu->lys
		del C:G	TTTG.GAAT	1626	
83	L-13	G:C -> A:T	TTGCGAATA	1627	glu->lys
		del C:G	TTTG.GAAT	1626	
105	L-15	C:G -> T:A	TATCCGAAC	1196	pro->leu
		G:C -> T:A	AATGGTGCA	1348	val->leu
83	B-4	C:G -> A:T	GCTGCATAA	664	his->asn
		del 1046 bp	ATAA.CAGG	670-1716	
83	B-5	C:G -> A:T	GCTGCATAA	664	his->asn
		del 1046 bp	ATAA.CAGG	670-1716	
23	B-6	G:C -> A:T	GAATGAATC	1399	glu->lys
		del A:T	TCCG.ACAA	1397	

5.5 DISCUSSION

We investigated the molecular basis of spontaneous LacZ mutants isolated *from* liver and brain of mice from transgenic mouse strain 35.5. This transgenic mouse strain harbours the LacZ transgene, as part of a bacteriophage lambda shuttle vector, on the X-chromosome and was previously characterized by high somatic mutation frequencies in liver and brain (Gossen et al., 1990).

The spectrum of spontaneous mutations in the 36 mutant LacZ genes analyzed in this study is characterized by three major features: (1) a predominance of single basepair mutatons; (2) tissue-specific mutation spectra, with transition mutations predominant in liver and transversion mutations predominant in brain; and (3) a high incidence of mutant LacZ genes, isolated from both liver and brain, containing multiple mutations.

The low number of deletion events observed may be explained by the relatively small size of the LacZ target gene (about 3000 basepairs). In spite of subtle differences reported in other small mutational target genes, like the hamster APRT gene (about 2600 basepairs; Phear et al., 1989) and the LacI transgene (about 1200 basepairs; Kohler et al, 1991), mutational spectra in genes with relatively small sizes are generally characterized by a high percentage of single basepair mutations (Phear et al, 1989; Ikehata et al, 1989). Moreover, since in our transgenic mice the LacZ transgene is incorporated into a bacteriophage lambda vector, and recovery of these vectors by in vitro packaging requires a minimum length of 42 Kb, deletions larger than 5 Kb will not be detectable in this system.

Major sources for spontaneous mutations in vivo are DNA modifications due to depurination (Loeb, 1990) and deamination of 5-methylcytosine (Ehrlich et al., 1990). Evidence that depurination is an important source of spontaneous mutations is the increased rate of misincorporation of nucleotides opposite abasic sites by DNA polymerases (Kunkel et al., 1983;

Schaaper et al., 1983). Since deoxyadenosine is preferentially incorporated by DNA polymerases, mutations due to depurination should be characterized by substitutions by deoxyadenosine. Such a mechanism may in part be responsible for the high incidence of C:G -> A:T transversion mutations observed in brain DNA. Spontaneous deamination of 5-methylcytosine, resulting in C:G -> T:A transitions, has been shown to be involved in hot spots for mutation in both E. coli (Coulondre et al., 1978) and mammalian cells (Rideout et al., 1990). It is, however, unlikely that this mechanism plays an important role in spontaneous mutagenesis at the LacZ transgene in liver and brain of mice from strain 35.5. Although all copies of the LacZ transgene are heavily methylated in liver and brain, only 4 of the 11 C:G -> T:A transitions observed were located within a CpG dinucleotide, the major site of methylation in mammalian cells (Ehrlich and Wang, 1981).

Our results contrast with results obtained by Douglas et al. (manuscript in preparation), who analyzed 75 spontaneous LacZ mutants isolated from mice of transgenic mouse strain 40.6 (Gossen et al., 1989). First, the majority of mutants isolated from strain 40.6 were G:C -> A:T transitions located within CpG sites, including a hot spot at the sequence 5'-CGCGCGCG-3'. Mutations at this sequence were not observed in mutant LacZ genes isolated from strain 35.5. Secondly, most of the mutations analyzed from strain 35.5 were located in a region of about 500 basepairs, encoding the active region of the ß-galactosidase protein, whereas mutations in mutant LacZ genes isolated from strain 40.6 were, except for the methylation hot spot, randomly spread throughout the LacZ gene. The major difference between these two transgenic mouse strains is that mice of strain 40.6 carry the LacZ transgene on one of the autosomes. The results, therefore, suggest that both the susceptibility for spontaneous mutagenesis and the contribution of different sources of spontaneous mutation are highly dependent on the chromosomal position of the LacZ transgene.

With respect to our observation that some of the LacZ mutants are characterized by the presence of two mutations, it

should be noticed that similar mutations have been described for other genes (de Jong et al, 1988, Ikehata et al., 1989). A molecular mechanism, however, has not yet been established. Multiple dispersed mutations have recently been observed in mutant APRT genes isolated from a human colorectal carcinoma cell line (Harwood et al., 1991). It was proposed that the multiple mutations observed could be the result of error prone DNA synthesis during normal replication or long-patch excision repair of spontaneously occurring DNA lesions, ultimately leading to increased spontaneous mutation frequencies. The observation that two of our mutants, containing a double mutation, were isolated twice during independent packaging experiments suggest that part of these mutations are caused by the same mechanism and were formed during early developmental stages of liver and brain. The latter is also supported by the finding that in liver and brain of two animals (no. 30 and 83), mutations in different LacZ mutants were found to be identical.

Although DNA sequence analysis of these 35 LacZ mutants does not reveal a specific pattern which may explain the increased susceptibility to spontaneous mutagenesis of the LacZ transgene in strain 35.5, the data presented here are consistent with our earlier report in which we proposed that the high spontaneous mutation frequency in liver and brain of mice from strain 35.5 was due to the location of the LacZ transgene at a highly unstable chromosomal region making it more susceptible for spontaneous mutagenesis. DNA sequence data obtained sofar indicate that spontaneous mutagenesis at the LacZ transgene is highly dependent on the chromosomal position of the LacZ gene and, in addition, is tissue specific. With respect to the latter, it is tempting to speculate that mutations observed reflect error prone DNA replication or repair of different spontaneously occurring DNA lesions due to different physiological conditions and metabolic processes in liver and brain cells.

5.6 References

Brash, D.E., Rudolph, J.A., Simon, A., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J., & Ponten, A. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc. Natl. Acad. Sci. USA. **88**, 10124-10128.

Bressac, B., Kew, M., Wands, J. & Ozturk, M., (1991) Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature, **350**, 429-431.

Cook and Lederberg (1962) Recombination studies of lactose non-fermenting mutants of Escherichia coli K-12. Genetics **47**, 1335-1353.

Cooper, D.N., and M. Krawzcak (1990) The mutational spectrum of single base-pair substitutions causing human genetic disease: patterns and predictions. Hum. Genet. **85**, 55-74.

Coulondre, C., Miller, J.H., Farabagh, P.J., & Gilbert, W. (1978). Molecular basis of base substitutions hotspots in Escherichia coli. Nature, **275**, 775-780.

Ehrlich, M. & Wangh, R.Y. (1981) 5-Methylcytosine in eukaryotic DNA. Science (Washington DC) **212**, 1350-1357.

Ehrlich, M., Zhang, X.Y. & Inamdar, N.M. (1990) Spontaneous deamination of cytosine and 5-methylcytosine residues in DNA and replacement of 5-methylcytosine residues with cytosine residues. Mutat. Res. **238**, 277-286.

Gossen, J.A., de Leeuw, W.J.F., Tan, C.H.T., Lohman, P.H.M., Berends, F., Knook, D.L., Zwarthoff, E.C. & Vijg, J. (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying gene mutations in vivo. Proc. Natl. Acad. Sci. (U.S.A.) **86**, 7971-7975.

Gossen, J.A., de Leeuw, W.J.F., Verwest, A., Lohman, P.H.M., & Vijg, J. (1991) High spontaneous mutation frequencies in a LacZ transgene integrated on the mouse X-chromosome. Mutat. Res. **250**, 423-429.

Harwood, J., Tachibana, A. & Meuth, M. (1991) Multiple dispersed spontaneous mutations: a novel pathway of mutation in a malignant human cell line. Mol. Cell. Biol. **11**, 3163-3170.

Ikehata, H., Akagi, T., Kimura, H., Akasaka, S. & Katao, T. (1989) Spectra of spontaneous mutations in a cDNA of the human HPRT gene integrated in chromosomal DNA. Mol. Gen. Genet. **219**, 349-358.

De Jong, P.J., Growsowski, A.J., Glickman, B. (1988) Spectrum of spontaneous mutation at the APRT locus of Chinese hamster ovary cells: an analysis at the DNA sequence level, Proc. Natl. Acad. Sci. USA **85**, 3499-3503.

Jones, P.A., Rideout, W.M., Shen, J., Spruck, C.H., & Tsai, Y.S. (1992) Methylation, mutation and cancer, BioEssays, **14**, 33-36.

Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, J.A., Putman, D.L., and Short, J.M. (1991) Spectra of spontaneous and mutagen-induced mutations in the LacI gene in transgenic mice, Proc. Natl Acad. Sci. USA **88**, 7958-7962.

Kunkel, T., Schaaper, R.M. & loeb, L. (1983) Depurination-induced infidelity of DNA synthesis with purified DNA replication proteins in vitro, Biochemistry, **22**, 2378-2384.

Loeb, L.A. (1990) Endogenous carcinogenesis: Molecular oncology into the twenty-first century, Cancer Res. **49**, 5489-5496.

Newton, W.A., Beckwith, J.R., Zipser, D., Brenner, S. (1965) Nonsense mutants and polarity in the Lac Operon of Escherichia coli. J. Mol. Biol. **14**, 290-296.

Phear, G., Armstrong, W., & Meuth, M. (1989) Molecular basis of spontaneous mutation at the APRT locus of hamster cells. J. Mol. Biol. **209**, 577-582.

Rideout, W.M., Coetzee, G.A., Olumi, A.F., & Jones, P.A. (1990) 5-Methylcytosine as an Endogenous mutagen in the human LDL receptor and p53 genes. Science, **249**, 1288-1291.

Schaaper, R.M., Kunkel, T. & Loeb, L. (1983) Infidelity of DNA synthesis associated with bypass of apurinic sites. Proc. Natl.Acad. Sci. USA **80**, 487-491.

CHAPTER 6

PLASMID RESCUE FROM TRANSGENIC MOUSE DNA USING LACI REPRESSOR PROTEIN CONJUGATED TO MAGNETIC BEADS

Jan A. Gossen, Wiljo J.F. de Leeuw, Anco Molijn, and Jan Vijg

Accepted for publication in BioTechniques

6.1 Abstract

A method for the efficient rescue of Lac operator containing plasmids from transgenic mouse genomic DNA is described. The method is based on the high affinity of the LacI repressor protein for the Lac operator sequence. Using the LacI repressor protein conjugated to magnetic beads, more than 95% of plasmid sequences could be purified from restriction enzyme digested genomic DNA. After circularization, the plasmids were introduced into E.coli by means of electroporation. Since the plasmid was cloned into a bacteriophage lambda vector, the efficiency of plasmid rescue could easily be compared with *in vitro* packaging. Our results indicate that plasmid rescue is about 25 times more efficient. Application of this method should be especially useful with transgenic mouse models harbouring LacZ plasmid shuttle vectors for studying spontaneous or induced mutations *in vivo*.

6.2 Introduction

In recent years the use of marker genes integrated in the mammalian genome has found many applications in the study of genome organization and gene expression (7,11). Especially in studies on transgenic animals, efficient retrieval of vector sequences has become desirable. The best example in this respect is the recent use of transgenic mice, harbouring shuttle vectors with bacterial reporter genes in their genome, for studying spontaneous or induced mutations *in vivo* (2,3,9, for a review, see 6). Thus far, only bacteriophage lambda-based shuttle vectors have been effectively used in such studies (2,9). This type of shuttle vector can be rescued from genomic DNA of transgenic animals by means of *in vitro* packaging, when integrated in multiple copies in a head-to-tail arrangement. Due to a high degree of methylation the use of E.coli host strains incapable of recognizing and subsequently degrading methylated DNA, like the E.coli C strain, was found to be essential

in this form of rescue (4). Grant et al. showed that the same was true for plasmids and that the use of E.coli strains lacking methylation-dependent restriction enzymes greatly improved rescue efficiencies (8).

Plasmid rescue involves the excision of the linear plasmid from genomic DNA, followed by circularization under dilute conditions. The latter is the main reason why plasmid rescue compares unfavourably with in vitro packaging of bacteriophage lambda DNA; multiple transformations have to be performed to rescue the same number of vectors that can be rescued in a single in vitro packaging experiment.

This present work describes a method to isolate from mammalian DNA specific sequences containing the Lac operator sequence. The system is based on the high affinity of the LacI repressor protein for the Lac operator sequence, a 28 bp sequence located in front of the LacZ gene [dissociation constant of the repressor-operator complex is about 10^{-13}; (10)]. Transgenic mice, harbouring tandemly integrated bacteriophage lambda vectors with a LacZ-containing plasmid, were used to compare in vitro packaging with plasmid rescue. The latter was performed either by using the LacI repressor protein conjugated to magnetic beads or directly from genomic DNA.

6.3 Materials and methods

6.3.1 lambda-pl288 shuttle vector

The plasmid pUR288 (a kind gift of Dr. L. Dorssers, Daniel den Hoed Kliniek, Rotterdam, The Netherlands (14)) was partially digested with the restriction enzyme EcoRI (Gibco BRL) and the entire plasmid was cloned into the unique EcoRI site of lambda-gt10 (Promega). The construct is schematically depicted in Fig.1. After *in vitro* packaging, phage were plated on Y1090 host cells in the presence of isopropyl ß-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl ß-D-galactoside (Xgal) and selected for ß-galactosidase expression. A single blue plaque was isolated and phage DNA was isolated as described elsewhere (15).

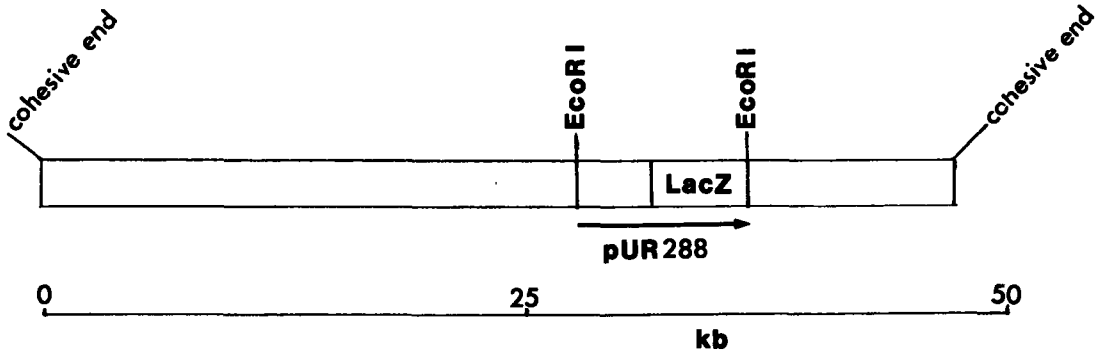


Figure 1. Schematic depiction of the lambda-pl288 shuttle vector. Indicated is the pUR288 plasmid which was cloned into the unique EcoRI site of the lambda-gt10 vector (not on scale).

6.3.2 Transgenic mice

About 200 copies of the lambda-pl288 shuttle vector were microinjected into fertilized CD2.F2 (BAlb/c x DBA/2) mouse oocytes which were then implanted into pseudo-pregnant BCBA foster mice. The offspring was analyzed for the presence of the transgene by means of Southern blot analysis of tail DNA using a ^{32}P labelled lambda-pl288 probe.

6.3.3 Preparation of LacI repressor protein-magnetic beads

To 1 ml Dynal M450 magnetic beads coated with sheep anti-mouse IgG (4×10^8 magnetic beads/ml), 130 µl anti-ß-galactosidase (2.3 mg/ml; Promega) was added. The mixture was incubated for 45 minutes at room temperature. The magnetic beads were then pelleted on a magnetic stand (DYNAL) and the clear supernatant removed. The beads were washed 3 times for 5 minutes in 1 ml PBS/0.1% BSA. Between the wash steps the beads were pelleted on the magnetic stand and the clear supernatant removed. After the last wash step the pellet was resuspended in 900 µl PBS/0.1% BSA, and 75 µl ß-galactosidase/ LacI-repressor fusion protein (2.0 mg/ml; Promega, see also ref. 12) was added and incubated for 2 hours at room temperature. Beads were pelleted and washed 2 times with 1 volume PBS buffer and once with 1 volume storage buffer (20 mM Tris.Cl (pH 7.6), 2 mM EDTA, 5% glycerol, 1 mg/ml acetylated BSA, 0.02% Na-azide). Finally, the beads were resuspended in 1 ml storage buffer (20 mM Tris.Cl (pH 7.6), 2mM EDTA, 5% glycerol, 1 mg/ml acetylated BSA, 0.02% Na-azide) and stored at 4°C.

6.3.4 Plasmid rescue

Transgenic mouse liver DNA was digested with the restriction enzyme Hind III under conditions recommended by the manufacturer (Gibco BRL) and subsequently diluted to a final concentration of

0.5 μg/μl. Restriction enzyme digestion of transgenic mouse DNA with this enzyme generates a 5.5 kb fragment containing the complete pUR288 plasmid. LacI-repressor magnetic beads (20 μl) were pelleted on a magnetic stand, the supernatant removed, and 8 μl 5 x binding buffer (100 mM Tris.Cl pH 7.6, 10 mM EDTA, 25% glycerol), 12 μl H_2O, and 20 μl Hind III-digested genomic DNA were added (for a schematic outline of the plasmid rescue protocol, see Fig. 2). The mixture was incubated for 1.5 hours at room temperature. The beads were then washed twice with 40 μl 20 mM Tris.Cl (pH 7.6), 2 mM EDTA, 5% glycerol, resuspended in 40 μl IPTG elution buffer (10 mM Tris.Cl (pH 7.6), 2 mM EDTA, 20 mM NaCl, 135 μg/ml IPTG) and incubated for 20 minutes at room temperature. The IPTG eluate containing the plasmids was stored at 4 °C.

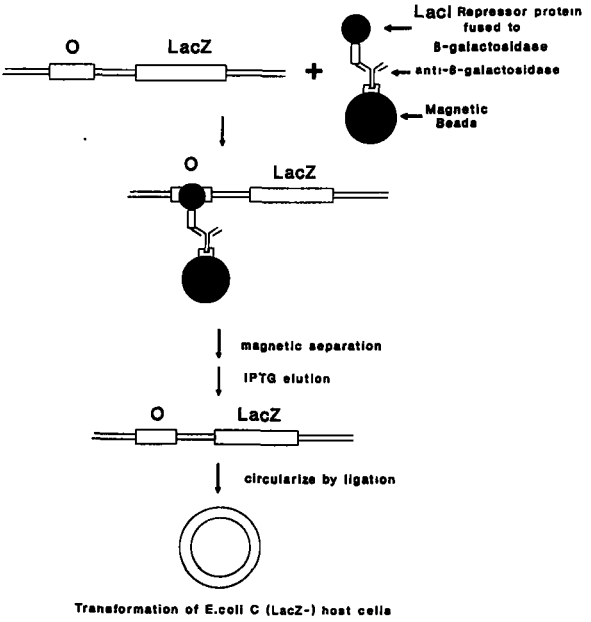


Figure. 2. Schematic outline of plasmid rescue using LacI-repressor magnetic beads (not on scale).

6.3.5 Circularization of plasmids by ligation

a. After LacI-magnetic bead purification

Linear pUR288 DNA rescued from genomic DNA (2 µl IPTG eluate), or 20 pg linearized control plasmid DNA was circularized by adding 0.04 unit T4 DNA ligase (BRL), 1 µl 10 x ligation buffer (500 mM Tris.Cl pH 7.6, 100 mM $MgCl_2$, 10 mM ATP, 10 mM DTT), and incubated for 2 hours at 16°C. DNA was subsequently precipitated by adding 5 µg glycogen (Boehringer), 1/10 volume 3 M NaAc (pH 4.9) and 2.5 volume ice-cold 100% ethanol. DNA was resuspended in 1 to 50 µl of 10 mM Tris.Cl/0.1 mM EDTA.

b. Directly from genomic DNA

Transgenic mouse DNA, digested with the restriction enzyme Hind III, was diluted to a concentration of about 0.5 µg/ml in ligation buffer (50 mM Tris.Cl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT). Ligations were performed in a total volume of 400 µl at 16 °C for 2 hours using 10 units T4 DNA ligase/ml. DNA was precipitated by adding 5 µg glycogen (Boehringer), 1/10 volume 3 M NaAc (pH 4.9) and 2 volumes ice-cold 100% ethanol. DNA was resuspended in 1 to 50 µl of 10 mM Tris.Cl/0.1 mM EDTA.

6.3.6 Electro-transformation

Electroporation of plasmids was essentially performed as described by Dower et al. (1).

Preparation of electro-competent cells: E.coli C $LacZ^-$ cells were grown to OD_{600}=0.4, placed on ice for 30 minutes, centrifuged at 1500g and washed once with 1 volume H_2O, once with 1/2 volume H_2O, once with 1/2 volume 10% glycerol and finally resuspended in 1/250 volume 10% glycerol. Aliquots of 40 µl were placed in a ethanol dry-ice bath for 15 minutes and stored at - 80 °C.

Electroporation: Electroporations were performed by adding 1-2 μl of circularized plasmid to 40 μl electro-competent E.coli C LacZ⁻ cells. Electroporation conditions were 25 μF and 2.5 kV for the Gene Pulser apparatus and 200Ω for the Pulse Controller using 0.2-cm cuvettes (Biorad). After electroporation, 1 ml SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added immediately and the cells were incubated in a shaking waterbath at 225 RPM for 1 hour at 37°C. Cells were then plated on LB agar plates containing 50 μg ampicillin per ml.

6.3.7 In vitro packaging

In vitro packaging of 1 μg genomic liver DNA was performed using Gigapack Gold II extracts under conditions recommended by the manufacturer (Stratagene). The phage titer was determined by plating small aliquots of the packaging extract on E.coli C (LacZ⁻) cells.

6.4 Results and discussion

Isolation of specific DNA sequences, like bacterial marker genes, from mammalian DNA has become a valuable tool in molecular biology, e.g., for investigating chromosomal regions adjacent to transgene integrations, for studying gene mutations in vivo. The most commonly used method for rescuing marker genes from mammalian DNA is in vitro packaging of bacteriophage lambda shuttle vectors (2,3,9). In the present study an alternative method, plasmid rescue, is described and compared to in vitro packaging. The pUR288 plasmid used for rescue experiments contains a pBR322 origin of replication, an ampicillin resistance gene and the complete LacZ gene. This plasmid was cloned into the unique EcoRI site of the lambda-gt10 vector and subsequently micro-injected into fertilized CD2.F2 oocytes. A number of transgenic mice were obtained, one of

which was harbouring approximately 40 copies of the lambda-pl288 shuttle vector and was used in the present study.

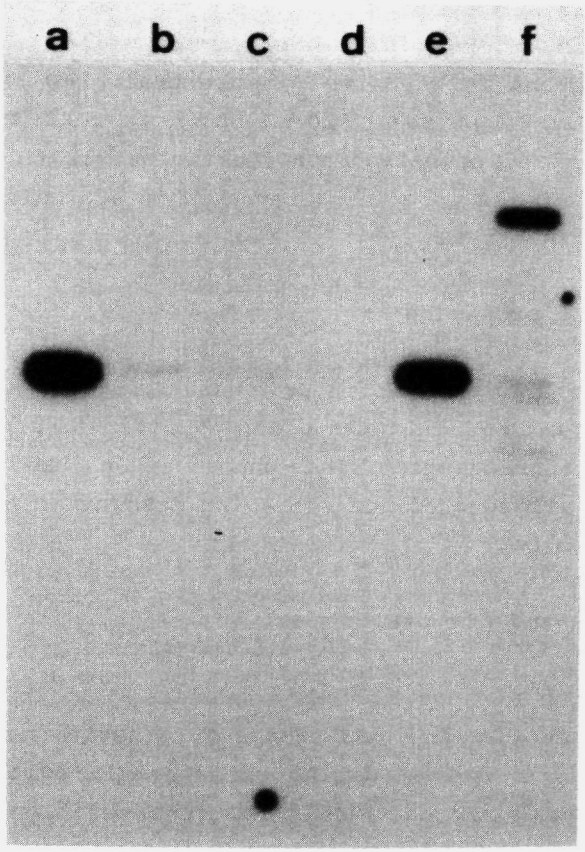


Figure. 3. Rescue of the pUR288 plasmid from Hind III digested liver DNA analyzed by means of Southern blot analysis. Lanes are: (a) 1 µg Hind III digested genomic liver DNA; (b) 1 µg Hind III digested liver DNA after incubation with LacI repressor magnetic beads and separation; (c,d) supernatants obtained after washing LacI repressor magnetic beads; (e) supernatant obtained after incubation of LacI repressor magnetic beads with IPTG; and (f) ligation product.

To recover the LacZ marker gene from transgenic mouse DNA three different methods were used: (i) plasmid vectors were rescued from genomic mouse DNA digested with the restriction enzyme HindIII, using LacI repressor-magnetic beads; (ii) plasmid vectors were rescued from HindIII digested genomic DNA by ligation at low DNA concentrations; and (iii) <u>in vitro</u> packaging of bacteriophage lambda-vectors using total genomic mouse DNA.

Rescue of the plasmid vectors from transgenic mouse DNA using LacI repressor-magnetic beads, was performed by first conjugating the LacI repressor-ß-galactosidase fusion protein via anti-ß-galactosidase antibodies to IgG coated magnetic beads. The LacI repressor protein-magnetic beads complex was then incubated with Hind III-digested genomic DNA after which the pUR288 plasmids were recovered using a magnetic particle concentrator (Fig. 2). As shown in Fig. 3 (lane a, b and e) rescuing pUR288 plasmid sequences from genomic DNA is highly efficient; almost all integrated plasmids appeared to be recovered upon their release from the beads.

Table 1. Plasmid and bacteriophage lambda rescue efficiencies from transgenic mouse liver DNA

Method	T4-ligase (Weiss units)	# colonies/ plaques	rescue efficiency c(p)fu/μg
Plasmid rescue[1]			
+ purification[2]	1.0	500	50
	0.1	2,187,750	218,750
	0.02	2,375,000	237,500
	0.004	1,132,250	131,250
	0.0008	1,125,000	112,500
- purification[3]	5.0	900	3,000
	0.5	67,500	225,000
	0.05	25,500	85,000
	0.005	6,000	20,000
In vitro packaging[4]	-	78,750	10,500

1) transformation efficiency 10^{10} cfu/μg plasmid DNA
2) 10 μg Hind III digested genomic DNA was used for a single rescue experiment using LacI magnetic beads
3) 0.3 μg Hind III digested DNA was ligated in a total volume of 0.4 ml.
4) packaging efficiency 1 x 10^9 pfu/μg lambda DNA

The next step involved circularization of the linear pUR288 plasmid by ligation, under conditions favouring circularization (< 5 μg DNA/ml (13)). Circularized plasmids were then used for transformation of E.coli C ($LacZ^-$) host cells by means of electroporation. Initial rescue efficiencies were only in the order of 50 cfu/μg DNA. To test if the linear plasmids were circularized by the T4-ligase enzyme, Southern blot analysis was performed. The results obtained (Fig. 3, lane f), showed a decreased mobility pattern, typical for circular plasmid DNA when run in the presence of ethidium bromide. The individual components of the ligation mixture were then tested for inhibitory effects during electroporation. The results obtained, indicated a dramatic increase of transformation efficiency, up to 4,000-fold, when using decreasing amounts of the T4-ligase enzyme. Similar results were obtained when control pUR288 plasmid was used. Such a strong inhibitory effect of T4-ligase during electroporation was also found by Ymer (17), who showed that heat-denaturing of the ligase enzyme prior to electroporation resulted in increased electroporation effiencies. Generally, optimal transformation efficiencies yielding up to 237,500 cfu/μg genomic DNA were obtained when using 0.02 Weiss unit T4-ligase per 10 pg pUR288 DNA (Table 1). This is 71% of the theoretically maximum 320.333 (assuming a transformation efficiency of 10^{10} cfu/μg plasmid DNA).

Plasmid rescue from transgenic mouse DNA by means of circularization at low DNA concentrations was found to be equally efficient. In general, about 60,000 cfu could be obtained in a single electroporation experiment when ligations were performed at DNA concentrations of 0.50 μg/ml using 0.5 Weiss unit T4-ligase. A major advantage of the LacI repressor-magnetic beads system, however, is that plasmid sequences are easily enriched from large amounts of genomic DNA in a single step.

As shown in Table 1, <u>in vitro</u> packaging of 1 μg total genomic liver DNA resulted in about 10,000 pfu (500 pfu/μg/copy integrated). Theoretically, the maximum number of vectors that can

be rescued from 1 μg genomic DNA is 320,000 (assuming a packaging efficiency of 10^9 pfu/μg lambda DNA). The overall packaging efficiency is therefore only 3.1%. This is lower than expected and probably due to degradation (shearing) of genomic DNA during isolation; about 40 copies of the lambda-pl288 vector are integrated in a head-to-tail fashion in the mouse genome (2,000,000 bp in total) and double strand breaks are therefore likely to occur during isolation of genomic DNA.

In conclusion, a major advantage of plasmid rescue using LacI repressor-magnetic beads is the high effective yield of vector copies in one single experiment. An important application of this form of plasmid rescue would be in studying mutagenesis in vivo. Using this system large numbers of vectors can be rescued in one single experiment from any given tissue and studied for the presence of mutations. Upon the transfer of circular plasmids to a galactose sensitive E.coli host strain mutation detection can now be based on selection against the non-mutant LacZ mutational target genes (5).

A more general application of the magnetic bead approach would be to conjugate DNA binding proteins, like transcription factors or proteins involved in regulation of gene expression, to magnetic beads (16). This would greatly facilitate the cloning and characterization of thus far unknown regulatory DNA sequences.

6.5 References

1 **Dower, W.J., Miller, J.F., and Ragsdale, C.W.** (1988). High efficiency transformation of E.coli by high voltage electroporation. Nucleic Acids Res. 16, 6127-6145.

2 **Gossen, J.A., de Leeuw, W.J.F., Tan, C.H.T., Lohman, P.H.M., Berends, F., Knook, D.L., Zwarthoff, E.C., and Vijg, J.** (1989). Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying gene mutations in vivo. Proc. Natl. Acad. Sci. (U.S.A.) 86, 7971-7975.

3 **Gossen, J.A., de Leeuw, W.J.F., Verwest, A., Lohman, P.H.M., and Vijg, J.** (1991). High somatic mutation frequencies in a LacZ transgene integrated on the mouse X-chromosome. Mutation Res. 250, 423-429.

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

5 **Gossen, J.A., Molijn, A.C., Douglas, G.R., Vijg, J.** (1992) Application of galactose-sensitive E.coli strains as selective hosts for LacZ$^-$ plasmids. Nucleic Acids Res. 20, 3254.

6 **Gossen, J.A. and Vijg, J.** (1993) Transgenic mice as model systems for studying gene mutations in vivo. Trends in Genetics, 9, 27-31.

7 **Gossler, A., Joyner, A.L., Rossant, J., and Skarnes, W.C.** (1989). Mouse embryonic stem cells and reporter constructs to detect developmental regulated genes. Science 244, 463-465.

8 **Grant, S.G.N., Jessee, J., Bloom, F.R., and Hanahan, D.** (1990). Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc. Natl. Acad. Sci. U.S.A. 87, 4645-4649.

9 **Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, J.A., Putman, D.L., and Short, J.M.** (1991). Spectra of spontaneous and mutagen-induced mutations in the LacI gene in transgenic mice. Proc. Natl. Acad. Sci. USA 88, 7958-7962.

10 **Levens, D. & Howley, P.** (1985) Novel method for identifying sequence-specific DNA-binding proteins. Molec. Cell. Biol. 5, 2307-2315.

11 **Mansour, S.L., Thomas, K.R., Deng, C., and Capecchi, M.R.** (1990). Introduction of a LacZ reporter gene into the mouse int-2 locus by homologous recombination. Proc. Natl. Acad. Sci. USA 87, 7688-7692.

12 **Müller, U & Kania, J.** (1974) Lac repressor can be fused to ß-galactosidase. Nature, 249, 561-562.

13 **Perucho, M., Hanahan, D., Lipsich, L., and Wigler, M.** (1980). Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature 285, 207-210.

14 **Rüther, U. & Müller-Hill, B.** (1983) Easy identification of cDNA clones. EMBO J. 2, 1791-1795.

15 **Sambrook, J., Fritsch, E.R., and Maniatis, T.** (1989). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 2nd Ed.

16 **Sompayrac, L & Danna, K.** Method to identify targets of DNA binding proteins. Proc. Natl. Acad. Sci. USA, 87, 3274-3278.

17 **Ymer, S.** (1991). Heat inactivation of DNA ligase prior to electroporation increases transformation efficiency. Nucleic. Acids Res. 19, 6960.

ACKNOWLEDGMENTS

We thank Dr. G.R. Douglas for stimulating comments during the course of this study. The research described is supported by PBTS Grant BIO 91076 from the Dutch Ministry of Economic Affairs.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7 SUMMARY AND GENERAL DISCUSSION

The study of spontaneous and induced mutations in vivo in mammalian genes has thusfar been hampered by the lack of techniques to readily identify and characterize mutated genes. Many different methods for mutation detection have been developed to obtain a more fundamental insight into the chain of molecular events that ultimately lead to mutations. However, most of these methods can only be applied to cultured cells and therefore do not allow comparative analysis of mutations in various organs and tissues of experimental animals. To date, these problems have been solved only partially and for a very limited number of loci, e.g. HPRT and HLA-A in mouse or human lymphocytes. This thesis describes how transgenic mice harbouring multiple copies of the bacterial LacZ gene as a target for mutagenesis in all body cells, can be applied for studying gene mutations in vivo, and overcome many of the limitations of previously described mutation detection systems.

7.1 Spontaneous mutations

Of a total of 20 different transgenic founder mice initially obtained, 3 were chosen for further analysis. Two of these strains, termed 20.2 and 40.6, were chosen because of the high copy number of the LacZ target gene (heterozygous mice of these strains carried about 80 and 40 copies of the LacZ transgene, respectively) which would facilitate the rescue of high numbers of vectors per μg of genomic DNA. The third strain (termed 35.5) was chosen, firstly because the LacZ target gene was found to be integrated in the X-chromosome and secondly, in view of the initial observation that spontaneous mutation frequencies in the LacZ gene in liver were up to 100 times higher than those in strains 20.2 and 40.6.

Strains 20.2 and 40.6, described in Chapters 2 and 3,

were characterized by low spontaneous mutation frequencies. In mice of strain 20.2 (heterozygous) the frequencies were about 0.6×10^{-5} in brain and 0.7×10^{-5} in liver. It should be noted, however, that in this first study both the number of animals and the total number of plaques analyzed per tissue type were low. Obviously, more data would be required to accurately determine the spontaneous mutation frequency. Strain 20.2, however, was not used in further studies due to the fact that the breeding performance of these transgenic animals was very poor (average litter size of 2.5). In addition, it was found impossible to obtain homozygous animals, which might be indicative of an insertional mutagenesis event, i.e. integration of the bacteriophage lambda vector into a gene essential for development of the early embryo. Because of this, a second transgenic mouse line (40.6) was used in further experiments.

More detailed studies on spontaneous mutation frequencies in bone marrow, skin, and liver of mice from strain 40.6 revealed that these were in the order of 3×10^{-5}. Such a mutation frequency is in the same order of magnitude as those observed in animal and human T-lymphocytes using the HPRT clonal assay (Horn et al., 1984; Albertini et al., 1985; Turner et al., 1985). Initially, when up to 200,000 plaques were analyzed per tissue, and low numbers of lacZ mutants were isolated, data obtained indicated large inter-animal variations in spontaneous mutation frequencies. More accurate data, however, were obtained when the total number of plaques analyzed per tissue was increased to 1,000,000. Interestingly, spontaneous mutation frequencies in testis were found to be about 4-5 times lower (0.7×10^{-5}). Such a difference may reflect differences in, e.g, the rate of cell turnover, exposure to endogenous mutagens, DNA repair, between the organs and tissues analyzed. However, it should be realized that the numbers of animals and plaques analyzed are still relatively small, and additional data are required in order to clearly indicate whether these differences are significant.

Similar studies performed by Kohler et al. (1991),

employing the bacterial LacI gene as the mutational target gene, showed that spontaneous mutation frequencies in spleen were in the order of 1.7 x 10^{-5}. Also, in these studies mutation frequencies in male germ cells were found to be lower, 0.6 x 10^{-5}. These results suggest that mutation frequencies for the LacZ and LacI mutational target gene in vivo were not significantly different. Yet, there are a number of important differences between these two strains. First, the two strains were constructed against different genetic backgrounds (C57Bl/6 versus CD2), which may e.g. result in differences in exposure to endogenous mutagens or DNA repair. Secondly, since integration of microinjected DNA in the mouse genome occurs at random, the chromosomal position of the mutational target genes is likely to vary considerably between the two transgenic mouse strains. Indeed, the observations described in Chapter 4 underline the possible influences of chromosomal position effects on spontaneous mutation. In this particular transgenic mouse strain (strain 35.5) mutation frequencies were up to 100 times higher than those in strains 20.2 and 40.6. Interestingly, in brain a significant difference between mutation frequencies in heterozygous and homozygous animals was found; 5.1 and 15.5 x 10^{-5}, respectively. The question arose whether the lambda vector could have integrated and inactivated a gene with an influence on mutation rate, e.g. DNA repair, replication level of endogenous mutatgens. The finding that the lambda-gt10LacZ concatemer was integrated on the X-chromosome, and mutation frequencies in brains of male and heterozygous female mice were not different, however, did not support this notion. Further experiments, such as DNA sequence analysis of LacZ mutants isolated from brains of both hemizygous and homozygous mice, are therefore required to explain the particular high mutation frequency in homozygous animals of strain 35.5.

In addition to the high mutation frequencies observed in strain 35.5, it was also shown that 3′ regions flanking the transgene were characterized by a high incidence of deletion events in male germ cells. In view of these results it was

suggested that the high spontaneous mutation frequency in this particular strain was due to the location of the lambda-gt10LacZ concatemer at a highly unstable chromosomal region, making it more susceptible to both germinal and somatic mutagenesis.

7.2 Chemical-induced mutations

The applicability of LacZ transgenic mice for detecting chemical-induced mutations was demonstrated in Chapters 2 and 3. These initial studies were focused on the detection of induced mutations caused by treatment of mice with relatively high doses of model carcinogens such as ENU, chlorambucil or procarbazine. A major question that arose immediately is whether or not lesions present in the DNA are fixed into mutations after rather than before rescue, during propagation in E.coli. A number of experiments addressed this question. First, transgenic mice treated with various doses of ENU were analyzed at several time points after treatment. Increased mutant frequencies (as compared to background) in post-mitotic cells, such as in brain tissue, were only observed 7 days after treatment but not after 1 day. The latter would be expected when the mutations found their origin in E.coli rather than in the mouse. Secondly, DNA sequence analysis of mutant LacZ genes isolated from brain DNA of mice treated with ENU showed that the pattern of mutations were different from those observed in ENU-treated E.coli. These results strongly suggest that the mutations analyzed found their origin in the mouse.

The results described in Chapters 2 and 3 indicated organ-specific and dose-dependent mutation induction. After treatment of the LacZ transgenic mice with different doses of these mutagens, mutation frequencies up to 50 times the background value were observed in organs and tissues like liver, brain and bone marrow. Similar results have been obtained by Kohler et al. (1991) using LacI transgenic mice.

It should be noted, however, that the results obtained cannot easily be compared due to differences in experimental set-up. This raises the more general issue, i.e. how mutagenicity studies with transgenic mice of this type should be performed to yield reliable and comparable results. Important parameters which need to be more precisely defined are: (1) the number of reporter gene copies (i.e. number of plaques) to be analyzed per DNA sample in order to accurately determine (induced) mutation frequencies; (2) the number of animals to be analyzed per chemical and; (3) the number of dose and time points to be analyzed per chemical.

With respect to the first point, results on the mutagenicity of ENU and chlorambucil in liver, brain, bone marrow and testis of mice from strain 40.6 (Chapter 3), indicated the expected increased mutation frequencies after treatment of the mice with these mutagens. However, relatively low numbers of plaques (<100,000 plaques per tissue) were studied, and the result was that not too many LacZ mutants were obtained. Evidently, the total number of plaques analyzed per tissue must be increased to at least 500,000 (see also Myhr, 1991), in order to significantly detect a chemical-induced increase of the mutation frequency above the background. Therefore, the results obtained are preliminary and may change when the total number of plaques analyzed is increased.

With respect to the last two points, the data shown in Chapter 2 on the mutagenicity of procarbazine, cyclophosphamide and acrylamide in bone marrow, and the data on the mutagenicity of MNNG and DMBA in skin show that chemical-induced mutation frequencies were highly dependent on the harvest time, and in addition, showed inter-animal variations up to 50%. In this respect it should be noted that most of the chemicals are administered by intraperitoneal injection. In general, differences in mutation frequencies could be due to a number of factors, such as metabolic activation, transport via the lymph and blood, distribution, uptake by the various organs and tissues or toxicity to organs surrounding the site

of injection. Certain of these factors will be different when other routes of administration are chosen. In view of these considerations it appears that, testing a single chemical for its mutagenic potential would require at least three different dose and time points with a minimum of 3 animals per dose/time point for each mode of administration, and would require the analysis of at least 500,000 plaques per tissue.

Studies on the mutagenicity of ENU, a well-known germ cell mutagen as determined in the mouse specific locus test (Favor, 1988), initially indicated only low increases of the mutation frequency in germ cells of male transgenic mice (Chapter 3). In the mouse specific locus test the offspring from matings between treated males and untreated females are analyzed for phenotypic changes. These matings were performed during about 8 weeks after exposure to the mutagen, which is sufficient to cover the entire spermatogenic cycle. These experiments showed that the highest mutation frequencies were observed when matings were performed at least 43 days after ENU treatment. Those conditions clearly differ from experiments performed by us and others (Kohler et al., 1991), in which genomic DNA was isolated from the whole testis, only 7 days after exposure to the mutagen. More recent results obtained by Douglas (personal communication) indicate much higher mutation frequencies when the LacZ transgenes (analogous to the mouse specific locus test) are isolated from sperm DNA of mice from strain 40.6 about 50 days after treatment with ENU. These results suggest that ENU predominantly induces mutations in stem-cell spermatogonia and not in mature or maturing sperm cells. Although these are as yet preliminary data, it underlines the importance of allowing sufficient time between treatment and rescue of the reporter genes for fixation of mutations, since this is likely to be different for each mutagen tested and also depending on the organs and tissues to be analyzed.

7.3 Mutational spectra

Analysis of both spontaneous and chemical-induced LacZ mutants, isolated from various organs and tissues, show that a variety of mutations can be detected. The predominant type of mutations observed were single basepair changes, resulting in amino acid changes or in the formation of stop codons. In addition, frameshift and deletion type mutations were detected. Insertion type mutations were not observed. The high percentage of basepair substitutions and the low number of deletion events observed may be explained by the relatively small size of the LacZ reporter gene; the size of the LacZ gene is 3 kb whereas the average size of a mammalian gene, where introns are customary, is about 100 kb. Similar mutational spectra, however, have been obtained in studies employing the hamster APRT gene (2500 bp; Phear et al, 1989) or HPRT cDNA (1300 bp; Ikehata et al, 1989), each having a size similar to that of the LacZ transgene. Moreover, since the rescue of the lambda-gt10 LacZ transgenes from transgenic mouse DNA is performed by *in vitro* packaging, and this process requires that the lambda vector has a size varying from 42 to 52 kb, deletions or insertions larger than $\pm$ 5 kb are not detectable in these systems. The use of plasmid shuttle vectors, as described in Chapter 6, may overcome part of this limitation. Plasmid vectors with a size up to 40 kb can be rescued from genomic DNA by using LacI-magnetic beads (see Chapter 6). In principle, this should allow the rescue of plasmids containing large insertions. In addition, since rescue of a plasmid vector is only depending on the presence of a bacterial origin of replication and a gene encoding antibiotic resistance, plasmid sequences flanking a large deletion within the plasmid concatemer can still be rescued.

With respect to spontaneous mutations, analysis of mutant LacZ genes isolated from liver, brain and bone marrow of mice from strain 40.6 showed that the majority of mutations were G:C->A:T transitions (Table 1). Since these mutations were mainly present within CpG dinucleotides, they probably

result from spontaneous deamination of 5'-methylcytosine. The latter has been hypothesized to be a major mechanism of spontaneous mutagenesis at CpG sites in vivo (Ehrlich and Wangh, 1981; Ehrlich et al., 1990) and these sites appear to be hot-spots for mutation in eukaryotic genes such as the low-density lipoprotein receptor gene and p53 tumour-suppressor gene (Rideout et al., 1991; Jones et al., 1992).

The results described in Chapter 5 on the characterization of spontaneous LacZ mutants isolated from liver and brain of mice from strain 35.5 showed remarkable differences with the spectra obtained with strain 40.6, which are likely to be due to the chromosomal position of the LacZ transgene (Table 1). This particular transgenic mouse strain, which carries the LacZ transgene at the X-chromosome, was found to be characterized by high somatic mutation frequencies (Chapter 4). Again, the mutant lacZ genes analyzed were characterized by a high incidence of C -> T transitions. However, only 25% were located within a CpG site, indicating that spontaneous deamination of 5-methylcytosine is not a major mechanism for spontaneous mutagenesis at this locus. Also, most of the mutations analyzed from this strain were located in a region of only 500 bp in contrast to LacZ mutations analyzed from strain 40.6 which were evenly distributed throughout the LacZ gene. These results suggest that both the susceptibility to spontaneous mutagenesis and mutational spectra are dependent on the chromosomal position of the LacZ transgene.

Table 1. Spontaneous mutations in LacZ transgenes isolated from different organs and tissues of two different transgenic mouse strains with different integration sites of the LacZ transgenes.

	strain 40.6* (autosomal)	strain 35.5 (X-chromosome)
Transition	43	14
GC => AT	35 (30)**	12 (4)**
AT => GC	8	2
Transversion	14	8
GC => TA	7	6
GC => CG	3	0
AT => TA	2	2
AT => CG	2	0
Deletion	8	9
Double mutation	4	3
Total	61	28

* Data from Douglas et al. (personal communication)
** In brackets, mutation within 5'CpG dinucleotide

Interestingly, the preliminary results shown in Chapter 3 on the DNA sequence analysis of procarbazine-induced mutations in bone marrow, suggest that these are different from spontaneous mutations (Table 2). Unlike spontaneous mutations, procarbazine-induced mutations were predominantly transversions. Similar results were obtained by Douglas et al. (personal communication) on ENU-induced mutations in bone marrow of mice from strain 40.6 (Table 2). Related data were presented by Kohler et al. (1991), who analyzed a number of LacI mutants isolated from the spleen of transgenic mice treated with benzo-a-pyrene (BaP). Unfortunately, the number of spontaneous LacI mutants sequenced in the latter study was too low to clearly distinguish BaP-induced from spontaneous mutations. However, the results obtained indicated that all LacI mutants isolated from BaP-treated mice resulted from either a transversion or frameshift mutation, a type of mutation previously shown to be characteristic for BaP in <u>in vitro</u> assays (Eisenstadt et al., 1982).

Table 2. Nature of spontaneous versus procarbazine- and ENU-induced LacZ mutations in bone marrow of transgenic mice of strain 40.6.

	spontaneous* (n=20)	procarbazine induced (n=8)	ENU* induced (n=8)
Transition	16	3	3
GC => AT	15 (14)**	1 (0)**	2 (1)**
AT => GC	1	1	1
Transversion	2	6	5
GC => TA	2	0	1
GC => CG	0	1	0
AT => TA	0	3	4
AT => CG	0	2	0
deletion	2	0	0

* Data from Douglas et al. (personal communication)
** In brackets, mutation within 5'CpG dinucleotide

7.4 Future perspectives

In general, the results described in this thesis indicate that various organs and tissues can be analyzed for mutations in the LacZ transgenic mouse models constructed. These models, therefore, allow to correlate mutations directly with the induction of DNA damage and its repair, and with important physiological processes to which mutations are thought to be causally related e.g. aging (Vijg, 1990: Kirkwood, 1990), atherosclerosis and cancer (Bishop, 1988; Loeb, 1989; Harris, 1990). With respect to aging, significant increases in mutation frequencies, up to ten-fold, have already been demonstrated in both the HPRT and HLA-A test for human lymphocytes (Turner et al., 1985; Grist et al., 1992). For cancer, evidence has recently been obtained that specific DNA-damaging agents leave characteristic mutational fingerprints (Runnebaum et al., 1991), skin (Brash et al., 1991, Bressac et al., 1991; Hsu et al., 1991, Harris, 1991;

Vogelstein and Kinzler, 1992). Transgenic mouse models can now be applied to significantly extend such studies, since they allow comparative analysis of mutations in many organs and tissues of the same animal. Data on mutational loads can be correlated with patterns of age-related multiple pathology, particular forms of age-related functional decline and tumour incidence, and they would for the first time allow to confirm such a hypothesis directly for the in vivo situation. The availability of methods to positively select mutant LacZ genes, for both the bacteriophage lambda en plasmid based transgenic mouse models, now facilitates the analysis of larger numbers of animals in a more extensive way, via the determination of mutation frequencies in various organs (Gossen et al., 1992a; Gossen et al., 1992b).

The observed differences in spontaneous mutation frequencies in LacZ transgenes integrated at different chromosomal positions described in Chapter 4, may raise the question whether or not other factors such as the transcriptional activity of a gene, may influence the susceptibility for spontaneous and/or chemical-induced mutagenesis. It has been reported that the repairability of chemical-induced lesions is different in non-transcribed ("inactive") regions of the genome versus transcribed ("active") regions (Mellon et al., 1986; Mullenders et al., 1992). Since the transgenic mice described in this thesis contain the LacZ bacterial reporter gene as a target for mutagenesis, which is not expressed in any organ or tissue, it is at this moment not clear whether the mutation frequencies analyzed are representative for those of endogenous gene sequences. Transgenic mice in which the bacterial reporter gene LacZ is fused to the mouse HPRT gene, a gene that is being expressed in various organs and tissues, are presently being constructed (de Leeuw, personal communication). Such a transgenic mouse model may provide additional insight in the relationship between the susceptibility to mutagenesis and the transcriptional activity of a gene.

In order to test the applicability of the transgenic

mouse systems described in this thesis as a short-term mutagenicity assay, a number of validation studies are now in progress. An important advantage of the use of transgenic mice is that it will no longer be required to test a chemical near the maximum tolerated dose as is done in the rodent long-term carcinogenicity assay; in principle studies can be performed using much lower doses without the necessity to increase the number of animals or the duration of the experiment. Such an experimental approach would be more representative for the human situation, would overcome side effects like sustained tissue injury due to high dose treatments which may lead to chemicals being falsely identified as carcinogens (Ames and Gold, 1990). In addition, for risk-assessment this would no longer require to extrapolate data obtained in mice using high doses to the much lower dose ranges to which humans are usually exposed.

Additional applications of transgenic mouse mutation models would be to generate mouse models which, in addition to bacterial reporter genes, would also harbour genes encoding (human) drug-metabolizing enzymes, e.g. P-450. Such transgenic mouse models would combine metabolic with mutagenic endpoints and may provide a sensitive system to study the complex pathways of mutagen metabolism in humans. Alternatively, potential risk factors such as defects in genes encoding DNA repair enzymes or the p53 tumour suppressor gene, could be studied via the inactivation of these genes by gene targeting techniques. The possibility to directly correlate risk factors with mutagenic endpoints may allow to clearly identify the role of each of these enzymes in the processes which ultimately lead to mutations.

7.5 References

Albertini, R.J. (1985) Somatic gene mutations in vivo as indicated by the 6-thioguanine-resistant T-lymphocytes in human blood, Mutation Res. 150, 411-422.

Ames, B.N. & Gold, L.S. (1990) Chemical carcinogenesis: too many rodent carcinogens, Proc. Natl. Acad. Sci. USA 87, 7772-7776.

Ames, B.N. & Gold, L.S. (1990a) Too many rodent carcinogens: Mitogenesis increases mutagenesis, Science (Perspective) 249, 970-971.

Bishop, J.M. (1987) The molecular genetics of cancer, Science 235, 305-311.

Brash, D.E., Rudolph, J.A., Simon, J., Lin, A., McKenna, G.J., Baden, H.P., Halperin, A.J., Ponton, J. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma, Proc. Natl. Acad. Sci. USA 88, 10124-10128.

Bressac, B., Kew, M., Wands, J. & Ozturk, M., (1991) Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa, Nature 350, 429-431.

Ehrlich, M. & Wangh, R.Y. (1981) 5-Methylcytosine in eukaryotic DNA. Science 212, 1350-1357.

Ehrlich, M., Zhang, X.Y. & Inamdar, N.M. (1990) Spontaneous deamination of cytosine and 5-methylcytosine residues in DNA and replacement of 5-methylcytosine residues with cytosine residues. Mutation. Res. 238, 277-286.

Eisenstadt, E., Warren, J., Porter, J., Atkins, D., Miller, J.H., (1982) Carcinogenic epoxides of benzo[a]pyrene and cyclopenta[cd]pyrene induced base substitutions via specific transversions, Proc. Natl. Acad. Sci. USA 7, 1945-1949.

Favor, J. (1989) Risk estimation based on germ-cell mutations in animals, Genome 844-852.

Gossen, J.A., Molijn, A.C., Douglas, G.R., Vijg, J. (1992a) Application of galactose-sensitive E.coli strains as selective hosts for LacZ$^-$ plasmids, Nucleic Acids Res. 20, 3254.

Gossen, J.A., de Leeuw, W.J.F., Molijn, A.C., Vijg, J. (1992b) A selective system for LacZ$^-$ phage using a galactose-sensitive E.coli host, BioTechniques, *in press*.

Grist, S.A., McCarron, Kutlaca, A., Turner, D.R., and Morley, A.A., (1992) In vivo human somatic mutation: frequency and spectrum with age, Mutation Res. 266, 189-196.

Harris, C.C. (1990) Chemical and physical carcinogenesis: advances and perspectives for the 1990s, Cancer Res. (suppl) 51, 5023-5044.

Horn, P.L., Turker, M.S., Ogburn, C.E., Disteche, C.M., Martin, G.M. (1984). A cloning assay for 6-thioguanine resistance provides evidence aqainst certain somatic mutational theories of aging, J. Cell. Physiol. 121, 309-315.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., & Harris, C.C. (1991) Mutational hotspot in the p53 gene in human hepatocellular carcinomas, Nature 350, 427-428.

Itehata, H., Kimura, H., Kato, T. (1989) Shuttle vector for the analysis of mutational events in mammalian chromosomal DNA, Mutat. Res. 210, 237-247.

Jones, P.A., Rideout, W.M., Shen, J-C., Spruck, C.H., Tsai, Y.C. (1992) Methylation, mutation and cancer, BioEssays 14, 33-36.

Kirkwood, T.B.L. (1989) DNA, mutations and aging, Mutation Res., 219, 1-7.

Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Sorge, Putman, D.L., and Short J.M. (1991) Spectra of spontaneous and mutagen-induced mutations in the LacI gene in transgenic mice, Proc. Natl. Acad. Sci. USA 88, 7958-7962.

Loeb, L.A., (1989) Endogenous carcinogenesis: Molecular oncology into the twenty-first century - Presidential address, Cancer Res. 49, 5489-5496.

Mellon, I., Bohr, V.A., Smith, C.A., Hanawalt, P.C. (1986) Preferential DNA repair of an active gene in human cells, Proc. Natl. Acad. Sci. USA 83, 8878-8882.

Mullenders, L.H.F., Vrieling, H., Venema, J., van Zeeland, A.A. (1991) Hierarchies of DNA repair in mammalian cells: biological consequences, Mutation res. 250, 223-228.

Phear, G., Armstrong, W., Meuth, M. (1989) Molecular basis of spontaneous mutations at the APRT locus of Hamster cells, J. Mol. Biol. 209, 577-582.

Rideout, W.M., Coetzee, G.A., Olumi, A.F., Jones, P.A. (1990) 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes, Science 249, 1288-1291.

Turner, D.R., A.A. Morley, M. Haliandros, R. Kutlaca, B.J.Sanderson (1985) In vivo somatic mutations in human lymphocytes frequently result from major gene alterations, Nature London 315, 343-345.

Vijg, J. (1990) DNA sequence changes in aging: How frequent, how important? Aging. 2 105-123.

Vogelstein, B. & Kinzler. (1992) Carcinogens leave fingerprints, Nature 355, 209-210.

SAMENVATTING

DNA mutaties worden verondersteld een belangrijke rol te spelen bij het onstaan van kanker, ontwikkelingsstoornissen, genetische ziekten en veroudering. DNA mutaties kunnen bijvoorbeeld ontstaan t.g.v. foutief herstel van DNA schade, tijdens DNA replicatie of t.g.v. het blootstaan aan endogene en/of exogene DNA beschadigende stoffen (radicalen, UV-straling, chemische stoffen etc.). Het onderzoek naar spontane en geinduceerde mutaties in zoogdiergenen in vivo is echter tot nu toe zeer beperkt geweest door de afwezigheid van gevoelige technieken voor het identificeren en karakteriseren van mutaties. Alhoewel de afgelopen jaren een groot aantal nieuwe mutatiedetectie methoden is ontwikkeld kunnen de meesten hiervan slechts worden toegepast op gekweekte cellen. Vergelijkend onderzoek naar mutaties in meerdere weefsels en organen van zoogdieren is met deze methoden echter in veel gevallen niet mogelijk. Technieken waarmee dit in beperkte mate wel mogelijk is zijn de de zgn. HPRT en HLA-A testen. M.b.v. deze testen kunnen mutaties in het HPRT of HLA-A gen in bloedcellen worden geanalyseerd.

Een belangrijke voorwaarde voor het detecteren van mutaties in verschillende weefsels en organen van zoogdieren is dat een doelwitgen met hoge efficientie kan worden geisoleerd uit chromosomaal DNA en vervolgens eenvoudig kan worden onderscheiden van niet gemuteerde doelwitgenen. Het onderzoek zoals beschreven in dit proefschrift toont aan, dat transgene muizen met in elke lichaamscel een aantal kopieën van het bacteriële mutatie doelwitgen LacZ kunnen worden gebruikt voor het onderzoek naar mutaties in vivo.

Bij de aanvang van dit onderzoek was de technologie voor het maken van transgene muizen relatief nieuw en transgene muizen voorzien van een bacterieël mutatie doelwitgen bestonden nog niet. Besloten werd een bacteriofaag lambda vector te nemen welke, voorzien van het bacteriele LacZ gen als mutatie doelwitgen, werd overgebracht naar de geslachtslijn van muizen. Dit laatste werd uitgevoerd d.m.v. microinjectie van bevruchte

eicellen. Bacteriofaag lambda vectoren kunnen m.b.v. een enzym extract relatief efficiënt uit chromosomaal DNA worden geïsoleerd en overgebracht naar E.coli bacterien. Selectie van de gemuteerde LacZ genen vindt plaats middels het meten van de activiteit van het intacte LacZ genproduct ß-galactosidase. De mutatiefrequentie in een weefsel of orgaan kan vervolgens worden bepaald als de ratio tussen het aantal kleurloze (gemuteerde) en blauwe (niet gemuteerde) plaques in het bacteriedek.

De eerste resultaten m.b.t. het terugwinnen van LacZ genen uit chromosomaal DNA van transgene muizen waren echter bijzonder teleurstellend. De oorzaak hiervan was dat bacteriofaag lambda vectoren in transgene muizen in hoge mate gemethyleerd bleken te zijn. Wanneer dit DNA werd overgebracht naar E.coli K12 bacteriën werd dit onmiddellijk door gastheer restrictie systemen als vreemd DNA herkend en afgebroken. Door gebruik te maken van E.coli C, een bacteriestam die niet in staat is tot gastheer restrictie, konden de bacteriofaag lambda vectoren echter met hoge efficienties teruggewonnen worden.

In totaal werden 20 verschillende transgene muizen verkregen waarvan er 3, (20.2, 35.5 en 40.6; zie Hoofdstukken 2, 3, en 4) werden geselecteerd voor verdere studies. Southern blot analyse van lever DNA geknipt met verschillende restrictie enzymen toonde aan dat in al deze stammen de bacteriofaag lambda vectoren kop-staart en zonder detecteerbare deleties en/of herrangschikkingen in het muizegenoom waren geintegreerd. De eerste mutatie experimenten werden uitgevoerd met muizen van stam 20.2. Zowel spontane als door ethylnitrosourea geinduceerde mutaties in lever en hersenen werden geanalyseerd en gekarakteriseerd. Na behandeling van de transgene muizen met verschillende doses ethylnitrosoureum werd een dosis-afhankelijke toename van de mutatie frequentie gemeten in de hersenen. Het dient echter opgemerkt te worden dat uitgebreidere studies, zoals beschreven in Hoofdstuk 3, aantoonden dat voor het nauwkeurig bepalen van spontane of geinduceerde mutatie frequenties een veel groter aantal LacZ genen diende te worden geanalyseerd. Deze studie toonde aan dat de variatie in spontane mutatie frequenties tussen verschillende dieren afnam naarmate het totaal aantal

geanalyseerde LacZ genen per orgaan of weefsel werd vergroot van 200.000 to circa 1.000.000. Spontane mutaties in LacZ genen geisoleerd uit verschillende weefsels en organen van stam 40.6 kwamen voor met een frequentie van circa 3 x 10^{-5}. De gevonden waarden voor testis daarentegen waren gemiddeld circa 4 á 5 keer lager. Dit zou kunnen duiden op verschillen in het blootstaan aan endogene en/of exogene DNA beschadigende agentia of verschillen in DNA herstel tussen de weefsels. Uitgebreidere studies zijn echter noodzakelijk om dit verschil te bevestigen.

De toepasbaarheid van LacZ transgene muizen als model systeem voor het testen van potentieel mutagene stoffen werd getest door muizen van stam 40.6 te behandelen met een aantal bekende mutagene stoffen (Hoofdstuk 3). Alhoewel de resultaten verkregen na behandeling van de muizen met ENU of chlorambucil inderdaad de verwachte verhoogde mutatie frequenties toonden in lever, beenmerg en testis, was het totaal aantal geanalyseerde LacZ genen per weefsel relatief laag. Dientengevolge was ook het aantal geïsoleerde LacZ mutanten klein. Gezien de resultaten verkregen m.b.t. spontane mutatiefrequenties moet het aantal te analyseren LacZ genen per weefsel ten minste verhoogd worden tot 500.000 voordat er sprake is van een significante toename in de mutatiefrequentie boven de achtergrond. Tevens toonden de resultaten m.b.t. de mutageniciteit van procarbazine, cyclofosfamide en acrylamide in beenmerg en de resultaten m.b.t. de mutageniciteit van MNNG en DMBA in de huid aan, dat de gevonden geinduceerde mutatiefrequenties sterk afhankelijk waren van het tijdstip van isolatie. Bovendien werd een grote variatie in geïnduceerde mutatiefrequenties tussen dieren waargenomen. In het algemeen echter toonden de resultaten orgaan specifieke en dosis afhankelijke mutatiefrequenties aan in verschillende weefsels en organen. Verschillen in mutatiefrequenties tussen dieren kunnen echter ontstaan t.g.v. verschillen in transport, opname door organen of weefsels of t.g.v. toxiciteit in organen en weefsels nabij de plaats van toediening. Deze mogelijke variabelen in aanmerking nemend vereist het zorgvuldig testen van potentieel mutagene stoffen de analyse van tenminste 3 doses en tijdpunten, waarbij tenminste 3 dieren en minimaal 500,000 LacZ

genen moeten worden geanalyseerd per weefsel.

In tegenstelling tot de transgene stammen 20.2 en 40.6, werden aanzienlijk hogere spontane mutatiefrequenties (tot circa 100 x hoger) in lever en hersenen van muizen van stam 35.5 waargenomen (Hoofdstuk 4). In transgene muizen van deze stam bleek de bacteriofaag lambda concatameer te zijn geintegreerd in het X-chromosoom. Daarnaast werd gevonden dat (i) mutatie-frequenties in hersenen van homozygote muizen significant hoger zijn dan die in heterozygote muizen, (ii) in mannelijke geslachtscellen de 3' flankerende gebieden nabij de bacteriofaag lambda concatemeer worden gekarakteriseerd door een hoge frequentie van deletie type mutaties, en (iii) dat LacZ mutanten geisoleerd uit de lever vnl. worden gekarakteriseerd door transitie type mutaties, terwijl LacZ mutanten geisoleerd uit de hersenen vnl. werden gekarakteriseerd door transversie type mutaties (Hoofdstuk 5). Helaas kon op basis van deze gegevens geen eenduidige conclusie worden getrokken m.b.t. het mechanisme van de verhoogde mutatiefrequentie in deze stam is. De resultaten suggereren echter dat de lambda concatameer is geintegreerd in een chromosomaal instabiel gebied, waardoor het gevoeliger wordt voor zowel somatische als kiemcel mutagenese.

Een alternatieve methode voor het terugwinnen van LacZ genen uit chromosomaal DNA van transgene muizen is beschreven in Hoofdstuk 6. Deze methode is gebaseerd op de sterke binding tussen het LacI repressor eiwit en de LacZ operator sequentie. Door het LacI repressor eiwit te koppelen aan magnetische bolletjes konden LacZ bevattende plasmide sequenties met hoge efficientie worden geisoleerd uit chromosomaal DNA van transgene muizen. M.b.v. deze methode kunnen grote hoeveelheden LacZ bevattende plasmiden in één stap worden geisoleerd. Gecombineerd met de ontwikkeling van een bacteriestam die niet in staat is tot groei wanneer het een intact LacZ gen heeft opgenomen biedt deze methode grote voordelen t.o.v. het gebruik van bacteriofaag lambda vectoren zoals beschreven in de hoofdstukken 2, 3 en 4. Hierdoor wordt de toepassing van transgene mutatie modellen voor het onderzoek naar zowel spontane als geinduceerde mutaties <u>in vivo</u> sterk vereenvoudigd.

LIST OF PUBLICATIONS

Publications in reviewed journals.

Gossen, J.A. and J. Vijg (1988) E.coli C: a convenient host strain for cloning highly methylated DNA, Nucleic Acids Res. 16, 9343.

Gossen, J.A., W.J.F. de Leeuw, C.H.T. Tan, P.H.M. Lohman, F. Berends, D.L. Knook, E.C. Zwarthoff and J. Vijg. (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying gene mutations in vivo. Proc. Natl. Acad. Sci. (U.S.A.) 86, 7971-7975.

Gossen, J.A., W.J.F. de Leeuw, A. Verwest, P.H.M. Lohman and J. Vijg (1991). High somatic mutation frequencies in a LacZ transgene integrated on the mouse X-chromosome, Mutation Res. 250, 423-429.

Gossen, J.A., Molijn, A.C., Douglas, G.R., Vijg, J. (1992) Application of galactose-sensitive E.coli strains as selective hosts for LacZ$^-$ plasmids, Nucleic Acids Res. 20, 3254.

Gossen, J.A. & Vijg, J.A. (1993) Transgenic mice a model system for studying gene mutations in vivo, Trends in Genetics, 9, 27-31.

Gossen, J.A., de Leeuw, W.J.F., Molijn, A.C., Vijg, J. A selective system for LacZ$^-$ phage using a galactose-sensitive E.coli host, BioTechniques, In press.

Gossen, J.A., W.J.F. de Leeuw, and Vijg, J. DNA sequence analysis of spontaneous mutations at a LacZ transgene integrated on the mouse X-chromosome, Mutagenesis, In press.

Gossen, J.A., W.J.F. de Leeuw, A.C. Molijn, Vijg, J. Plasmid rescue from transgenic mouse DNA using LacI repressor protein conjugated to magnetic beads, BioTechniques, In press.

LIST OF ABBREVIATIONS

APRT	adenine phosphoribosyl transferase
BaP	benzo-(a)-pyrene
bp	basepair
CHL	chloroambucil
DMBA	9,10 dimethyl benz(a)anthracene
DMSO	dimethylsulfoxide
EBV	Eppstein-Bar virus
ENU	ethylnitrosourea
HPRT	hypoxanthine phosphoribosyltransferase
kb	kilobase (pair)
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
p-Gal	phenyl-ß-D-galactosidase
X-Gal	5-bromo-4-chloro-3-indolyL-ß-D-galactoside

NAWOORD

Dit proefschrift zou niet tot stand zijn gekomen zonder de hulp van velen die hieraan ieder op hun eigen wijze hebben bijgedragen.

Jan Vijg wil ik bedanken voor zijn bijzonder enthousiaste en stimulerende begeleiding van het onderzoek tijdens de afgelopen jaren. De vele discussies over het onderzoek en de te publiceren resultaten waren bijzonder leerzaam.

Wiljo wil ik bedanken voor zijn enorme inzet en bijzondere betrokkenheid bij het onderzoek tijdens de afgelopen jaren. Mede dank zij hem is dit proefschrift in deze vorm tot stand gekomen.

Cecilia, Aart, Petra, Arjen en Anco dank ik voor hun bijdragen aan de verschillende hoofdstukken van dit proefschrift en voor hun plezierige samenwerking. Verder dank ik alle collegae van het Instituut voor Experimentele Gerontologie en Ingeny B.V. voor hun medewerking en collegialiteit.

Mijn promotor Paul Lohman dank ik voor zijn nimmer aflatende steun, vertrouwen en geduld tijdens de laatste fase van het promotie onderzoek. Met name Frits Berends wil ik bedanken voor het bijzonder kritisch doorlezen van de verschillende hoofdstukken van dit proefschrift. Zijn kundig commentaar heb ik zeer op prijs gesteld.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 16 augustus 1961 te Ruinerwold. Na het behalen van het HAVO diploma in 1978 en het Atheneum diploma in 1980, werd een aanvang gemaakt met de studie Chemie aan de Rijks Universiteit te Groningen. Het kandidaats examen werd afgelegd in 1983 en het doctoraal examen, met als specialisatie moleculaire biologie, in 1986. In augustus van datzelfde jaar begon hij met het promotie-onderzoek dat beschreven is in dit proefschrift. Dit onderzoek betrof een samenwerkings project tussen het Instituut voor Experimentele Gerontologie en het Medisch Biologisch Laboratorium, welke beide onderdeel uitmaken van de hoofdgroep Gezondheids onderzoek TNO. Vanaf oktober 1991 is hij werkzaam bij Ingeny B.V. waar het promotie onderzoek werd afgerond. In het kader van een samenwerkingsproject is hij momenteel werkzaam aan de Harvard universiteit te Boston.