# DNA damage and mutations in *λlacZ* mice

# induced by alkylating agents

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| | |
|---|---|
| AGT | $O^6$-alkylguanine-DNA-alkyl transferase |
| AP | apurinic/apyrimidinic |
| B(a)P | benzo(a)pyrene |
| bp | base pair |
| b.w. | body weight |
| DEN | diethyl nitrosamine |
| *dlb* | Dolichos biflorus |
| DMN | dimethylnitrosamine |
| DMSO | dimethyl sulfoxide |
| CS | Cockayne's syndrome |
| ELISA | enzyme-linked immunosorbent assay |
| EMS | ethyl methanesulphonate |
| ENNG | *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine |
| ENU | *N*-ethyl-*N*-nitrosourea |
| HPLC | high pressure liquid chromatography |
| *hprt* | hypoxanthine-guanine phosphoribosyltransferase |
| i.p. | intraperitoneal |
| ISB | immunoslotblot |
| kb | kilo bases |
| Mab | monoclonal antibody |
| MNNG | *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine |
| MNU | *N*-methyl-*N*-nitrosourea |
| N7-EtG | N7-ethylguanine |
| NER | nucleotide excision repair |
| $O^6$-EtG | $O^6$-ethylguanine |
| PCR | polymerase chain reaction |
| TTD | trichothiodystrophy |
| XP | xeroderma pigmentosum |

# *Chapter 1*

## General introduction

### 1.1 Introduction

Carcinogenic effects of chemical agents have been suspected as early as 1761, with the observation of a high incidence of nasal cancer in users of tobacco snuff (cited in Redmond, 1970). Much later, the discovery of the mutagenic effects of exogenous factors such as radiation (Muller, 1927) and chemicals (Auerbach *et al.*, 1947) on the genetic material of *Drosophila* laid the foundation for the role of DNA in the aetiology of cancer, which had already been postulated by Boveri (1914). Research into the processes underlying mutagenesis and carcinogenesis has since become more extensive, utilising a large variety of agents including radiation, polycyclic aromatic hydrocarbons and alkylating agents.

The cells of which organisms consist are frequently exposed to numerous chemicals. Some of these are capable of directly interacting with the DNA, while others are genotoxic only as metabolites that are the result of metabolic processes within these cells or elsewhere in the organism. Examples of the latter include benzo(a)pyrene and the alkylnitrosamines, which must be bioactivated by P450 isoenzymes before gaining genotoxic properties. The differences between cells with respect to metabolic activity result in tissue-specific effects of certain mutagens.

It is now generally accepted that the induction of changes in the genetic code (mutations) by carcinogens represents an early step in the initiation of chemically induced cancer. The induction of mutations can be preceded by an interaction of the carcinogen with the DNA, resulting in the alteration of its chemical structure (adduct formation). Such carcinogens are termed genotoxic. Some agents covalently modify the DNA by forming mono-adducts, by the transfer of small groups (*eg.* simple alkylating agents) or of much larger moieties such as those derived from polycyclic hydrocarbons. Bifunctional agents, on the other hand, are able to interact at two positions of the DNA molecule thereby forming intra- or inter-strand crosslinks, while cross-links with proteins can also be formed by these agents. Other agents such as X-rays are clastogenic, inducing, among others, chromosomal aberrations believed to be caused by double strand breaks resulting in large deletions in the DNA. The DNA lesions can often be removed by cellular repair mechanisms. Those lesions still present during DNA replication may lead to a fixed mutation. Erroneous DNA repair is another source of mutations. Mutations can vary from a single base-pair substitution to deletions and chromosomal rearrangements. The nature of these mutations and their position in the genome largely dictate their phenotypic effect on a cell. A mutation in an oncogene or a tumour-suppressor gene, for instance, may ultimately lead to tumour

viruses or agents that interfere with spindle formation. They are called non-genotoxic.

With the aim of assessing and preventing human exposure to both natural and man-made carcinogens, extensive research has been done on developing test-systems to predict the carcinogenic potential of chemical agents. A large number of short-term *in vitro* assays available now employ prokaryotic and mammalian cells. These systems do not determine the carcinogenic potency of the chemical directly but instead assess the induction of certain genetic alterations such as gene mutations, micronuclei and chromosomal aberrations, which are considered as possible indicators of carcinogenicity. The bacterial and mammalian cells employed *in vitro* only mimic to a certain extent the *in vivo* situation in organs and tissues as it exists in mammals such as man. In addition, the restricted number of cell types that can be tested also limits the usefulness of *in vitro* tests. A compound does not have a similar effect on all cell types since its mutagenicity is influenced by such factors as transmembrane transport, metabolic capabilities and repair capacity. Also entirely lacking in *in vitro* systems is the ability to study different routes of exposure (*eg.* oral, topical), an issue very relevant to man. Due to these differences, results of *in vitro* assays are of limited predictive value for carcinogenic potency of chemicals, warranting the need to look at genetic (cancer-related) endpoints *in vivo*.

It is possible to determine mutations in a number of endogenous genes *in vivo* in rodents, which is a closer approximation of the human *in vivo* situation than is achieved in *in vitro* assays. A number of such short-term *in vivo* tests measure the rate of gene mutation at the hypoxanthine phosphoribosyl transferase (*hprt*) locus (Clive *et al.*, 1972, 1988), the *dlb*-1 locus (Winton *et al.*, 1988) and in 7-loci in the mouse spot assay (Russel *et al.*, 1979). Chromosomal damage can also be monitored *in vivo* in lymphocytes or bone marrow in the chromosome aberration and micronucleus induction assays (Heddle, 1973). In humans, 2 loci exist in which DNA-mutant frequencies can be determined, *viz. hprt* and HLA-A (Strauss and Albertini, 1978; Kavathas *et al.*, 1980). Indirect evidence can also be obtained in the form of "mutated" protein molecules such as glycophorin A and haemoglobin A detectable in human erythrocytes (Langlois *et al.*, 1987; Osterman-Golkar *et al.*, 1976). Unfortunately, these tests are confined to a limited number of cell types such as fibroblasts, lymphocytes and erythrocytes restricting their predictive capacity. Moreover, these loci can only function as biological dosimeters after human mishaps.

The rodent cancer bioassay is an *in vivo* test-system used to check directly for tumour development during life-time chemical exposure. This is a long-term test that requires the use of many animals. Currently, this assay is routinely performed in both sexes of rats and mice, and a large number of chemicals tested are found to induce tumours in one of the two species or sexes. (Ames, 1989; Ames *et al.*, 1993; Ashby and Purchase, 1993). Consequently these tests are considered to be hypersensitive. A possible explanation for this may be the high, toxic doses employed. Injury to tissues as a result of treatment with genotoxins may act as a mitogenic stimulus to regenerate damaged tissue. The resulting increase in cell turn-

over, with a concomitant increase in DNA replication of the adduct-containing DNA, unavoidably leads to an increase in mutation fixation. At lower doses the toxic effects will be reduced or absent. Consequently, carcinogenic agents administered at high doses do not necessarily induce the same effects when administered at doses to which humans may be exposed in their environment. Therefore a short-term *in vivo* test system is needed that is able to measure effects at low doses and in which all routes of exposure and all tissues can be studied.

A number of rodent strains exist that have a higher susceptibility to develop a specific type of cancer. Such hypersensitive rodents include the Fischer 344 rats that experience a high incidence of lymphoma and C3H/HeJ mice that suffer from a higher rate of hepatoma than other mouse strains (Shisa and Hiai, 1985; Drinkwater and Guster, 1986). These inbred rodents have been found not only to have a higher incidence of spontaneous tumours but also after exposure to genotoxins. Although these rodents can be used in carcinogenicity assays, the increased tumour susceptibility is limited to a few organs and tissues.

Recently, genetically modified animals have become available as mammalian *in vivo* test systems for mutagenesis and carcinogenesis (Goldworthy *et al.*, 1994). Three categories can be distinguished. The first group consists of animals in which certain enzymes involved in metabolic activation or detoxification of chemicals, or in DNA-damage repair, are "knocked out" or are over-expressed. In principle, animals in which repair or detoxification genes have been inactivated offer a "hypermutable" animal in which the level of certain type of DNA lesions is increased after genotoxic treatment. The outcome is a higher tumour frequency that can be measured already after lower and hence more relevant doses. The second category includes animals in which oncogenes have been activated or tumour suppressor genes inactivated. Consequently these animals will form tumours much more rapidly than do normal animals. Similar to the "knockouts" this reduces not only the time needed to induce tumours, but also the number of animals required. Although they are very useful for studies on the mechanisms involved in mutagenesis and carcinogenesis, the same objections can be raised as those mentioned for *in vitro* assays; an unnatural situation is created in these genetically modified animals because the influence of the increased expression or absence of these gene products on other cellular processes remains unknown. The third category consists of genetically altered animals that harbour multiple genetic targets (reporter genes) for mutagenesis and allow the determination of mutagenic effects of test compounds in essentially all organs and tissues. The reporter genes in these animals are inactive and do not confer a growth (dis)advantage and hence do not influence essential cellular processes.

The present work deals with the processes involved in mutagenesis, through the use of a transgenic mouse model harbouring multiple copies of a target gene in all tissues. The transgenes in these animals are transcriptionally inactive and do not appear to influence the

parameters were expected to add to our understanding of the mechanisms involved in tissue specific mutagenesis and hence carcinogenesis *in vivo*. This was investigated for a specific class of genotoxic agents, namely those that introduce alkyl groups in the DNA.

## 1.2 Alkylating agents

Alkylating agents are abundantly present in our environment. Among them, the *N*-nitroso compounds form a major group of environmental mutagens man is exposed to; they have been implicated in human carcinogenesis (Bartsch and Montesano, 1984). These compounds are found in the diet in the form of dimethyl nitrosamine (DMN) and are also formed in the acid environment in the human stomach during the reaction of ingested nitrite and secondary or tertiary amines (Bartsch and Montesano, 1984; Groenen and Busink, 1988). DMN, diethyl nitrosamine (DEN) and ethylene oxide are examples of industrial mutagens that have given rise to occupational exposure.

Alkylating agents are relatively simple compounds that can interact with the proteins and nucleic acids of the cell. In their interaction with DNA, a wide variety of lesions are formed which include simple DNA adducts and inter- and intra-strand crosslinks. Usually, each alkylating agent forms multiple DNA lesions, which, depending on the extent of alkylation, are capable of initiating a wide range of biological effects. These effects also depend on the type of lesions formed and whether these lesions can potentiate the fixation of mutations (*i.e.*, are mutagenic) or block the polymerase during DNA-replication or transcription (*i.e.*, are cytotoxic). Cytotoxicity is often associated with the formation of inter-strand crosslinks.

Some agents such as *N*-methyl-*N*-nitrosourea (MNU) and *N*-ethyl-*N*-nitrosourea (ENU) are monofunctional and induce mono-adducts. Bifunctional alkylating agents can, in addition to mono-adducts, form intra- and inter-strand cross-links by interacting with bases in the same or opposite DNA strand. A number of the later kind of agents are used in chemotherapy such as bis(chloroethyl)nitrosourea and *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea and their functioning is expected to relate to the cytotoxic properties of cross-links (Singer and Grunberger, 1983). Also sulphur mustard ("mustard gas") and nitrogen mustards are such bifunctional agents. Cyclophosphamide is an example of an indirect bifunctional agent and is a chemotherapeutic drug that has to be metabolized to form phosphoramide mustard (Singer and Grunberger, 1983).

In mechanistic studies certain mono-functional alkylating agents are frequently used as model compounds. These can be divided into two groups, alkyl sulphates and *N*-nitroso compounds. They are listed in Table 1. Model alkylating agents usually induce the addition of methyl or ethyl groups to the base groups and the phosphate backbone of the DNA. Those mentioned in Table 1 are direct acting agents with the exception of the dialkyl nitrosoamines DMN and DEN, which are metabolically activated by cytochrome P450

(CYP)-dependent monooxygenases prior to becoming DNA-reactive (Figure 1)(Yang *et al.*, 1985; Guengerich and Shimada, 1991). The distribution of the DNA damage in the organs and tissues depends on the presence of the P450 enzymes. Consequently, rodent livers exposed to agents such as DMN develop lesions predominantly in the neighbourhood of the central vein of the liver lobule where the P450 system is present. Due to the short half-life of the active metabolite (10 seconds), no diffusion to other organs occurs. The DNA-damage distribution in cross sections of livers subjected to DMN shows a pattern in the form of "islands" located directly around the central vein (Meer *et al.*, 1986). Other agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) are also capable of reacting directly with the genetic material but at a very low rate. The presence of cellular thiols (*eg.* cysteine) catalyses their decomposition (Figure 1).

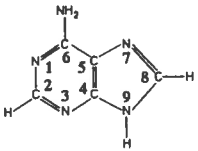
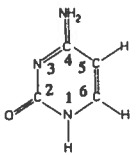
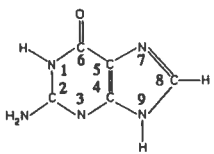
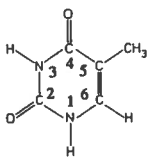
Table 1: *Commonly used model alkylating agents*

| Type | example | abbreviation |
|---|---|---|
| alkyl sulphates | dimethyl sulphate | DMS |
| | diethyl sulphate | DES |
| | methyl methanesulphonate | MMS |
| | ethyl methanesulphonate | EMS |
| *N*-nitroso compounds | *N*-methyl-*N*-nitrosourea | MNU |
| | *N*-ethyl-*N*-nitrosourea | ENU |
| | *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine | MNNG |
| | *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine | ENNG |
| | dimethyl nitrosamine | DMN |
| | diethyl nitrosamine | DEN |

### *1.2.1 Reaction with DNA*

Alkylating agents are capable of reacting with most nitrogen (N) and oxygen (O) atoms present in the four DNA bases. N-nitroso compounds, for example, first break down into their alkylating species as depicted in Figure 1. The rate of the reactions with the various atoms and the mechanism involved can be predicted with the Ingold concept of nucleophilic substitution and the Swain-Scott equation for relative reaction rates (Swain and Scott, 1953). The Swain-Scott constant (s) is the reaction rate of alkylating agents with nucleophilic reagents in water relative to the reaction rate of methylbromide, which has been designated a value of 1. Alkylating agents with a relatively high s value approaching 1, such as MMS and EMS, react via an $S_N2$ pathway and tend to react with the strongest nucleophilic centres in the DNA, namely the N7-atom of guanine and the N3 atom of

Table 2: *Adduct spectra of various alkylating agents in rat liver or cultured cells expressed as a percentage of total alkylation.*[1]

| | | MNU | ENU | DMN | DEN | MMS | EMS | DMS[2] | DES[2] | MNNG |
|---|---|---|---|---|---|---|---|---|---|---|
| | *s*[3] | 0.42 | 0.26 | 0.42[4] | 0.42 | 0.83 | 0.70 | 0.86 | 0.64 | 0.42 |
|  | Adenine | | | | | | | | | |
| | N1- | 0.3 | nd[5] | 0.8-0.9 | - | 1.0 | - | - | nd | - |
| | N3- | 1.1-3.6 | 3.2-4.3 | 2.3-5.0 | 3.7 | 9.4 | 3.3 | 15.5 | 7.4 | 8.6 |
| | N7- | 0.8 | 0.6-0.8 | 0.7-1.7 | - | 0.9 | - | 4.0 | nd | - |
|  | Cytosine | | | | | | | | | |
| | $O^2$- | nd | 1.3-2.2 | nd | - | - | - | - | nd | - |
| | N3- | nd | nd | 0.6 | - | - | - | - | nd | - |
|  | Guanine | | | | | | | | | |
| | N3- | 0.6-1.6 | 1.4-1.9 | 0.6-0.9 | - | 0.7 | - | - | nd | - |
| | $O^6$- | 3.6-10.0 | 5.5-7.2 | 6.1-7.8 | 5.6 | nd | 1.5 | 0.4 | 2.0 | 9.2 |
| | N7- | 70.0-86.6 | 13.5-19.6 | 66.8-74.5 | 15.0 | 85.5 | 70.0 | 80-84.5 | 70.1 | 82.2 |
|  | Thymine | | | | | | | | | |
| | $O^2$- | nd | 5.9-8.9 | 0.2 | 6.0 | - | - | - | nd | - |
| | N3- | nd | nd | 0.2-0.4 | - | - | - | - | nd | - |
| | $O^4$- | 1.8 | 0.8-3.0 | nd | 0.7 | - | - | - | 0.6 | - |
| | Phosphotriesters | | | | | | | | | |
| | | 13.4 | 58.4-61.9 | 9.0-12.0 | - | 1.0 | - | - | 19.7 | - |

*[1] table constructed by using data from Beranek (1990); [2] cultured cells; [3] s: Swain-scott constant; data taken from Beranek (1990); [4] estimated value; [5] nd: not detectable*

ENU, react via an $S_N1$ type reaction and have been found to alkylate not only the strongly nucleophilic N-atoms but also the less nucleophilic O-atoms in the bases such as $O^6$-guanine and $O^2$- and $O^4$-atoms of thymine, but also the O-atoms in the phosphate groups connecting nucleosides in the backbone of the DNA. The ratio $O^6$-alkylguanine/N7-alkylguanine is lower for agents with a high s-value than for those with a low s-value. The Swain-Scott constant should therefore be taken as a means of predicting the selectivity of alkylation of the different sites in the DNA by various alkylating agents (Vogel and Nivard, 1994). T h e reaction of the various alkylating agents with single-stranded (ss) DNA does not differ much from that with double-stranded (ds) DNA, with the exception of a higher degree of alkylation of the N1-adenine and N3-adenine in ss-DNA (Singer and Grunberger, 1983). These 2 N-atoms partake in the hydrogen bonding between thymine and adenine and hence the difference is most probably due to steric hindrance in ds-DNA.

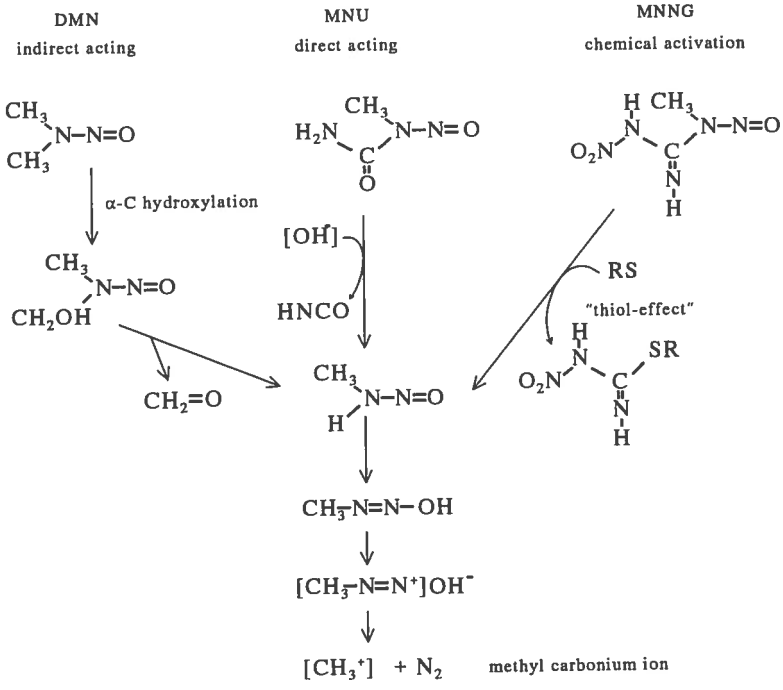



Figure 1: *The formation of alkylating intermediates by various methylating N-nitroso compounds via different mechanisms*

to estimate the dose to which an organism has been exposed. In mechanistic studies, to determine the role of adducts in mutagenesis and carcinogenesis, correlations can be made between, for instance, the level of DNA damage and the mutation-induction frequency or the tumour incidence. A number of techniques available for measuring DNA-lesion frequency in alkylated DNA are briefly described in Table 3.

Immunochemical methods are frequently used in the determination of alkyl-DNA damage owing to their relative simplicity. In the immunoslotblot (Nehls *et al.*, 1984) or ELISA (Harris, 1982) assays only microgram quantities of DNA are required and sensitivities of up to 1 adduct in $10^8$ nucleotides have been attained (Phillips, 1990). Antibodies against carcinogen-DNA damage can also be employed for the *in situ* localisation of DNA adducts within cells, *e.g.*, by use of fluorescence detection. A major obstacle is to obtain specific antibodies. Small DNA adducts are not very immunogenic, which makes it difficult to raise antibodies against them. Also cross-reactivity of antibodies between structurally similar adducts complicates the quantification of individual DNA adducts (Phillips, 1990).

Other systems for the determination of DNA damage make use of physicochemical methods. HPLC is very frequently used in the fractionation of hydrolysates of adduct-containing DNA samples. The difficulty in this procedure is found mainly in determining the right conditions for adequate separation of the damaged from normal DNA bases in the DNA hydrolysate. Another disadvantage is the relatively large quantity of DNA needed compared to the immunological assays. Data obtained with the combination of gas chromatography and mass spectrometry (GC-MS) are very limited but this technique has shown to be very sensitive in the detection of isotopically labelled adducts (Koch *et al.*, 1987; Phillips, 1990).

The $^{32}$P-postlabelling assay is in principle a very sensitive assay to determine DNA adducts. It requires only a few $\mu$g of DNA. However, the method has been developed and worked out mainly for aromatic and lipophilic adducts and not quite as well for simple alkyl adducts. The reason for this is that small alkyl moieties do not have a significant effect on the chromatographic properties of nucleosides, making it difficult to separate the modified from the unmodified nucleosides on a chromatogram (Randerath *et al.*, 1981; Reddy *et al.*, 1984). Despite the high sensitivity of $^{32}$P-postlabelling, immunochemical methods remain the choice for alkyl-adduct detection not only because the assays are less laborious, but also because of the specificity of the detection.

*iques used to determine DNA-alkylation products in total genomic DNA.*

| | Description | Adduct | Reference |
|---|---|---|---|
| nation | $^{3}$H-thymidine-labelled DNA heat depurinated. AP sites cleaved with NaOH and DNA placed on alkaline sucrose gradient. $^{3}$H measurements in fractions. Measure number of AP-induced breaks | N-alkyl-purines | Scicchitano and Hanawalt, 1989 |
| methods | Use of mono- and polyclonal antibodies for the detection of adducts in the ELISA or immunoslotblot | $O^{6}$-alkylguanine<br>$O^{4}$-alkylthymine<br>$O^{2}$-alkylthymine<br>N7-alkylguanine | Nehls *et al.*, 1984<br>van Delft *et al.*, 1991 |
| | $^{32}$P-labelled DNA on PEI cellulose plates | N7-alkylguanine<br>N3-alkyladenine | Ledoux *et al.*, 1991 |
| | Treatment with normal or radioactively labelled alkylating agents. Digestion and measurement of HPLC peaks using radioactive, electrochemical or fluorescence detection | $O^{6}$-alkylguanine<br>$O^{4}$-alkylthymine<br>$O^{2}$-alkylthymine<br>N7-alkylguanine | Ryan *et al.*, 1986<br>den Engelse *et al.*, 1987<br>van Delft *et al.*, 1993 |
| graphy and<br>ıetry | use of deuterated alkylating agents to distinguish between natural and carcinogen-induced adducts | N7-alkylguanine<br>N3-alkyladenine | Shuker *et al.*, 1984 |

To maintain the integrity of the DNA in the cell, repair mechanisms exist to rid the DNA of lesions. Exposure to alkylating agents leads to the formation of a broad spectrum of DNA adducts, some of which are potentially mutagenic when not removed. A number of repair pathways exist for the removal of DNA damage with varying specificities with respect to the type of lesions repaired. Most of our knowledge regarding the different repair pathways has been obtained through extensive studies in organisms such as *E. coli* and yeast (Walker, 1985). Four repair pathways are known at present that deal with the removal of DNA-alkylations or alkylation-induced base pair mismatches. These are the alkyltransferase-mediated $O^6$-alkylguanine repair; the repair *via* glycosylases referred to as base excision repair; nucleotide excision repair capable of removing nearly all type of lesions and the post-replicative mismatch repair pathways.

### *1.3.1 Alkyl transferase-mediated repair*

An inducible repair pathway responding to treatment with alkylating agents, known as the adaptive response, was discovered in *E. coli* (Samson and Cairns, 1977). Exposure to to low levels of alkylating agents induces a response that enables the organism to survive a subsequent exposure to higher concentrations of a similar agent which would otherwise have been lethal (Schendel and Robins, 1978). Upon stimulation of the adaptive response, an increased expression of a number of genes including the *ada* gene is observed. The *ada* gene encodes an alkyltransferase enzyme (ADA) capable of removing the alkyl adduct from the $O^6$-alkylguanine and $O^4$-alkylthymine lesions (McCarthy and Lindahl, 1985). The removal of the former occurs more efficiently than that of the latter. The DNA-alkyltransferase protein acts via a suicide mechanism in which the alkyl group is transferred from the adducted base to a cysteine residue in one of the two active sites of the protein (Lindahl *et al.*, 1988). The reaction is stoichiometric: one molecule of alkyltransferase binds one alkyl group, inactivating the protein. After the interaction the original base is regenerated (Olsson and Lindahl, 1980; Foote *et al.*, 1980). The second active site confers the capacity to accept an alkyl group residing in the phosphates of the DNA backbone (McCarthy and Lindahl, 1985). Upon transfer of the alkyl group from the DNA to the cysteine residue, the ADA protein is capable of transcriptionally activating the *alk*A and *aid*B genes (Lindahl *et al.*, 1988). Although the function of the *aid*B gene is not known, the *alk*A gene encodes a DNA glycosylase that can repair $O^2$-alkylpyrimidines and N3-alkylpurines by base excision repair (see 1.3.3)(Evensen and Seeberg, 1982; Karran *et al.*, 1982). A low level of resistance to alkylating agents is also present in the absence of an adaptive response, conferred by the OGT protein, encoded by the constitutively expressed *ogt* gene; it can remove the alkyl group from $O^6$-alkylguanine and $O^4$-alkylthymine (Rebeck *et al.*, 1988).

A protein similar to the bacterial *ada* and *ogt* gene products, which repairs alkylated $O^6$-guanine atoms, is expressed in mammalian cells (Pegg, 1983). However, the mammalian

$O^6$-alkyl-guanine-DNA-alkyl transferase (AGT), unlike its bacterial counterpart, is unable to repair alkylations in the phosphate backbone (Dolan *et al.*, 1984; Yarosh *et al.*, 1984). Recently, AGT protein from rat and humans have been shown to repair $O^4$-methylthymine in methylated poly(dA:dT) *in vitro*, but with 200-fold lower efficiency compared to $O^6$-methylguanine (Zak *et al.*, 1994). Similar work carried out with purified mouse methyltransferase, however, does show this protein to be capable of $O^4$-methylguanine repair with the same efficiency as $O^6$-methylguanine in oligonucleotides containing $O^4$-methylthymine paired with adenine or guanine (Kawate *et al.*, 1995). The difference in $O^4$-methylthymine repair rates between the rat and human AGT and the mouse AGT may be due to differences in sequence context in which the $O^4$-methylthymine was presented to the protein but may more likely stem from species differences. Whether AGT also possesses $O^4$-methylthymine repair capabilities *in vivo* remains to be seen. Efficient repair of $O^4$-ethylthymine by AGT seems unlikely in view of the persistence of this lesion in rat liver ($t_{½}$ = 14 days) (den Engelse *et al.*, 1987).

Expression of AGT in mammalian cells is not ubiquitous and neither is its induction. AGT levels are not induced in MNU- and DEN-pretreated hamsters and mice or following partial hepatectomy (Pegg 1983; Saffhill *et al.*, 1985; Dumenco *et al.*, 1991). In contrast, rat

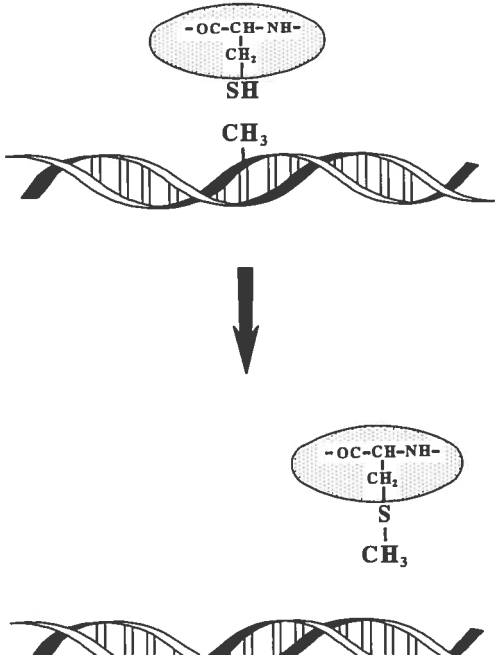



Figure 2: *Alkyl transferase-mediated repair. A cysteine group in the AGT molecule accepts an alkyl group attached to the*

et al., 1981). Although repair of O-ethylguanine is rapid in liver homogenates of rodents (den Engelse *et al.*, 1987), certain types of cells in this organ do not contain high levels of the protein. Heterogeneous expression was found in liver cells, with high levels in Kupffer cells and low levels in sinusoidal, endothelial, hepatic artery cells and connective tissue of the liver (Wani *et al.*, 1993). In humans the level of AGT in the various organs decreases in the order of liver > brain > lung (Citron *et al.*, 1991). A difference between species with respect to AGT content was also found. The level in human organs was higher than that in the corresponding organ of the rat which in turn was higher than the corresponding mouse values (Gerson *et al.*, 1986).

### *1.3.2 Base-excision repair*

Alkyltransferases lack affinity for alkyl lesions other than $O^6$-alkylguanine and $O^4$-alkylthymine in *E. coli* and $O^6$-alkylguanine (and possibly $O^4$-alkylthymine) in mammalian cells. Repair of some alkylations at other O- and N-atoms (*e.g.* N7-alkylguanine, N3-alkyladenine) is predominantly carried out by the process known as base-excision repair. This repair system depends on the presence of glycosylases that are each restricted in their substrate specificity. DNA glycosylases cleave the *N*-glycosidic bond between the adducted base and the sugar moiety, forming an apurinic or apyrimidinic (AP) site. The AP-site is excised from the DNA through the action of AP endonucleases that hydrolyse the phosphodiester bonds 5' and 3' to the AP-site. Some glycosylases, however, besides releasing the modified base, can hydrolyse the phosphodiester bond 3' to the AP-site. One example is the pyrimidine-dimer-DNA glycosylase, T4 endonuclease V. The DNA sequence is restored with the opposite strand acting as a template directing the action of DNA polymerase $\beta$, $\delta$ or $\epsilon$. DNA ligase restores the remaining gap (Figure 3)(Sancar and Sancar, 1988; Lindahl, 1990).

A well-characterized glycolase is the uracil-DNA glycosylase that has been isolated from a number of sources including *E. coli*, human placenta and fibroblast cells (Sancar and Sancar, 1988). This protein catalyses the removal of uracil that has been formed by the spontaneous deamination of cytosine. The spontaneous deamination product of adenine is hypoxanthine. The removal of this base is carried out by the hypoxanthine-DNA glycolase found in *E. coli*, calf thymus and HeLa cells (Sancar and Sancar, 1988).

In *E. coli*, two glycosylases have been found that remove N-alkylated products formed after treatment with alkylating agents or after methylation by the intracellular methylator *S*-adenosylmethionine (Rydberg and Lindahl, 1982). The first is N3-methyladenine-DNA glycosylase I that is constitutively expressed at the *tag* gene. Upon induction by alkylating agents the second glycosylase, N3-methyladenine-DNA glycosylase II encoded by the *alk*A gene is expressed (Lindahl, 1988). The TAG protein is only able to remove N3-alkyladenine, a toxic lesion capable of blocking DNA replication (Boiteux *et al.*, 1984). The *alk*A gene product has a broader specificity, allowing it to repair in addition to N3-alkyladenine other

lesions such as N3-alkylguanine, $O^2$-alkylthymine, $O^2$-alkylcytosine and at a lower rate N7-alkylguanine (Lindahl, 1988). N7-alkylguanine, one of the adducts formed by alkylating agents can give rise to AP-sites as a result of spontaneous hydrolysis of the glycosidic bond. The alkali-catalysed imidazole ring-open form of this lesion is also formed. The resulting formamidopyrimidine can be released from the DNA through the action of FaPy glycosylase (Sancar and Sancar, 1988)

Due to the lack of mammalian cells with a defined mutation in one of the glycosylase repair genes, much less is known regarding *N*-methyl-purine glycosylases. The mammalian equivalent of *N*-methyl-purine glycosylase has been cloned and overexpressed under the control of a retroviral or SV40 promoter in Chinese Hamster Ovary (CHO) cells (Ibeanu *et al.*, 1992). The *N*-methyl-purine glycosylase was found to release N3-methyladenine, N7-methylguanine and 3-methylguanine from the DNA of cells treated with alkylating agents.

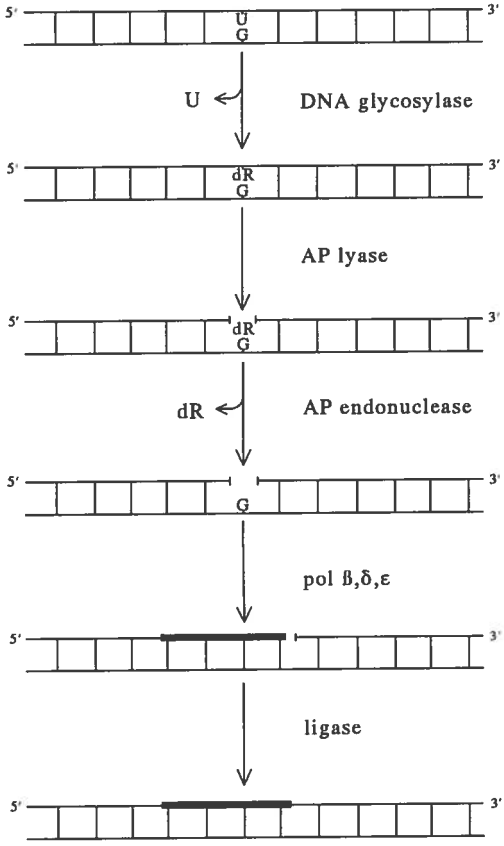



Figure 3: *Base excision repair in mammalian cells. In this example, a deaminated cytosine (U) serves as the base damage.*

Nucleotide excision repair (NER) is present in both bacterial and mammalian cells and is responsible for the removal of DNA alterations such as ultra violet (UV) radiation-induced cyclobutane pyrimidine dimers (CPD), (6-4) photoproducts, chemically-induced bulky adducts, crosslinks (Friedberg, 1985) and $O^4$-alkylthymine (Klein *et al.*, 1994). In *E. coli*, NER is carried out by the UvrA-D proteins; it involves at least five different enzymatic steps. A heterotrimer of two UvrA and one UvrB molecules scans the DNA for distortions in the helix (Grossman and Yeung, 1990). At the damaged site, the UvrA dissociates and the UvrBC complex attaches close to the damaged site and makes two incisions: one at 8 bases 5' and one at 5 bases 3' to the lesion. The damage-containing oligonucleotide is displaced by the concerted actions of DNA polymerase I and Helicase II (UvrD) and a new strand is synthesized (Lin and Sancar, 1992). DNA ligase completes the sequence of events.

In mammalian cells the mechanism of NER is expected to be essentially analogous to that in *E. coli*. However, the number of proteins involved is much larger and little DNA-sequence homology exists between *E. coli* NER genes and their mammalian counterparts. A wealth of information about mammalian NER has been obtained through the study of the human NER-related diseases xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD). Patients suffering from XP have a higher sensitivity to UV radiation manifested as a predisposition to skin cancer (Hoeijmakers, 1993). In these patients a defect in the step preceding or involving the incision of the DNA, 5' and 3' of the adduct, is believed to be the cause of the reduced NER activity. The defect can be alleviated through the introduction of endonucleases capable of making the incisions (de Jonge *et al.*, 1985). Patients suffering from TTD also have a reduced NER capacity. In CS-patients, normal NER for the total genomic DNA is present but the increase in UV-sensitivity associated with this disease is believed to be due to a lack of preferential removal of the damages from actively transcribed regions of the genome, disabling normal RNA synthesis and DNA replication (see 1.3.5)(Venema *et al.*, 1991; Hoeijmakers, 1993; Hanawalt, 1994). Additional information regarding the genes involved in NER has been obtained from the study of mutant rodent cell lines. Significant phenotypic overlap exists between human XP and CS syndromes and the rodent NER mutants, indicating similar aetiologies (Hoeijmakers, 1993).

The ability of cells derived from different patients to complement the repair defect of cells from other patients has led to the discovery of at least eight XP- and two CS-complementation groups. The study of mutant CHO cell lines has revealed at least 11 NER complementation groups. The existence of these complementation groups has permitted the isolation of the cDNAs for different NER-related genes. The human NER activity is believed to require at least 17 proteins (Sancar, 1994).

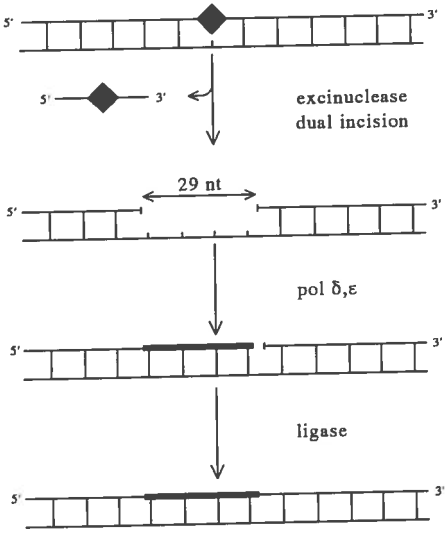



Figure 4: *Nucleotide excision repair in mammalian cells.*

Although the different repair pathways in mammalian cells seem to act independently, interactions between the alkyltransferase-mediated repair pathway and NER have been shown *in vitro* with respect to repair of $O^6$-alkylguanine lesions. In cell lines displaying different repair phenotypes, a synergistic effect has been observed between the alkyltransferase-mediated and NER pathways (Bronstein *et al.*, 1992a and b). Huang *et al.* (1994) go even further in saying that human NER has a wider specificity which includes bulky adducts, AP-sites, $N^6$-methyladenine, $O^6$-methylguanine, and mismatches, although each with different degrees of efficiency. They envision a repair complex whose binding to DNA is influenced by conformational changes in the DNA helix induced by the presence of adducts. The binding activates the repair machinery. The excision-repair pathway is believed to also function as a "back-up" for damage repair by alkyltransferase, glycosylase and AP-endonuclease.

### *1.3.4 Mismatch repair*

Unlike the aforementioned repair pathways, mismatch repair does not function to rid the DNA of lesions but rather removes mismatched base pairs from the DNA. Mismatch repair operates post-replicatively and processes mismatches induced, *e.g.*, as a result of spontaneous deamination of 5-methylcytosine to thymine or mismatches that arise from slippage during replication of miscoding lesions. It is a repair mechanism that has been well-

complex and excises a patch of mismatch-containing ss DNA of up to several kilobases in length. The gap is filled and the nick ligated. In *E.coli*, methylation of the parental DNA strand serves as a signal to determine the target strand. The inactivation of *mut*S and *mut*L results in an increase in the spontaneous mutation frequency.

Much less is known about the mammalian equivalent of *mut*S and *mut*L genes. Recently, however, the human *h*MSH2 protein, sequentially homologous to the *E. coli mut*S gene, has been purified and found to bind to mismatch-containing DNA (Fishel *et al.*, 1994). Cells deficient in mismatch-repair tend to be tolerant to alkylating agent-induced cytotoxicity (Branch *et al.*, 1993) believed to be due to the lack of abortive $O^6$-alkylguanine:thymine mismatch repair (Goldmacher *et al.*, 1986). Part of the toxicity induced by $O^6$-EtG in normal repair-proficient cells has been postulated to involve the difficulty with which the cell can repair $O^6$-alkylguanine:thymine mismatches *via* the mismatch repair machinery (Goldmacher *et al.*, 1986). Mismatch repair-deficient cells also acquire a mutator phenotype postulated to play a role in the acceleration of the multistep cancer process (Loeb, 1994). Homozygous *msh*2-deficient mice, although not displaying any developmental abnormalities, do develop lymphomas at an early age (de Wind *et al.*, 1995).

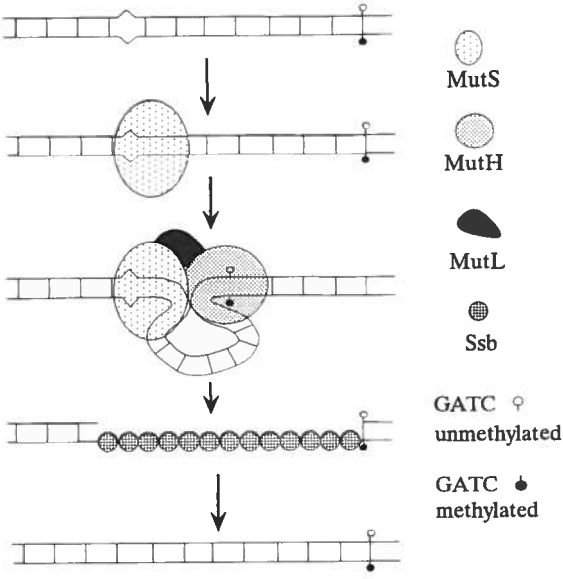



Figure 5: *Mismatch repair in E. coli. MutS binds to the mismatch and recruits a methylation specific endonuclease (MutH) and MutL to stabilize the complex. A nick is introduced in the unmethylated strand followed by strand degradation. The single-strand gap that is formed is protected by single-strand binding protein (Ssb). The process is completed by DNA replication to fill the gap.(after Jiricny, 1994).*

### 1.3.5 *Gene- and strand-specific repair*

In the past, repair studies *in vivo* and *in vitro* have been done on total chromosomal DNA because of technical limitations and it was assumed that all the DNA in a cell was equally accessible to genotoxic agents and repair complexes. The DNA within eukaryotic cells is highly compressed, necessitating a complex organisation of the genetic material. Structural heterogeneity of the DNA, however, exists. Chromosomal DNA consists largely of transcriptionally inactive, tightly packed heterochromatin. This has been postulated to be less accessible to repair complexes compared to the transcriptionally active, and hence "open", euchromatin. A higher sensitivity of euchromatin compared to heterochromatin to DNase I digestion, corroborates the higher accessibility of the transcriptionally active DNA (Weintraub and Groudine, 1976). Transcription, replication and repair of DNA is associated with higher-order loop structures known as the nuclear matrix which has been found to have a more 'open' structure. This heterogeneity in the DNA structure may have consequences for the accessibility of the DNA not only to repair complexes but also to DNA-damaging agents (Bohr, 1991).

Differences in the rate of formation and repair of various DNA lesions in transcriptionally active *versus* inactive DNA have been found. The initial level of DNA damage encountered in the total genome is lower than that in the actively transcribed portions of the genome in the case of $O^6$-methylguanine (Ryan *et al.*, 1986), $O^6$-ethylguanine (Thomale *et al.*, 1994), N7-nitrogen mustard adducts (Wassermann *et al.*, 1990), pyrimidine dimers (Ruven *et al.*, 1993) and cisplatin intrastrand adducts (Jones *et al.*, 1991). The higher level of repair encountered in active genes is termed preferential repair. The phenomenon of gene-specific or preferential repair was first demonstrated in the *dhfr* gene in CHO cells by Bohr *et al.*, (1985). They found a higher initial removal rate of UV-induced pyrimidine dimers from the transcriptionally active *dhfr* gene relative to the total genomic, largely inactive, DNA. The region subject to preferential repair in which the *dhfr* gene is situated is approximately 60-80 kb (Bohr *et al.*, 1986), the size of loops associated with the nuclear matrix, and is larger than the transcriptional unit of the *dhfr* gene (30 kb).

The preferential removal of adducts is, however, not limited to UV-induced DNA damage in the *dhfr* gene in CHO cells. Other examples of heterogeneous repair have been found for pyrimidine dimers in the *hprt* and *ada* genes (Vrieling *et al.*, 1991; Ruven *et al.*, 1993), $O^6$-ethylguanine adducts in the $\beta$-globin gene (Thomale *et al.*, 1994) and alkali-labile sites in *dhfr* and *c-fos* sequences (LeDoux *et al.*, 1991). In principle two kinds of preferential repair exist. The first is transcription-independent such as that seen with AGT and base excision repair. The second is transcription-coupled repair associated with NER. In the *dhfr* gene the heterogeneous repair of UV-induced dimers was found to occur only in the transcribed strand, whereas DNA lesions in the non-transcribed strand were repaired at the same rate as in the rest of the genome (Mellon *et al.*, 1987). This strand bias has also been

et al., 1993). The effect of the level of transcription has also been investigated. In [illegible] and human metallothionein genes, the efficiency of gene-specific repair of pyrimidine dimers can be increased by inducing transcription (Okumoto and Bohr, 1987; Leadon and Snowden, 1988). A higher level of repair was also found for N-methylpurines in the transcriptionally active insulin gene compared to the inactive gene (Ledoux *et al.*, 1990). The transcription-dependent repair model depends on the ability of a bulky adduct to block transcription. The blocks in turn act as a signalling complex that recruits the repair excinuclease (Hanawalt, 1993). As a result, bulky lesions should initiate a larger induction of the transcription-dependent repair pathway than the smaller adducts. Furthermore, the normal transcription-independent repair pathway, whether mediated by glycolase, transcription-independent NER or transferase, should lack any strand bias. Nevertheless, relatively small adducts such as $O^6$-alkylguanine, N7-alkylguanine and N3-alkyladenine that are repaired by a transcripional-independent pathway are preferentially removed from active relative to transcriptionally silent DNA. This is possibly due to an increased accessibility of the open loci to the AGT and glycosylase proteins (Bronstein *et al.*, 1992a; Wang *et al.*, 1995). The fact that these adducts do not from a transcription block explains the lack of strand bias in repair. The importance of preferential repair is illustrated in patients suffering from Cockayne's syndrome associated with mental retardation, dwarfism and hypersensitivity to UV radiation. In these patients preferential repair is absent, although they are proficient in the repair of the total genomic DNA (Venema *et al.*, 1990).

The data presented above has been determined in large stretches of DNA (5-20 kb) encompassing a whole gene or part of a gene. When Gao *et al.*, (1994) looked at cyclobutane pyrimidine dimer repair at the nucleotide level, a 15-fold difference in repair rate within the nontranscribed strand of the phosphoglycerate kinase 1 gene (a housekeeping gene) was found. This indicates that the repair rate in whole genes is only an average of the actual repair rate of each individual nucleotide and that "hot-spots" for DNA damage are actually slow regions of repair (Kunala and Brash, 1992; Tornaletti and Pfeifer, 1994).

### *1.3.6 Methods to determine gene-specific damage*

The apparent lack of homogeneous repair of the genome necessitated the development of methods to determine DNA damage at the gene level. The available methods for measuring adducts in total genomic DNA are unable to differentiate between specific sequences of interest. The first such method was set up by Bohr *et al.* (1985) to determine pyrimidine dimers levels in the *dhfr* gene (Figure 6). The method is based on lesion-specific enzymes that are capable of incising the DNA near or at the position of the lesion in question. T4 endonuclease V is one such enzyme that generates single-strand breaks at pyrimidine dimers in DNA. The incised DNA is separated from the rest of the DNA by alkaline gel electrophoresis and transferred onto a nylon membrane. Labelled (radioactive or otherwise) DNA in the form of the transcribed or non-transcribed strand of

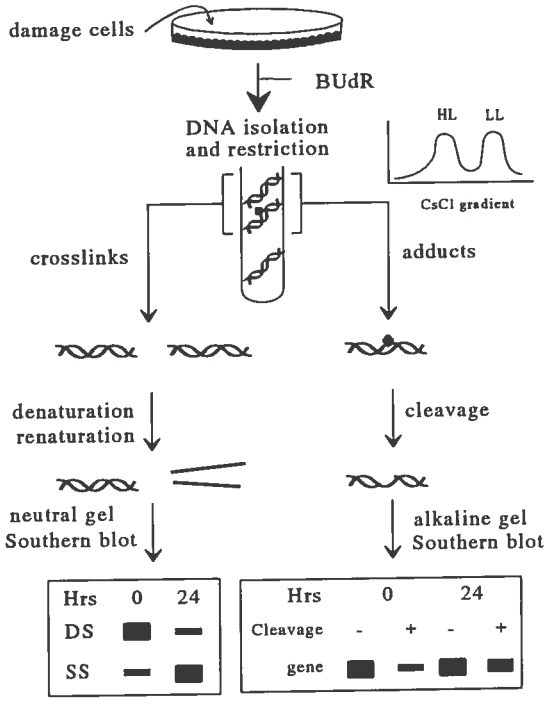



Figure 6: *Method for determining gene-specific repair developed by Bohr et al.(1985).*

a particular sequence is hybridized to the DNA on the blot. In this fashion the number of incisions (cut *versus* uncut DNA) in either strand can be determined. This approach to measuring gene-specific adduct levels is not limited to pyrimidine dimers. The use of the *E. coli* UvrABC excision complex, with its wide substrate specificity, allows the quantification of UV-induced (6-4)photoproducts, benzo(a)pyrene and *N*-acetoxy-2-acetylaminofluorene-induced lesions (Thomas *et al.*, 1988; Tang *et al.*, 1989). Repair of N-alkylpurines (N7-alkylguanine and N3-alkyladenine) can be determined in a similar fashion. Alkylated DNA is heat-depurinated followed by alkaline treatment to induce strand breaks at the apurinic sites (Scicchitano and Hanawalt, 1989; Wasserman *et al.*, 1990). Detection of N3-alkyladenine is possible by use of N3-methyladenine-DNA glycosylase I to form AP-sites cleaved by T4 endonuclease V. The DNA is denatured with NaOH and placed on an agarose gel. To ensure that none of the AP-sites originate from N7-alkylguanine, the DNA is heated in the presence of methoxyamine after the T4 endonuclease V treatment. The AP-sites that result from the heat-labile N7-alkylguanine are protected from further alkaline degradation by the methoxyamine (Wang *et al.*, 1995).

Antibody-based systems for determining specific DNA lesions in defined sequences

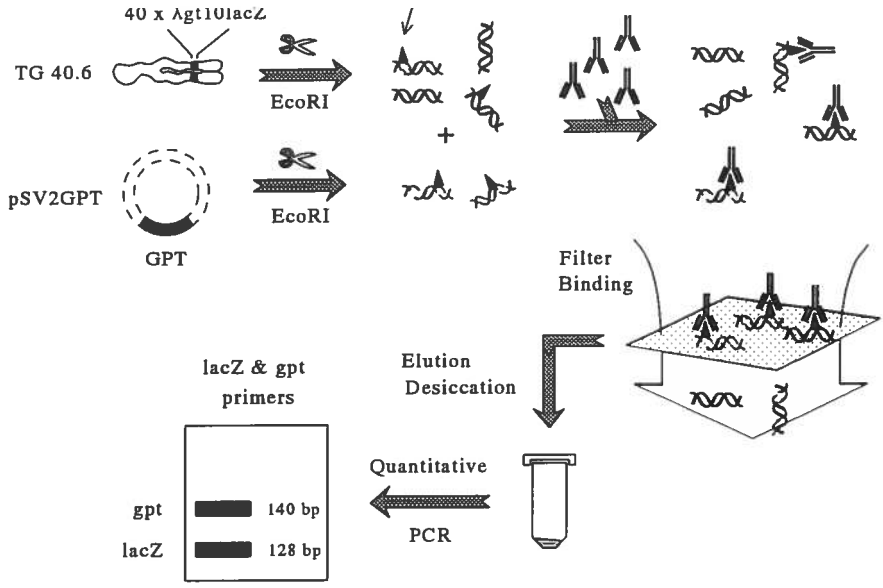



Figure 7: *Immunoaffinity-based method used to determine adduct levels in lacZ in λlacZ mice.*

was employed to analyse the preferential repair in the $\beta$-actin gene in rat hepatoma cells (Hochleitner *et al.*, 1991; Thomale *et al.*, 1994). This assay involves the selective enrichment of $O^6$-ethylguanine-containing DNA fragments with monoclonal antibodies, followed by the quantification of specific DNA sequences of interest by the polymerase chain reaction (PCR) as illustrated for a different application in Figure 7. In principle any adduct can be assayed in this manner provided that specific antibodies directed against the adduct are available.

Another approach to determine the level of gene-specific damage is through the use of PCR to amplify sequences of interest. Govan *et al.* (1990) made use of the ability of UV-induced dimers to block *Taq* polymerase elongation during PCR. The assay allows one to study shorter pieces (100-500 bp in the study by Govan *et al.*, 1990) of DNA segments as opposed to 5-20 kb fragments studied in other genes. This opens the possibility of studying repair regions within genes. In principle this strategy can also be applied to other bulky adducts. Recently Tornaletti and Pfeifer (1994) and Gao *et al.* (1994) have started looking at repair in active and inactive genes at the nucleotide level with the ligase-mediated PCR technique (Figure 8). Genomic DNA exposed to UV radiation, for instance, is treated with T4 endonuclease V to induce nicks with a 5'-phosphate group. A specific primer is annealed to denaturated genomic DNA and primer extension is performed with polymerase. Linker

DNA is subsequently ligated to the generated blunt ends. The mixture is then amplified by PCR with a linker-specific primer and a primer overlapping the first primer. The PCR product is run on a denaturing polyacrylamide gel and electroblotted to a nylon filter. A gene-specific probe is hybridized to the filter and all positions nicked appear as bands. The intensity of the band is an indication of how often a cyclobutane pyrimidine dimer forms at a particular nucleotide (Pfeifer *et al.*, 1992). This method can also be applied to other types of DNA damage as long as enzymes or chemical agents are available that nick the DNA at the damaged position in the DNA.

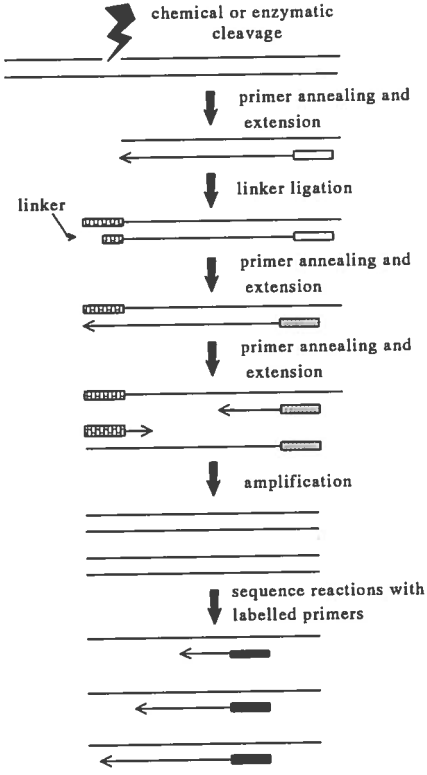



Figure 8: *Principle of ligase-mediated polymerase chain used by Gao et al. (1994) to determine the repair rate of lesions at the nucleotide level. See text for details.*

## 1.4 Mutation induction

When the genetic material is exposed to genotoxicants, DNA lesions can be introduced. These structural changes can have an adverse effect on the DNA coding

mechanisms such as mismatch repair (see 1.3.4), the misincorporated nucleotide can serve as a template during a second round of DNA replication leading to a permanent mutation. The original miscoding lesion, when not adequately repaired, remains potentially mutagenic. Another consequence of unrepaired DNA lesions is cytotoxicity, which occurs when they block transcription and replication.

The type of mutation formed depends, among others, on the adduct. As the exposure of DNA to an agent results in the formation of a spectrum of adducts, a mutation spectrum specific for that agent will be formed. Mutations can consist of simple base pair substitutions in which a purine (guanine and adenine) or a pyrimidine (thymine and cytosine) is replaced by the other purine or pyrimidine, respectively. Such changes are referred to as transition mutations. Another substitution is that of a purine by a pyrimidine or *vise versa*, which is called a transversion mutation. Deletions/insertions of DNA sequences of various sizes are also found. When they result in a shift ($\neq 3n$) in the reading frame of the protein encoded, such genetic changes are referred to as frameshift mutations. Larger aberrations can occur as large deletions or insertions. The following sections are limited to gene mutations caused by alkylating agents and do not discuss mutations at the chromosome level in depth.

### *1.4.1 O-alkylation-induced mutagenesis*

Alkylating agents with a low Swain-Scott constant such as ENU and ENNG, result in high levels of O-alkylation of the DNA bases relative to total alkylation. These compounds have been found to be more mutagenic and more carcinogenic than those reacting mainly with the N-atoms. A linear correlation has been found to exist between the Swain-Scott constant or initial $O^6$-/N7-alkylguanine ratio and the carcinogenic potential in rodents (Barbin and Bartsch, 1989; Vogel *et al.*, 1990). The $O^6$-atom of guanine, the $O^4$- and $O^2$-atoms of thymine together with the phosphodiester bonds are the main targets for O-alkylation (see 1.2.2). Already in 1969, Loveless predicted the possible role of $O^6$-alkylguanine in the mutagenic and carcinogenic effect of alkylating agents (Loveless, 1969). On the bases of experiments carried out *in vitro* this lesion has been shown to cause the incorporation of a wrong nucleotide during RNA synthesis (Gerchman and Ludlum, 1973) and DNA synthesis (Abbott and Saffhill, 1979). In mammalian cell lines, the $O^6$-ethylguanine and $O^4$-ethylthymine adducts were found to induce misincorporation which results in the induction of respectively GC → AT and TA → CG transition mutations (Ellison *et al.*, 1989; Klein *et al.*, 1990). NMR studies on $O^6$-alkylguanine-containing oligonucleotides have shown that $O^6$-ethylguanine:thymine mispairs are not formed because of the stability of the hydrogen bonds between the two bases (Kalnik *et al.*, 1989a,b) as previously thought (McHenry, 1988). Rather, it appears that a reduction in DNA-helix distortion may play a role in the preferential incorporation of a thymine opposite $O^6$-ethylguanine instead of cytosine during DNA replication (Swann, 1990). Similar work carried out on $O^4$-

ethylthymine-containing oligonucleotides indicates that the $O^4$-alkylthymine:adenine base pairs (Kalnik *et al.*, 1989a,b) adopt a wobble conformation whereas the $O^4$-alkylthymine:guanine mispairs retain the Watson-Crick alignment in which the N1 of the purine is juxtaposed to the N3 of the pyrimidine (Swann, 1990).

The relatively less abundant but highly persistent $O^2$-alkylthymine adduct is also capable of mispairing *in vitro* (Grevatt *et al.*, 1992). $O^2$-ethylthymine is able to pair with thymine, eventually resulting in a TA → AT transversion (Grevatt *et al.*, 1992). This adduct has also been found to block DNA synthesis *in vitro* when pairing with thymine, which may explain its cytotoxic property *in vivo* (Grevatt *et al.*, 1992). TA → AT transversions have been implicated in the activation of the oncogenes *neu* and c-Ha-*ras*, respectively, by ENU (Perantoni *et al.*, 1987) and DEN (Stowers *et al.*, 1988) *in vivo*.

Relative to the alkylated base-oxygens, little research has been put into the study of possible mutagenic effects of the alkylphosphotriesters, the most abundant O-alkylation product. It has been postulated that these lesions in the backbone of the DNA could interfere with the functioning of proteins that act through an interaction with the DNA such as polymerases, nucleases or other DNA-binding proteins (den Engelse *et al.*, 1986).

Table 4: *Summary of in vitro mutagenic lesions in mammalian cells.*

| lesion | mechanism | mutation | ref. |
|---|---|---|---|
| $O^6$-alkylguanine | miscoding | GC→AT | Ellison *et al.*, 1989 |
| $O^4$-alkylthymine | miscoding | TA→CG | Grevatt *et al.*, 1992 |
| $O^2$-alkylthymine | miscoding | TA→AT | Klein *et al.*, 1990 |
| $O^2$-alkylcytosine | ? | ? | |
| N7-alkylguanine | AP-sites ? | chromosomal aberration | |
| | ring-open form | random | Tudek *et al.*, 1992 |
| N3-alkylguanine | transcription block | - | Saffhill *et al.*, 1985 |
| N3-alkyladenine | miscoding | AT→GC | Klungland *et al.*, 1992 |
| N3-alkylthymine | miscoding | TA→AT | Grevatt *et al.*, 1991 |
| AP-sites | random insertion | random | Cabral-Neto *et al.*, 1994 |

Although N-alkylation products predominate among [illegible] treatment with an alkylating agent, these lesions tend not to induce many gene mutations, although a correlation does exist between N-alkylation and chromosomal aberrations and sister chromatid exchanges (Natarajan *et al.*, 1984). N7-alkylguanine lesions are chemically unstable and are a source of AP-sites following spontaneous depurination. Also, N7-alkylguanine is in itself not a miscoding lesion (Abbott and Saffhill, 1979), but the formation of the imidazole ring-open form of the base or further processing by glycosylases to yield AP-sites does give this adduct mutagenic potential (Tudek *et al.*, 1992). Formamidopyrimidine-DNA-glycosylases present in *E. coli* (Bioteux *et al.*, 1992) and in mammalian cells (Laval *et al.*, 1990) remove ring-open lesions. When these are left unattended, such lesions have been found to inhibit DNA synthesis *in vitro* indicating a role in cell toxicity (Boiteux and Laval, 1983). Alkylation of the N3-position of cytosine and the N1-position in purines, despite their position within the base-pairing region, inhibit DNA synthesis and are as a result toxic to the cell (Saffhill *et al.*, 1985). N3-alkylthymine in addition to being cytotoxic has also been implicated in TA → AT transversion mutagenesis (Grevatt *et al.*, 1991) whereas N3-alkyladenine has been found to cause miscoding and the formation of AT → GC transition mutations (Klungland *et al.*, 1992).

As a consequence of glycosylase action during repair of N-alkyl products, an AP-site is formed. In bacteria, dAMP is preferentially inserted by DNA polymerase opposite an AP-site, a process referred to as the A-rule (Loeb and Preston, 1986). Although some investigators also apply this rule to mammalian cells, only in *E. coli* has the A-rule been confirmed to operate. The use of shuttle vectors containing AP-sites has shown the four bases to be inserted opposite the AP-site with equal frequency in monkey COS cells (Gentil *et al.*, 1992; Cabral-Neto *et al.*, 1994). Since the A-rule has also been found not to be applicable to other monkey cells (Kamiya *et al.*, 1992) and *Saccharomyces cerevisiae* (Kunz *et al.*, 1994), its relevance to eukaryotic cells is doubtful.

CHO cell lines expressing human N-methylpurine-DNA glycosylase cDNA, display an increased removal of N7-methylguanine and N3-methyladenine following MNNG and MMS treatment. However, the level of chromosomal aberrations increased and the survival rate decreased. The hypothesis was put forward that increased levels of AP-sites occurring in the DNA can result in overlapping repair patches upon processing of the AP-site by AP endonucleases and exonucleases. Double strand breaks may be the result, inducing the formation of aberrations leading to increased cytotoxicity (Kaina *et al.*, 1993).

### *1.4.3 Mutagenesis and mitogenesis*

DNA-replication independent mutations can occur as a consequence of the spontaneous deamination of 5-methylcytosine yielding thymine, which causes the incorporation of an adenine during DNA transcription. For the fixation of the majority of mutations, however, DNA synthesis is a prerequisite. The DNA polymerase $\alpha$ incorporates

a nucleotide not coded for in the original DNA strand opposite a miscoding or non-instructive (*eg.* AP-site) lesion. Toxic effects of agents can injure the tissue, thereby initiating cell division to replace the lost cells. In addition some agents have indirect mitogenic activity that results in increased cell proliferation. The use of high doses of a mutagen can up-regulate cell proliferation and thus increase the chance of mutation fixation. For this reason, agents that stimulate cell division (mitogens), although not mutagenic by themselves, can be indirectly mutagenic as a consequence of fixation of endogenously formed DNA damage (Ames *et al.*, 1993). Experimental evidence points to an increase in the spontaneous mutation rate *in vivo* in the *hprt* gene of dividing T-cells (Nicklas *et al.*, 1988). In contrast, in transgenic marker mice (see below) treated with mitogenic substances no such increase has been observed in the liver (Lefevre *et al.*, 1994). On the other hand, a direct effect of proliferation on mutagenesis has been revealed by the work of Shane *et al.* (1995). Proliferation induced in ENU-treated transgenic mice by a partial hepatectomy led to an additional 4-fold increase in the mutant frequency compared to ENU-treated mice not hepatectomized. In addition, no increase in mutant frequency was observed in organs lacking actively proliferating cells, such as brain, despite the presence of DNA adducts (Chapter 5).

## 1.5 Model systems used for short-term mutagenicity testing

A large battery of tests exist for the evaluation of genotoxic agents in terms of their mutagenic properties. Although these assays do not measure tumourigenicity as the genetic end-point, their use is based on the general assumption that the induction of DNA damage and mutations are causally related to cancer.

A widely used short-term assay developed for the evaluation of genotoxic effects is the "Ames test", which makes use of bacteria such as *Salmonella typhimurium* combined with rat liver microsomal enzymes to meet possible metabolic requirements (Ames *et al.*, 1975). This assay, in which reverse mutations in histidine auxotrophs are scored, is capable of identifying agents that induce base-pair substitution and frameshift mutations. Since the reverse mutation has to occur at a predetermined spot, multiple strains each containing a different mutation must be applied simultaneously in order to prevent the detection of only a limited set of agents with a particular sequence preference (Levin *et al.*, 1982).

*Drosophila* was one of the first animal models in which mutagenesis was studied *in vivo* (Auerbach *et al.*, 1947). Its short generation time and the possibility to look in both somatic and germ cells made it an attractive system to study mutagenesis. One mutagenicity assay in *Drosophila* was the sex-linked recessive lethal test in which induced mutations could be scored in the second generation. The low sensitivity of this assay has led to its replacement with other tests such as the wing mosaic system (Graf *et al.*, 1983) and the *white/white*$^+$ eye mosaic system (Vogel and Nivard, 1993).

defined as clastogenic effects. Clastogenicity is [illegible]
carcinogenicity; it is determined in a short-term mutagenicity assay termed the micronucleus induction (MN) test (Mavournin *et al.*, 1990). Micronuclei are small bodies, separate from daughter nuclei, present in mitotically dividing cells. They contain complete chromosomes or fragments of chromosomes. In mice, micronuclei can easily be detected in newly formed polychromatic erythrocytes (PCE) that form after the extrusion of the nuclei from erythroblast cells. In bone marrow, PCE form 6 hours after mitosis and remain in the bone marrow for 12 to 24 hours. Thereafter they are present in the peripheral blood for the same time period (Mavournin *et al.*, 1990). Measurement of micronuclei in PCE is thus possible in bone marrow and slightly later in the peripheral blood shortly after exposure to a clastogenic agent. Chromosomal aberrations are also induced by some clastogenic mutagens. A multitude of aberrations exist such as gaps, breaks, deletions and translocations, which are visualized in metaphase preparations of treated cell populations. Cross-linking agents or poly-functional alkylating agents have been found to be strong inducers of these type of aberrations (Brusick, 1987).

Short-term *in vitro* gene-mutation tests that make use of mammalian cells determine the mutant frequency in a selectable marker gene such as the *hprt* gene. The *hprt* gene is part of the salvage pathway for dATP synthesis. Its location on the X-chromosome provides a hemizygous and a functionally hemizygous situation in the male and female cells, respectively. A mutation in the single (functional) copy of the gene renders the cell resistant to the utilisation of toxic purine analogues. Other suitable loci for use as selection markers are the thymidine kinase (*tk*), the adenine phosphoribosyl transferase (*aprt*) and the xanthine phosphoribosyl transferase (*xprt*) genes (Meuth, 1990). All these genes code for non-essential nucleotide salvage enzymes and as such can be selected for in a manner analogous to *hprt*. The *tk*, *aprt* and *xprt* genes are, however, autosomal which restricts their use to functionally heterozygous cells *in vitro*. In these mammalian gene- mutation assays the mutant frequency (MF), expressed as the ratio of mutated cells against the total number of surviving cells, is measured. Despite the fact that not all mutations lead to an mutated phenotype, an increase of the MF can still be taken as a measure of the mutagenic potential of a chemical or agent. In addition to the MF, the nature of the mutations in these loci can be determined by sequencing the cDNA obtained by PCR amplification of cytoplasmic RNA (Vrieling *et al.*, 1989). Unfortunately not all mutations can be sequenced, since mutations that block RNA transcription or that involve splice sites may go uncharacterized (Meuth *et al.*, 1990).

Despite the usefulness of mammalian cells in studying mutagenesis *in vitro*, these cells are still unable to adequately imitate the *in vivo* situation. The effect on mutagenesis of variables such as culture conditions, proliferation rates and lack of cell-cell contact in these systems is unknown. In addition, differences between the *in vitro* and *in vivo* environment with respect to distribution of chemicals, metabolism, repair capabilities and scavenging effects, stress the need to look at mutagenesis *in vivo*.

*Drosophila* provide an *in vivo* system in which sequences, such as the *vermilion* gene, can be studied. The *vermilion* gene which encodes an enzyme involved in eye pigmentation can be used as a mutational target, and mutations scored phenotypically in treated flies (Searles and Voelker, 1986). Because the gene has been isolated and sequenced, the mutation spectra of genotoxins can be obtained by DNA sequencing.

In rodents the *hprt* locus can also be studied *in vivo* in skin fibroblasts taken from a granuloma pouch (Maier, 1984). This assay involves the creation of an air bubble on the backs of young adult rats to induce proliferation of skin fibroblasts. After treating the animal with a mutagen the fibroblasts are isolated and subcultured *in vitro*. Next to determination of the MF at the *hprt* locus (Maier and Schawalder, 1988), the molecular characterisation of the mutants is possible (Mohn *et al.*, 1991; Jansen *et al.*, 1992; Jansen *et al.*, 1994a,b). In addition to short-term effects, long-term effects can also be studied in this assay, *e.g.*, the formation of fibrosarcomas. Other cell types in which gene mutations can be determined *in vivo* are lymphocytes (*hprt*; Skopek *et al.*, 1992); the gut (*dlb*-1 locus; Winton *et al.*, 1988) and skin (mouse spot assay; Russell *et al.*, 1979).

Another means available for measuring the MF *in vivo* is the restriction site mutation assay (RSM) (Myers and Parry, 1994). This assay is based on base changes induced by mutagens at restriction sites. Such mutated sequences will no longer be recognised by the restriction endonuclease and cleavage will not occur. Restriction-fragment-length polymorphisms are the result, which can be detected by PCR. Despite the fact that this assay can be applied to any tissue, it is limited to a very small sequence substrate and it is insensitive. The lack of sensitivity stems from the difficulty of digesting DNA by restriction endonucleases with 100% efficiency.

The use of the above methods, with the exception of RSM, is limited to somatic cells. The measurement of gene mutations induced in germ-line cells is possible through the screening of the offspring from treated male mice, as is done in the 7-locus specific locus test (Russell *et al.*, 1979). By scheduling mating at different time points after treatment, and determining mutations in the 7 loci based on morphological traits in their offspring, mutagenesis in different stages of spermatogenesis can be investigated. Mutation induction in germ cells can also be measured in offspring by electrophoretic analysis of proteins (Lewis *et al.*, 1991) and by changes in enzyme activity (Charles and Pretsch, 1987).

## 1.6 Transgenic rodent models

Lohman *et al.* (1987) were the first to describe the potential of using transgenic rodents to study the mechanism of mutagenesis and carcinogenesis *in vivo*. Since then transgenic marker mice and rats have been developed in which exogenous target genes for mutagenesis have been inserted into the genome. The animals, also referred to as marker

This transgenic mouse strain developed by Gossen et al. (1989) contains 80 copies of the λgt10*lac*Z shuttle vector in all somatic cells (Figure 9). They occur as two 40-mer concatemers, in a head-to-tail fashion at a single site on both homologues of chromosome 3 (Gossen *et al.*, 1989; Myhr, 1991; Swiger *et al.*, 1994). Each individual prophage DNA, having an approximate size of 47 kb, contains the 3126 bp *E. coli lac*Z gene of the lactose operon. To determine the mutations in the *lac*Z genes, the prophages must be individually

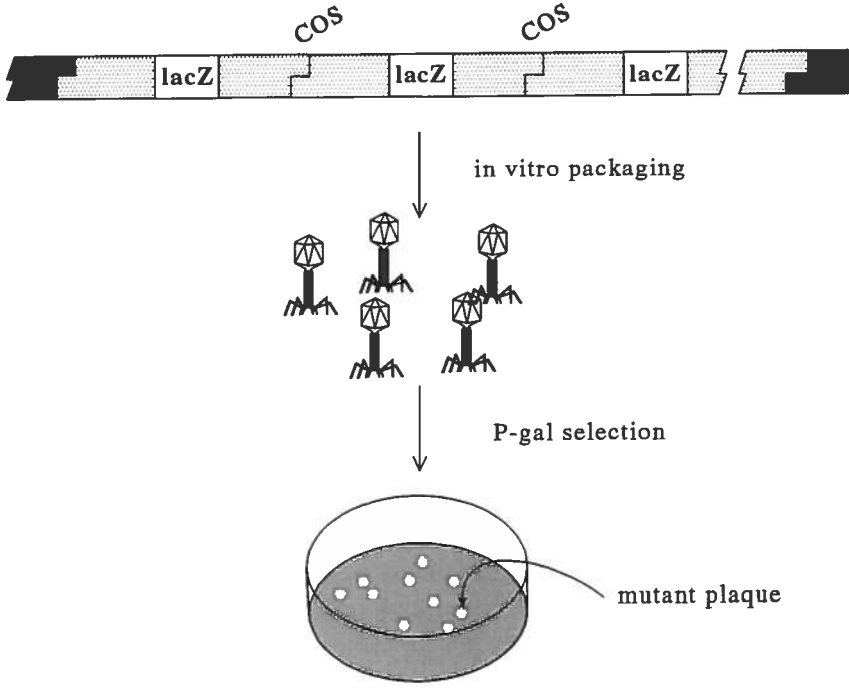



Figure 9: *Mutagenicity testing in λlacZ transgenic mice (Muta™Mouse). λ phages are rescued from total genomic DNA isolates and phages screened in the P-gal positive selection assay. (see Chapter 2)*

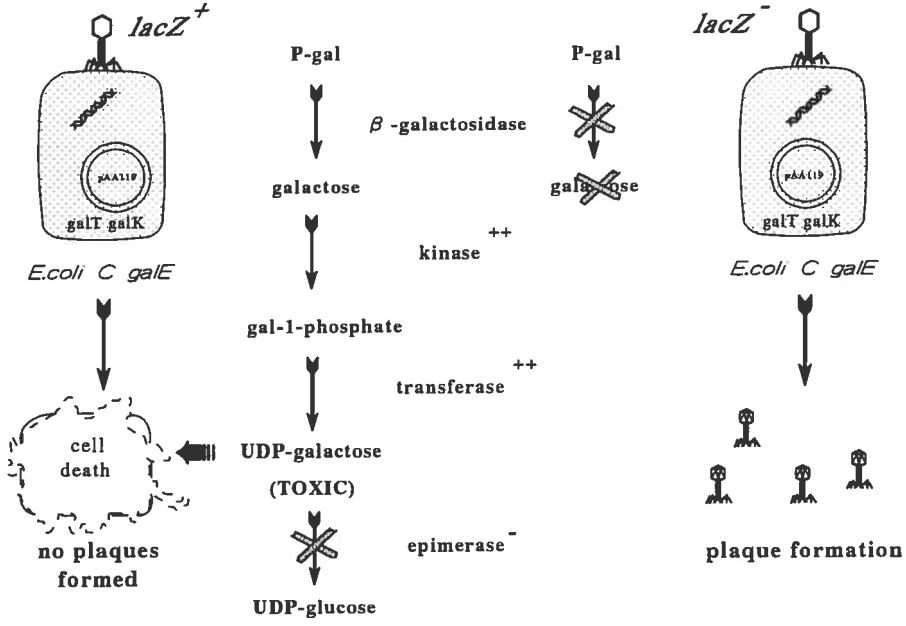
excised and recovered from a total genomic DNA isolate. The recovery of the shuttle vectors is achieved by *in vitro* packaging with the use of commercially available packaging extracts. Enzymes present in these extracts cleave the COS sites at both ends of each

[1]trade mark of Hazelton Laboratories

prophage and package the DNA into a viable $\lambda$ phage particle. The individual phages can be tested for their ability to code for the active *lac*Z gene product: $\beta$-galactosidase. This is done by plating the phages on a suitable *E. coli* strain. On plates containing 5-bromo-4-chloro-3-indolyl-$\beta$-*D*-galactoside (X-gal), a substrate for $\beta$-galactosidase, the phages carrying a wild-type *lac*Z gene will form blue plaques, whereas phages with a mutated *lac*Z gene, resulting in (partially) inactivated $\beta$-galactosidase, remain colourless or are light blue in colour. The MF is expressed as the ratio of mutant *lac*Z phages to the total number of phages.

Initial problems with this assay concerned the rescue efficiency of the phages from the DNA. The highly methylated state of the transgene DNA inhibited efficient packaging (Gossen *et al.*, 1989). The rescue efficiency was improved by plating the phages on an *E. coli* C strain, which is less capable of restricting foreign DNA and through the use of packaging extracts derived from an *E. coli* strain lacking a host restriction system (Gossen *et al.*, 1988).

A further improvement of this system was made with the introduction of the positive selection system described in detail in Chapter 2. Basically, the positive selection system involves the suppression of non-mutated phages while mutated phages are permitted to



galactopyranoside (P-gal), releasing the galactose moiety. In a *E. coli* C *galE*::Tn*10* host, which harbours a plasmid overexpressing galactose kinase and galactose transferase, galactose is metabolised to UDP-galactose through the actions of these two enzymes. Due to the *galE*$^-$ genotype of the cells, UDP-galactose cannot be converted by the epimerase enzyme to UDP-glucose. The accumulation of UDP-galactose is toxic to the cell and thus propagation of $\beta$-galactosidase-expressing $\lambda$ phages is aborted (Figure 10). The new assay obviates the need to screen large numbers of plaques on the basis of their colour, which is very tedious and expensive, and rather inaccurate. The number of phages screened is limited to 14,000 phages per 500 cm$^2$ plate. The new system enables one to screen over $1x10^6$ phages per 9cm petri dish (64 cm$^2$).

Additional information regarding the location and the molecular nature of the mutation can be determined in the transgenic system. Although the *lac*Z gene is more than 3 kb in size, the length needed to be sequenced can be reduced by roughly determining the position of the mutation prior to sequencing, with the $\alpha, \beta, \omega$ complementation assay. The *lac*Z protein consists of 3 regions designated $\alpha, \beta$ and $\omega$. Although the confines of these regions are not that well-defined, the regions have been designated as $\alpha$ (base positions 9-276), $\beta$ (277-1800) and $\omega$ (1801-3096) (Douglas *et al.*, 1994). A mutation in one of the regions leads to a reduced or a total absence of $\beta$-galactosidase activity. Two $\beta$-galactosidase proteins, each with a mutation in a different region can complement each other *in trans* to restore the enzyme activity. The complementation assay employs three *E. coli* strains DH5$\alpha$ ($\alpha^-,\beta^+,\omega^+$), W4680 ($\alpha^+,\beta^-,\omega^+$) and Hfr 3000x90 ($\alpha^+,\beta^+,\omega^-$) (Gossen *et al.*, 1993a) in which $\beta$-galactosidase complementation occurs between the host protein and the phage encoded $\beta$-galactosidase. When the region mutated in the *E. coli host* strain differs from that in the phage, active protein will be produced. When the region has been determined, sequencing can ensue either by cloning the *lac*Z sequence into M13 (Gossen *et al.*, 1993a) or by direct PCR of the mutant phages (Chapter 5).

### *1.6.2 Big Blue™ rodents*[2]

The Big Blue system includes transgenic mice, rats and cell lines. The mice harbour 80 copies of the 1080 bp *E. coli lac*I target gene per diploid genome, coding for the lactose operon repressor protein, on a lambda shuttle vector, in addition to an $\alpha$-complementing *lac*Z reporter gene (Kohler *et al.*, 1991a,b)(Figure 11). A number of Fischer 344 rat lineages have been constructed with varying copy-numbers ranging from 1-2 copies to 200 copies per haploid genome (Dycaico *et al.*, 1994). A rat cell line is also available with *λlac*I shuttle vectors stably integrated at two sites, with a total of 50-70 copies per polyploid cell (Wyborski *et al.*, 1995). Like in the *lac*Z mouse, the transgene DNA can be excised from the

[2]trade mark of Stratagene

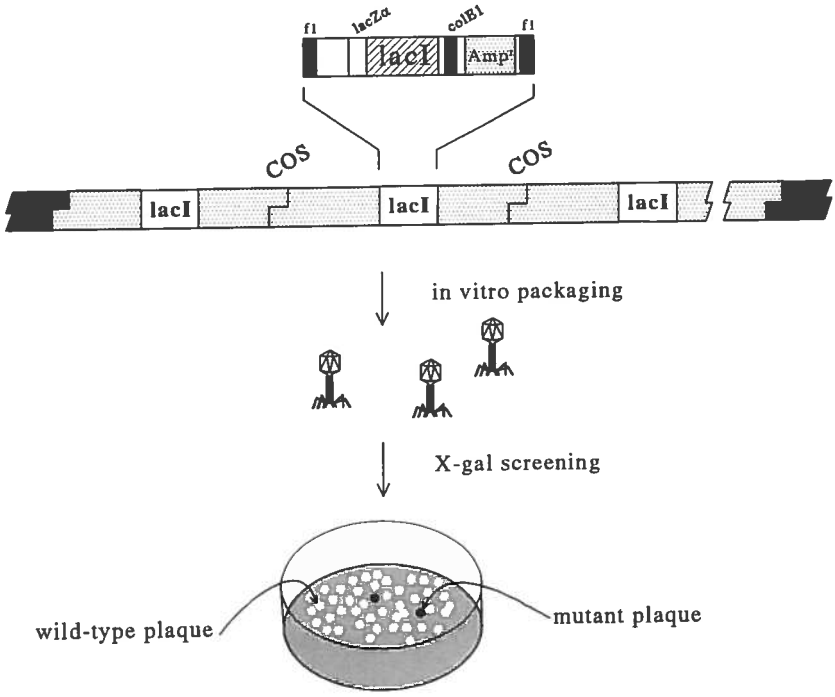



Figure 11: *Mutagenicity testing in λlacI transgenic mice (Big Blue™).*

chromosomal DNA with the use of packaging extracts. Screening of the phages is carried out on X-gal plates using a suitable strain of *E. coli*. The assay relies on the ability of the repressor protein to prevent the transcription of the lactose operon. A mutation in the *lac*I target gene may inactivate the repressor function thus allowing the production of β-galactosidase. As a consequence, mutant phages isolated from *lac*I transgenic mice produce a blue colour in the presence of X-gal, which facilitates their detection in a background of white plaques. A positive selection assay in which propagation of non-mutant phages is suppressed, also exists for screening *λlac*I phages. The selection is carried out with an *E. coli* host containing an integrated *c*I$^+$ gene coding for the λ repressor protein (Lundberg *et al.*, 1993). Infecting λ particles will remain in a lysogenic state maintained by *c*I as opposed to the lytic state normally encountered. Phages encoding a mutated *lac*I will not be able to repress either the phage-encoded *lacZα* or the mutated *lac* operon ($\alpha^-$,b$^+$,$\omega^+$) present in the cell. Consequently, complementation can occur between the phage *lacZα* and the host *lacZ*. Grown on mimimal medium plates containing lactose, only cells expressing an active β-

For the molecular characterisation of the mutations in the *lac*I gene, the target region can be excised as a phagemid using M13 helper phages that act on the partial f1 origins (Kohler *et al.*, 1991a). Upon reinfection of *E. coli* with these phagemids and subsequent selection with ampicillin, a colE1-containing plasmid can be obtained in large quantities. The length of the DNA that needs to be sequenced is less than for the *lac*Z mice. Another advantage of the *lac*I mice is the existence of a large mutation database. Almost all known mutations leading to inactivating amino acid changes have been determined (Provost *et al.*, 1993).

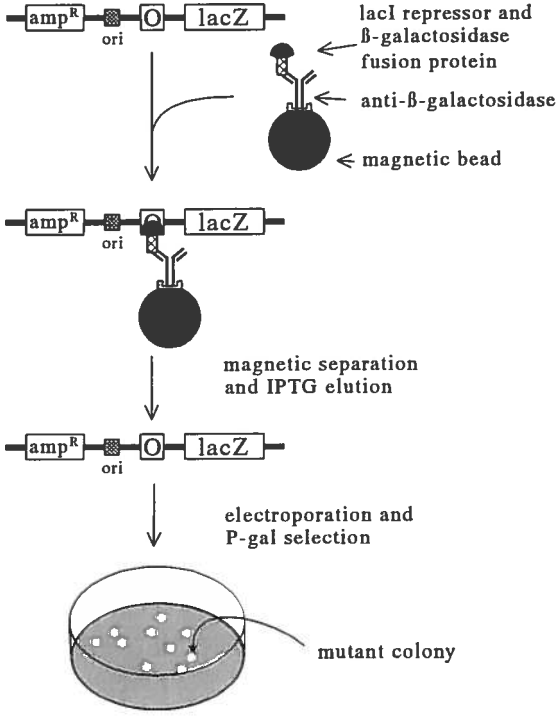



Figure 12: *Mutagenicity testing in plasmid based Xenomouse™ (after Gossen et al. 1994)*

### *1.6.3 Xenomouse™* [3]

A third transgenic marker-mouse strain commercially available is that harbouring 40 copies/diploid genome of the pUR288 plasmid (5000 bp) containing the operator region and the *lac*Z gene (Figure 12)(Gossen *et al.*, 1995; Martus *et al.*, 1995). Upon excision of the pUR288 plasmids from the chromosomal DNA by restriction endonucleases, the plasmids can be "fished out" with the help of *lac*I repressor protein attached to magnetic beads. The repressor protein binds to the *lac* operator region (O) of the plasmids which are then isolated and transferred to *E. coli* by electroporation. Apparently, with this method a higher rescue efficiency can be achieved than by packaging in λ-phages, obviating the need for high molecular weight chromosomal DNA (Gossen, 1993b). An advantage of this system is the ability of detecting large deletion mutants (Gossen *et al.*, 1995). Mutant *lac*Z-containing plasmids can also be scored with the P-gal positive selection method.

### *1.6.4 Transgene versus endogenous sequences*

A number of studies involving various compounds have been performed in the *lac*Z and *lac*I transgenic mice (see Morrison and Ashby, 1994). Treatment with alkylating agents such as MNU, ENU and DEN have shown an MF induction in organs such as the liver, bone marrow, skin and germ cells (Morrison and Ashby, 1994; van Delft and Baan, 1995). A comparison was also made between mutation induction in the *lac*I and the *hprt* loci of splenic T-cells in the same animal. Skopek *et al.* (1995) treated 3 week old mice with a single i.p. injection of ENU and measured the MF in both loci. Similar MF's were measured in both loci, at different ENU doses. The spontaneous MF, however, was 5-20-fold higher in the *lac*I transgene than in *hprt*.

In a comparison between somatic mutations induced at the *lac*I and the *dbl*-1 loci in the hemizygous *lac*I/*dbl*-1$^a$ mice, which were obtained by crossing homozygous *lac*I mice with homozygous *dbl*-1$^a$ mice, ENU led to equal inductions at both loci (Tao *et al.*, 1993). Upon exposure to X-rays, the MF increase at the *dbl*-1 locus was higher than that observed for *lac*I (Tao *et al.*, 1993). Large deletions are probably missed with *lac*I transgenic mice. During packaging when the DNA flanked by two COS-sites is encapsulated into a phage particle, the length of the DNA must be between 42 to 52 kb for successful packaging. Large deletions or insertions in the 47 kb shuttle vector can therefore not be detected. In mice carrying the pUR288 plasmid, insertions or deletions in the plasmid sequence will only lead to a change in the size of the plasmid. Plasmids with a length of up to about 40 kb could be isolated (Gossen, 1993). With respect to deletion/insertion mutants, a replicating plasmid can be formed as long as the sequence near the deletion is flanked by an *ori* and an ampicillin resistance gene. Treatment of *lac*Z plasmid-based transgenic mice has shown a

1995). Interestingly, the spontaneous MF in the liver, using [illegible]
roughly twice that seen in the λ shuttle vector-based transgenic mouse models. In about 50% of these mutants, deletions were observed, the majority of which were between 1-3 kb in length. The reason why the frequency of such mutations is lower in the λ shuttle vector-based models, most likely can be found in the disruption of essential λ genes required for propagation.

## 1.7 Mutation spectra of alkylating agents in different test systems

The molecular characterisation of the mutations induced by treatment with alkylating agents, or other agents for that matter, provides information pertaining to the role of pre-mutagenic lesions and neighbouring DNA sequences in mutagenesis. The mutation spectrum found after treatment with alkylating agents is dependent upon the chemical compound used and also on the metabolic and repair capability of the organ, tissue or cell line being investigated. The following data is a summary of the mutation spectra produced by MNU and ENU in *E. coli* and mammalian cells *in vitro* and *in vivo*.

In *E. coli*, treatment with agents such as MNU (Table 4) induces mainly GC → AT transition mutations in the *lac*I gene, which are attributed to the $O^6$-methylguanine adduct (Burns *et al.*, 1988a). Treatment with ENU, which forms adducts on other base oxygens than only the $O^6$-guanine, still produces primarily GC → AT transitions but a higher level of AT → GC transitions and TA → AT transversions are encountered (Table 5). The increase in AT → GC and TA → AT is believed to be due to the $O^4$-ethylthymine and $O^2$-ethylthymine respectively (Burns *et al.*, 1988b).

The mutation spectrum of similar agents *in vitro*, with the *hprt* gene as the target for mutagenesis, has been determined in both human and CHO cells. The spontaneous mutations at this locus consist mainly of base substitutions (64%) and splice mutations (33%) (Zhang *et al.*, 1991). Similar to *E. coli*, MNU-induced mutations consisted mainly of GC → AT transitions (Zhang and Jenssen, 1991; Akagi *et al.*, 1993), although about a third of the mutations found were at splice sites. A greater difference between *E. coli* and the *hprt* locus of mammalian cells is seen upon treatment with ENU (Table 5). A reduction in GC → AT transitions and an increase in AT → GC and TA → AT mutations are observed. The TA → AT transversions originate from $O^2$-ethylthymine, a lesion that is repaired in *E. coli* by the *alk*A-encoded glycosylase. How this lesion is repaired in mammalian cells is not known. Little information is available concerning the origin of GC → TA and TA → GC transversions formed in appreciable numbers after ENU treatment.Available data obtained from alkylated oligonucleotides *in vitro* has not identified the responsible lesion. In this case, the role of adducts such as $O^2$-alkylcytosine and N7-alkyladenine, which have received much less attention relative to other O- and N-alkylation products, should be looked into.

Table 4: *Mutation spectra formed in vitro and in vivo upon treatment with MNU expressed as a percentage of all mutations sequenced.*

| | *in vitro* | | *in vivo* | |
|---|---|---|---|---|
| Gene<br>Cell type | *lac*I<br>*E.coli* | *hprt*<br>V79<br>(hamster) | *hprt*<br>VH12<br>(mouse) | *hprt*<br>fibroblasts<br>(rat) |
| GC→AT | 95 | 58 | 81 | 58 |
| AT→GC | 0 | 3 | 0 | 5 |
| GC→TA | 1 | 3 | 2 | 5 |
| TA→AT | 1 | 2 | 0 | 0 |
| TA→GC | 0 | 0 | 0 | 0 |
| GC→CG | 0 | 3 | 0 | 0 |
| other[#] | 3 | 35 | 15 | 32 |
| No. mut.[$] | 104 | 31 | 53 | 19 |
| reference | a | b | c | d |

*a: Burns et al., 1988a; b: Zhang and Jenssen, 1991; c: Akagi et al., 1993; d: Jansen et al., 1994a; [#]: includes insertions, deletions and mutations at splice sites; [$]: number of mutants sequenced.*

The differences between mutation spectra in bacterial and mammalian cells stresses the importance of using mammalian cells in mutagenicity testing. Part of the differences is due to inherent differences between the cell types. For instance splice-site mutations, which form a substantial portion of *hprt* mutations, are not present in bacteria. More important, however, are the differences in trans-membrane transport, metabolism, DNA repair and proliferation rate between bacterial and mammalian cells.

Differences in gene-mutation data obtained at the *hprt* locus *in vivo* and *in vitro* after treatment with alkylating agents show that the mutation spectra do not differ much between the two (Tables 4 and 5)(Skopek *et al.*, 1992; Jansen *et al.*, 1994, 1994b, 1995), although a slight reduction in $O^6$-ethylguanine-mediated (GC → AT) and an increase in $O^2$-ethylthymine-mediated (TA → AT) mutagenesis is seen *in vivo* compared to *in vitro* (Table 5) in ENU-treated mammalian cells.

The need to study mutagenesis in various organs and tissues triggered the development of new mutagenesis models such as the transgenic marker mice, discussed above. The use of different dose regimes, agent concentrations, expression times and organs, however, complicates proper comparisons of the data now available. In addition, the small number of mutants characterized in some cases, makes comparisons difficult. In Table 6 mutation spectra obtained in several organs of the *lac*Z and *lac*I mice are shown. An initial

*as a percentage of all mutations sequenced*

| | *in vitro* | | | *in vivo* | | |
|---|---|---|---|---|---|---|
| Gene | *lacI* | *hprt* | *hprt* | *hprt* | *hprt* | *hprt* |
| Cell type | *E.coli* | lymphoblast (human) | fibroblast (rat) | T-cells (mouse) | fibroblast (rat) | T-lymphocytes (rat) |
| GC→AT | 77 | 19 | 20 | 4 | 12 | 11 |
| AT→GC | 17 | 31 | 11 | 27 | 9 | 17 |
| GC→TA | 1 | 15 | 3 | 2 | 7 | 2 |
| TA→AT | 1 | 23 | 34 | 55 | 40 | 54 |
| TA→GC | 3 | 12 | 9 | 12 | 16 | 4 |
| GC→CG | 0 | 0 | 0 | 0 | 0 | 0 |
| other[#] | 1 | 0 | 23 | 0 | 16 | 9 |
| No. mut.[$] | 109 | 26 | 35 | 51 | 43 | 46 |
| reference | a | b | c | d | e | f |

*a: Burns et al., 1988b; b: Bronstein et al., 1991; c: Jansen et al., 1994b; d: Skopek et al., 1992; e: Jansen et al., 1994a; f: Jansen et al., 1995; [#]: includes insertions, deletions and mutations at splice sites; [$]: number of mutants sequenced.*

Table 6: *Mutation spectra formed in different tissues of lacZ and lacI transgenic mice treated with MNU or ENU expressed as a percentage of all mutations sequenced.*

| | *lacZ* mice | | | | | | *lacI* mice | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tissue | liver | liver | bm | bm | germ | germ | spln | spln | spln | germ | germ | germ |
| Agent | ctrl | ENU | ctrl | ENU | ctrl | ENU | ctrl | MNU | ENU | ctrl | ENU | ENU |
| Time | | 10 d | | 10 d | | 55 d | | 6-17 d | 3 d | | 3 d | 90 d |
| GC→AT | 44 | 30 | 71 | 20 | 38 | 33 | 41 | 91 | 38 | 85 | 61 | 27 |
| AT→GC | 26 | 10 | 5 | 10 | 4 | 16 | 9 | 1 | 0 | 0 | 0 | 15 |
| GC→TA | 4 | 40 | 14 | 20 | 21 | 3 | 19 | 5 | 37 | 8 | 4 | 12 |
| TA→AT | 7 | 10 | 0 | 50 | 4 | 42 | 4 | 0 | 13 | 0 | 30 | 46 |
| TA→GC | 0 | 0 | 0 | 0 | 4 | 7 | 4 | 0 | 0 | 0 | 0 | 0 |
| GC→CG | 4 | 10 | 0 | 0 | 13 | 0 | 7 | 2 | 0 | 0 | 0 | 0 |
| other[#] | 15 | 0 | 10 | 0 | 17 | 0 | 16 | 0 | 13 | 8 | 4 | 0 |
| No. mut.[$] | 27 | 10 | 21 | 10 | 24 | 31 | 90 | 99 | 8 | 13 | 23 | 26 |
| reference | a | b | a | b | c | c | d | g | e | f | f | f |

*ctrl: control mice; bm: bone marrow; spln: spleen; Douglas et al., 1994; b: Douglas et al., 1993; c: Douglas et al., 1995; d: de Boer et al., 1996; e: Kohler et al., 1991a; f: Provost and Short, 1994; g: Provost et al., 1993. [#]: includes insertions, deletions and mutations at splice sites. [$]: number of mutants sequenced.*

these mice are comparable to that found at the *hprt* locus *in vivo* (Gossen *et al.*, 1993; Kohler *et al.*, 1991a; Douglas *et al.*, 1994). In the germ cells of both types of *lac* mice a similar mutation spectrum was obtained at the late expression times after ENU-treatment (Table 6)(Douglas *et al.*, 1995; Provost and Short, 1994). The spontaneous spectra do, however, differ. Such variations may still be due to the low number of mutants sequenced (24 in *lac*Z *vs.* 13 in *lac*I mice).

## 1.8 Alkylating agent-induced tumourigenesis

Barbin and Bartsch (1989) and Vogel *et al.* (1990) determined a relation between the nucleophilic selectivity of a series of monofunctional agents and their carcinogenic potency in rodents. This relation was not found to apply to bifunctional agents. This is the basis for the use of monoalkylators in carcinogenicity studies. Monofunctional compounds with a low selectivity that have the tendency to alkylate more at oxygen atoms in the DNA bases, were found to be more potent carcinogens than those that primarily result in N-alkylation products. One of the lesions responsible for this is $O^6$-alkylguanine.

### *1.8.1 The role of $O^6$-EtG repair*

The link between the presence of $O^6$-alkylguanine and tumourigenesis was made by Goth and Rajewsky (1974a) who found that the treatment of pregnant rats with ENU resulted in tumours of the nervous system in all offspring. Analysis of the tissues showed a lack of $O^6$-ethylguanine repair in the brain in contrast to the liver even though N7-guanine and N3-adenine lesions were repaired at the same rate in the brain and liver. A similar observation was made with rats treated repeatedly with MNU; these subsequently accumulated $O^6$-methylguanine in the brain, a little in the kidney and none in the liver. The accumulation was explained in terms of AGT depletion in the brain and at a later stage in the kidney (Pegg, 1983). This correlated well with the tumour induction in these tissues (Kleihues and Margison, 1974). When the AGT in the liver is exhausted by high doses of alkylating agents, a dose-dependent increase in liver tumours is observed (Peto *et al.*, 1984). The mutation signature of $O^6$-alkylguanine, a GC → AT transition (see 1.5.3), has often been found in codon 12 of the H-*ras* protooncogene in alkyl-induced rat mammary tumours (Zarbl *et al.*, 1985). In transgenic mice overexpressing the human AGT gene, the development of thymic lymphomas, the most common MNU-induced tumours in mice, did not occur after MNU treatment (Dumenco *et al.*, 1993).

### *1.8.2 Mutation spectra in activated oncogenes*

Comparative studies on the persistence of $O^6$-methylguanine in different rat tissues with tumour incidence after MNU treatment showed the persistence to be highest in brain

was established that *neu* activation could proceed only by replacement of valine at position 644 by glutamic acid or glutamine (Bargmann and Weinberg, 1988). Therefore, the *neu*-activating TA → AT mutation most likely reflects biological selection rather than preferential mutagenesis. In mammary tissue, where a slow rate of $O^6$-alkylguanine repair exists, tumours induced by MNU were shown to contain activated H-*ras* with a GC → AT transition mutation (Zarbl *et al.*, 1985). Skin tumours induced by MNU or MNNG also contain GC → AT transitions in c-Ha-*ras* (Brown *et al.*, 1990).

Mutations induced in oncogenes by ethylating agents are more diverse than those induced by methylating agents. Activated H-*ras* genes in liver tumours as a result of DEN treatment have been found to consist of 50% GC → TA, 20% AT → GC and 30% TA → AT mutations (Stowers *et al.*, 1988). The lack of GC → AT mutations is consistent with the high $O^6$-ethylguanine-repair capacity of the liver. The mutation spectrum of the liver suggests that $O^2$- and $O^4$-alkylthymine are the main mutagenic lesions in this case. Lung tumours induced by both ENU and DEN, on the other hand, contain more GC → AT mutations (20%) and 70% $O^4$-ethylthymine-induced AT → GC transversions (You *et al.*, 1992). This is believed to be due to the lower $O^6$-ethylguanine repair capacity of the lung.

The above data clearly indicate a relation between the induced adducts, the capacity to repair $O^6$-alkylguanine and the incidence of particular mutations in activated oncogenes. A difference between the mutation spectra in the oncogenes and *hprt in vivo* (see 1.7) is the lower frequency of TA → AT transversions and the higher incidence of AT → GC transitions in the oncogenes. This difference between *hprt* and oncogenes suggests that also lesions other than $O^6$-alkylguanine, $O^2$-alkylthymine and $O^4$-alkylthymine are responsible for mutations in oncogenes.

## 1.9 Outline of the thesis

The primary aim of the work presented in this thesis was to establish a link between alkylating agent-induced DNA lesions and mutations, in different tissues *in vivo,* with the use of the *lac*Z transgenic marker mouse model. The animals were treated with alkylating agents to induce DNA adducts. Alkylating agents are able to induce a variety of biological effects that have been well described in the literature. Through the use of different ethylating agents, each inducing a specific adduct spectrum, it is possible to study the mutagenic effects of specific adducts in the DNA. The choice of the model ethylating agents ENU, EMS and DEN for our studies was based on the following arguments. From literature it is known that the $O^6$-ethylguanine adduct, a major O-alkylation product induced by several ethylating agents, is a premutagenic lesion *in vitro*. To test whether this is also true *in vivo*, ENU was chosen not only for its ability to react directly with the DNA thereby forming adducts in all tissues it comes in contact with, but also because ENU is primarily an oxygen alkylator

inducing among others a relatively high level of $O^6$-ethylguanine adducts. The use of EMS, another direct acting agent, was based on the relatively low proportion of oxygen and high level of nitrogen adducts it induces compared to ENU. As a result, an effect attributed to the $O^6$-ethylguanine would be seen with ENU but not with EMS. To test for tissue-specific effects, DEN was used. This agent needs to be metabolically activated prior to inducing adducts, and as such is only expected to be mutagenic in tissues capable of metabolic activation. Its active molecular species is identical to that formed by ENU. Measurements were limited to three organs; namely the liver, brain and bone marrow. The liver was chosen not only for its metabolic capacity but also for its DNA-repair capacity which has been well documented. The effect of cell proliferation was looked at by observing the effect of the ethylating agents in the brain compared to that in the bone marrow, in addition to comparing these tissues to the liver in relation to their different repair capacities.

The measurement of the mutant frequency (MF) in the organs of the treated transgenic mice was initially carried out on plates containing the $\beta$-galactosidase substrate X-gal. The limit of 14,000 phages per plate and the laborious and tricky scoring procedure, hampered rapid progress and spurred the search for an alternative. The introduction of the P-gal positive selection system (Chapter 2) was an essential step in improving the phage-screening process. It turned out to provide a drastic saving in time and effort. Most previous data had, however, been obtained with the X-gal procedure. It was essential, therefore, to determine whether the data obtained with the two systems was comparable. To this end, MF data from ENU- and benzo(a)pyrene-treated mice determined with the X-gal colour screening procedure and the P-gal positive selection assay was compared (Chapter 3).

Although the positive selection system permitted the rapid measurement of the MF in the various tissues, the question pertaining to the relevance of the exogenous *lac*Z DNA for endogenous DNA remained. The *lac*Z in which mutations are scored is of bacterial origin, it is present in tandem copies and is not transcribed; as such it might differ from the endogenous mouse DNA with respect to the rate of DNA lesion induction, repair and mutation fixation. The relevance of the transgene DNA for the total genome was investigated with respect to $O^6$-ethylguanine formation and repair in the brain and liver DNA in ENU-treated mice (Chapter 4).

To investigate the mutagenicity of the $O^6$-ethylguanine and N7-ethylguanine adducts *in vivo*, mice were treated separately with EMS, DEN and ENU and the MF's were measured in liver, brain and bone marrow. In addition, the $O^6$-ethylguanine and N7-ethylguanine levels in the total genomic DNA were measured with monoclonal antibodies directed specifically against these adducts. This allows the comparison of the mutation induction with $O^6$- and N7-ethylguanine adduct levels and the repair profiles. Additional information concerning the molecular nature of the mutations induced by ENU in the liver and bone marrow DNA was obtained by sequencing mutant *lac*Z genes (Chapter 5).

The implications of the data presented in this thesis and that obtained from the

and other gene-mutation assays will be addressed.

# *Chapter 2*

## An improved selection method for *λlacZ*⁻ phages based on galactose sensitivity

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*Transgenic Research* **3**: 67-69.

**Abstract**

The determination of the *lacZ* mutant frequency in *λgt10lacZ* phage vectors isolated from the transgenic mouse strain 40.6 (Muta™Mouse), requires the screening of large numbers of phages on β-galactosidase activity. Existing methods rely on distinguishing a few white plaques on X-gal containing plates amongst a multitude of blue ones, which is both time-consuming and expensive. The new screening method described here employs the galactose sensitive *E. coli C lacZ recA galE* strain into which a multicopy plasmid has been introduced which results in over-expression of the *galK* and *galT* genes. In the presence of phenyl-β-D-galactopyranoside, a substrate for β-galactosidase, this leads to the suppression of *λlacZ*⁺ phage propagation without affecting the ability of *λlacZ*⁻ phages to form plaques. With this method it is possible to screen 1.5 x$10^6$ phages on a single 9-cm petri-dish. Furthermore the need for blue/white screening has been eliminated.

**Introduction**

Transgenic mice harbouring a reporter gene in a shuttle vector have been developed as animal models for mutagenesis studies. The transgenic mouse strain 40.6 (Muta™Mouse) has incorporated into its genome 80 copies of a *λgt10lacZ* shuttle vector in the form of two 40-mer tandem repeats (Gossen *et al.,* 1989; Myhr, 1991). The *E. coli lacZ* gene, coding for β-galactosidase, functions as the target sequence for scoring mutations. The increase in *lacZ* mutant frequency is an indication of the mutagenicity of a compound in question. Until recently, the detection of phages with a mutated *lacZ* was carried out by infecting *E. coli C* with phages 'rescued' *in vitro* from chromosomal DNA on media containing X-gal, a substrate for β-galactosidase. Plaques formed by *λlacZ*⁺ phages contain active β-galactosidase and as a result are blue in colour, while *λlacZ*⁻ plaques remain white. This procedure has proven to be very laborious as only a relatively small number of phages can be screened at one time and also because the detection of white plaques in a sea of blue plaques is a tedious process. This prompted us to search for an alternative method to discriminate between *λlacZ*⁺ and *λlacZ*⁻ phages.

selection of vectors containing a mutated *lacZ* gene. An *E. coli galE* strain is unable to complete the Leloir pathway for galactose metabolism as it lacks the epimerase enzyme (Adyha, 1987). This strain, when grown in the presence of galactose, is unable to convert the toxic UDP-galactose intermediate, formed through the enzymic actions of the galactose kinase (galK) and transferase (galT), into harmless UDP-glucose. As a result of UDP-galactose accumulation, bacteriolysis ensues. The formation of galactose in these cells can be brought about by the introduction of *lacZ* containing vectors and growth in the presence of phenyl-$\beta$-D-galactopyranoside (P-gal), another substrate for $\beta$-galactosidase. After the release of the galactose moiety from P-gal by $\beta$-galactosidase, the toxic UDP-galactose is formed resulting in the survival of only the *lacZ*$^-$ containing cells. Recently, a galactose-sensitive *E. coli C lacZ galE* strain (Gossen *et al.,* 1992) has been constructed which, in the

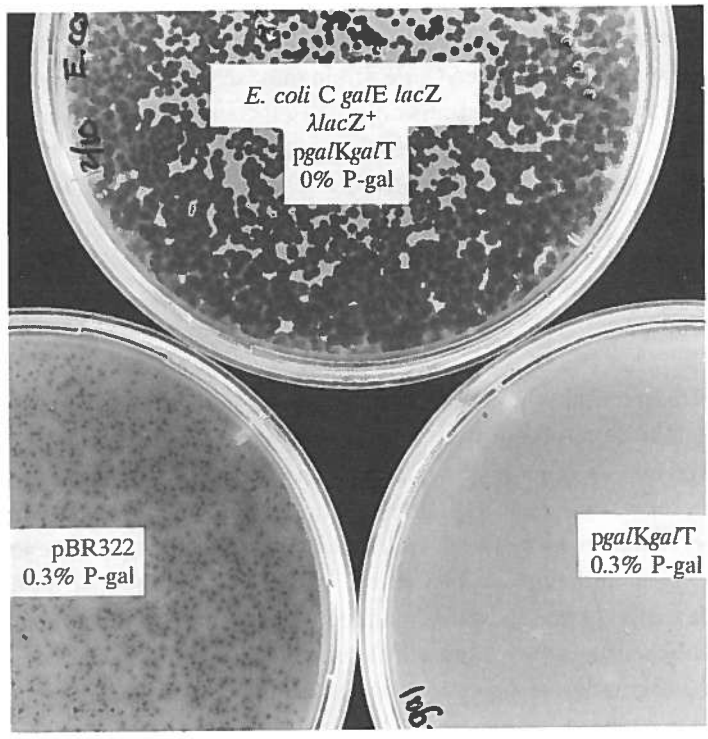


Figure 1: *Suppression of propagation of λgt10lacZ$^+$ phages in the presence of P-gal by the E.coliC lacZ recA galE strain harbouring a galK and galT expressing plasmid (pAA119) or the control pBR322. Both pAA119 (bottom right) and pBR322 (bottom left)-containing cells were infected with equal numbers of λgt10lacZ$^+$ phages and plated out on P-gal-containing medium. The same procedure was also carried out with the pAA119-containing E.coli but in the absence of P-gal in the medium (top).*

presence of P-gal, selects against plasmids expressing *lacZ* (Gossen *et al.,* 1992) and *λlacZ* phages (Gossen *et al.,* 1993b) derived from transgenic mice described in Gossen *et al.* (1993a).

The *E. coli C lacZ galE* strain was made *recA* by P1 transduction from PC4014 (Fabagen, The Netherlands), to minimize possible recombination between plasmid and host DNA. The application of this strain with the procedure described in Gossen *et al.* (1993b), however, appeared not to be successful for the screening of *λgt10lacZ* phages from 40.6 transgenic mice as the propagation of *λgt10lacZ*$^+$ phages could not be adequately suppressed. In spite of numerous variations in growth conditions, such as the use of different media (rich and poor and with various carbon sources), different growth temperatures and variations in the length of infection periods, small plaques were still formed and complete lysis occurred at phage numbers higher than $10^4$ phages per 9-cm petri dish. The lack of success is most probably due to the fact that the amount of UDP-galactose accumulating in the *E. coli* is insufficient to cause swift cell death, thus enabling progeny virus release in a substantial number of infections.

Table 1: *The selection against λgt10lacZ*$^+$ *phages by the E. coli C lacZ recA galE strain containing pAA119.*

| number of phages added | | number of plaques observed | |
|---|---|---|---|
| | | [P-gal] | |
| *lacZ*$^+$ | *lacZ*$^-$ | 0% | 0.3% |
| 150 | 0 | 146 | 0 |
| 0 | 100 | 97 | 112 |
| 150 | 100 | 260 | 100 |
| $1.5x10^3$ | 100 | lysis | 93 |
| $1.5x10^4$ | 100 | lysis | 111 |
| $1.5x10^5$ | 100 | lysis | 103 |
| $1.5x10^6$ | 100 | lysis | 137 |
| $1.5x10^7$ | 100 | lysis | lysis |

In order to make this *E. coli* strain more sensitive to galactose by over-expressing the *galK* and *galT* genes, a multicopy plasmid containing the *gal* operon with a deletion in *galE* but with intact *galK* and *galT* (pAA119; Dreyfus *et al.*, 1985) was transferred to the *E. coli C lacZ recA galE* host. To evaluate the effect of the addition of this plasmid, the new strain was initially compared with a pBR322-containing control in its ability to suppress plaque formation by *lacZ*$^+$ phages. The bacteria were grown at 37°C to OD = 0.6 (709 nm) in LB

% T-gal (Fluka)) was added. The mixture was poured onto 10 ml agar plates (22, 10 mM $MgSO_4$ and 0.3 % fructose) and incubated overnight at 37°C. As shown in Figure 1 a drastic improvement in the suppression of $\lambda gt10lacZ^+$ phages was observed in the presence of pAA119 compared to the pBR322 containing control strain. The use of media such as LB, NZYM and LB:M9 (3:1) (Sambrook *et al.*, 1989) was not found to influence phage suppression. The next step in testing the effectiveness of the new strain in suppressing $lacZ^+$ phages was a reconstruction experiment with a fixed number of $lacZ^-$ phages and increasing numbers of $lacZ^+$ phages. The results presented in Table 1 demonstrate that up to $1.5 \times 10^6$ $\lambda lacZ^+$ phages can be suppressed completely on a 9-cm dish without affecting plaque formation by $lacZ^-$phages.

In summary, it appears that the use of the *E. coli C lacZ recA galE* host strain with increased *galK* and *galT* expression, due to the presence of a *galK galT* multicopy plasmid, enables the rapid and convenient selection of $\lambda gt10lacZ^-$ phages derived from 40.6 transgenic mice. As a result, compared to the earlier X-gal method, the time and costs needed to determine *lacZ* mutation frequencies have been drastically reduced.

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# *Chapter 3*

## Comparison of the X-gal- and P-gal-based systems for screening of mutant *λlacZ* phages originating from the transgenic mouse strain 40.6

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**Abstract**

The recent introduction of the phenyl-β-D-galactopyranoside (P-gal) based positive selection system for screening of *λlacZ* phages originating from the *λlacZ* transgenic mouse (Muta™Mouse) has made the determination of mutant frequencies (MF) a much simpler task. Previously, MF data from these mice have been collected by means of the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) colour screening procedure. To determine whether data obtained with the two systems are comparable, the MF in λ phages recovered from liver and brain of transgenic mice treated with *N*-ethyl-*N*-nitrosourea (ENU) and liver of benzo(a)pyrene (B(a)P) treated mice was determined with both procedures. For the livers of mice treated with ENU, both methods yielded approximately the same MF values. No induction of mutants, relative to the control animals, was seen after 1.5 h, but a clear 4-fold increase was measured with both assays at the 14 d time point. No induction of mutants was found in the brain with either method. In the B(a)P-treated mice, both methods showed a substantial induction in MF after 21, 28 and 35 d. The values generated by the X-gal and P-gal methods were not significantly different, with the exception of the 35 d post-treatment point that appeared higher in the X-gal assay. When the mutants isolated by use of the X-gal method were tested in the P-gal assay, a number of these did not turn up as mutants, and the significance disappeared. In conclusion, the data obtained with the two screening procedures agree to such an extent as to permit a direct comparison between the earlier results generated with X-gal and P-gal values generated with the new positive selection method. This is likely to apply also to other organs and mutagens than those studied here.

The transgenic mouse strain 40.6 (Muta™Mouse) (Gossen *et al.*, 1989; Myhr, 1991) is an animal model that can be used for short-term *in vivo* tests to determine the mutagenic potency of genotoxic compounds. Integrated into the genome of these animals are 80 copies of the λgt10*lac*Z shuttle vector in the form of two 40-mer concatemers. The *Escherichia coli lac*Z gene present in each of the prophages serves as a target sequence in which mutations are scored. A mutation in this sequence may lead to partial or complete inactivation of the *lac*Z gene product, β-galactosidase. To determine the mutant frequency (MF), the individual shuttle vectors are isolated from the chromosomal DNA through the use of commercially available *in vitro*-packaging extracts. The viable phage particles recovered are then tested on a suitable *E. coli* strain for the absence of *lac*Z expression. The MF is finally expressed as the ratio of λ*lac*Z⁻ phages *versus* the total number of phages analysed. Owing to the presence of the transgenes in all cells in an identical genomic position on chromosome 3 (Swiger *et al.*, 1994), strain 40.6 mice are highly suitable for comparative *in vivo* mutagenicity tests on different organs or tissues in one and the same animal.

The classic screening method for λ*lac*Z phages is based on the ability of β-galactosidase to metabolise X-gal to yield a blue-coloured product. In the presence of this substrate, phages with an active *lac*Z gene form β-galactosidase after infecting an *E. coli C lac*⁻ host, which results in a blue plaque. A white plaque is formed upon infection with a λ*lac*Z⁻ phage. The major drawback of this method is the difficulty in visually discerning a few white mutant plaques amongst thousands of blue ones. Recently, in our laboratory, a new method that overcomes this problem has been set up for the selection for λ*lac*Z⁻ phages originating from the transgenic mouse strain 40.6 (Mientjes *et al.*, 1994). In this positive selection system the *E. coli C lac*⁻ *recA galE* host harbours a plasmid expressing galactose kinase and transferase (pAA119; Dreyfus *et al.*, 1985). An infecting λ*lac*Z⁺ phage generates active β-galactosidase which breaks down phenyl-β-D-galactopyranoside (P-gal), present in the medium. Galactose, released as a result of the enzymatic action, is converted into UDP-galactose through the actions of the galactose kinase and transferase enzymes. The absence of galactose epimerase (*gal*E) will cause the accumulation of UDP-galactose and result in cell death. As a consequence, plaques cannot be formed. Only plaques derived from λ*lac*Z⁻ phages, which do not follow this pathway, will be visible.

Although Dean and Myhr (1994) have published comparative studies with the X-gal and P-gal assays, the data they present is rather limited and a critical evaluation of the results is not possible. In order to compare the two screening methods we determined λ*lac*Z⁻ MF in DNA from liver and brain of 40.6 mice treated with *N*-ethyl-*N*-nitrosourea (ENU) and in the liver of mice treated with benzo(a)pyrene (B(a)P), with the X-gal screening procedure and the P-gal positive selection system.

## Materials and methods

*Animal Treatment*

*ENU*: Female transgenic mice (strain 40.6) were obtained at 14 weeks (±2 weeks) of age from the TNO Centre for Animal Research (The Netherlands). The mice were injected intraperitoneally (i.p.) with 150 mg ENU/kg body weight (CAS: 759-73-9; Sigma, St Louis, MO, USA) dissolved in DMSO (Merck, Darmstadt, Germany), while the control animals received DMSO only (2 $\mu$l/g bw). At various time points after treatment animals were sacrificed by $CO_2$ asphyxiation after which the organs were collected, frozen on Dry Ice and stored at -20°C.

*B(a)P*: Male mice were treated i.p. with 100 mg B(a)P/kg bw (CAS: 50-32-8; Sigma) dissolved in olive oil. The control mouse was injected with olive oil (20 $\mu$l/g).

*DNA isolation*

High molecular-weight DNA was isolated from liver and brain in accordance with the protocol described by Roggeband *et al.,* (1993), except for the overnight incubation of the isolated chromatin, which occurred with 50 $\mu$g proteinase K (Merck)/ml instead of to 100 $\mu$g/ml. During brain DNA isolation, the tissue was directly homogenized in the sucrose/EDTA/Triton X-100 buffer. The remaining steps are the same as those for liver DNA.

*In vitro packaging and mutant lacZ phage screening*

A 5 $\mu$l (1.5-2 mg/ml) aliquot of each of the viscous DNA solutions was taken and the $\lambda$ prophages were 'rescued' by use of *in vitro* packaging extracts as prescribed by the manufacturer (Giga-Pack Gold II, Stratagene, La Jolla, CA, USA). The resulting phages were screened for $\beta$-galactosidase expression, as follows:

*X-gal:* A culture of *E. coli C* $\Delta$*lac* (Gossen *et al.*, 1989) was allowed to grow at 37°C in NZY medium (Sambrook *et al.,* 1989), supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$, to $OD_{709}$= 0.6. A 700 $\mu$l aliquot of the culture was infected with approximately $10^4$ phages at 37°C without shaking. After an infection period of 16 min, 35 ml prewarmed top agar consisting of 0.8% (w/v) agar in NZY medium with 0.2% maltose, 10 mM $MgSO_4$ and 125 mg X-gal (New Jersey Lab, Livingston, NJ, USA) was added. The solution was mixed briefly and poured onto a 22 cm x 22 cm plate containing 35 ml bottom agar (NZY medium with 1.6% agar, 0.2% maltose and 10 mM $MgSO_4$). After an overnight incubation at 37°C the plates were screened for white mutant plaques. The mutant plaques were sampled with a sterile Pasteur pipette and transferred to 500 $\mu$l SM-buffer (Sambrook *et al.*, 1989). To estimate the total number of phages tested, plaques were counted within 5 randomly chosen squares (2 cm x 2 cm). All mutant plaques collected were retested on a lawn of *E. coli C lac*$^-$ on X-gal-containing plates to verify that they were indeed mutants.

*P-gal*: *E. coli C* $\Delta$*lac rec*A *gal*E (Mientjes *et al.*, 1994) harbouring pAA119 (Dreyfus *et al.*, 1985) was grown to $OD_{709}$ =0.6 in LB medium (Gibco BRL, Gaithersburg, Maryland, USA) containing 0.2% maltose, 10 mM $MgSO_4$ and 60 $\mu$g/ml ampicillin. Phages were added to 700 $\mu$l cell suspension and incubated for 16 min at 37°C, after which 5 ml top-agar (containing LB, 10 mM $MgSO_4$, 60 $\mu$g/ml ampicillin, 0.3% fructose, 0.3% P-gal (Fluka, Buchs, Switzerland)) was added. The mixture was poured onto 10 cm petri dishes containing 10 ml bottom agar (LB, 10 mM $MgSO_4$, 0.3% fructose and 1.6% agar) and incubated overnight at 37°C. The resulting mutant plaques were picked out and tested on X-gal plates to confirm their mutant status. The number of phages screened was estimated on dilution plates containing X-gal.

*Statistical analysis*

Probability calculations were performed with the Student's t-test (ENU) and the Fisher's 2x2 exact

Two groups of transgenic mice were injected with ENU (150 mg/kg) and sacrificed 1.5 h and 14 d later respectively (at least 2 animals per time point). Liver and brain DNA were subsequently isolated and the λ-prophages packaged *in vitro*. The mutant frequency in the *lac*Z transgene was determined by screening the λ*lac*Z phages with respect to their ability to produce active β-galactosidase in infected *E. coli* bacteria. With one group the X-gal method was applied and with the other the P-gal procedure. In both groups control mice were run through the same procedure.

In the liver (table 1), the lowest MF was obtained in the control animals with both the X-gal and P-gal method. A slight, but insignificant increase relative to the control animals was observed 1.5 h after treatment; at 14 d, however, a large induction was seen (X-gal: $P=0.01$; P-gal: $P<0.01$; Student's t-test). When the results of the two methods are compared, the MF obtained with X-gal was found to be consistently lower than the corresponding MF measured with P-gal, but the difference was not statistically significant.

The MF determined in DNA isolated from the brain of ENU-treated transgenic mice showed approximately the same values for the X-gal screening as for the P-gal assay (table 2). The values obtained were not found to differ significantly between the control and the 1.5 h or 14 d post-treatment animals. So, no increase in MF was evident in the brain, not even after 14 d.

In a separate experiment, four groups of B(a)P-treated transgenic mice, one of which was followed for 35 d, were analysed (table 3). The liver DNA isolated from these mice was divided into two portions, for the X-gal and P-gal studies respectively. The MF values for the control and the 1 d, 21 d and 28 d post-treatment mice were not found to differ significantly between the X-gal and P-gal assays, although the treated animals showed higher X-gal values. A significantly higher X-gal MF relative to P-gal MF was observed for the 35 d time point ($P<0.01$; Fisher's 2x2 exact test). Both assays showed an induction in MF in treated mice at 21 d and later (X-gal $P<0.01$; P-gal $P<0.01$).

In view of possible differences in selectivity between the two screening methods, the mutants isolated with the X-gal procedure were re-tested to see whether they would also have been found with the P-gal selection system. In the case of the ENU-treated animals, 3 of the 52 mutants from the 14 d mice did not form plaques on plates containing P-gal. When the X-gal mutants that do not form plaques on P-gal are excluded, the 14 d MF still does not differ significantly between X-gal and P-gal. In the case of the B(a)P-treated animals, 2, 1 and 8 mutants (13%, 6% and 28% respectively) from respectively the 21 d, 28 d and 35 d post-treatment animals, could not be confirmed with P-gal. When mutants that were not scored as mutants on P-gal were omitted, the corresponding X-gal and P-gal values were all found not to differ significantly.

Table 1: *Comparison of the X-gal and P-gal methods for ENU-induced mutagenesis in the liver.*

| Treatment | Time | No. phages | No. mut. | | MF x$10^{-6}$ | | Avg. ±SD[a] |
|---|---|---|---|---|---|---|---|
| **Method: X-gal** | | | | | | | |
| DMSO | 1.5 h | 52045 | 5 | | 96 | | 46 ±31 |
| DMSO | 1.5 h | 48249 | 2 | | 42 | | |
| DMSO | 1.5 h | 43459 | 1 | | 23 | | |
| DMSO | 1.5 h | 51780 | 0 | | <19 | | |
| DMSO | 1.5 h | 103532 | 5 | | 48 | | |
| ENU | 1.5 h | 101636 | 3 | | 30 | | 68 ±54 |
| ENU | 1.5 h | 84826 | 9 | | 106 | | |
| ENU | 14 d | 102123 | 11 | (9)[b] | 108 | (88)[c] | 171 ±77 |
| ENU | 14 d | 84419 | 23 | (22) | 272 | (261) | (163 ±78)[d] |
| ENU | 14 d | 52813 | 6 | | 114 | | |
| ENU | 14 d | 63439 | 12 | | 189 | | |
| **Method: P-gal** | | | | | | | |
| DMSO | 1.5 h | 145000 | 7 | | 48 | | 55 ± 24 |
| DMSO | 1.5 h | 66336 | 7 | | 106 | | |
| DMSO | 1.5 h | 311049 | 14 | | 45 | | |
| DMSO | 1.5 h | 233159 | 15 | | 64 | | |
| DMSO | 28 d | 661770 | 39 | | 59 | | |
| DMSO | 28 d | 970425 | 42 | | 43 | | |
| PS | 28 d | 349350 | 18 | | 52 | | |
| PS | 28 d | 355300 | 9 | | 25 | | |
| ENU | 1.5 h | 534750 | 44 | | 82 | | 80 ± 4 |
| ENU | 1.5 h | 626715 | 48 | | 77 | | |
| ENU | 14 d | 145520 | 43 | | 296 | | 220 ± 49 |
| ENU | 14 d | 98480 | 25 | | 254 | | |
| ENU | 14 d | 242991 | 50 | | 206 | | |
| ENU | 14 d | 231021 | 52 | | 225 | | |
| ENU | 14 d | 242478 | 43 | | 177 | | |
| ENU | 14 d | 399285 | 65 | | 163 | | |

*a: mean ± standard deviation; b: mutants that could also be detected with the P-gal method (see text); c: MF determined with mutants also detected with P-gal; d: average MF excluding mutants not detected with P-gal*

*the brain.*

| Treatment | Time | No. phages | No. mut. | MF x$10^{-6}$ | Avg. ±SD[a] |
|---|---|---|---|---|---|
| **Method: X-gal** | | | | | |
| DMSO | 1.5 h | 69455 | 6 | 87 | 44 ±37 |
| DMSO | 1.5 h | 38214 | 0 | <26 | |
| DMSO | 1.5 h | 49556 | 0 | <20 | |
| ENU | 1.5 h | 72847 | 1 | 14 | 29 ±21 |
| ENU | 1.5 h | 93437 | 3 | 43 | |
| ENU | 14 d | 88705 | 4 | 45 | 58 ±28 |
| ENU | 14 d | 87925 | 8 | 91 | |
| ENU | 14 d | 25388 | 1 | 39 | |
| **Method: P-gal** | | | | | |
| DMSO | 1.5 h | 137313 | 5 | 36 | 48 ± 27 |
| DMSO | 1.5 h | 85671 | 6 | 70 | |
| DMSO | 1.5 h | 103253 | 9 | 87 | |
| DMSO | 1.5 h | 163718 | 8 | 49 | |
| DMSO | 28 d | 919770 | 20 | 22 | |
| DMSO | 28 d | 278070 | 6 | 22 | |
| ENU | 1.5 h | 64142 | 1 | 16 | 29 ± 12 |
| ENU | 1.5 h | 109749 | 5 | 46 | |
| ENU | 1.5 h | 386880 | 11 | 28 | |
| ENU | 1.5 h | 667740 | 18 | 27 | |
| ENU | 14 d | 52041 | 6 | 115 | 48 ± 37 |
| ENU | 14 d | 46087 | 1 | 22 | |
| ENU | 14 d | 156612 | 4 | 26 | |
| ENU | 14 d | 243660 | 10 | 41 | |
| ENU | 14 d | 137919 | 9 | 65 | |
| ENU | 14 d | 148521 | 3 | 20 | |

*a: mean ± standard deviation*

Table 3: *Comparison of the X-gal and P-gal methods for B(a)P-induced mutagenesis in the liver.*

| Mouse No. | Treatment | Time | No. phages | No. mut. | | MF x$10^{-6}$ | |
|---|---|---|---|---|---|---|---|
| **Method: X-gal** | | | | | | | |
| 1 | Olive oil | | 43063 | 3 | | 70 | |
| 2 | BP | 1 d | 79898 | 8 | | 100 | |
| 3 | BP | 21 d | 49635 | 16 | (14)[a] | 320 | (280)[b] |
| 4 | BP | 28 d | 54789 | 17 | (16) | 310 | (292) |
| 5 | BP | 35 d | 47384 | 29 | (21) | 610 | (443) |
| **Method: P-gal** | | | | | | | |
| 1 | Olive oil | | 382442 | 3 | | 86 | |
| 2 | BP | 1 d | 272146 | 14 | | 51 | |
| 3 | BP | 21 d | 81567 | 21 | | 257 | |
| 4 | BP | 28 d | 99522 | 21 | | 211 | |
| 5 | BP | 35 d | 170905 | 55 | | 322 | |

*a: mutants that could also be detected with the P-gal method; b: MF determined with mutants also detected with P-gal.*

## Discussion

Several *in vivo* mutagenesis studies have been carried out with the transgenic mouse strain 40.6, but most data published until now have been obtained with the X-gal-based screening method to discriminate between $\lambda lacZ^+$ and $\lambda lacZ^-$ phages (Morrison and Ashby, 1994). With the introduction of the P-gal selection method, the determination of the MF has become considerably less labour intensive. Because the X-gal screening and P-gal selection methods are different in their mode of action, this may have an effect on the outcome of the experiments. For this reason we compared data on mutant induction with both methods. The results presented here for the liver and brain of ENU-treated and for the liver of B(a)P-treated mice, suggest that data generated with the X-gal and P-gal systems are comparable.

The X-gal and P-gal assays both yielded approximately the same MF values in the liver DNA of ENU-treated mice. The X-gal values were consistently lower than the P-gal values but the difference was not statistically significant. Both systems showed similar levels of mutant induction after 14 d. The reason for the higher P-gal values may be that a number of mutant plaques will go undetected on the X-gal plates because of the difficulty in their detection. An increase in the number of phages tested per plate has been shown to reduce the efficiency of mutant plaque detection with the X-gal system (Gossen, 1993). This is

Because no plaques are missed here, a higher MF value is expected relative to the MF measured with X-gal. A similar effect has previously been found for the MF in bone-marrow of ENU-treated *lac*Z transgenic mice where the X-gal value was lower than the corresponding P-gal value (Dean and Myhr, 1994). In the brain, no differences in absolute MF values were evident when using either one of the screening methods. The absence of mutant induction in the brain may be ascribed to the low level of DNA synthesis, relative to the liver. As a result, DNA adducts induced by the genotoxic treatment are not fixed into mutations (Data not shown. Manuscript in preparation)

In the livers of mice treated with B(a)P, both the X-gal and P-gal values indicate a clear induction of mutants at 21 d after treatment and later. The MF determined with X-gal in the treated animals were higher than those observed with P-gal, albeit not significantly so, with the exception of the 35 d animal. Such an effect has also been seen in the lungs of B(a)P-treated mice, where the MF determined with X-gal was higher than the corresponding P-gal value (Dean and Myhr, 1994). The reason why B(a)P, in contrast to ENU, induces a higher MF with X-gal than with P-gal is not entirely clear. One possibility may be that the two agents induce different types of mutations. The specificity of $\beta$-galactosidase is likely to be different for the two substrates X-gal and P-gal and, therefore, different types of *lac*Z mutations induced by the two agents can have a differential effect on the $\beta$-galactosidase protein.

The mutant status of the phages isolated with the P-gal assay is routinely checked on X-gal- containing plates to determine $\beta$-galactosidase activity relative to a wild type *lac*Z control. For suppression of phage propagation to occur in the P-gal assay, a threshold level of $\beta$-galactosidase must be present to produce enough UDP-galactose to result in cell death. Some mutant plaques detected with the X-gal method are blue in colour but less intense than the wild type control, because they have a relatively high intracellular $\beta$-galactosidase level compared to white mutants. They may, as a result of this, not appear in the presence of P-gal. This was checked by testing mutants originally obtained with the X-gal procedure with respect to their ability to form plaques in the P-gal assay. In the ENU experiment, 6% of the X-gal mutants found at 14 d after treatment, did not score as mutants in the P-gal assay. In the case of B(a)P- treated mice, 13%, 6% and 28% of the mutants scored on X-gal for respectively the 21 d, 28 d and 35 d animals were found not to produce plaques on P-gal-containing plates. When the mutants that scored negative in the P-gal assay were omitted from the X-gal data, the difference between the X-gal and P-gal data for the 35 d mouse was no longer statistically significant.

In summary, it can be concluded that the X-gal colour screening method and the P-gal positive selection procedure for the detection of $\lambda$ phages with a mutated *lac*Z gene are not completely equivalent, because of the risk of overlooking mutants with X-gal screening and the chance of missing in the P-gal method those mutants that have some residual $\beta$-galactosidase activity and are picked up with X-gal. Nevertheless, these differences do not

appear to have a substantial influence on the outcome of the MF determinations. The differences in MF values with both assays become insignificant when considered in relation to the accuracy with which the MF can be established in practice in mutagenicity studies with strain 40.6 mice. Consequently, although this work is limited to the analysis of only a small number of tissues, the similarity in X-gal and P-gal values gives confidence that a direct comparison of earlier X-gal results with newly obtained P-gal data is justified for other mutagens and organs.

**Acknowledgements**

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# *Chapter 4*

## Formation and Persistence of $O^6$-Ethylguanine in Genomic and Transgene DNA in Liver and Brain of λlacZ Transgenic Mice Treated with *N*-ethyl-*N*-nitrosourea

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*Submitted to Carcinogenesis*

**Abstract**

*λlacZ* transgenic mice are suitable for short-term mutagenicity studies *in vivo*. Mutagenicity in these mice is determined in the *lacZ* transgene. Since the *lacZ* gene is of bacterial origin the question has been raised whether DNA-adduct formation and repair in the transgene are comparable to those in total genomic DNA. Mice were treated with *N*-ethyl-*N*-nitrosourea (ENU) and sacrificed at several time points following treatment. Some mice were pretreated with $O^6$-benzylguanine to inactivate the repair protein $O^6$-alkylguanine-DNA alkyltransferase (AGT). $O^6$-ethylguanine ($O^6$-EtG) was determined in *lacZ* in liver and brain by means of a monoclonal antibody-based immunoaffinity assay. In addition, $O^6$-EtG and N7-ethylguanine (N7-EtG) were assayed in total genomic DNA of liver and brain with an immunoslotblot procedure. In liver, the initial $O^6$-EtG level in total genomic DNA was 1.6 times that in *lacZ*. The extent of repair of $O^6$-EtG during the first 1.5 h after treatment was 2.1 times that in *lacZ*. At later time points, $O^6$-EtG repair was the same. N7-EtG repair in genomic DNA was evident. In contrast to the liver, little repair of $O^6$-EtG in total genomic and lacZ DNA occurred in the brain while N7-EtG was repaired. No initial difference in $O^6$-EtG levels were found in *lacZ* and genomic brain DNA. These findings indicate that in the liver, total genomic DNA is more accessible than *lacZ* to ENU and/or the AGT protein, during the first 1.5 h following treatment. Because the difference in $O^6$-EtG levels in the transgene and genomic DNA in the liver is restricted to the first 1.5 h after treatment, while the fixation of mutations occurs at later time points, $O^6$-EtG-induced mutagenesis most likely is also very similar in both types of DNA.

Mammalian short-term *in vivo* mutagenesis studies have become possible with the development of transgenic animals carrying multiple copies of a reporter gene (Gossen *et al.* 1989; Myhr 1991; Kohler *et al.*, 1991). The transgenic mouse strain 40.6 (Muta™Mouse)harbours 80 copies of the *λgt*10*lac*Z shuttle vector in each somatic cell in the form of two 40-mer concatemers. The *Escherichia coli lac*Z gene located within each *λ* prophage codes for *β*-galactosidase and serves as the indicator sequence for mutations.

Whether mutations induced in the transgene DNA reflect those in total genomic DNA is a critical question for assessing the value of transgenic marker mice in mutagenesis studies. The accessibility of foreign DNA sequences for genotoxic agents and repair proteins may be different from that of the rest of the genomic DNA, due to differences in conformation and transcriptional status. This contention is confirmed by the observation that increased levels of alkylation and enhanced repair of certain adducts have been found in the relatively less compact, transcriptionally active chromatin compared to the more condensed, inactive chromatin, both *in vitro* and *in vivo* (Nehls and Rajewsky, 1985; Ryan *et al.* 1986; Boffa *et al.*, 1992). At the gene level, repair of DNA damage induced by agents such as UV has been extensively studied *in vitro*. Bohr *et al.* (1985) first observed the preferential repair of UV-induced pyrimidine-dimers in transcriptionally active DNA *versus* inactive DNA. The same was shown to occur *in vivo* in the transcriptionally active *hprt* and *ada* genes as opposed to the inactive *c-mos* and *Hp* sequences of mouse epidermal cells (Ruven *et al.*, 1993). This phenomenon is not limited to UV-damage, as illustrated by the preferential repair of cisplatin adducts in the actively transcribed *dhfr* gene compared to the non-transcribed *c-fos* gene (Jones *et al.*, 1991). Similar observations were made for the mutagenic DNA-alkylation product $O^6$-ethylguanine ($O^6$-EtG) in the actively transcribed *β*-actin gene in comparison to the inactive IgE gene and to total genomic DNA of ENU-treated rat hepatoma cells (Thomale *et al.*, 1994). Not all types of adducts, however, are subject to preferential repair in transcriptionally active genes. The DNA alkylation products N7-methylguanine and N3-methylguanine are repaired with equal efficiency in both the active *dhfr* domain and the inactive sequences further downstream of the gene (Scicchitano and Hanawalt, 1989; LeDoux *et al.*, 1991).

Given the fact that transgenic DNA sequences present in the *lac*Z transgenic mice are not actively transcribed, it is necessary, to determine possible differences between transgene and genomic DNA with respect to adduct formation and repair. This will make interpretation of mutagenesis data collected with these mice more reliable. Here we address this question with respect to $O^6$-EtG in *lac*Z transgenic mice.

$O^6$-EtG, together with $O^2$- and $O^4$-ethylthymine, are premutagenic lesions formed *in vitro* upon exposure of cells to ethylating agents. These adducts are thought to exert their mutagenic effect by mispairing during DNA replication (Ellison *et al.*, 1989; Klein *et al.*, 1990; Grevatt *et al.*, 1992). Repair of $O^6$-alkylguanine in DNA is accomplished largely by the

$O^6$-alkylguanine-DNA alkyltransferase (AGT) protein. The alkyl group is transferred from the $O^6$-atom of guanine to a cysteine residue in the active site of the AGT protein. This leads to the restitution of the original guanine base and inactivation of the AGT protein (Pegg, 1983). AGT is also capable of interacting with the base analogue $O^6$-benzylguanine which also leads to the inactivation of the protein. Pretreatment of cells with $O^6$-benzylguanine ($O^6$-BzG) results in the depletion of the pool of cellular AGT (Dolan *et al.*, 1990). This allows the study of the initial repair of $O^6$-EtG in DNA in the absence of repair during the alkylation period.

To determine whether a difference in $O^6$-EtG formation and repair exists between transgene DNA and total genomic DNA, we applied a recently developed method (Hochleitner *et al.*, 1991), using genomic DNA digested with *EcoRI* and mixed with a known amount of internal standard DNA (linearized ethylated pSV2*gpt* plasmid). $O^6$-EtG containing DNA fragments are isolated by use of anti-$O^6$-EtG monoclonal antibodies (Mab), followed by the quantification of the *lac*Z and *gpt* DNA by quantitative PCR. $O^6$-EtG levels at *lac*Z loci were determined in liver and brain DNA of transgenic mice at various time points after treatment with ENU, and in mice pretreated with $O^6$-BzG followed by exposure to ENU. In addition, the $O^6$-EtG and N7-ethylguanine (N7-EtG) contents in total genomic DNA were determined with immunoslotblot (ISB)(Nehls *et al.*, 1984). Liver and brain DNA was analysed because these tissues exhibit large differences with respect to their rates of DNA synthesis (Craddock *et al.*, 1984) and $O^6$-EtG repair (den Engelse *et al.*, 1987).

## Materials and Methods

*Transgenic mice and treatment*

Female transgenic mice (strain 40.6) were obtained at 14 weeks (±2 weeks) of age from the TNO Centre for Animal Research (The Netherlands). The mice were injected intraperitoneally (i.p.) with ENU (Sigma, St. Louis, MO, USA), 150 mg/kg body weight (b.w.) dissolved in DMSO (Merck, Darmstadt, Germany), while the control animals received DMSO (2 ml/kg b.w.). In the ENU-treated group, a total of 4 animals were sacrificed after 1.5 h and two animals each after 24 h, 72 h and 14 d. The control animals were sacrificed after 1.5 h and 14 d. Two hours prior to ENU treatment, 4 mice were injected i.p. with 10 mg/kg b.w. $O^6$-BzG dissolved in DMSO. At various time points after treatment animals were sacrificed by $CO_2$ asphyxiation after which the organs were collected, placed on dry ice and stored at -20°C.

*DNA isolation*

High molecular weight DNA was isolated from liver and brain in accordance with a protocol described by Roggeband *et al.* (1993), except for the incubation of the isolated chromatin, which was for 16 h at 20°C in the presence of proteinase K (50 μg/ml; Merck). In the case of DNA isolation from the brain, the tissue was directly homogenized in the sucrose/EDTA/Tween-20 buffer. The remaining steps were the same as those for liver DNA. The DNA was finally dissolved up in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). After incubation with RNAse A (0.1 mg/ml; Sigma) and T1 (100 U/ml; Boehringer Mannheim, Mannheim,

procedure (Nehls *et al.*, 1984). For the assay of N7-EtG, DNA was pretreated as follows: DNA was diluted in potassium phosphate buffer (10 mM; pH 7) to a concentration of 50 $\mu$g/ml and sonicated for 5 sec (Ultrasonics W-370, USA, with microtip; output level 2.5). To 200 $\mu$l DNA, 4.7 $\mu$l 3 M NaOH was added and the solution was incubated at 37°C for 30 min to convert N7-EtG bases into their ring-opened form. The reaction was stopped by the sequential addition of 6 $\mu$l 1 M $K_2HPO_4$ and 15 $\mu$l 1 M HCl. The pH of the mixture was checked to be 7.4. To this mixture 275 $\mu$l phosphate-buffered saline (PBS) was added, and the solution was heated to 100°C for 10 min to denature the DNA and placed on ice for 5 min. After addition of 500 $\mu$l 2 M ammonium acetate the DNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm. For determination of $O^6$-EtG, DNA was sonicated, heated to 100°C for 10 min and placed on ice for 5 min. After addition of an equal volume of 2 M ammonium acetate the DNA concentration was determined. For both the $O^6$- and the N7-EtG assay, 1 $\mu$g genomic DNA isolated from the liver or brain from each animal, along with calibration standards of *in vitro* ethylated DNA of known $O^6$- and N7-EtG content, were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using the Manifold II blotting device (Schleicher and Schuell). All slots were washed with 200 $\mu$l 1 M ammonium acetate and the membranes were baked for 2 h at 80°C. Preincubation of the nitrocellulose blots was carried out for 1 h, under gentle agitation, in 16 ml PBS containing 0.5% (w/v) skimmed milk and 0.1% (v/v) Tween-20, at room temperature. The blots were washed twice in PBS/0.1% Tween-20 and then incubated with Mab ER-6 (Rajewsky *et al.*, 1980) specific for $O^6$-EtG (60-fold diluted culture supernatant) or Mab N7E-026 (van Delft *et al.*, 1991) specific for ring-opened N7-EtG (25,000-fold dilution of 100 $\mu$g/ml protein A-purified antibody) in 16 ml PBS/0.1% Tween-20/0.5% skimmed milk, for 2 h at room temperature. The blots were washed 3x 5 min in PBS/0.1% Tween-20 followed by incubation with the peroxidase-labelled second antibody (ER-6: rabbit anti-rat; N7E-026: rabbit anti-mouse; Dakopatts a/s, Denmark) in PBS/0.1% Tween-20/0.5% skimmed milk for 1 h at room temperature. The blots were washed again 3x5 min in PBS/0.1% Tween-20, and treated with the ECL western blotting reagents (Amersham, Buckinghamshire, England). Chemiluminescence was detected with sensitive film (Hyperfilm-ECL, Amersham) and evaluated by densitometry (Ultroscan XL, LKB, Brommo, Sweden).

*$O^6$-EtG content in the lacZ transgene*

The procedure for the gene-specific measurements has been described previously (Hochleitner *et al.*, 1991); it consists of the following steps:

*Enrichment of $O^6$-EtG-containing DNA fragments*

Isolated genomic DNA was digested with EcoRI to cut out the individual lacZ sequences from the concatemers of $\lambda$gt10*lac*Z shuttle vectors. Completeness of digestion was checked by separating aliquots of the digests on a 1% agarose gel. One $\mu$g aliquots of digested DNA were placed in separate siliconized 1.5 ml Eppendorf tubes and 1 pg pSV2*gpt* plasmid containing an average of 1 $O^6$-EtG per plasmid molecule was added to each tube. The plasmid served as an internal calibration standard during the entire procedure.

After drying the samples *in vacuo*, the DNA was dissolved in 70 $\mu$l STE (50 mM Tris pH 7.5, 100 mM NaCl and 1 mM EDTA). To each sample, 30 $\mu$g of Mab ER-6 (1 $\mu$g/$\mu$l) was added and the mixture incubated for 45 min at room temperature. Antibody-DNA complexes were separated from non-complexed DNA by passage through a nitrocellulose filter (Protran BA 85, Schleicher and Schuell). Membrane-bound DNA was released and eluted by washing the filters three times with 300 $\mu$l 5% (v/v) n-butanol in 5 mM Tris pH 7.5/0.1 mM EDTA. The eluent was dried under vacuum. The dried DNA and constituents were redissolved in 100 $\mu$l 0.1 M NaCl containing 30 $\mu$g Mab ER-6 and 1 $\mu$g of DNase-free BSA.

The procedure of antibody-binding and elution was performed three times in total. For the third elution, the butanol/Tris/EDTA-buffer was replaced with 5% (v/v) n-butanol in 5 mM KCl. Standards for the quantification of *lac*Z DNA were prepared by adding various amounts of EcoRI-digested genomic DNA to a constant amount of pSV2*gpt* plasmid. All standards and enriched experimental samples were purified by passage over an octylsepharose (Phamacia) column. The collected eluent was dried and subsequently subjected to quantitative PCR analysis.

*lacZ and gpt amplification by PCR*

Co-amplification of short sequences within the *lac*Z (127 bp) and *gpt* (148 bp) DNA, enriched as described above, was carried out in a PCR reaction (Saiki *et al.*, 1985). The PCR conditions were as follows: 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM of each of the dNTP's, 0.11 MBq $\alpha$-$^{32}$PdCTP, 0.4 $\mu$M of each oligonucleotide (see below) and 20 U/ml Taq DNA polymerase (Promega, Madison, WI, USA). The PCR profile consisted of 30 sec denaturation at 94°C; 1 min primer hybridization at 65°C and primer extension for 3 min at 72°C. This program was run for 20 cycles in a Thermal Cycler (Perkin-Elmer/Cetus). The *lac*Z and *gpt* oligonucleotides used in the PCR reactions were prepared by means of an automated DNA synthesiser (Applied Biosystems). The sequence of the oligonucleotides was as follows: *lac*Z P1 5' dTCCGCCGTTTGTTCCCACGGAGAAT; *lac*Z P2 5' dACAGATGAAACGCCGAGTTAACGCC; *gpt* P1 5'dCGTTACTGGCGCGTGAACTGGGTAT; *gpt* P2 5' dTCACGAATCGCAACCGCAGTACCAC.

*Calculation of $O^6$-EtG content in lacZ*

Fifteen $\mu$l of each PCR reaction mixture was run on a 6% non-denaturing polyacrylamide gel. The separate bands were quantified by $^{32}$P-counting using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA). The *lac*Z/*gpt* signal ratio of each sample, as measured from the gel, was compared to a standard curve, an example of which is shown in Figure 1. Interpolation provides the amount of DNA present in the tube prior to PCR. By dividing the ng equivalent of genomic DNA in the PCR mixture by the DNA content per cell (5 pg) and multiplying this with the number of *lac*Z copies per cell (80), one obtains the number of *lac*Z copies present containing an $O^6$-EtG adduct. It is reasonable to assume that each antibody-complexed *lac*Z sequence contains 1 $O^6$-EtG residue. Therefore, the number of $O^6$-EtG adducts in *lac*Z in the initial 1 $\mu$g of genomic DNA at the start of the experiment, is equivalent to the number of bound *lac*Z sequences. Dividing this value by the number of nucleotides making up the *lac*Z loci (1 $\mu$g/5 pg per cell x 80 copies/cell x 6256 nt per *lac*Z molecule) gives the number of $O^6$-EtG per *lac*Z nt. This is converted into $O^6$-EtG/$10^6$ nt.

For the determination of $O^6$-EtG residues in the transgenic *lac*Z genes, genomic DNA from ENU-treated mice was first digested with EcoRI which cuts out a 3128 bp fragment comprising all but the first 25 bp of the *lac*Z gene. After addition of a fixed amount of an $O^6$-EtG labelled plasmid (pSV2*gpt*, 5200bp, on average 1 $O^6$-EtG per plasmid) the $O^6$-EtG-containing DNA fragments were concentrated via repeated Mab binding and filtration. The ratio of *lac*Z to *gpt* fragments in the enriched samples was then determined via co-amplification of a short portion of either gene by PCR. To calculate the $O^6$-EtG content of the *lac*Z loci in the original genomic DNA sample, a calibration curve was constructed. To this end, various amounts of EcoRI-digested genomic DNA were subjected to PCR together with a constant amount of pSV2*gpt* plasmid (Figure 1a and b). This calibration curve was used to determine the ng equivalent of genomic DNA in the experimental sample, from which the number of $O^6$-EtG-containing *lac*Z genes per cell were calculated (see Materials and Methods for further details).

*PCR analysis*

PCR conditions for co-amplification of *lac*Z and *gpt* were optimized as to $Mg^{2+}$ concentration and annealing temperature. The specificity of the primers was checked by allowing the PCR reaction to reach saturation (40 cycles). No unspecific PCR products were detected when the reaction mixture was analysed by gel electrophoresis (data not shown). A PCR cycle number of 20 was chosen where the two products were still in the exponential phase of amplification.

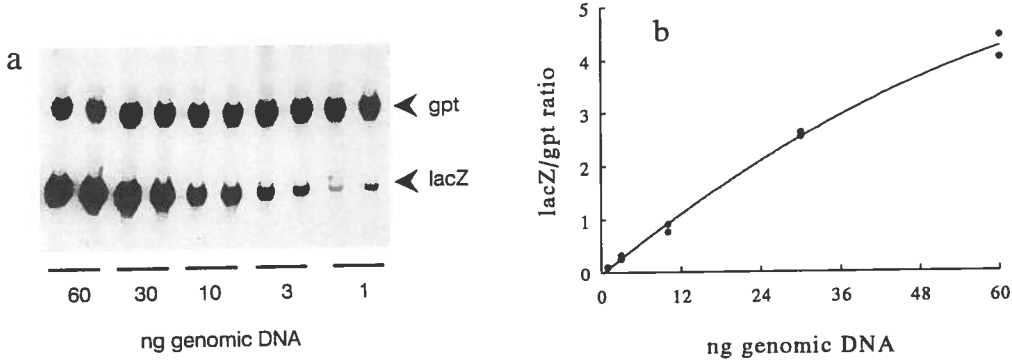


Figure 1: *a) Autoradiogram of a polyacrylamide gel containing PCR samples used in the construction of a calibration curve. b) An example of a calibration curve. Ratios of lacZ/gpt signals of the $^{32}$P-labelled PCR products were plotted against the ng genomic DNA input.*

*O⁶-EtG in genomic and lacZ DNA*

*lac*Z-transgenic mice were treated with ENU (150mg/kg). At various time points after treatment, O⁶-EtG was determined in both total genomic DNA and in *lac*Z isolated from liver and brain. The O⁶-EtG and N7-EtG contents in genomic DNA and O⁶-EtG in *lac*Z DNA were determined in duplicate 2-4 and 4-9 times, respectively, in liver and brain DNA of each animal. The SD in genomic and *lac*Z DNA was on average ± 25%. In total genomic DNA from liver, the average O⁶-EtG content found at 1.5 h post-treatment (Figure 2a) was 6.6 ± 2.8 O⁶-EtG/10⁶ nt in the four mice studied. At 24 h, the two mice exhibited 8.6 and 2.4 O⁶-EtG/10⁶ nt in the liver DNA respectively. O⁶-EtG levels at 3 d approached

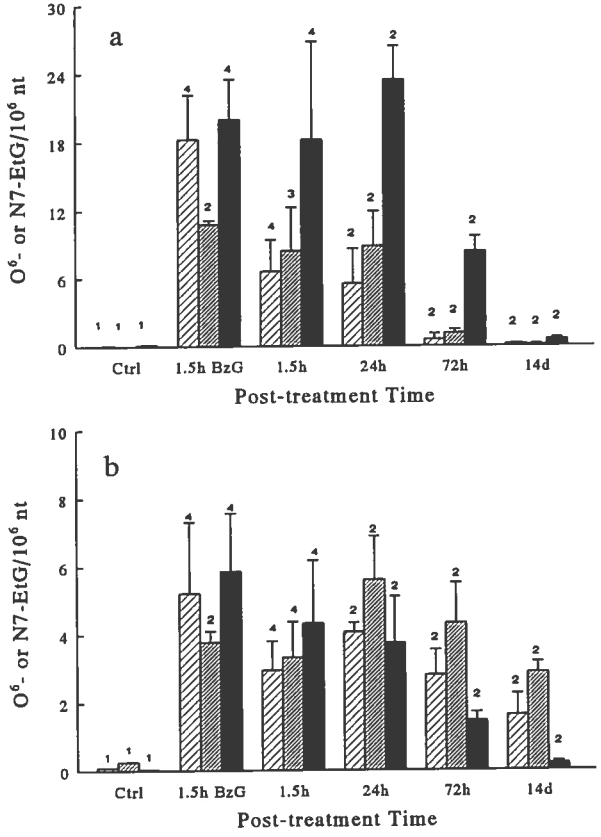


Figure 2: *O⁶-EtG levels in genomic and lacZ sequences and N7-EtG levels in genomic DNA isolated from (a) liver and (b) brain of mice treated with O⁶-BzG/ENU or ENU only. The data are presented as means with standard deviations when more than 2 animals were used and the range when 2 animals were employed. The numbers above the error bars indicate the number*

treatment (0.2 and 0.2 $O^6$-EtG/$10^6$ nt). In the $O^6$-BzG pre-treated mice decreased to
after ENU-treatment, an average $O^6$-EtG content of 18.3 ±3.9/$10^6$ nt was determined, *i.e.* a 2.8-fold increase compared to the mice that received only ENU. At the *lac*Z loci, the level of $O^6$-EtG was slightly higher than in the corresponding genomic DNA at all time points except for the $O^6$-BzG-treated mice (Figure 2a). The level detected at the 1.5 h time point amounted to 8.4 ±3.8 $O^6$-EtG/$10^6$ nt. No reduction was measured after 24 h (12.0 and 5.7 $O^6$-EtG/$10^6$ nt), whereas most $O^6$-EtG had been removed after 3 d (0.7 and 1.4 $O^6$-EtG/$10^6$ nt). The levels observed at 14 d post-treatment approached the background level.

In the total DNA isolated from the brain tissue, the $O^6$-EtG level at 1.5 h after ENU-treatment averaged 2.9 ±0.9/$10^6$nt for the four animals tested (Figure 2). No reduction was noticed after 24 h: values of 4.4 and 3.8 $O^6$-EtG/$10^6$ nt were found in two mice. After 3 and 14 d the average adduct values were 2.8 ±1 and 1.6 ±0.9 $O^6$-EtG/$10^6$ nt, respectively. In contrast to the liver samples, brain DNA from the $O^6$-BzG-pretreated mice showed a smaller increase in genomic $O^6$-EtG levels (5.2 ±2.1 $O^6$-EtG/$10^6$ nt) at the 1.5 h time point compared to non-pretreated mice. The level of $O^6$-EtG in the *lac*Z loci was again slightly higher than that in genomic DNA. There was no difference in $O^6$-EtG content in *lac*Z between animals treated with ENU only (3.3 ±1.1 $O^6$-EtG/$10^6$ nt) and those having received $O^6$-BzG and ENU (3.8 ±0.5 $O^6$-EtG/$10^6$ nt). No significant overall decrease was found to occur during the 14 d post-treatment (1.5 h: 3.3 ±1.1; 24 h: 5.6 ±1.8; 3 d: 4.3 ±1.7 and 14 d: 2.9 ±0.5 $O^6$-EtG/$10^6$ nt).

*N7-EtG content in genomic DNA of liver and brain*

The level of N7-EtG could only be measured in genomic DNA. In the liver, maximum values were obtained at 1.5 h (18.2 ±8.6 N7-G/$10^6$ nt) and 24 h (23.4 ±4.2 N7-EtG/$10^6$) after ENU treatment (Figure 2). More than 50% of the adducts had disappeared after 3 d (8.3 ±2 N7-EtG/$10^6$ nt), whereas nearly all N7-EtG had been removed at 14 d. In the BzG-pretreated mice an average of 20.0 ±3.5 N7-EtG per $10^6$ nt was measured. In brain DNA, N7-EtG levels were 4.3 ±1.8 N7-EtG/$10^6$ nt after 1.5 h and averaged 3.8 ±2 N7-EtG/$10^6$ nt at 24 h after ENU administration (Figure 2). In contrast to $O^6$-EtG, N7-EtG was removed from brain DNA. About half of the adducts had disappeared by day 3 (1.5 ±0.4 N7-EtG/$10^6$ nt) and barely detectable levels were found after 14 d (0.2 ±0.01 N7-EtG/$10^6$ nt).

**Discussion:**

In view of their prokaryotic origin, their repetitiveness and the sheer size of the transcriptionally silent transgene concatemers present in the genome of the transgenic mouse 40.6, it is possible that the configuration of this DNA and its interaction with nuclear proteins is different from that in the rest of the DNA. These factors may have profound effects on the reactivity of an agent towards exogenous *versus* endogenous DNA.

To address this question a recently developed method for the measurement of $O^6$-EtG in single-copy genes (Hochleitner *et al.*, 1991) was employed. The level of $O^6$-EtG was measured in total genomic DNA and in *lac*Z sequences of liver and brain cells isolated from *lac*Z transgenic mice treated with ENU. As a control N7-EtG was measured in the overall genome of both tissues. The results demonstrate that 1.5 h after ENU exposure, substantial levels of $O^6$- and N7-EtG can be measured in liver DNA, which hardly decrease in the subsequent 22.5 h. This indicates a very slow removal of the adducts during this time interval. However, the effect of pretreatment of the mice with $O^6$-BzG, which inactivates most of the suicidal AGT protein present in the cell, suggests a very strong reduction, in the absence of $O^6$-BzG in the $O^6$-EtG level shortly after formation of these lesions, attributable to the cellular AGT pool. Evidently, the AGT pool becomes rapidly exhausted, and further

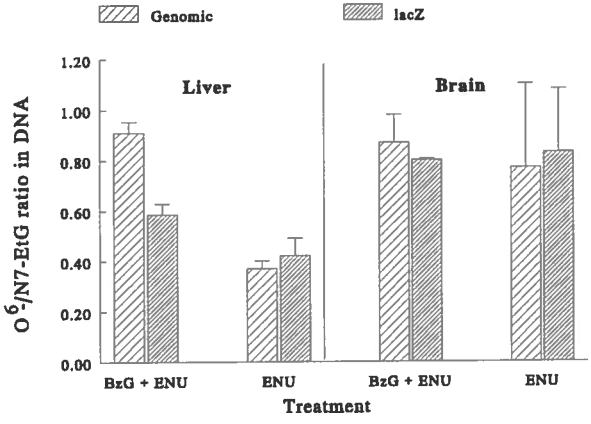


Figure 3: *Comparison of $O^6$-EtG content in genomic and lacZ DNA 1.5 h after treatment with ENU or with $O^6$-BzG/ENU. $O^6$-EtG values have been normalised to the genomic N7-EtG levels to correct for inter-animal variation.*

removal of $O^6$-EtG is then achieved by *de novo* synthesized AGT. As expected, N7-EtG levels were not affected by $O^6$-BzG because this lesion is removed by glycosylases and is not a substrate for AGT. The lack of repair of N7-EtG over the first 24 h opened the possibility to adjust the $O^6$-EtG results with respect to inter-animal variation, which in particular after i.p. dosing can be considerable, by expressing these data relative to the N7-EtG level in the

DNA than in the *lac*Z genes (1.6x; P= 0.001, Student t-test). In non-pretreated mice, 60% ($P < 0.001$) and 28% ($P = 0.06$) lower levels in genomic and *lac*Z DNA are seen, respectively, relative to the pretreated mice, which indicates a 2.1-fold faster repair in genomic *versus lac*Z DNA. These findings indicate that genomic DNA, representing transcriptionally active as well as inactive DNA, is generally more accessible both to ENU and to the AGT repair protein than the inactive and exogenous transgene DNA sequences.This is not entirely in agreement with the result obtained with rat hepatoma cell lines *in vitro* (Thomale *et al.*, 1994). Here, no difference in the initial $O^6$-EtG content and repair was found between total genomic DNA and transcriptionally inactive IgE gene in cells pre-treated with $O^6$-BzG followed by ENU. On the other hand, the data obtained in the present study at the 24 h time point and later no longer indicate a difference between *lac*Z and total genomic DNA with regard to the efficiency of $O^6$-EtG repair.

The biphasic repair profile of $O^6$-EtG in genomic DNA of liver cells, with 60% of the lesions being repaired within the first 1.5 h and 30% between 24 h and 3 d after treatment, has previously been reported in rats for $O^6$-methylguanine (Herron *et al.*, 1981) and $O^6$-EtG (Goth and Rajewsky, 1974b). This phenomenon is likely due to a rapid depletion of the cellular reserve of AGT by the suicidal reaction with alkyl groups at the $O^6$-atom of guanine and the slow recovery of AGT activity over the following days.

In DNA from brain tissue of the same animals little or no effect of $O^6$-BzG and no significant reduction in $O^6$-EtG content was detected over the first 72 h, although there was a clear elimination of N7-EtG (Figure 2b). No differences in either $O^6$-EtG formation or repair were evident from the normalized data (Figure 3). In previous studies carried out on the brain of adult rats, no or very slow repair of $O^6$-EtG ($t_{½}$ 44 d) was found, but a faster repair rate of N7-EtG was measured ($t_{½}$ 2.5-16 d) (den Engelse *et al.*, 1987; Goth and Rajewsky, 1979; Goth and Rajewsky, 1974b).

The level of $O^6$-EtG in genomic DNA of the liver, 1.5 h after treatment, was found to be approximately twice the level detected in the brain. Some difference in $O^6$-EtG contents of various organs such as the liver, brain, testis and bone marrow was also found by others at 2 h after $^3$H-ENU treatment (den Engelse *et al.*, 1987; van Zeeland *et al.*, 1985). This difference, together with the inter-animal variation we found, may be ascribed to the route of administration: the local distribution of ENU in the abdominal cavity after administration of the chemical, may play a role. This effect was in part accounted for in our studies, when $O^6$-EtG values were normalized to the N7-EtG level determined in the total genomic DNA.

The role of cell proliferation should also be considered as the levels of alkylation products in the DNA of replicating cells will be diluted. Cell proliferation in the liver of *lac*I transgenic mice is low with approximately 0.07% of the hepatocytes taking part in the cell-division process (Mirsalis, 1994). Had excessive cell proliferation been induced as a result

of ENU treatment, a reduction in adduct levels would have been expected due to DNA replication. However, we found that in the liver approximately 54% of N7-EtG was lost after 72 h compared to 90% of O⁶-EtG. With the chemical half-life of N7-EtG, *in vitro*, being ≈150-225 h (Goth and Rajewsky, 1974b), about 20% of the N7-EtG initially formed is lost owing to the inherent instability of this alkylation product in DNA. Therefore, 34% (54% - 20%) of the N7-EtG must be removed through repair by DNA glycosylases (Margison and Pegg, 1981) or diluted by cell-proliferation. As O⁶-EtG is much more chemically stable in DNA than N7-EtG, the loss of this lesion (more than 90% within 72 h) should be predominantly attributed to repair, whereas the proliferation effect must have been relatively small.

The results presented here indicate that the initial repair (during the first 1.5 h after ENU-treatment) of O⁶-EtG in the liver is higher in total genomic DNA than in *lac*Z sequences. Thereafter, further repair of the transgene does not appear to be different from that seen in total genomic DNA. In the brain, no repair of O⁶-EtG was detected in either genomic or *lac*Z DNA although brain cells are proficient in the removing N7-EtG and contain AGT (data not shown). These findings are of importance for the use of these mice for mutagenicity testing. Since the *lac*Z sequences present in the shuttle vectors is used as an idicator target in which mutations are scored, it is essential that transgene and genomic DNA are similar with regard to formation and repair of DNA adducts. As observed in the present study, the difference between transgene DNA and genomic DNA appears to be restricted to a somewhat different initial O⁶-EtG level (*i.e.* a lower level of O⁶-EtG in *lac*Z, the effect of which is mitigated by less efficient repair) at a very early stage in the process of mutation induction. As the fixation of mutations is most likely to occur after the 1.5 h time point because only a small fraction of replication competent cells are in S-phase during this time period, mutation analysis in the *lac*Z DNA from the livers of 40.6 mice probably provides data that are significant for the vast majority of the genomic DNA. Further studies may be necessary to determine the repair of mutagenic lesions in the *lac*Z DNA in comparison to transcriptionally active DNA sequences. Studies are presently being carried out to investigate the repair profiles of DNA lesions in the *lac*Z DNA that are not repaired by AGT.

**Acknowledgements**

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# *Chapter 5*

## DNA adducts, mutant frequencies and mutation spectra in various organs of *λlacZ* transgenic mice after exposure to ethylating agents

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*Submitted to Cancer Research*

**Abstract**

Transgenic marker mice offer a suitable *in vivo* system for mechanistic studies on mutagenesis. In these mice, both DNA-adduct and mutation-induction data can be obtained for any organ or tissue, allowing one to investigate tissue-specific relations between DNA adducts and mutagenesis. In the experiments described here, *λlacZ* transgenic mice were i.p. treated with the model mutagens *N*-ethyl-*N*-nitrosourea (ENU), diethylnitrosamine (DEN) and ethyl methanesulphonate (EMS). In the DNA of liver, bone marrow and brain obtained from mice sacrificed at several time points after treatment (from 1.5 h to 28 d), $O^6$-ethylguanine and N7-ethylguanine levels were determined in total genomic DNA in addition to the mutant frequency (MF) in the *lacZ* loci. In the liver of ENU- and DEN-treated mice, the bulk of $O^6$-ethylguanine was removed from the DNA at 3 days after treatment, while the MF increase continued thereafter, with a maximum at 28 days (6-fold). This suggests that $O^6$-ethylguanine is not a major premutagenic lesion in the liver. After EMS treatment no $O^6$-ethylguanine or MF increase were observed in liver DNA whereas high levels of N7-ethylguanine were present. In bone marrow, ENU and EMS were found to be mutagenic. A maximum induction (23-fold) in the ENU-treated mice occurred at 3 days post-treatment when the $O^6$-ethylguanine level had dropped below the detection limit. EMS treatment led to N7-ethylguanine formation and some $O^6$-ethylguanine accompanied by a small MF increase. The findings suggest that $O^6$-ethylguanine may be a major premutagenic lesion at the earlier time points in bone marrow. No adducts or mutation induction were observed in bone marrow of DEN-treated mice. No induction of mutations was observed in the brain of either ENU- or EMS-treated mice although $O^6$- and N7-adducts were present. To determine the origin of the mutations responsible for the highest MF increase in liver and bone marrow of ENU-treated mice, *lacZ* mutant phages originating from these organs were sequenced. The resulting mutation spectra show that the major contributors to the mutations in the liver were GC → AT (19%) transitions and GC → TA (29%) and

ethylguanine which seems of less importance to mutagenesis in liver than in bone marrow.

## Introduction

Model alkylating agents are widely used to study the role of DNA adducts in the complex process of mutagenesis and carcinogenesis. These agents are capable of forming a large number of different types of DNA lesions, some of which are mutagenic and others cytotoxic, inducing a wide variety of biological responses. Alkylating agents react either directly or after metabolic activation with the nucleophilic nitrogen and oxygen atoms in DNA bases and with oxygen in the backbone phosphates. By exposing animals to different alkylating agents with different adduct spectra, comparative analysis of adduct, mutation induction and mutation spectra data can provide information regarding the mutagenicity of individual DNA adducts in different tissues. Presently, such data is limited due to the small number of studies performed.

Mechanistic studies on mutagenesis have often been performed *in vitro* with the use of bacterial and mammalian cells. The *in vivo* and *in vitro* situations, however, differ with regard to such cellular processes as metabolism, DNA repair and proliferation. Recently, mutagenicity studies have been carried out *in vivo* on the *hprt* gene in lymphocytes, bone marrow and skin fibroblasts of rats and mice (Skopek *et al.*, 1992, 1994; Jansen *et al.*, 1994, 1995). The *dlb*-1 gene is another endogenous locus available for mutagenicity testing *in vivo* (Winton *et al.*, 1988). These tests are restricted to a small number of highly proliferative tissues and as such do not provide data relevant to mutagenesis or carcinogenesis in other tissues.

The availability of transgenic marker-mouse strains that carry multiple copies of a reporter gene in a shuttle vector in all cells, provides a system in which the mutagenic effect of genotoxins on the DNA can be studied in all organs and tissues of a treated animal (Gossen *et al.*, 1989; Myhr, 1991; Kohler *et al.*, 1991). Transgenic marker mice such as the *lac*Z mouse strain 40.6 (Muta™Mouse) and the BigBlue™ *lac*I mouse, carrying the *E. coli lac*Z and *lac*I gene respectively, have since their introduction been used to test numerous agents with respect to their mutagenic capability (Morginson and Ashby, 1994; Mirsalis, 1994; Gorelick, 1995). It was hoped that mutagenicity data obtained with transgenic systems would show a correlation with the carcinogenic properties of the compound tested. A comparison with the Salmonella/microsome test has demonstrated that the transgenic mouse assays yield data that predict carcinogenicity more reliably than do their bacterial counterparts for the same group of chemical agents (Gorelick, 1995).

After exposure of DNA to methylating and ethylating agents, the predominant N-alkylation products are N7-alkylguanine and N3-alkyladenine while the O-alkylations

result in alkylphosphotriesters in the DNA backbone and $O^6$-alkylguanine, $O^2$- and $O^4$-alkylthymine and $O^2$-alkylcytosine (Beranek, 1990). The preference of a particular agent for nitrogen or oxygen atoms in the DNA bases is predicted by the Swain-Scott constant associated with the compound (Lawley, 1974). Agents with a high Swain-Scott constant tend to form primarily N-alkylation products. A lower Swain-Scott constant predicts a relatively high O-alkylation level. Once induced, these products become the substrate of cellular repair processes of various kinds, such as specific dealkylation by the $O^6$-alkylguanine-DNA-alkyltransferase (AGT) (Pegg, 1983) or removal by the more general action of the nucleotide excision repair (NER) system believed to be involved in repair of $O^4$-ethylthymine (Klein *et al.*, 1994).

Unrepaired DNA lesions may give rise to mutations. $O^6$-ethylguanine ($O^6$-EtG) and $O^4$- and $O^2$-ethylthymine have been demonstrated to be mutagenic by mispairing with respectively thymine, guanine and thymine, which following DNA replication results in GC → AT and TA → CG transition and TA → AT transversion mutations, respectively (Ellison *et al.*, 1989; Klein *et al.*, 1990; Grevatt *et al.*, 1992). N3-alkylthymine and N3-alkyladenine have also been implicated in TA → AT and TA → CG mutagenesis (Grevatt *et al.*, 1991; Klungland *et al.*, 1992). N7-alkylguanine is not a miscoding lesion and is not directly mutagenic (Abbott and Saffhill, 1979). When N7-alkylguanine is converted into its ring-open form or when it is removed by glycosylases, the subsequent creation of an apurinic (AP)-site may be mutagenic leading to various types of base pair substitutions (Tudek *et al.*, 1992). N3-alkyladenine, in addition to being mutagenic, can block DNA synthesis and is as a result toxic to the cell (Saffhill *et al.*, 1985).

To study the mutagenicity of alkyl-DNA lesions in different tissues *in vivo*, we used the 40.6 *λlacZ* transgenic mouse strain as an animal model and various ethylating agents as model mutagens. The 40.6 strain carries 80 copies of the *Escherichia coli lacZ* gene per diploid genome, which act as the target for mutagenesis. The *lacZ* genes are positioned in phage *λ* shuttle vectors situated as 40-mer concatamers on chromosome 3 (Gossen *et al.*, 1989; Swiger *et al.*, 1994). The mutant frequency (MF) in the *lacZ* gene can be measured by packaging the individual prophages into viable phages and screening the phages with respect to their inability to produce the active *lacZ* gene product, *β*-galactosidase. For this screening, the positive selection assay developed for this purpose was used (Mientjes *et al.*, 1994). This so called P-gal assay permits the propagation of mutant *λlacZ* phages, while that of wild-type phages is repressed.

Here, we report our studies on tissue-specific mutagenesis, in *λlacZ* transgenic mice, induced by three alkylating agents, *viz.* *N*-ethyl-*N*-nitrosourea (ENU), diethylnitrosamine (DEN) and ethyl methanesulphonate (EMS). The choice of ENU and EMS as model agents was based on the large difference in the $O^6$/N7-EtG adduct ratio in DNA after treatment with these agents (Beranek, 1990), and was meant to obtain

with respect to DNA-replication rates and $O^6$-EtG-repair capacity. Bone marrow with its high cel-turnover rate was chosen to determine the effect of cell proliferation on mutation fixation and the effect of absence of metabolic activation of DEN.

In transgenic mice, the $O^6$-EtG and N7-ethylguanine (N7-EtG) content in the liver, bone marrow and brain was assayed at different time periods after treatment. In the DEN-treated mice only the liver and bone marrow were investigated. Adducts were assayed with the immunoslotblot procedure using specific monoclonal antibodies directed against the two lesions. To correlate these adducts to mutation fixation in the same mice, we determined the MF at the *lac*Z locus in these tissues by use of the P-gal positive selection assay. Finally, mutant *λlac*Z phages obtained from the liver and bone marrow of ENU-treated mice were studied as to the nucleotide-sequence alteration in *lac*Z to obtain information regarding the adducts responsible for the mutation induction seen in these organs.

## Materials and Methods

*Animal treatment*

Female transgenic mice (strain 40.6) were obtained at 14 weeks (±2 weeks) of age (average weight ± 20 g) from TNO Centre for Animal Research (The Netherlands). The mice were injected intraperitoneally (i.p.) with ENU (150 mg/kg body weight; Sigma, St Louis, MO, USA) dissolved in DMSO (Merck, Darmstadt, Germany), or DEN (66mg/kg bw; Fluka Chemie AG, Buchs, Zwitzerland) dissolved in 0.15 M saline solution. Mice were also treated with EMS (250 mg/kg; Pfalt and Bauer inc., Flashing, N.Y., USA) dissolved in DMSO. The control animals for the ENU and EMS-treated groups received DMSO (2 μl/g bw) while the control mice in the DEN group were injected with saline (2 μl/g bw). At various time points after treatment, animals were sacrificed by $CO_2$ asphyxiation after which the organs were collected, frozen on Dry Ice and stored at -20°C.

*DNA isolation*

High molecular-weight DNA was isolated from liver and brain in accordance to the protocol described by Roggeband *et al.* (1993), except for the overnight incubation of the isolated chromatin, which occurred with 50 μg proteinase K (Merck)/ml instead of 100 μg/ml. During brain DNA isolation, the tissue was directly homogenized in the sucrose/EDTA/Triton X-100 buffer. The remaining steps were the same as those for liver DNA. The isolation of DNA from bone marrow proceeded at the proteinase K incubation step. The DNA was taken up in 0.1 mM Tris.HCl (pH 7.4) and 0.01 mM EDTA, stored at 4°C and used for *in vitro* packaging.

For adduct level determinations, DNA remaining after *in vitro* packaging was incubated with RNAse A (0.1 mg/ml; Sigma) and T1 (100 U/ml; Boehringer, Mannheim, Germany) for 2 h at 37°C. DNA was once more extracted with phenol\chloroform, washed with chloroform prior to alcohol precipitation and taken up in potassium phosphate buffer (10 mM, pH 7.0).

*In vitro packaging and mutant lacZ phage screening*

A 5 μl (1.5-2 mg DNA/ml) aliquot of each of the viscous DNA solutions was taken and the λ prophages were 'rescued' by use of *in vitro* packaging extracts as prescribed by the manufacturer (Giga-

Pack Gold II, Stratagene). The MF in the liver, brain and bone marrow were measured with the positive selection system ("P-gal assay") originally described by Mientjes *et al.* (1994).

*$O^6$- and N7-EtG levels in genomic DNA*

The quantification of $O^6$- and N7-EtG adduct levels in total genomic DNA was carried out with the immunoslotblot assay as described previously (Mientjes *et al.*, 1996).

*DNA sequencing of lacZ*

The region ($\alpha$,$\beta$,$\omega$) of $\beta$-galactosidase in which the mutation probably was located was determined with the $\beta$-galactosidase protein complementation assay (Gossen *et al.*, 1993). The three *lacZ* complemation strains of *E. coli*, DH5$\alpha$ ($\alpha^-$,$\beta^+$,$\omega^+$), W4680 ($\alpha^+$,$\beta^-$,$\omega^+$) and Hfr 3000x90 ($\alpha^+$,$\beta^+$,$\omega^-$) were grown to an $OD_{709}$=0.5 and mixed with 5 ml molten LB-agar containing LB (Gibco BRL, Gaithersburg, Maryland, USA), 0.8% agarose, 0.2% maltose, 10 mM $MgSO_4$, 1.5 mg X-gal (New Jersey Lab, Livingston, NJ, USA) and 0.1 mg IPTG (New Jersey Lab). The mixture was poured onto agar plates (LB, 10mM $MgSO_4$, 0.2 maltose and 1.6% agarose). 5 $\mu$l mutant phage suspension obtained with the P-gal assay was spotted on each of the bacterial lawns together with wild-type control phages. The mutation was assumed to be located in the region that corresponds to the mutated region of the non-complementing strain.

Table 1: *Primers used to sequence lacZ*

Primers for *lacZ* amplification

| Primer | Position[1] | Sequence |
|---|---|---|
| U1 | -77 | 5'-biotin-dAGGCACCCCAGGCTTTACAC with S6 (for S1-S5) |
| U3 | 1273 | 5'-biotin-dGGCATGGTGCCAATGAATCG with S7 (for S5-S6) |
| U2 | 1640 | 5'-biotin-dCGATGGGTAACAGTCTTGGC with S11 (for S7-S11) |

*lacZ* sequence primers

| Primer | Position[1] | Sequence |
|---|---|---|
| S1 | 367 | 5'-dCCGTGGGAACAAACGGCGGA |
| S2 | 687 | 5'-dGCTGATTTGTGTAGTCGGTT |
| S3 | 1029 | 5'-dAATCCGCACCTCGCGGAAAC |
| S4 | 1369 | 5'-dGGTGATTACGATCGCGCTGC |
| S5 | 1589 | 5'-dAGCCATTTTTTGATGGACCA |
| S6 | 1909 | 5'-dAACGGAACTGGAAAAACTGC |
| S7 | 2179 | 5'-dGCCACTGCTGCCAGGCGCTG |
| S8 | 2378 | 5'-dGCACGGGTGAACTGATCGCG |
| S9 | 2699 | 5'-dCGCTCTGCTACCTGCGCCAG |
| S10 | 2960 | 5'-dCCTTCTTCCGCGTGCAGCAG |
| S11 | 37 bp in right arm $\lambda$ | 5'-dTATGAGTATTTCTTCCAGGG |

directly to PCR. Three different sets of primers (Pharmacia, Uppsala, Sweden) were used to span the entire *lac*Z sequence. Depending on which sequence primers were required, amplification was carried out with one biotin-labelled primer for the upper strand and one of the sequence primers for the lower strand (Table 1). The 60-$\mu$l PCR reaction mixture contained 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Triton X-100, 2.1 mM $MgCl_2$, 0.2 mM of each dNTP (Pharmacia), 0.4$\mu$M of each oligonucleotide primer and 20 U/ml Taq DNA polymerase (Promega, Madison, WI, USA). PCR was carried out with a PTC-100 thermal controller (M.J. Research, Watertown, MA, USA). The PCR profile for the 40-cycle amplification was as follows: 94°C 1.5 min; 53°C 1 min and 72°C 2 min. The PCR product was purified by gel electrophoresis on a 1% low-melting Nusieve agarose gel (FMC BioProducts, Rockland, ME, USA). The amplified product was isolated from the agarose as described by Menichini *et al.* (1991) using streptavidin-coated magnetic beads (Dynal A.S., Oslo, Norway). The biotin-labelled DNA strand was sequenced with a set of *lac*Z primers (Table 1) using T7 DNA polymerase (Pharmacia) and $\alpha$-$^{35}$S-dATP (Amersham, Buckinghamshire, England).

*Statistical analysis*

The probability calculations were carried out with the Student's t-test with the Solo (4.0) computer program (BMDP Statistical Software, Los Angeles, CA, USA).

## Results

Female transgenic mice were given a single i.p. injection of one of the alkylating agents ENU, EMS or DEN. At different time intervals after treatment, animals were sacrificed and the DNA extracted from the liver, brain and bone marrow (for brain, after ENU and EMS only). In this DNA the levels of $O^6$-EtG and N7-EtG adducts were measured with the immunoslotblot procedure using specific monoclonal antibodies directed against the two lesions. The adduct levels presented below are expressed as adducts per $10^6$ nucleotides ± the standard deviation. In the same DNA samples, the MF was determined. To obtain information regarding the origin of the induced mutations in ENU-treated mice, 25 liver and 23 bone marrow mutations were characterised by DNA sequencing.

The amount of agent used in the experiments were, per kg body weight, 150 mg ENU ($\approx$1/3 $LD_{50}$) and 66 mg DEN (1/2 $LD_{50}$). In two separate experiments, mice were treated with EMS (250 mg/kg; 1/2 $LD_{50}$). In the second EMS experiment, mice were treated with different a batch of EMS which led to unintentionally high adduct levels and the premature death of 8 of the 12 mice. Two animals were sacrificed 4 h after treatment and 2 after 14 d. Since the data obtained from these mice was relevant with respect to a comparison of adduct levels after various periods in the different organs and to the relation between these levels and the mutation induction measured, these results have been included in the following.

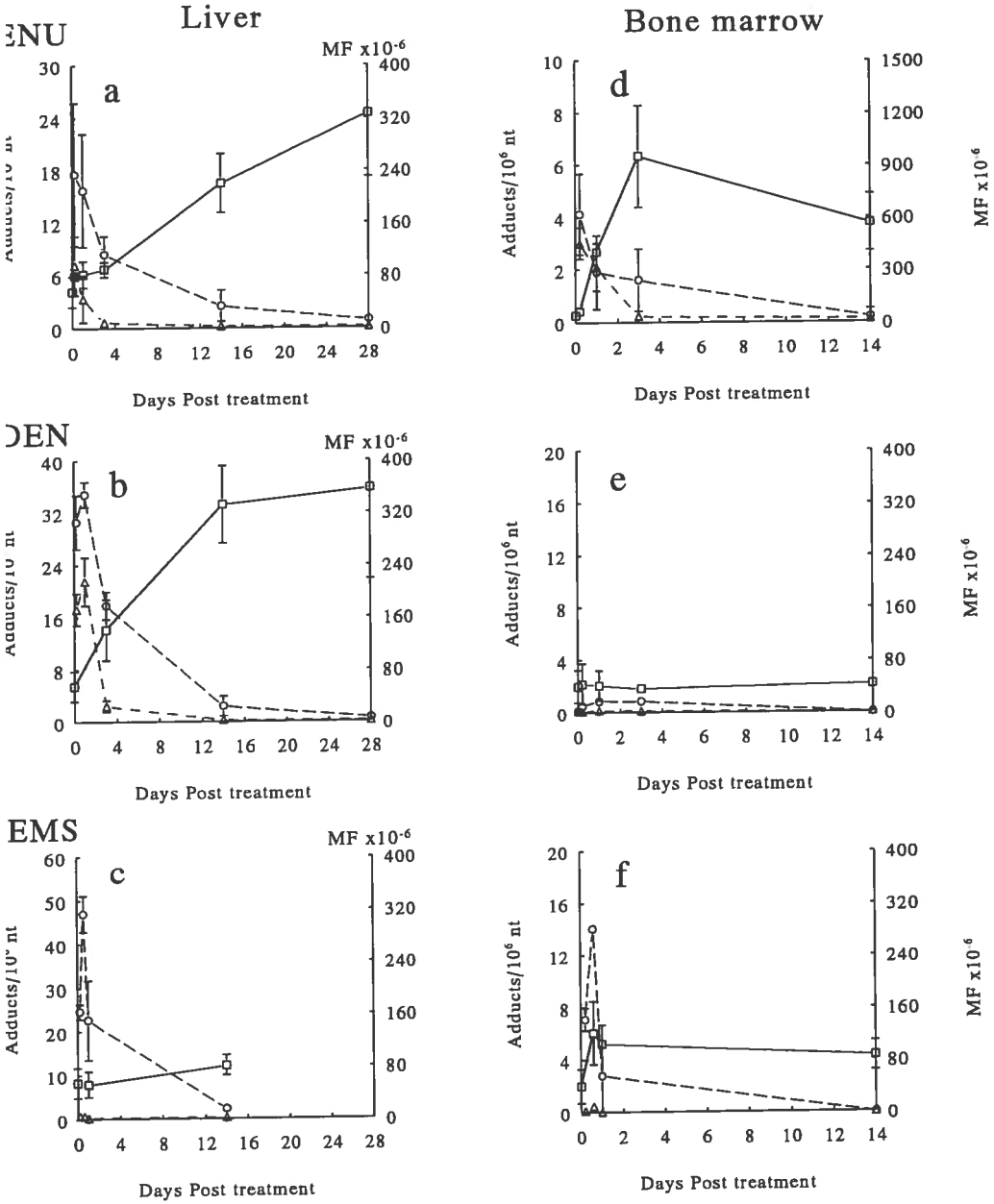


Figure 1: *$O^6$-EtG and N7-EtG content in the genomic DNA and the mutation-induction frequencies in lacZ in liver after ENU (a), DEN (b) and EMS (c) treatment and in bone marrow after ENU (c), DEN (d) and EMS (e) treatment. For the EMS-treated animals the*

EtG in the liver was highest at 1.5 h after treatment (7.0 ± 3.0 $O^6$-EtG and 17.5 ± 6.7 N7-EtG, respectively) (See Figure 1a). About 50% and 90% of the $O^6$-EtG lesions had been removed from the DNA at respectively 24 h and 3 d after treatment (3.4 ± 2.6 and 0.6 ± 0.3, respectively). In the same period the level of N7-EtG was reduced by about 5% and 50% (24 h: 16.7 ± 6.0; 3 d: 8.4 ± 2.1). At the 14 d and 28 d time points, background levels of $O^6$-EtG were seen while small amounts of N7-EtG were still present (14 d: 2.5 ±1.8; 28 d: 0.9 ±0.1).

In DEN-treated mice the quantity of both $O^6$-EtG and N7-EtG in the liver DNA was higher than that found in the corresponding ENU-treated mice (Figure 1b). At 1.5 h after treatment 17.2 ±2.8 $O^6$-EtG and 30.6 ±4.7 N7-EtG lesions were present in total genomic DNA. This level was maintained over the first 24 h ($O^6$-EtG: 21.5 ±4.3; N7-EtG: 34.9 ±2.2). It was followed by a substantial reduction to 2.5 ± 0.9 $O^6$-EtG and 17.8 ± 2.1 N7-EtG by day 3. At 14 d all $O^6$-EtG had disappeared. Most of the N7-EtG lesions had been removed at 14 d (2.4 ±1.8) with none remaining at 28 d. No $O^6$-EtG could be detected in the liver DNA of the first group of EMS-treated mice (Figure 1c). In the second EMS experiment, 2.8 ± 0.2 was determined 4 h after treatment and none after 14 d. The N7-EtG levels at 1.5 h, 4 h and 24 h after treatment in the first experiment were 24.7 ±1.6, 47.0 ±4.1 and 22.7 ±9.1, respectively. At day 14, 2.3 ±0.4 were still present. In the second EMS experiment a much higher level of 237 ±51 and 16.2 ±2.9 were measured at 4 h and 14 d, respectively.

*Bone marrow*: In the bone-marrow DNA of ENU-treated mice (Figure 1d) the initial (1.5 h) quantities of $O^6$-EtG (3.0 ±0.6) and N7-EtG (4.1 ± 1.6) lesions were lower than those in the corresponding liver samples. A slight reduction in $O^6$-EtG was seen 24 h after treatment (2.1 ±0.9) but the lesion had been removed completely by day 3. About half of the N7-EtG lesions were removed between 1.5 h and 24 h (24 h: 1.9±1.4). Little further reduction was observed at day 3 (1.6 ±1.2). By day 14 all N7-EtG were absent. In bone marrow of the DEN-treated animals, no elevated level of either adduct relative to the control mice was measured at any of the time points (Figure 1e).

In bone marrow of EMS-treated mice, $O^6$-EtG was detectable only in the animals of the second experimental group at 4 h after treatment (3.2 ±0.2). The N7-EtG level in these animals was 109 ±8. In the first experiment, EMS treatment resulted in 7.1 ±0.8 N7-EtG at 1.5 h and 14.1 ±0.2 at 4 h after treatment (Figure 1f). A large portion of these adducts had been removed after 24 h (2.8 remaining) and none were found after 14 d.

*Brain*: In brain DNA examined after ENU administration (Figure 2a), both $O^6$-EtG and N7-EtG were highest at 1.5 h post-treatment with 2.8 ±0.6 and 3.3 ±1.7, respectively. Gradual reduction in $O^6$-EtG was seen during the first 3 d (24 h: 3.0 ± 1.0; 3 d: 2.3 ±0.8) while levels of 1.2 ±0.8 and 0.8 ±0.3 were measured after 14 and 28 d, respectively. N7-EtG levels were 2.7 ±1.4, 1.4 ±0.6 at 24 h and 3 d, respectively, and below the detection limit at 14 d and 28 d.

In the brain of EMS-treated mice (Figure 2b), $O^6$-EtG was present at relatively low levels of 2.1 ±0.3, 1.6 and 0.6 ±0.2 at respectively 1.5 h, 4 h and 24 h. A higher level was found in the second EMS experiment (4 h: 4.4 ±4.0; 14 d: 3.8 ±0.7). The N7-EtG level observed in the brain DNA of EMS-treated mice at 1.5 h, 4 h, 24 h and 14 d were 114.5 ±4.3, 77.6 ±3.0, 16.9 ±3.8 and 3.6 ±2.1, respectively. The levels in the second experiment were 288 ±4.9 and 23.7 ±4.7 at 4 h and 14 d, respectively, after treatment. Adducts in DNA from brain of DEN-treated animals were not investigated.

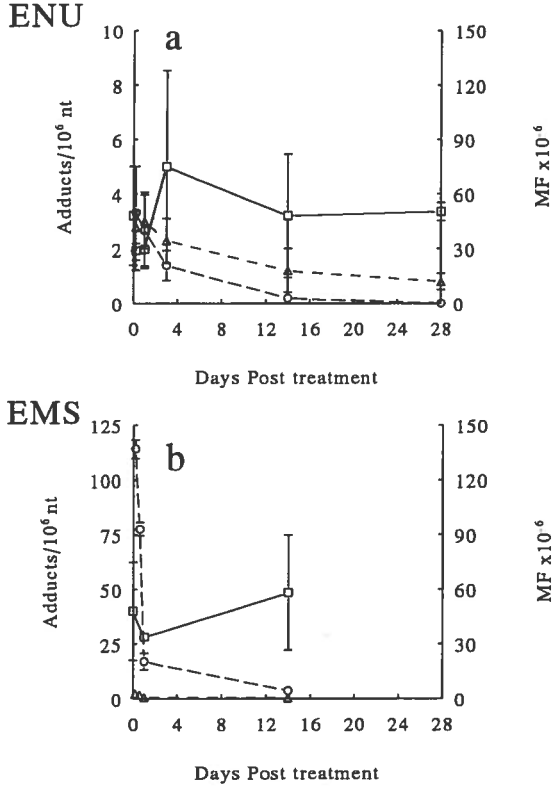


Figure 2: *$O^6$-EtG and N7-EtG content in the genomic DNA and the mutation-induction frequencies in lacZ in brain after ENU (a) and EMS (b) treatment. For the EMS-treated*

In the determinations of the MF in different organs, we [illegible]
200,000 *λlacZ* phages or more than 30 mutants per mouse for each organ studied. In a number of cases this goal could not be achieved due to the small amount of DNA isolated from the sample. This occurred predominantly in the bone marrow from mice treated with ENU or DEN, to such an extent that no MF assays could be performed at 28 d after treatment, possibly due to cytotoxic effects. Data per animal are presented in Tables 2-4.

*Liver*: In the liver DNA of ENU-treated mice a 1.4- and 1.5-fold increase in MF was measured at respectively 1.5 h and 24 h after treatment (Figure 1a). A 1.6-fold increase was seen after 3 d ($P=0.02$; Student's t-test) compared to the controls and a 5.8-fold enhanced mutation induction after 14 d ($P<0.01$). The further 1.5-fold increase in MF between day 14 and 28 was not found to be significant ($P=0.09$). A similar mutation induction curve was observed in the mice treated with DEN (Figure 1b). The MF at 3 d after treatment was 2.6-fold as high as that in the controls. At 14 d after treatment it was 6-fold higher ($P<0.01$). The MF increased slightly to 6.5-fold the control level at 28 d. Animals subjected to EMS did not experience a statistically significant increase in MF in the liver after 14 d (Figure 1c) not even when the animals from the second experiment are included.

*Bone marrow*: In the bone marrow DNA of animals subjected to ENU treatment (Figure 1d), a 1.6-fold, but statistically not significant increase was seen at 1.5 h after treatment. A clear 10-fold increase was observed at 24 h ($P<0.01$). The largest induction relative to the control mice was detected at 3 d post-treatment (23x; $P<0.01$). The MF seemed to decrease somewhat between days 3 and 14. In the mice that received DEN (Figure 1e), at no time point after treatment a significant increase in MF was observed. Due to the small amount of DNA obtained from the treated mice, only a relatively small number of phages could be screened for DEN-injected animals at 14 d and none for both ENU and DEN at 28 d. After treatment with EMS, a 3-fold mutation induction was observed at 4 h ($P<0.01$)(Figure 1f). Data from only 1 mouse could be obtained 24 h post-treatment. After 14 days the 4 mice (both EMS experiments) showed a 2.1-fold increase relative to the controls ($P<0.01$).

*Brain*: At none of the time points after treatment with ENU or EMS was a significant increase in MF detected in the brain DNA (Figure 2a,b). The MF was not determined in brain DNA of DEN-treated mice.

Table 2: *Mutation frequencies in liver DNA of ENU, DEN and EMS-treated mice*

| Treatment | Time | No. Phages | No. Mut. | MF x$10^6$ | Avg. ±SD |
|---|---|---|---|---|---|
| DMSO | 1.5 h | 145000 | 7 | 48 | 55 ± 24 |
| DMSO | 1.5 h | 66336 | 7 | 106 | |
| DMSO | 1.5 h | 311049 | 14 | 45 | |
| DMSO | 1.5 h | 233159 | 15 | 64 | |
| DMSO | 28 d | 661770 | 39 | 59 | |
| DMSO | 28 d | 970425 | 42 | 43 | |
| saline | 28 d | 349350 | 18 | 52 | |
| saline | 28 d | 355300 | 9 | 25 | |
| ENU | 1.5 h | 534750 | 44 | 82 | 79 ± 3 |
| ENU | 1.5 h | 626715 | 48 | 77 | |
| ENU | 24 h | 334305 | 34 | 102 | 82 ± 20 |
| ENU | 24 h | 469395 | 29 | 62 | |
| ENU | 3 d | 92160 | 8 | 87 | 89 ± 13 |
| ENU | 3 d | 103600 | 11 | 106 | |
| ENU | 3 d | 75120 | 7 | 93 | |
| ENU | 3 d | 436050 | 31 | 71 | |
| ENU | 3 d | 253337 | 22 | 87 | |
| ENU | 14 d | 145520 | 43 | 296 | 220 ± 49 |
| ENU | 14 d | 98480 | 25 | 254 | |
| ENU | 14 d | 242991 | 50 | 206 | |
| ENU | 14 d | 231021 | 52 | 225 | |
| ENU | 14 d | 242478 | 43 | 177 | |
| ENU | 14 d | 399285 | 65 | 163 | |
| ENU | 28 d | 284202 | 132 | 465 | 328 ± 120 |
| ENU | 28 d | 409203 | 101 | 247 | |
| ENU | 28 d | 398772 | 108 | 271 | |
| DEN | 3 d | 169680 | 16 | 94 | 141 ± 47 |
| DEN | 3 d | 357840 | 67 | 187 | |
| DEN | 14 d | 144480 | 45 | 312 | 333 ± 68 |
| DEN | 14 d | 141120 | 54 | 383 | |
| DEN | 14 d | 199920 | 49 | 245 | |
| DEN | 14 d | 204960 | 80 | 390 | |
| DEN | 28 d | 204802 | 119 | 581 | 357 ± 160 |
| DEN | 28 d | 252000 | 92 | 365 | |
| DEN | 28 d | 722500 | 174 | 241 | |
| DEN | 28 d | 269450 | 65 | 241 | |
| EMS | 24 h | 404414 | 27 | 67 | 53 ± 14 |
| EMS | 24 h | 179037 | 7 | 39 | |
| EMS | 14 d | 215802 | 16 | 74 | 82 ± 15[#] |
| EMS | 14 d | 139878 | 9 | 64 | |
| EMS[%] | 14 d | 257124 | 23 | 90 | |
| EMS[%] | 14 d | 273558 | 27 | 99 | |

*#: MF determined with animals from both EMS experiments; %: second EMS experiment.*

| Treatment | Time | No. Phages | No. Mut. | MF x$10^6$ | Avg. ±SD |
|---|---|---|---|---|---|
| DMSO | 1.5 h | 198900 | 4 | 20 | 41 ± 23 |
| DMSO | 1.5 h | 200940 | 14 | 70 | |
| DMSO | 1.5 h | 151202 | 3 | 20 | |
| DMSO | 1.5 h | 147030 | 3 | 20 | |
| DMSO | 14 d | 100068 | 5 | 50 | |
| DMSO | 14 d | 331080 | 12 | 36 | |
| saline | 28 d | 39100 | 3 | 77 | |
| saline | 28 d | 90610 | 3 | 33 | |
| ENU | 1.5 h | 171080 | 16 | 94 | 64 ± 25 |
| ENU | 1.5 h | 186120 | 14 | 75 | |
| ENU | 1.5 h | 260313 | 11 | 42 | |
| ENU | 1.5 h | 318060 | 14 | 44 | |
| ENU | 24 h | 20520 | 8 | 390 | 400 ± 10 |
| ENU | 24 h | 19494 | 8 | 410 | |
| ENU | 3 d | 216657 | 147 | 679 | 951 ± 338 |
| ENU | 3 d | 263133 | 370 | 1406 | |
| ENU | 3 d | 152520 | 154 | 1010 | |
| ENU | 3 d | 329220 | 233 | 708 | |
| ENU | 14 d | 215385 | 156 | 724 | 569 ± 190 |
| ENU | 14 d | 99950 | 40 | 400 | |
| ENU | 14 d | 47096 | 35 | 743 | |
| ENU | 14 d | 320050 | 131 | 409 | |
| DEN | 1.5 h | 12920 | 0 | - | 44 ± 38 |
| DEN | 1.5 h | 148750 | 13 | 87 | |
| DEN | 1.5 h | 279280 | 5 | 18 | |
| DEN | 1.5 h | 189125 | 5 | 26 | |
| DEN | 24 h | 270385 | 20 | 74 | 42 ± 27 |
| DEN | 24 h | 380375 | 13 | 34 | |
| DEN | 24 h | 289850 | 3 | 11 | |
| DEN | 24 h | 362100 | 17 | 50 | |
| DEN | 3 d | 272067 | 8 | 29 | 37 ± 8 |
| DEN | 3 d | 608960 | 27 | 44 | |
| DEN | 14 d | 8624 | 0 | - | 44* |
| DEN | 14 d | 43806 | 0 | - | |
| DEN | 14 d | 17444 | 0 | - | |
| DEN | 14 d | 22769 | 1 | 44 | |
| EMS | 4 h | 186915 | 13 | 70 | 121 ± 48# |
| EMS | 4 h | 93585 | 16 | 171 | |
| EMS% | 4 h | 432600 | 66 | 153 | |
| EMS% | 4 h | 225339 | 21 | 93 | |
| EMS | 24 h | 143480 | 15 | 105 | 105* |
| EMS | - | - | - | - | |
| EMS | 14 d | 95625 | 7 | 73 | 86 ± 23# |
| EMS | 14 d | 155905 | 10 | 64 | |
| EMS% | 14 d | 63672 | 6 | 94 | |
| EMS% | 14 d | 17472 | 2 | 115 | |

**: MF determined in one animal only; #: MF determined with animals from both EMS experiments; %: second EMS experiment.*

Table 4: *Mutation frequencies in brain DNA of ENU and EMS-treated mice*

| Treatment | Time | No. Phages | No. Mut. | MF x$10^6$ | Avg. ±SD |
|---|---|---|---|---|---|
| DMSO | 1.5 h | 137313 | 5 | 36 | 48 ± 27 |
| DMSO | 1.5 h | 85671 | 6 | 70 | |
| DMSO | 1.5 h | 103253 | 9 | 87 | |
| DMSO | 1.5 h | 163718 | 8 | 49 | |
| DMSO | 28 d | 919770 | 20 | 22 | |
| DMSO | 28 d | 278070 | 6 | 22 | |
| ENU | 1.5 h | 64142 | 1 | 16 | 29 ± 12 |
| ENU | 1.5 h | 109749 | 5 | 46 | |
| ENU | 1.5 h | 386880 | 11 | 28 | |
| ENU | 1.5 h | 667740 | 18 | 27 | |
| ENU | 24 h | 411060 | 16 | 39 | 30 ± 9 |
| ENU | 24 h | 539400 | 11 | 20 | |
| ENU | 3 d | 50181 | 7 | 140 | 75 ± 61 |
| ENU | 3 d | 26994 | 3 | 111 | |
| ENU | 3 d | 150753 | 1 | 7 | |
| ENU | 3 d | 186465 | 8 | 43 | |
| ENU | 14 d | 52041 | 6 | 115 | 48 ± 37 |
| ENU | 14 d | 46087 | 1 | 22 | |
| ENU | 14 d | 156612 | 4 | 26 | |
| ENU | 14 d | 243660 | 10 | 41 | |
| ENU | 14 d | 137919 | 9 | 65 | |
| ENU | 14 d | 148521 | 3 | 20 | |
| ENU | 28 d | 663090 | 33 | 50 | 51 ± 6 |
| ENU | 28 d | 157449 | 7 | 45 | |
| ENU | 28 d | 229338 | 13 | 57 | |
| EMS | 24 h | 124061 | 4 | 32 | 34 ± 2 |
| EMS | 24 h | 142358 | 5 | 35 | |
| EMS | 14 d | 174164 | 9 | 52 | 58 ± 32[#] |
| EMS | 14 d | 106875 | 11 | 103 | |
| EMS[%] | 14 d | 280475 | 8 | 29 | |
| EMS[%] | 14 d | 323554 | 16 | 50 | |

*#: MF determined with animals from both EMS experiments; %: second EMS experiment*

| Mutant | Clone | Colour[1] | Region[2] | Mutation | Position | Sequence | A.A. change |
|---|---|---|---|---|---|---|---|
| L1 | L15.1 | Cl | ω | CG→TA | 1313 | GCGCTGGCTAC | Trp→STOP |
| L2 | L15.2 | Cl | β | TA→AT | 1190 | TTCGCATTATC | His→Leu |
| L3 | L15.3 | Lb | β | CG→AT | 1823 | GTTCTGTATGA | Cys→Phe |
| L4 | L15.4 | Cl | β | AT→TA | 1720 | TCGTCTGGGAC | Trp→Arg |
| L5 | L15.7 | Cl | ω | CG→TA | 2714 | GCTCGGATTAG | Gly→Glu |
| L6 | L15.13 | Cl | ω | GC→TA | 2840 | CGGGACGCGCG | Thr→Lys |
| L7 | L15.15 | Cl | β | CG→AT | 1661 | TGGCGGTTTCG | Gly→Val |
| L8 | L15.17 | Cl | ω | TA→AT | 2389 | TGGATAACGAC | Asn→Tyr |
| L9 | L15.18 | Cl | β | CG>AT | 1090 | GTCACGAGCAT | Glu→Stop |
| L10 | L15.20 | Cl | ω | CG→AT | 2666 | GATTGGCCTGA | Gly→Val |
| L11 | L15.21 | Cl | β | TA→GC | 1828 | GTATGAACGGT | Asn→His |
| L12 | L15.22 | Cl | ω | AT→TA | 2717 | CGGATTAGGCT | Leu→STOP |
| L13 | L15.24 | Bl | β | C→AG | 1334 | CGAACGCGTAA | FS |
| L14 | L13.1 | Cl | β | ΔT | 1307 | CGATGATCCGC | FS |
| L15 | L13.2 | Bl | ω | TA→CG | 2390 | GGATAACGACA | Asn→Ser |
| | | | | TA→GC | 2073 | GAACTACCGCA | Leu→Leu |
| L16 | L13.3 | Cl | ω | CG→TA | 2744 | CGACCGCCTTA | Arg→His |
| L17 | L13.4 | Cl | ω | TA→AT | 2557 | AGGGGAAAACC | Lys→Stop |
| L18 | L13.5 | Bl | ω | CG→TA | 2392 | ATAACGACATT | Asp→Asn |
| L19 | L13.6 | Cl | β | AT→CG | 1523 | CCCGATGTACG | Met→Arg |
| L20 | L13.7 | Cl | ω | CG→AT | 2714 | GCTCGGATTAG | Gly→Val |
| L21 | L13.8 | Cl | β | GC→AT | 1187 | CTGTTCGCATT | Ser→Leu |
| L22 | L14.5 | Cl | ω | TA→AT | 2393 | TAACGACATTG | Asp→Val |
| L23 | L14.8 | Cl | β | AT→TA | 1398 | GGGAATGAATC | Asn→Lys |
| L24 | L14.10 | Cl | β | GC→AT | 380 | GACGGGTTGTT | Gly→Asp |

[1]: *Cl* : *clear*
*Lb* : *light blue*
*Bl* : *blue*

[2]: *region as predicted by β-galactosidase-complementation assay*
*FS* : *frameshift mutation*
Δ : *deletion*

Table 6: *Bone marrow mutants in ENU-treated (150mg/kg) mice, 3 d after exposure*

| Mutant | Clone | Colour[1] | Region[2] | Mutation | Position | Sequence | A.A. change |
|---|---|---|---|---|---|---|---|
| BM1 | BM7.3 | Cl | $\alpha\beta$ | GC→AT | 1212 | CTGTGGTACAC | Trp→Stop |
| BM2 | BM7.4 | Cl | $\alpha\beta$ | GC→AT | 1187 | CTGTTCGCATT | Ser→Leu |
| BM3 | BM7.6 | Bl | $\omega$ | CG→TA | 2714 | GCTCGGATTAG | Gly→Glu |
| BM4 | BM7.7 | Cl | $\omega$ | CG→AT | 2440 | GGGTCGAACGC | Glu→Stop |
| BM5 | BM7.15 | Cl | $\omega$ | GC→AT | 2374 | TCACCCGTGCA | Arg→Cys |
| BM6 | BM8.1 | Bl | $\beta$ | GC→CG | 387 | TGTTACTCGCT | Tyr→Stop |
| BM7 | BM8.5 | Cl | $\alpha\beta$ | AT→TA | 1476 | CAGTATGAAGG | Tyr→Stop |
| BM8 | BM8.9 | Cl | $\omega$ | TA→AT | 2819 | GAGCGAAAACG | Glu→Val |
| BM9 | BM8.10 | Cl | $\alpha\beta$ | AT→GC | 1402 | ATGAATCAGGC | Ser→Pro |
| BM10 | BM8.14 | Bl | $\alpha\beta$ | CG→TA | 590 | GCGCTGGAGTG | Trp→Stop |
| BM11 | BM8.20 | Cl | $\alpha\beta$ | CG→TA | 1252 | TGGATGAAGCC | Glu→Lys |
| BM12 | BM5.1 | Cl | $\alpha$ | Δ115bp | 25-140 | ACTGG/GGCCC | FS |
| BM13 | BM5.2 | Bl | $\alpha$ | GC→AT | 1111 | ATGGTCAGGTC | Gln→Stop |
| BM14 | BM5.3 | Cl | $\alpha\beta$ | CG→TA | 1212 | CTGTGGTACAC | Trp→Stop |
| BM15 | BM5.5 | Cl | $\alpha$ | AT→TA | 945 | CTCTATCGTGC | Tyr→Stop |
| BM16 | BM5.6 | Cl | $\omega$ | GC→AT | 2467 | ATTACCAGGCC | Gln→Stop |
| BM17 | BM5.10 | Cl | $\alpha\beta$ | AT→TA | 625 | ATATGTGGCGG | Trp→Arg |
| BM18 | BM5.17 | Cl | $\omega$ | AT→GC | 2869 | ACCAGTGGCGC | Trp→Arg |
| BM19 | BM5.20 | Cl | $\beta$ | CG→AT | 409 | TTGATGAAAGC | Glu→Stop |
| BM20 | BM6.1 | Cl | $\alpha$ | CG→TA | 1385 | CATCTGGTCGC | Trp→Stop |
| BM21 | BM6.2 | Cl | $\alpha\beta$ | GC→TA | 281 | CCCCTCAAACT | Ser→Stop |
| BM22 | BM6.3 | Cl | $\beta$ | TA→AT | 1582 | TCAAAAAATGG | Lys→Stop |
| BM23 | BM6.7 | Cl | $\beta$ | GC→CG | 281 | CCCCTCAAACT | Ser→Stop |

[1]: *Cl* : *clear*
*Lb* : *light blue*
*Bl* : *blue*

[2]: *region as predicted by β-galactosidase-complementation assay*
*FS* : *frameshift mutation*
Δ : *deletion*

was observed at 28 d and 3 d post-treatment, respectively. These time points were chosen for collection of mutant phages for DNA-sequence analysis. Of the mutant plaques obtained from each of these ENU-treated mice (liver: 3; bone marrow: 4) a number were selected randomly to be subjected to the β-glactosidase-complementation assay. In this assay a first, rough approximation of the position of the mutation within the *lac*Z gene can be obtained, by determining which of three *E. coli* strains carrying a well-defined *lac*Z mutation can complement the phage-encoded mutated β-galactosidase. In this manner, the mutation can be assigned to one of the three regions of the gene, *i.e.* the α-region (position 9-276), the β-region (277-1800) or the ω-region (1801-3096). Although the confines of these regions have not been established with absolute certainty, this rough localisation greatly facilitates the subsequent sequencing to identify the exact nature of the mutation. Of the liver mutants, 12 were predicted to have a mutation in the β-region and 12 in the ω-region. Upon sequence analysis all mutations except one (L1) were found in the predicted region (Table 5). For the bone marrow-derived mutants (Table 6), the prediction of the complementation assay was less accurate. Although all 6 ω-region predictions were correct, of the 17 mutations found in the β-region, only 4 had been predicted correctly. For 3 of the remaining 13, the complementation assay had indicated the α-region, for the other 9 an undecisive α or β location had been the result. One mutation was found in the α-region as predicted.

Table 7: *Summary of the lacZ mutations sequenced from liver and bone marrow DNA*

| | Liver | | Bone marrow | |
|---|---|---|---|---|
| | number | % | number | % |
| Transitions | | | | |
| GC→AT | 6 | 24 | 10 | 43 |
| TA→CG | 1 | 4 | 2 | 9 |
| Transversions | | | | |
| GC→TA | 6 | 24 | 3 | 13 |
| GC→CG | 0 | 0 | 2 | 9 |
| TA→GC | 3 | 12 | 0 | 0 |
| TA→AT | 7 | 28 | 5 | 22 |
| Deletion | 1 | 4 | 1 | 4 |
| Insertion | 1 | 4 | 0 | 0 |
| TOTAL | 25 | 100 | 23 | 100 |

In the liver the most frequent mutations were TA → AT and GC → TA (or the equivalent AT → TA and CG → AT) transversions and GC → AT transitions (or CG →TA) comprising 28%, 24% and 24% of the mutations sequenced, respectively (Table 7). Of the GC → AT mutations, half were at CpG sites. *In vivo,* methylation of cytosine is often found at such CpG sites, which may give rise to spontaneous GC → AT transitions as a consequence of deamination of methylcytosine to thymine. In the bone marrow GC → AT transitions clearly dominated with 43% of the total mutations, only 20% of which were at CpG sites. TA → AT transversions made up the next most frequent class of mutations in bone marrow (22%). The positions of the mutations in the *lac*Z sequence also differed between the two tissues. In the liver 11 and 14 mutations were found in respectively the $\beta$- and $\omega$-regions (one mutant carried 2 $\omega$-mutations). In the bone marrow 1 mutation was localised in the $\alpha$-region, 16 in the $\beta$-region and 6 in the $\omega$-region. 'Hot-spots' for mutations in *lac*Z were searched for in the combined liver and bone marrow data. In the $\beta$-region position 281 was mutated twice; 3 mutations were observed at or close to position 1187 and 2 at position 1212. Two additional 'hot-spots' were localised proximal to positions 2391 and 2714, each with 4 mutations at or near (±3bp) the site. None of the mutations characterised either in the liver or in bone marrow originating from the same animal occurred at the same position in *lac*Z. Therefore, it is assumed that all mutations analysed arose from independent events.

**Discussion**

Differences exist in cellular processes such as metabolism, DNA repair and rate of cell proliferation between cells of different organs and tissues. Since these processes are of fundamental influence on the conversion of DNA adducts into fixed mutations, proper insight in the mutagenic effects of chemicals in higher organisms can only be obtained from *in vivo* studies in various organs. To contribute to these studies, *lac*Z transgenic mice were treated with different ethylating agents and adduct levels as well as the mutant frequency in *lac*Z were analysed in liver, bone marrow and brain DNA. In addition, a total of 48 liver and bone marrow *lac*Z mutations were sequenced to determine possible differences in mutation spectra between the two tissues. The agents ENU and EMS were selected because of the strong difference in their preference for ethylating either O- or N-atoms in DNA. In experiments aimed at establishing a correlation between DNA alkylation and mutation induction, the levels of $O^6$-EtG and N7-EtG were determined as each is a predominant base adduct in its category. Since O-alkylated bases were expected to be the major premutagenic lesions, the role of metabolic activation in mutation induction by ethylating agents was studied by comparing ENU with DEN, which has a

whereas O⁶-EtG was hardly detectable. As a consequence, only a few DNA samples — in particular those from brain - allowed calculation of the O⁶/N7 ratio, which amounted to about 0.02. The highest adduct levels were measured in brain DNA followed by liver, but only bone marrow, with the lowest degree of alkylation, showed a significant (2-fold) increase in MF. These results are in agreement with data by Suzuki *et al.* (1993) who did not observe induction of mutants in the liver either after EMS administration, in contrast to a 1.8-fold increase in bone-marrow mutations at 7 d after treatment.

Much higher O⁶/N7 ratios were measured shortly after ENU administration, with values of about 0.40 for liver DNA and 0.73 for bone marrow. These ratios have been influenced by the action of the repair protein AGT, which rapidly removes O⁶-alkyl groups from DNA in a suicidal reaction (Pegg, 1993). In earlier experiments with ENU-injected transgenic mice that had been pretreated with O⁶-benzylguanine to deplete all AGT activity, liver and brain DNA were found to have an initial O⁶/N7 ratio of around 0.9 (Mientjes *et al.*, 1996). The higher value for bone marrow compared to liver in the present study, probably is due to the higher AGT content of the liver. In brain, where no rapid repair of O⁶-EtG takes place, the initial ratio was about 0.85. Presumably, the stability of this adduct in brain also explains why only in that tissue O⁶-EtG could be detected after the EMS treatment.

In the ENU-treated mice, the well measurable levels of O⁶-EtG went together with a considerable increase of the MF in liver (6x) and bone marrow (23x). Since the corresponding N7-EtG levels were clearly below those induced by EMS, which did not raise the MF in *lac*Z, these results underline the dominant role of adducts formed at O-atoms in the induction of gene mutations by alkylating agents.

The results obtained with DEN illustrate the decisive rolt that is played by metabolic conversion in mutagenesis. Whereas in liver DNA both O⁶-EtG and N7-EtG were induced to levels about twice those caused by ENU, with a concomitant increase in MF (3x) at 3 d, in bone marrow neither adduct formation nor induction of *lac*Z mutants could be detected. This is in line with the absence, in the latter tissue, of the cytochromes p450 2A6 and 2E1 (Camus *et al.*, 1993; Genter and Recio, 1994) which are responsible for activation of DEN in the liver.

Various authors have presented data indicating O⁶-alkylguanine as an important premutagenic lesion, involved in carcinogenesis as well. It has been quantitatively correlated to the induction of thymic tumours in mice (Frei *et al.*, 1978). Sequence analysis of rat mammary tumours induced by *N*-methyl-*N*-nitrosourea demonstrated GC → AT mutations in the *Ha-ras* proto-oncogene, which were attributed to O⁶-methylguanine lesions (Zarbl *et al.*, 1985). O⁶-EtG has been connected to mutagenesis in the *hprt* gene of V79 Chinese Hamster cells (van Zeeland *et al.*, 1985). The role of AGT in carcinogenesis provided additional indications: depletion of this protein specific for O⁶-alkylguanine repair resulted in an increase in liver tumours (Peto *et al.*, 1984). It appears

justified, therefore, to look for a correlation between the $O^6$-EtG levels measured and the *lac*Z mutations scored in the same animal tissues.

In the livers of the ENU- and DEN-treated mice, the $O^6$-EtG levels attained their maximum already within 24 h after treatment, whereas at 3 d more than 90% of the lesions had been removed from the DNA. The adducts present during the initial period may have participated in mispairing, leading to mutation fixation. Indeed, at day 3, a significant increase in the MF was observed. After this period, however, with the bulk of $O^6$-EtG removed, the induction of *lac*Z mutations still went on, for some weeks. This continued increase in MF after disappearance of most of these lesions strongly suggests that adducts other than $O^6$-EtG were responsible for the majority of the mutations that arose between day 3 and day 28. In case of ENU treatment about 75% of the mutants scored at day 28 must have arisen after day 3. Among the 25 mutations sequenced from this population, only 24% were GC → AT transition, the mutation assumed to originate from $O^6$-alkylguanine (Ellison *et al.*, 1989). This low value is in line with the mechanism suggested above.

In bone marrow, the ENU treatment induced fewer adducts than in liver, but a much higher number of *lac*Z mutants was the result. In this tissue the maximum MF was observed already after 3 d. The high proliferation rate of bone marrow cells can be held responsible for this rapid fixation of mutations, while the inherent high cell turnover probably caused the subsequent drop in MF. Rapid fixation together with a slower repair

Table 8: *Summary of the corrected lacZ mutation spectra in liver and bone marrow*

| | LIVER | | | Bone marrow | | |
|---|---|---|---|---|---|---|
| | % spont.[1] | Corrected No. mutants[2] | Corr. % | % spont. | Corrected No. mutants | Corr. % |
| Transitions | | | | | | |
| GC→AT | 44 | 4 | 19 | 71 | 9 | 41 |
| TA→CG | 26 | 0 | 0 | 5 | 2 | 9 |
| Transversions | | | | | | |
| GC→TA | 4 | 6 | 29 | 14 | 3 | 14 |
| GC→CG | 4 | 0 | 0 | 0 | 2 | 9 |
| TA→GC | 0 | 3 | 14 | 0 | 0 | 0 |
| TA→AT | 7 | 7 | 33 | 0 | 5 | 23 |
| Deletion | 15 | 0 | 0 | 10 | 1 | 5 |
| Insertion | 0 | 1 | 5 | 0 | 0 | 0 |
| TOTAL | 100 | 21 | 100 | 100 | 22 | 100 |

transitions. Indeed, among the 25 *lacZ* plaques randomly selected for sequence analysis from the mutants isolated at 3 d after ENU administration, this transition mutation dominated (43%). In bone marrow, even the low levels of $O^6$-EtG induced by EMS went together with a significant MF increase. Also in this case presumably the high proliferation activity played a decisive role.

The requirement of cell proliferation for the occurrence of mutation fixation, probably explains the complete absence of an MF increase in brain DNA, notwithstanding the well measurable adduct levels after ENU and EMS treatment and the relative persistence of $O^6$-EtG in this tissue.

For a proper comparison of the mutation spectra in liver and bone marrow in relation to the ENU-induced adducts, a correction for the spontaneous mutations was considered. In liver DNA isolated after 28 d, a 6-fold increase in the MF was observed relative to the control value, in bone marrow, at 3 d, the increase was 23-fold. This implies that statistically 1/6 of the 25 sequenced liver mutations was spontaneous in origin and in bone marrow 1/23. Douglas *et al.* (1994) have published sequence data of spontaneous mutations in the *lacZ* gene in liver and bone marrow of transgenic mice (Table 8). They found that 44% of the liver mutants carried a GC → AT transition and 26% TA → CG. Correcting our liver data for the spontaneous mutations shows that the contribution of ENU-induced, *i.e.* $O^6$-EtG-attributable GC → AT transitions, can only become lower than the uncorrected value.

In bone marrow, the GC → AT transitions amounted to 71% of the spontaneous mutations sequenced (Douglas *et al.*, 1994; Table 8). Correction of our bone marrow data has little consequence for the mutation spectrum. The overall conclusion remains that the contribution of the $O^6$-EtG-induced GC → AT transitions to the total ENU-induced *lacZ* mutations is roughly twice as large in bone marrow as in liver. The large proportion of liver mutants that arose after removal of most $O^6$-EtG adducts, probably plays a role in this difference. Nevertheless, even in bone marrow, where earlier collection, slower $O^6$-EtG repair and faster fixation owing to rapid proliferation resulted in a higher MF, at a lower adduct content, over half of the mutants were not GC → AT.

The other relatively frequently formed mutations, both in liver and bone marrow, were the TA → AT and GC →TA transversions. The former conversion may be ascribed to the $O^2$-ethylthymine adduct (Grevatt *et al.*, 1992), which is removed from the DNA very slowly *via* an as yet unidentified mechanism. To our knowledge, no alkylation product has been linked to the GC → TA mutation. $O^2$-ethylcytosine, a persistent lesion formed at 1/3 of the level of $O^6$-EtG upon ENU treatment (Beranek, 1990), appears a possible candidate. A mispairing of $O^2$-ethylcytosine with thymine would bring about such a sequence change. Attribution to the "A-rule", operating at AP-sites resulting from N7-EtG, is not justified since this mechanism has only been confirmed to operate in *E. coli* (Loeb and Preston, 1986; Gentil *et al.*, 1992; Cabral-Neto *et al.*, 1994). Additional

research will be needed to identify the premutagenic lesion giving rise to this mutation. The TA → CG transitions found in bone-marrow DNA probably stem from $O^4$-ethylthymine adducts (Klein *et al.*, 1990).

For the validation of the *lacZ* transgenic mouse as an *in vivo* mutagenicity assay, a central question is whether the target DNA properly represents the endogenous genes. As to the stability of the $O^6$-EtG, earlier studies (Mientjes *et al.*, 1996) have demonstrated comparable repair kinetics in *lacZ* and in total genomic DNA, in liver and in brain, but for a slower removal in liver DNA over the first 1.5 h after exposure. Data allowing a more direct comparison with an endogenous gene comes from *in vivo* studies on mutations in the *hprt* locus (Skopek *et al.*, 1992; Jansen *et al.*, 1994, 1995). In rat and mouse T-cells and in rat skin fibroblasts, ENU induced predominantly TA → AT transversions (40-45%) ascribed to $O^2$-ethylthymine, next to $O^4$-ethylthymine-attributable TA → CG transitions (9-27%) and only low levels of the $O^6$-EtG-induced GC → AT mutations (4-12%). The strikingly small contribution of GC → AT in *hprt* relative to *lacZ* may be due to a difference in repair of $O^6$-EtG. Owing to the fact that *hprt* is actively transcribed, the lesions will be removed from this gene in a preferential manner, analogous to the preferential repair of $O^6$-EtG in the $\beta$-actin gene. In rat hepatoma cells, $\beta$-actin was shown to have a faster repair rate of $O^6$-EtG than total genomic DNA (Thomale *et al.*, 1994). The accelerated repair of this adduct in *hprt* together with the late harvest time of the T-cells and fibroblasts (4-6 weeks after treatment) should have provided ample opportunity for the fixation into mutations of the more persistent $O^2$- and $O^4$-ethylthymine lesions.

On the other hand, in rats exposed to a high dose of EMS (325 mg/kg), the *hprt* mutations scored in T-lymphocytes were primarily GC → AT transitions (Jansen *et al.*, 1995), in accordance with the observations by Beranek (1990) that $O^2$- and $O^4$-ethylthymine are not induced by EMS in appreciable amounts. Furthermore, there is the remarkable difference between the *in vivo lacZ* and *hprt* mutation spectra after ENU treatment with respect to the GC → TA transversions, which are rare in *hprt* and relatively frequent in *lacZ*. Interestingly, a high proportion of this kind of mutation was observed in the activated H-*ras* oncogene in DEN-induced liver tumours (7/14), next to TA → AT (4/14) and TA → CG (3/14) (Stowers *et al.*, 1988), both of which were also found in *lacZ*. In addition, the earlier mentioned H-*ras* mutations are of relevance, *i.e.* the GC → AT transitions established in rat mammary tumours induced by methylating agents (Zarbl *et al.*, 1985). Although a definite conclusion cannot be drawn yet on the extent to which *lacZ* are comparable to endogenous genes with respect to mutation induction, the presently available data indicate sufficient similarities to justify an optimistic view.

compared to available data previously obtained with other test systems measuring gene mutations or other cancer-related end-points. The mutagenicity of ENU in liver and bone marrow and DEN in the liver coincides with the organs in which tumours are frequently found after treatment of adult rodents with the same agents (IARC 1978). ENU often leads to liver and lymphoreticular tumours, the latter believed to stem from cells migrating from the bone marrow. Another target are the tissues of the nervous system, where tumour formation has only been found to occur, however, in very young mice and not in adults. Interestingly, ENU and DEN, which are, according to out results, respectively mutagenic and non-mutagenic in bone marrow, score positive and negative, respectively, in the bone marrow micronucleus induction assay (Suzuki *et al.*, 1994). EMS which is not a liver or brain carcinogen, was not found to be mutagenic in these organs, in our studies. EMS was found to be mutagenic in bone marrow and also induced micronuclei in that tissue (Suzuki *et al.*, 1994). These data and that reviewed in Gorelick *et al.* (1995) show that the results of transgenic marker mice assays are in close concordance with those of rodent carcinogenicity tests. In addition, the present study indicates the applicability of these transgenic systems for mechanistic studies in various tissues of mice exposed to genotoxins.

# *Chapter 6*

## General discussion

### 6.1 Introduction

It is generally believed that the majority of chemically induced cancers in man is initiated by lesions in the DNA that result from interaction of chemically reactive species with the genetic material. The mutagenic properties of a number of these lesions result in their fixation into permanent changes in the genetic code during DNA replication. Depending on which DNA sequences are affected, mutations may initiate the cancer process. To minimise the occurrence of such events, putative carcinogens must be identified in order to reduce or avoid human exposure. It is also important that the mechanisms involved in mutagenesis are well understood as such knowledge can aid, for example, in the improvement of risk assessments.

The principal aim of the present study was to look at alkylation-induced mutagenesis *in vivo*, from a mechanistic point of view, in several organs of transgenic marker mice. The mutagenicity of these agents is expressed as the frequency with which they induce mutations in a target locus of the respective organ and is referred to as the mutant frequency (MF). This target locus consists of the *E. coli lac*Z gene, present in all cells of the transgenic mice used, incorporated in the genome as tandem repeats of $\lambda$ phages each containing one *lac*Z gene.

The work presented in this thesis initially involved the development and implementation of a positive selection assay to determine the MF in *lac*Z in phages recovered from the mouse DNA. This was followed by an evaluative study in which the new assay was compared to the assay previously used. Then, the transgenic marker mice were used to study ethylating agent-induced DNA damage and mutagenesis. Firstly, the formation and repair of an important premutagenic ethyl adduct, $O^6$-ethylguanine ($O^6$-EtG), was determined in the *lac*Z transgene in comparison to the total genomic DNA. This comparison was considered of great importance, since it is generally hoped that results from the transgenes can be extrapolated to the entire genome; possible differences in adduct processing between transgene and endogenous DNA, if observed, probably would also indicate differences in the process of mutagenesis.

A major portion of the work concerned a comparative study of the mutagenicity of three model ethylating agents, *viz. N*-ethyl-*N*-nitrosourea (ENU), diethyl nitrosamine (DEN) and ethyl methanesulphonate (EMS) in selected tissues of the transgenic marker mice. The mechanistic studies involved the quantification of the two major DNA-ethylation products, $O^6$-EtG and N7-ethylguanine (N7-EtG) in the genomic DNA, the measurement of the

absence of individual adducts with an increase in MF in the [illegible]
obtained in these studies with transgenic marker mice will be discussed in a broader context.

## 6.2 Gene-mutation assays: *in vitro vs in vivo*

Through the years, many systems have been devised to assay the mutagenicity of chemical agents. The use of bacterial and mammalian *in vitro* cell systems for this purpose is widespread. As has been repeatedly stated in this thesis, the information offered by the available *in vitro* assays in the screening of chemical agents on potential mutagenicity and/or carcinogenicity is limited in value when one wants to extrapolate the findings to whole animals. Part of the restriction stems from the small number of mammalian cell types suitable for such analyses. Differences that exist between various cell types with respect to detoxification/activation and DNA repair, to name a few, in addition to the absence, *in vitro*, of processes such as distribution and excretion, make it difficult to envision that these limited assays can serve as general indicators of mutagenicity and carcinogenicity for mammals like man. The advent of transgenic marker mice carrying a target sequence in all somatic and germ cells has expanded the scope of mutagenicity testing. These short-term *in vivo* test-systems provide the possibility to test agents *in vivo* and, more importantly, in a cell type or organ-specific manner. Thus, they have the capability of producing more relevant data pertaining to mutagenic and presumably carcinogenic effects in target organs. Although it should be realised that results obtained with the transgenic rodent models can, at best, provide an estimation of the actual damaging capacity of the agents in man, it is to be expected that these models will prove to be of great value to the genetic toxicologist, in particular with respect to mechanistic aspects.

## 6.3 Transgenic marker mice

Transgenic marker mice such as the 40.6 (Muta™Mouse) and Big Blue™ strains carry 80 copies of, respectively, a *lac*Z$^-$ and *lac*I-containing shuttle vector situated as two 40-mer concatemers in the mouse genome (Gossen *et al.*, 1989; Myhr, 1991; Kohler *et al.*, 1991). The Xenomouse™ harbours 40 copies of the *lac*Z containing plasmid per diploid cell (Gossen *et al.*, 1995). In these animals, the gene-mutation frequency in the *lac* sequences can be obtained for any organ, provided sufficient DNA can be isolated. With the collection of MF data at different time points in combination with data on DNA-adduct levels and the nucleotide sequence of the induced mutations, the entire process of adduct formation, repair and mutation fixation can be followed. Long-term carcinogenicity studies can also be performed in parallel when a correlation between the mutation load and tumour incidence is to be made.

### *6.3.1 Positive selection system*

The determination of the MF in the *lac* sequences in *lac*Z or *lac*I transgenic marker mice involves the packaging of the individual shuttle vectors, *in vitro,* into viable $\lambda$ particles. The phages are subsequently spotted onto a suitable *E. coli* strain and screened for the absence of an active *lac*Z or *lac*I gene product, respectively. For *lac*Z phages, the original method (X-gal-based) involved the visual discrimination of a few mutated white plaques amongst a multitude of blue ones; this has proven to be very laborious, time-consuming and rather inaccurate. The visual identification of mutant plaques appeared a tricky task that formed a source of variability, the extent of which was investigator dependent. In reconstruction experiments, Gossen (1993) found that the efficiency of visual detection decreased quite rapidly with an increase in plating density.

A major step forward was the introduction of the P-gal-based positive selection system (Chapter 2). This led to a drastic decrease in time and cost of MF determinations. With the original X-gal based system, approximately 14,000 phages could be screened on an *E. coli* lawn on a 500 $cm^2$ plate. With the P-gal system developed during our studies, a single plating on a 9-cm dish permits the screening of more than $10^6$ phages. The main difference between the two assays is the presence of selective pressure applied in the new method resulting in the propagation of only the *lac*Z mutants. The problem of variation introduced by the inaccurate visual identification of mutant plaques has been eliminated since all plaques formed in the presence of P-gal are mutants.

The introduction of the new method necessitated a validation study comparing the two systems, which is described in Chapter 3 of this thesis. In ENU-treated animals, the MF determined with X-gal tended to be slightly lower than that found with P-gal, whereas the opposite was true after treatment with benzo(a)pyrene. This may be due to differences in the type of mutations induced by the two agents. The specificity of $\beta$-galactosidase is likely to differ for the two substrates X-gal and P-gal and consequently different types of *lac*Z mutations induced by the two agents may have different effects on the $\beta$-galactosidase protein. Although minor variations were observed between the X-gal and P-gal derived data, they were sufficiently small and statistically insignificant so as to warrant direct comparison of the results of both assays. On the whole, the P-gal positive selection assay is much more efficient in terms of resources and hence cost and time compared to the X-gal-based method. It is presently being employed by most investigators who make use of the MutaMouse transgenic mouse strain.

### *6.3.2 Sources of variability in MF determinations*

The quantification of the MF in the *lac* sequences comprises a number of steps each of which contributes to a certain extent some variability to the final data. Studies carried out with Big Blue mice have shown that when DNA is packaged in a single *in vitro* packaging

et al. (1994, 1995) were not able to gather [illegible]
an increase in MF was observed with an increase in the length of time between DNA isolation and packaging. Such increases were also observed in the present work. In some instances, in which 12 month old isolated DNA stored at 4°C was packaged and the resulting MF data compared to that obtained with the same DNA shortly after isolation, MF values were up to 20-times higher in the former DNA sample. A possible explanation for this could be a high level of oxidative damage in DNA that has been stored at 4°C for extended periods of time (Piegorsch *et al.*, 1994). This source of variability can easily be avoided by limiting the time between DNA isolation and packaging to a few weeks. Yet another source of variability is that introduced by differences in batches of packaging extracts which can be circumvented by adhering to a "blocked design" of the study. In such studies all DNA samples within an experiment are isolated simultaneously and packaged with the same batch of extracts an equal number of times. The experiments described in this thesis were not carried out in this fashion since most results of the present study had been obtained before data on these possible sources of error were available.

With regard to the number of phages that should be screened for an accurate estimation of the MF, Tinwell *et al.*, (1994) determined that the cumulative MF, after repeated packagings, stabilised after testing 200,000 to 300,000 phages. Similarly, the same number of phages need to be tested in each of at least 5 animals for a two-fold increase over the background to be detectable with a statistical significance (Callahan and Short, 1994).

### *6.3.3 Role of DNA lesions in λ phages*

An important point that deserves clarification concerns the fate of the adducts still present in the shuttle vectors at the time of packaging. DNA lesions in *lac*Z are also premutagenic in *E. coli*. The question that remains to be answered, therefore, is whether an increased MF, observed shortly after treatment, is due to mutation fixation in the mouse or in *E. coli*. Should the DNA damage result in a mutation in *E. coli*, MF measurements taken shortly after treatment should be higher in treated than in control animals. Data presented in Chapter 5 do not support this notion. In neither liver nor brain DNA from mice treated with ENU or EMS at, respectively, 1.5 h and 24 h after treatment, was a significant increase in MF found, while $O^6$-EtG and N7-EtG lesions were detected in the DNA. In bone marrow of mice treated with EMS, an MF increase was evident at 4 h after exposure. In view of the high proliferation rate in bone marrow, early mutational events in the treated mice appears the likely explanation, because of the absence of mutation induction with the adduct-containing liver and brain DNA.

In the *lac*I mouse system, mutant plaques lack an active *lac*I repressor protein and thus appear blue on X-gal because repression of the *lac*Z gene is absent. It has been observed that some plaques appear "sectored". Sectored plaques contain blue-coloured

regions (*lac*I$^-$ phages) and colourless regions (*lac*I$^+$ phages). In the liver of untreated *lac*I mice the frequency with which sectored plaques occur is equivalent to 46% of the control MF. This frequency increased 10 d after treatment with ENU (Piegorsch *et al.*, 1995). It is believed that these plaques stem from mutations fixed in *E. coli* and not in the mouse.
In *lac*Z mice, mutant *lac*Z$^-$ phages plated onto X-gal-containing plates appear white. Therefore, if a mutation occurs in what was originally a wild type *lac*Z phage during plaque formation, the white "mutant sectors" that are formed may go undetected against a dark blue background. Most likely, only when mutations occur very early in phage DNA replication, *i.e.* during the initial infection (first infection that will lead to a plaque), will the mutation be visualised as a light blue plaque in the presence of X-gal. Similarly, in the P-gal assay, these "mixed-plaques" that produce a mixture of active and inactive $\beta$-galactosidase will often be repressed. However, early mutational events may lead to plaque formation in this system. This is believed to be the cause of the high MF observed in 12-month old DNA isolates, most probably caused by high amounts of oxidative damage accumulated during storage at 4°C. Although an increase in mutants formed in *E. coli* can occur in DNA with very high adduct levels, at the doses employed in our experiments no indication for the occurrence of such mutants was found (Chapter 5).

### *6.3.4 Spontaneous mutant frequency*

The mutagenicity data obtained with the *lac* transgenes have been found to agree with other gene mutation results. The mutation induction curves in *hprt* and the *lac*I loci in Big Blue were found to be very similar (Skopek *et al.*, 1995, 1995b). A difference noted, however, was the 10-fold higher background encountered in the *lac*I loci which reduces the sensitivity of mutagenicity assays based on this locus. Similar observations were made in germ cells of untreated *lac*Z mice in comparison to the specific locus test (van Delft and Baan, 1995). The higher background in the *lac* sequences relative to *hprt*, which hampers the detection of small increases in MF, is believed to be due to a number of factors. One of these is the higher methylated state of the *lac* sequences compared to the endogenous genes (Palmiter *et al.*, 1982), which results in a higher probability of methylcytosine deamination and hence GC → AT transitions at CpG sites. This would be strengthened by the 10-fold higher number of CpG sites in *lac*I compared to *hprt* (Skopek, 1995b). If this explanation is valid, it would be expected that the spontaneous frequency of GC → AT transitions is much higher in *lac*I than in *hprt*. In the spontaneous mutation spectrum in the spleen of *lac*I mice, 41% are GC → AT transitions (de Boer *et al.*, 1996). Indeed, the proportion of this type of transition in the spontaneous mutation spectrum in *hprt* obtained from splenic T-cells is lower (Walker *et al.*, 1994; Gorelick, 1995). Another source of the increased background MF in *lac* genes is the endogenously formed DNA damage that is believed to be less rapidly repaired in the inactive *lac* sequences. Spontaneous mutations

phages which screened with the P-gal assay (unpublished data). Since the plating [illegible] was performed in the absence of selective pressure, the frequency of 14 $x10^{-6}$ is an overestimation of the actual frequency of mutants formed during the P-gal assay itself. This process, therefore and thus forms only a minor portion of the mutants found in our studies.

## 6.4 Relevance of *lac*Z for host sequences

A relevant question that must be addressed when employing marker mice in mutagenicity tests concerns the representativeness of the exogenous DNA for the endogenous sequences. In the 40.6 transgenic mouse strain the two concatemers of 40 copies of the shuttle vector amount to 4.1 Mbp, corresponding to 0.1% of the diploid genome. Large stretches of foreign DNA may adopt an altered configuration and have different adduct formation and repair rates compared to endogenous sequences. Differences of this kind could have an effect on mutagenesis in the *lac* sequence.

### *6.4.1 $O^6$-EtG repair in lacZ versus overall genomic DNA*

The ability of a cell to remove a premutagenic lesion such as $O^6$-alkylguanine prior to DNA replication is vital in the prevention of mutagenesis. The $O^6$-alkylguanine-DNA-alkyltransferase (AGT) protein is one repair factor that plays an important role in $O^6$-alkylguanine mutagenesis, as is illustrated by, *e.g.,* the lack of *N*-methyl-*N*-nitrosourea (MNU)-induced thymic tumours in transgenic mice that over-express the human AGT gene in T-cells (Dumenco *et al.*, 1993). The level of endogenous AGT expression in T-cells in normal mice is low and apparently not sufficient to prevent mutation induction by MNU.

In the liver of 40.6 transgenic mice treated with ENU, the initial $O^6$-EtG level measured in genomic DNA in the absence of repair at 1.5 h after treatment, was found to be higher than the corresponding level in *lac*Z. In these mice, dealkylation by AGT had been suppressed. In "normal" mice, at 1.5 h after treatment, genomic and *lac*Z DNA showed an equal $O^6$-EtG level. Evidently, genomic DNA had been repaired at an higher rate than the *lac*Z sequences. This difference in repair did not continue, since at all later time points the levels of $O^6$-EtG in *lac*Z and genomic DNA were comparable. The similarity of $O^6$-EtG repair in *lac*Z and genomic DNA after the initial 1.5 h period suggests that mutations will be formed with equal frequency in the two DNA fractions. The difference in the liver, observed only during the first 1.5 h, will most likely have little effect on mutation induction as very few mutations will be formed within this short period. In the brain, the initial $O^6$-EtG level in *lac*Z and genomic DNA were similar. Very little repair was observed in the brain. These results indicate that $O^6$-EtG-induced effects found in the *lac*Z loci provide a good estimation of what happens in the average genomic sequences as far as they depend on the level of this adduct.

### 6.4.2 *$O^6$-EtG repair in active versus inactive genes*

The mutagenicity of adducts of importance with respect to carcinogenesis are those found in the small part (<1%) of the genome that consists of potentially transcribed sequences, both active and inactive. In rat hepatoma cells, Thomale *et al.*, (1994) found a higher initial $O^6$-EtG content in the actively transcribed $\beta$-actin sequence compared to the overall genomic DNA and transcriptionally inactive IgE sequences. The repair rate was also highest in the $\beta$-actin gene. The increase in $O^6$-EtG formation and the higher repair rate in active *vs.* inactive genes supports the idea that actively transcribed stretches of DNA are more accessible to both genotoxins and repair proteins (Boffa *et al.*, 1992; Thomale *et al.*, 1994). Such influences of transcriptional status on repair stress the need to perform a comparative study of repair in *lac* sequences and an active sequence, *e.g. dhfr gene*, to determine whether mutagenesis in the transgene is comparable to that found in trancriptionally active sequences.

### 6.4.3 *Mutagenesis in transgene versus host sequences*

In mammalian cells, $O^6$-EtG is largely repaired by interaction with the AGT. Differences between transcriptionally active *versus* inactive sequences have previously been observed for AGT-mediated $O^6$-EtG repair (Thomale *et al.*, 1994). Consequently, assuming that mutation induction due to the lower level of repair is higher in *lac*Z, the MF results obtained in *lac*Z will tend to be an overestimation of that pertaining to active genes. The sensitivity of a target gene depends on its "mutability". This is defined as the number of bases that can be mutated and, when mutated, will result in a functionally altered gene product. When comparing the *lac*I gene to the *hprt* sequence, the former was found to contain 329 and the latter 305 sites that lead to a mutant phenotype (Skopek, 1995b). Comparative analyses of ENU-induced mutagenesis in *hprt* and *lac*I (Walker *et al.*, 1994; Skopek *et al.*, 1992), have been performed. Little difference in the results was found. This outcome was a bit surprising considering the fact that the *hprt* is transcriptionally active and should experience an increased clearance of most lesions and a lower mutation rate.

Tao *et al.* (1993) compared the endogenous *dlb*-1 gene and *lac*I in hemizygous *lac*I/*dlb*-1 mice. Very little difference in ENU-induced mutagenesis was observed between the two loci. These two loci can not be directly compared since the gene(s) in the *dlb*-1 locus have not been characterised as has been done for *hprt*. The "mutability" of the *dlb*-1 gene(s) is thus unknown and therefore any conclusions drawn from the comparison regarding differences in repair rates would be speculative.

A comparison between *lac*Z germ cell data obtained with ENU-treated mice (van Delft and Baan 1995) and that generated with the specific locus test (SLT) (Favor *et al.*, 1990; Russell *et al.*, 1982) also indicated strong similarity. In this case, however, the set of genes studied in the SLT assay are assumed to be transcriptionally inactive in the germ cells [illegible] fertilization. Again the mutability of these genes are not

base excision or nucleotide excision repair pathways (NER). The aforementioned comparisons were carried out with chemicals that induce small lesions in the DNA. The extent to which NER is involved in repair of these lesions is relatively small compared to the repair of bulky lesions. Also for the more bulky lesions subject to NER, a difference with respect to repair is expected between the endogenous transcriptionally active gene and the inactive *lac*Z loci. It would be interesting, therefore, to carry out similar comparisons with agents inducing lesions predominantly repaired *via* the NER pathway, in the expectation that large differences in MF will be present in that case.

Another point is that the published studies in which a comparison was made between the *lac* genes and endogenous sequences were restricted to rapidly proliferating tissues, which may have had an effect on the MF. Work presented in this thesis (Chapter 5) has shown that differences in mutagenicity of particular adducts do exist between tissues as was, for instance, the situation in the liver and bone marrow of transgenic mice treated with ENU. Therefore, during the evaluation of the mutagenic properties of an agent, an emphasis must be put on determining its mutagenicity in several tissues differing, among others, in proliferation.

## 6.5 Mutagenicity of the model agents ENU, DEN and EMS

The 40.6 transgenic mouse strain was employed as an animal model to study ethyl-DNA adduct-mediated mutagenesis in the liver, bone marrow and brain of animals treated with the model alkylating agents ENU, DEN and EMS (Chapter 5). The choice of ENU and EMS as model agents was based on the large difference in $O^6$/N7-EtG adduct ratio formed in the DNA by the two agents (ENU: 0.4; DEN: 0.02; Beranek, 1990). DEN produces the same $O^6$/N7 ratio as ENU but requires metabolic activation. This allowed the study of organ-specific effects. The liver, bone marrow and brain were chosen because of their differences with respect to $O^6$-EtG repair, rate of cell proliferation and metabolic activity.

The determination of the mutation spectra, the sequence alterations in the DNA, after treatment of cells with a genotoxic agent, provides information regarding the adduct responsible for the increase in MF. Some adducts are difficult to detect and hence their presence can be revealed only indirectly by their mutational signature. The interpretation of these spectra is based on published work in which the mutagenicity of specific adducts has been determined *in vitro* through the use of plasmid DNA carrying a single adduct at a predetermined position. The DNA is introduced into mammalian cells, isolated after a number of cell divisions and the sequence is determined around the adducted position. The mutagenicity of adducts such as $O^6$-EtG, $O^2$- and $O^4$-ethylthymine have been determined *in vitro* in this fashion; these lesions have been found to induce GC → AT, TA → AT and TA → CG mutations, respectively (Ellison *et al.*, 1989; Grevatt *et al.*, 1992; Klein *et al.*, 1990).

Unfortunately this has been done for only a very limited variety of DNA lesions. This restricts the amount of information that can be obtained from mutation spectra with regard to the origin of mutations.

### *6.5.1 ENU, DEN and EMS mutagenesis in liver, bone marrow and brain*

Of the three agents employed, ENU and DEN were found to be liver mutagens. Other investigators, too, have shown these two agents to be mutagenic in the liver of both *lacZ* and *lacI* mice (Myhr, 1991; Hoorn *et al.*, 1993; Suzuki *et al.*, 1993 and 1994; Monroe *et al.*, 1993). EMS, which induces predominantly N7-EtG adducts, was not found to be mutagenic in the liver which is in accordance with the findings by Suzuki *et al.* (1994). $O^6$-EtG and N7-EtG levels in the DEN-treated mice were approximately twice those in the ENU-treated animals. The mutation induction curves for these agents were essentially parallel, as would be expected of two agents producing identical adduct spectra (Beranek, 1990). When comparing the mutation-induction curves in liver with the amounts of $O^6$-EtG and N7-EtG at various time points, the largest increase in MF was observed after the disappearance of most of the $O^6$-EtG lesions. The presence of high N7-EtG levels as a consequence of EMS treatment, was not accompanied by an induction of mutations. These findings suggest that the mutation induction is largely due to DNA lesions other than $O^6$-EtG and N7-EtG. DNA-sequence analysis of liver-derived mutant phages indicated that only 19% of the mutations were $O^6$-EtG-induced GC → AT transition mutations. The majority of mutations were presumably $O^2$-ethylthymine-induced TA → AT (33%) transversions and GC → TA (29%) mutations of unknown origin. The sequence data has been corrected for mutations arising spontaneously, such as those following deamination of methylcytosine at CpG sites, which yield thymine, and hence GC → AT transitions.

In bone marrow, ENU was strongly mutagenic (Chapter 5) as was also previously found (Myhr, 1991; Suzuki *et al.*, 1993 and 1994; Hoorn *et al.*, 1993). DEN, not metabolically activated in the bone marrow, did not form adducts or cause an increase in MF in agreement with previous findings (Suzuki *et al.*, 1994). Very high levels of N7-EtG were formed by EMS but only a slight increase in MF was observed. Similar results were also obtained by Suzuki *et al.* (1994). After exposure to ENU, the initial $O^6$-EtG content in the bone marrow was half that found in the liver. When the mutation induction curve for bone marrow was compared to the $O^6$-EtG and N7-EtG levels at several post-treatment periods, it was concluded that possibly $O^6$-EtG is a major premutagenic lesion in bone marrow, but other premutagenic lesions could be excluded. Sequence data obtained from the mutant phages retrieved from the bone marrow at 3 d after ENU treatment indicated that 41% of the mutations likely originated from $O^6$-EtG, which is a factor 2.2 higher than in the liver at 28 d after treatment. TA → AT (23%) and GC → TA (14%) transversions were the next most frequent types of mutations. In the ENU-treated mice a maximum mutation induction in bone marrow was observed at 3 d after treatment. The MF level stabilised between day

renewal within the bone marrow cell population [illegible]
induction in mutations was observed differed between liver (28 d) and bone marrow (3 d), most likely because of the relatively high proliferation rate in bone marrow that leads to the rapid fixation of mutations.

The apparent difference in mutagenicity of $O^6$-EtG in liver and bone marrow can be explained in terms of rates of DNA repair and cell proliferation. Repair of $O^6$-EtG lesions is expected to proceed at a higher rate in liver relative to bone marrow owing to higher levels of AGT in the liver (data not shown). According to the $O^6$-EtG measurements, all $O^6$-EtG had been repaired in the bone marrow at 3 d after treatment. Bone marrow has a high level of DNA replication (mitotic index = 4% [Ghosh *et al.*, 1990] compared to a labelling index of 0.07% in the liver [Mirsalis *et al.*, 1994]), as is also illustrated by the high mutation induction at 3 d, as a consequence, the low level of $O^6$-EtG measured may, in part, be attributed to a dilution effect due to the high cell turn-over. Thus, the shorter expected persistence of $O^6$-EtG in liver DNA coupled to a lower cell turn-over rate results in a lower level of mutation fixation compared to the bone marrow. This is in agreement with carcinogenesis data obtained in ENU-treated mice. In these animals thymic tumours that originate from T-cells formed in the bone marrow, are much more frequent than liver tumours (IARC, 1978). The need for DNA replication in mutagenesis and carcinogenesis is supported by the observed high incidence of brain tumours in the offspring of pregnant rats transplacentally exposed to ENU (Goth and Rajewsky, 1974). Adult rodents do not show this sensitivity, probably owing to a lack of cell proliferation in the brain. The apparent need for mitogenesis in mutation fixation was also observed in our studies on the brain DNA of transgenic mice. In genomic brain DNA, both $O^6$-EtG and N7-EtG were detected in mice treated with ENU, and N7-EtG in EMS-treated mice, at similar concentrations as found in the bone marrow, but no MF increase was noted. This again illustrates the important role of cell proliferation in mutagenesis.

## 6.6 Lesion-specific mutagenesis: *lacZ vs. hprt*

The mutagenic effects of DNA adducts are not only dependent on the location of these lesions in the DNA but also on their persistence. The latter is largely determined by the efficacy of repair, which varies for the different adducts and also depends on the location in the genome. The resulting persistence of some lesions can be an important determinant in their mutagenicity *via* miscoding or other mechanisms.

### *6.6.1 $O^6$-EtG as a mutagenic lesion*

The sequence data of the *lacZ* mutants from the liver (28 d after treatment) and bone marrow (3 d)(Chapter 5) indicated a higher proportion of $O^6$-EtG-mediated mutagenesis than was found in the *hprt* locus of lymphocytes (28-32 d), splenic T-cells (42

d) and skin fibroblasts (2 d + 7 d *in vitro*) taken from mice and rats treated with the same agent (Skopek *et al.*, 1992; Jansen *et al.*, 1994 and 1995). Despite the differences in expression time between the lymphocytes, splenic T-cells and skin fibroblasts, their mutation spectra were still very similar. The *hprt* mutation spectra were obtained at a minimum of 9 d after treatment when probably most adducts were absent from the DNA and no further fixation occurred. The *lac*Z mutation spectrum in the bone marrow was obtained shortly after treatment (3 d), and it can not be excluded that it may have contained a lower proportion of GC → AT mutations had the spectrum been determined after a longer expression time. Nevertheless, an assumed slower removal of $O^6$-EtG from *lac*Z relative to *hprt* correlates well with the higher GC → AT mutation frequency in *lac*Z.

### 6.6.2 *$O^2$-alkylthymine as a mutagenic lesion*

Another adduct formed in appreciable amounts after ENU-treatment *in vivo* is $O^2$-ethylthymine. Data obtained from *in vivo* studies point to $O^2$-alkylthymine as being the most effective mutagenic alkyl lesion. Although $O^6$-EtG *per se* is the more mutagenic of the two, in AGT-proficient cells the persistence of $O^2$-alkylthymine, which is not repaired by AGT, exposes this lesion to more rounds of DNA replication than $O^6$-EtG. The $O^2$-ethylthymine-induced TA → AT transversions (45-55%) found in *hprt* originated mostly from the non-transcribed strand (Jansen *et al.*, 1994 and 1995). In the *lac*Z locus (Chapter 5), about 25% of the mutations were TA → AT transversions after treatment with ENU. Although this is lower relative to the frequency determined in *hprt*, the higher incidence of GC → AT transitions in the *lac*Z locus, which may be attributed to less efficient $O^6$-EtG repair in this silent gene, reduces the relative amount of $O^2$-ethylthymine-induced mutations. The mechanism responsible for the removal of $O^2$-ethylthymine is as yet unknown, although the strand bias observed in *hprt* (Skopek *et al.*, 1992; Jansen *et al.*, 1994, 1995) implicates a strand-specific mode of repair, possibly by NER. Gene- and strand-specific repair studies, therefore, need to be carried out to elucidate the kinetics of $O^2$-alkylthymine removal in active sequences.

### 6.6.3 *$O^4$-ethylthymine as a mutagenic lesion*

*In vivo* data from rat liver shows the half-life of $O^4$-methylthymine to be much shorter (<4 h) than that of $O^4$-ethylthymine (14 d) (den Engelse *et al.*, 1986). Mouse AGT may play an important role in repair of $O^4$-methylthymine in view of effects recently found *in vitro* (Kawate *et al.*, 1995). The long half-life of $O^4$-ethylthymine, however, indicates a lack of efficient AGT repair for this lesion. Klein *et al.* (1990) demonstrated that the removal of $O^4$-ethylthymine proceeded through NER. Despite the prolonged presence of $O^4$-ethylthymine in total genomic DNA, the lack of AT → GC type mutations in *lac*Z and *hprt* after ENU-treatment suggests a low mutagenicity of this adduct *in vivo*.

marrow *lac*Z mutations, respectively, still requires attention, *viz.* GC → TA transversions. These mutations occurred at a lower frequency in the *hprt* locus of both T-lymphocytes and fibroblasts (Skopek *et al.*, 1992; Jansen *et al.*, 1994, 1995) than in our *lac*Z transgenic mice (Chapter 5). Interestingly, this mutation has been found in 50% of activated H-*ras* in mouse liver tumours following DEN treatment (Stowers *et al.*, 1988). Little information is available in the literature regarding the origin of these mutations. Possible candidates include $O^2$-ethylcytosine and apurinic/apyrimidinic-sites. $O^2$-ethylcytosine is found initially at a 1/4 of the level of $O^6$-EtG after ENU treatment (Beranek, 1990) but is much more persistent in genomic liver DNA ($t_{½}$= 3.6 d; den Engelse *et al.*, 1986). The difference in frequency of GC → TA mutations in the active *hprt in vivo versus* the inactive *lac*Z, suggests a gene-specific mode of $O^2$-ethylcytosine repair, possibly involving NER.

### *6.6.5 Mutagenicity of persistent lesions*

The measurement of adduct levels at various post-treatment times in combination with the MF induction is potentially a powerful tool to study the mutagenicity of an individual adduct. The methods to determine adduct levels with the use of HPLC or immunoslotblot have a detection limit around 1 adduct per $10^6$ nucleotides. A small number of lesions in specific sequences may persist below the detection limit and go undetected. These persistent lesions may impose a threat to the cell. From our present work, in the liver of ENU-treated mice, at day 3, more than 90% of the $O^6$-EtG adducts had been repaired and the MF increase at that time point was 34 mutants per $10^6$ phages relative to the control mice. At 28 d, the induction was 273 mutants per $10^6$ phages. Therefore, of the total induction at 28 d (assuming the absence of clonal expansion) at most 1/8 (34/273) of the mutants could have been $O^6$-EtG-induced. However, sequence analysis indicated that between 1/4 and 1/5 of the mutations fixed were GC → AT transitions. This could mean that either other lesions are responsible for the excess of these mutations or that the persistence and mutagenic effect of the "few" $O^6$-EtG left unrepaired at 3 d is higher. Differences in the repair (15-fold) of UV-induced DNA damage has been observed to occur between adjacent nucleotides (Gao *et al.*, 1994). It is thus believed that such "hot-spots' of mutations are not due to preferentially damaged nucleotides but to slower repair (Kunala and Brash, 1992; Tornaletti and Pfeifer, 1994).

## 6.7 Transgenic marker mice in mechanistic studies

A number of evaluation studies concerning the use of transgenic marker mice have been published recently (Mhyr, 1991; Mirsalis *et al.*, 1994; Morrison and Ashby 1994; Gorelick, 1995). In these papers issues such as proliferation, dosing regimens and fixation time are discussed in detail. In the present study relatively high doses close to 50% of the

$LD_{50}$ were employed. Although these do not resemble the doses humans are exposed to, these high doses are required in mechanistic studies to obtain a MF increase that is sufficiently high relative to the background and to obtain a reasonable induction of non-spontaneous mutants that can be sequenced. In the liver of ENU-treated mice, only a 6-fold increase in MF was observed at 28 days after treatment. This implies that 1 out of every 6 mutants is spontaneous in origin. A lower ENU dose, which results in a lower mutation induction, would require a higher number of mutants to be sequenced in order to achieve statistical significance. In the bone marrow where the increase in MF was 23-fold, a lower treatment dose would have been justified. The stabilisation of the MF between day 3 and 14, also observed in other studies (Mhyr, personal communication), could be due to the short life time of bone-marrow cells. After repair of the adducts has occurred, the rate at which mutant cells are formed is equivalent to rate of cell death or migration away from the bone marrow. Possible ENU-induced toxicity could also have played a role as reflected in the small amount of DNA that could be isolated from the cells at the later time points (Chapter 5). Hoorn *et al.* (1993) used lower doses of 20 and 50 mg/kg and did observe a linear increase up to 10 d. This in an indication that at lower less toxic doses, ENU can induce a linear increase in bone marrow. In EMS-treated mice, bone marrow DNA isolation was also less efficient at later time points. This may have the result of cytotoxic effects. The fact that no "jackpot" mutations (multiple mutations at one site in the same animal) were obtained does suggest that the haematopoietic stem cells were not involved, at least not during the period after which the mutants were isolated (3 d). Longer expression times may be required to pick up stem cell-derived mutants.

The fact that differences in mutagenicity of $O^6$-EtG between the liver and bone marrow indicates the importance of studying mutagenicity in various organs and tissues. The transgenic marker animals offer an excellent *in vivo* model in which this can be carried out.

## 6.8 Performance of marker mice

A question that remains to be discussed concerns the extent of agreement of transgenic mouse data with the results of other short-term *in vivo* mutation assays that are currently used to predict mutagenicity and carcinogenicity. One such test is the micronucleus (MN) induction assay carried out on the bone marrow and peripheral blood of mice (Mavournin *et al.*, 1990). Suzuki *et al.* (1994) determined the mutagenicity of the alkylating agents ENU, DEN and EMS in the liver and bone marrow of *lac*Z mice with the positive selection assay and the MN assay. In the bone marrow, ENU (100 mg/kg) and EMS (400 mg/kg) were found to induce both *lac*Z gene mutations and MN. The mutation induction was 7- and 6-fold, respectively, after a 7 d expression period. In the present study, a high induction in MF in the bone marrow (23-fold) was observed after application of ENU (150 mg/kg) and a much smaller 2-fold increase was observed with EMS (250 mg/kg)

The absence of mutation induction in the bone marrow, [illegible]
metabolism, should also apply to the MN test: indeed no induction in MN was observed (Suzuki *et al.*, 1994). Although for these alkylating agents the *lac*Z and MN data agree, it is not always the case. Transgenic *lac*Z mice treated with the known clastogen mitomycin C (MMC), gave a strong MN induction and little or no increase in *lac*Z mutations (Suzuki *et al.*, 1993, 1994). MMC is believed to predominantly produce large chromosomal aberrations that are probably not detectable in the marker mice. The latter phenomenon is analogous to the lower mutation induction measured in *lac*Z *versus dlb*-1 after X-irradiation, which is also believed to predominantly produce deletions (Tao *et al.*, 1993).

Table 1: *Performance of the λlacI and λlacZ transgenic mouse mutation assays in predicting rodent carcinogenicity (from Gorlick, 1995).*

| Characteristic | response | |
|---|---|---|
| Big Blue™ | | |
| Sensitivity | 67% | (12/18) |
| Specificity | 100% | (3/3) |
| + Predictivity | 100% | (12/12) |
| – Predictivity | 33% | (3/9) |
| Overall concordance | 71% | (15/21) |
| Overall concordance for the Salmonella/microsome test for this set of chemicals | 67% | (14/21) |
| Muta™Mouse | | |
| Sensitivity | 91% | (10/11) |
| Specificity | 100% | (1/1) |
| + Predictivity | 100% | (10/10) |
| – Predictivity | 50% | (1/2) |
| Overall concordance | 92% | (11/12) |
| Overall concordance for the Salmonella/microsome test for this set of chemicals | 83% | (10/12) |

Gorelick (1995) carried out a comparative study on data obtained with the two most common transgenic marker systems and Salmonella/microsome test (Sal) and carcinogenicity data. A variety of agents were used in the analysis and included alkylating agents and the non-mutagenic non-carcinogen acetone (Table 1; Gorelick, 1995). Carcinogens, defined as agents found to be carcinogenic in the mouse bioassay, were divided into mutagenic (Sal+) and non-mutagenic (Sal-). The same was done for the non-carcinogic substances. In the MutaMouse and Big Blue animals, respectively, 91% and 67% of the carcinogens tested

produced a positive response in the transgenes (sensitivity). The few non-carcinogens tested, both Sal+ and Sal-, did not lead to a response in either system, indicating a 100% specificity. With respect to the predictive capacity of the trangenic mice, all substances that led to a positive result in *lac* sequences were carcinogens. On the other hand, 50% and 33% of the substances that did not give a positive response in the transgenes, were non-carcinogens. The overall concordance of transgenic marker mouse data with that obtained in the mouse cancer bioassay is an impressive 92% and 71% for the *lac*Z and *lac*I mice, respectively.

Shown in Table 2 is a summary of the *lac*Z/I mutation data available on alkylating agents in comparison to MN induction and the rodent cancer bioassay. Also in this comparison, the mutagenicity of agents in different organs as determined in the transgenic marker mice confirms data obtained with both the MN and cancer bioassay. Such high concordance levels between the various assays places confidence in the applicability of the marker mice in mutagenicity testing.

## 6.9 Future prospects

Transgenic marker mice have removed the limitations with regard to the types of cells accessible to mutagenicity studies set by the previously available methods. These systems are still in their developmental stage and more work needs to be done so as to help us interpret results obtained in these rodents and also to improve our understanding of the underlying mechanisms of alkylation-induced mutagenesis. Mentioned below are a few suggestions for future studies.

### *6.9.1 Strand specific repair of $O^6$EtG*

The procedure followed to measure $O^6$-EtG levels in *lac*Z, discussed in Chapter 4, is only capable of detecting $O^6$-EtG adducts in double stranded DNA. At present, no methods are available to measure strand specific repair of $O^6$-EtG. It would be of great interest to modify the procedure to allow the detection of $O^6$-EtG-bearing single-stranded DNA. In combination with transgenic mice that are AGT deficient or mice lacking the *XPA* gene and thus functionally NER deficient (de Vries *et al.*, 1995; Nakano *et al.*, 1995), this procedure could determine the contribution of NER to $O^6$-EtG repair in transcriptionally active *versus* inactive DNA strands. In addition, in conjunction with the ligase-mediated polymerase chain reaction protocol (Gao *et al.*, 1994) (see 1.3.6), $O^6$-EtG repair can be investigated at the nucleotide level within active genes to determine the sequence context in which the more persistent adducts are located.

There is no doubt that transgenic marker mice p... g...
powerful tool to detect and study mutagenicity. It is at present the only system in which organ-specific mutagenic effects can be studied. In this respect, these animal models have achieved their goals. With regard to the sensitivity of these systems, however, they do fall short of the expectations. Due to the relatively high background levels of mutations in the silent transgenes, which are about 10-times that of other gene-mutation systems (*e.g., hprt*), small increases remain undetectable which hinders the detection of mutagenicity at the low doses humans are exposed to. Future designers of transgenic marker animals should construct their target gene such as to reduce the number of CpG sites. This reduces the frequency of spontaneous deaminations at 5-methylcytosine positions, a probable cause of high backgrounds. Also increasing the "mutability" of the target sequences such that more frequent phenotypic changes occur as a result of a mutational event more frequently.

Marker mice presently available do not permit the expression of the *lac*Z product *in situ*. The construction of transgenic mice with a reporter gene under control of a promoter such that the transgene is expressed *in situ* will permit mutation analysis *in situ* in tissue sections. Transgenic mice strains that harbour an active *lac*I-sequence that can repress a *lac*Z gene also present in the genome, could indicate mutations in the *lac*I locus by the formation of blue-coloured cells when tissue sections are bathed in a X-gal-containing solution. This system will allow one to study mutagenesis at the cellular level and obviate the need to study mutagenesis in organ homogenates as it presently practised. It could be used, for example, to study the effects of the varying AGT-repair capacity seen in different types of liver cells. A problem encountered when inserting foreign DNA into eukaryotic cells is the lack of homogeneous expression (Cui *et al.*, 1994). This has been attributed to the hypermethylation of the foreign DNA. It can be avoided by targeting the DNA to a transcriptionally-active site in the genome in combination with a reduction the number of CpG sites in the construct.

### *6.9.2 Crosses of marker mice with other transgenic mice*

Examples of other genetically altered mice that are of interest to the study of mutagenesis include those that are deficient in a repair protein. Mice lacking genes involved in NER (*XPA*-deficient, de Vries *et al.*, 1995; *XPC*-deficient, Sands *et al.*, 1995) have been found to be very susceptible to UV-induced mutagenesis and carcinogenesis. These animals provide a system that is more sensitive to mutagen exposure than wild type mice, owing to an impaired repair capacity. Crosses made between these and marker mice allows one to devise a system that is very sensitive and in which mutagenic events in the transgene can be observed in all organs and tissues. Although of great interest in mechanistic studies on mutagenesis, the problem that remains for mutagenicity testing is the unnatural situation that is created in the repair deficient mice. Unforseen influences of the repair deficiency on other cellular processes may exist.

### 6.9.3 *Extrapolation from rodent to man*

Data acquired with the various mutagenicity assays can be used to prevent human exposure. With respect to approaching the human system, mutagenicity assays have evolved considerably from *in vitro* assays in bacteria, *via* cultured mammalian cells to the *in vivo* situation in *Drosophila* and rodents. It must be taken into account that the data obtained is always an estimation of what can occur in humans since metabolic process such as DNA repair and chemical activation/detoxification differs between rodents and humans. Of interest for mechanistic studies will be the future design of transgenic rodents with human genes that are involved in DNA repair and chemical activation/detoxification.

## 6.10 Conclusions

The advantage of the marker mouse systems over existing *in vivo* gene-mutation assays is the possibility to detect mutagenic effects in any tissue from which sufficient DNA can be isolated. Not only does this provide a means to determine mutagenic activity of agents in somatic cells of importance to human cancers, but also in male germ cells, of concern to hereditary effects. Since the introduction of the *lac*Z mice in 1989 (Gossen *et al.*, 1989) and *lac*I mice in 1991 (Kohler *et al.*, 1991), many compounds have been tested through various application routes and in different organs in these animals. Comparison of the resulting data with that previously obtained from the rodent cancer bioassay indicate a 71% and 92% overall concordance for the *lac*I and *lac*Z mice, respectively (Gorelick 1995)(Table 1,2). The agreement of *lac* gene-mutation data with endogenous gene-mutation data (*e.g., hprt*) and those of genes included in the specific locus test only strengthens the argument in favour of the use of transgenic mice in routine *in vivo* mutagenicity testing.

Transgenic marker mice also provide a means to determine multiple genetic end-points such as MF in *lac* and *hprt* sequences, MN-induction, chromosomal aberrations and tumour induction, which limits the number of test animals needed and enables the direct correlation of these multiple-genetic end-points. The transgenic data available to date have been obtained with relatively strong mutagens at high doses, mainly because of the relatively high background MF. In the design of future marker mice, reporter genes containing less CpG sites should be chosen, with the aim of reducing the spontaneous MF. In addition, constructing rodents with transgenes that permit *in situ* detection of mutations will obviate the need to pass the genetic material through a bacterial host during the MF determination, eliminating any effects induced during DNA isolation, storage, packaging and processing inside the bacteria.

The 40.6 *λlac*Z transgenic mouse provides an *in vivo* system in which mechanistic studies on adduct formation and mutation induction can be performed. The presence of the transgenes in all tissues enables one to investigate organ-specific mutagenicity. The ability

based positive selection assay which has not only simplified mutagenicity testing [illegible] also reduced the cost of it. Present results also indicate that very little difference exists in the formation and repair rates of the premutagenic $O^6$-EtG lesion in *lacZ* and genomic DNA. This gives confidence for the relevance of the data obtained at the *lacZ* locus for mutagenic events elsewhere in the genome. Finally, the agreement of the ethylating agent-induced mutagenicity in our studies with that of other investigators and, more importantly, with the cancer incidence in target organs can only raise the prospects for the future use of transgenic marker rodents.

*)mparison of transgenic mouse, micronucleus and cancer data obtained after exposure to alkylating agents.*

| Tissue | lacI/Z | MN[1] | cancer[1] | *lac*Z/I references |
|---|---|---|---|---|
| liver | + | | + | |
| bone marrow | | + | + (thymus) | |
| spleen | + | | + | Kohler *et al.*, 1991 |
| germ cells | + | | | |
| brain | | | + (very young) | |
| liver | + | | + | |
| bone marrow | + | +[2] | + (lymphoreticular system) | |
| spleen | + | | + | Kohler *et al.*, 1991 |
| germ cells | + | | | |
| brain | – (adult) | | – (adult)/+ (very young) | present work |
| liver | + | | + | Mirsalis *et al.*, 1993; Tinwell *et al.*, 1994 |
| bladder | – | | | |
| liver | + | | + | Suzuki *et al.*, 1994; present work |
| bone marrow | – | –[2] | – | Suzuki *et al.*, 1994; present work |
| liver | – | | – | Mirsalis *et al.*, 1993 |
| bone marrow | | | – | |
| germ cells | + | | | van Delft *et al.*, 1995 |
| small intestine | + | | – | Tao *et al.*, 1993 |
| liver | – | | – | Suzuki *et al.*, 1994; present work |
| bone marrow | + | +[2] | – | Suzuki *et al.*, 1994; present work |
| germ cells | + | | | van Delft *et al.*, 1995 |
| brain | – | | – | present work |
| Liver | – | | – | Myhr, 1991 |
| skin | + | | + | Myhr, 1991; Brooks and Dean, 1995 |
| Stomach | + | | + | Brooks and Dean, 1995 |

*78; 2: Suzuki et al., 1994*

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## Summary

To reduce human exposure to chemical agents that may induce DNA damage and subsequently mutations and cancer, it is important to identify these chemicals. A number of test systems are available to determine whether a chemical is a putative mutagen or not, but these are mostly carried out *in vitro* in cultured bacterial or mammalian cells. The *in vivo* situation, however, differs greatly from that *in vitro* with respect to metabolism, DNA repair, proliferation rate and cell-cell contact, for example. The data obtained with the *in vitro* assays may therefore differ from that obtained *in vivo*. Many mutations have to be attributed to an initial chemical reaction with the genetic material. These mutations usually are changes at the nucleotide level such as base pair substitutions and small deletions or insertions. Mutations also can involve large stretches of DNA, in which case they often manifest themselves as chromosomal aberrations. All of these types of mutations can serve as indicators of mutagenicity. The work presented in this thesis is limited to gene-mutations, *i.e.*, alterations at the nucleotide level.

The transgenic marker mouse strain 40.6 (Muta™Mouse) is used as a short-term *in vivo* mutagenicity test system that can identify mutagenic agents by scoring gene mutations in any organ or tissue. Each diploid cell in these mice contains 80 copies of bacteriophage $\lambda$ DNA ($\lambda gt10$ shuttle vector). Each copy of the bacteriophage $\lambda$ DNA harbours the bacterial *lac*Z sequence, which encodes the enzyme $\beta$-galactosidase. Exposure of these mice to mutagenic agents will result in the formation of DNA lesions in the host DNA as well as in the *lac*Z transgenes. Some of these lesions will give rise to gene mutations. Mutations in *lac*Z will result in an altered $\beta$-galactosidase. The number of times that such an event occurs is referred to as the mutant frequency (MF) and, in relation to the exposure dose, is a measure of the mutagenic capacity of a chemical.

The shuttle vectors can be individually "rescued" from isolated genomic DNA as viable $\lambda$ phages by *in vitro* packaging. To determine whether a mutation has occurred in these $\lambda$ phages, they are tested for the functional integrity of the *lac*Z gene, *i.e.,* whether an active $\beta$-galactosidase can be produced. To this end, the phages are used to infect an *E. coli* host, in the presence of the substrate analogue X-gal which will be metabolized into galactose and a blue-coloured substance when active $\beta$-galactosidase is synthesized in the cells. Upon infection, $\lambda$ phages lyse the bacterial hosts and form plaques in the bacterial "lawn". Phages carrying an inactivating mutation in *lac*Z will form colourless instead of blue plaques. The MF used to be determined *via* this procedure, which is a very laborious and expensive process since it requires the time-consuming identification of white mutant plaques amongst a multitude of blue ones.

The introduction of a positive selection assay developed during our studies proved to be a tremendous improvement over the existing X-gal-based method. In this selection

screening of more than $1x10^6$ phages on a single and [illegible]
X-gal. An ever increasing number of researchers working with the MutaMouse now make use of this method.

Inherent to the introduction of a new system is the follow-up evaluation. A comparative study was carried out with the X-gal and the P-gal assays. A number of mice were treated with the model mutagen N-ethyl-N-nitrosourea (ENU) and the MF was determined in liver and brain DNA with both methods. In addition another group of mice was exposed to benzo(a)pyrene and the MF determined in the liver. In both experiments the values obtained with the X-gal and P-gal assays were not significantly different. It was found, however, that some mutants obtained with X-gal did not form plaques in the P-gal assay. Therefore, a different threshold level of *lac*Z activity exists for both the X-gal and P-gal assay above which phages will not appear as mutants.

One of our aims was to correlate, in organs of transgenic mice, the presence of DNA adducts with the induction of mutations in *lac*Z. In this context, it was important to establish whether or not this foreign gene could be compared to the genomic mouse DNA with respect to the formation of alkylation products and their removal (repair). This required the development of a procedure for the selective detection of adducts in *lac*Z sequences, which subsequently was applied in studies on the $O^6$-ethylguanine levels in liver and brain DNA at different time points after ENU-treatment. Measurements in *lac*Z were performed with an antibody-based protocol that consisted of the enrichment of $O^6$-ethylguanine-containing DNA and the subsequent quantification of the *lac*Z DNA through quantitative polymerase chain reaction. The amount of $O^6$-ethylguanine and also of N7-ethylguanine in the genomic DNA was measured in an immunoslotblot procedure using damage-specific monoclonal antibodies. In the liver the initial induction of $O^6$-ethylguanine in genomic DNA was found to be 1.6-times as high as that in *lac*Z DNA. In the brain about equal levels were observed. At 1.5 h after treatment, however, $O^6$-ethylguanine levels in liver genomic DNA and *lac*Z hardly differed. Evidently, repair in total genomic liver DNA during the first 1.5 h proceeded much faster than that in *lac*Z (removal of 60% and 28%, respectively). The repair profile after this initial 1.5 h was identical for the transgene and host sequences. No such difference was observed in brain DNA. Since the repair profile of the major premutagenic alkyl lesion, *i.e.* $O^6$-ethylguanine, is very similar in both total chromosomal DNA and *lac*Z in liver and brain, except for the initial 1.5 h during which hardly any mutation fixation is to be expected, it appeared likely that *lac*Z would mimic genomic DNA also with regard to the induction of mutations caused by this lesion. The MF measured in *lac*Z, therefore, was considered to yield good estimates of the rate of mutation formation in the total genomic DNA.

By determining the level of DNA damage and the induction of mutations it is possible to correlate the presence of specific DNA adducts to an increase in the MF. The type of mutations induced, as can be determined by DNA sequencing, on the other hand, provides more direct information as to the specific lesion responsible for the mutation. In

this manner the mutagenic potency of individual DNA lesions can be determined. In a comparative study, transgenic mice were treated with three ethylating agents, *viz.* ENU, diethyl nitrosamine (DEN) or ethyl methanesulphonate (EMS). Two major alkylation products are formed in DNA by these agents, *i.e.* $O^6$-ethylguanine and N7-ethylguanine. In treated animals, the levels of these adducts were determined in liver, brain and bone marrow DNA, in addition to the MF, at several time points.

$O^6$-ethylguanine and N7-ethylguanine assayed for in the genomic DNA were found to be present in the liver DNA after ENU- and DEN-treatment but only N7-ethylguanine was detected after exposure to EMS, in agreement with its strong preference of alkylating N-atoms in DNA bases. The presence of a MF increase in the liver after ENU- and DEN-treatment and not after EMS, strongly suggests that $O^6$-ethylguanine is an important premutagenic adduct whereas N7-ethylguanine is not. DNA-sequence analysis of liver-derived *lac*Z mutants 28 d after ENU-treatment indicated that 19% of the induced mutations was a GC → AT type mutation believed to be induced by the $O^6$-ethylguanine lesion. TA → AT transversions occurred most frequently (33%) followed by GC → TA (29%) and TA → GC (14%) transversion mutations. This indicates that $O^2$-ethylthymine, known to cause TA → AT mutations, though less abundant, is more mutagenic in the liver than $O^6$-ethylguanine.

In the bone marrow, adducts were only formed by ENU and EMS. DEN, an indirect acting agent requires P450 enzyme activation to form the DNA-reactive species, and the P450 isozyme responsible for metabolising DEN is absent in the bone marrow. ENU-treatment resulted in relatively low $O^6$-ethylguanine and N7-ethylguanine adduct levels but did induce a 23-fold increase in *lac*Z mutations at 3 d after treatment. At that time $O^6$-ethylguanine adducts were no longer detectable. EMS-treated mice only showed a 2-fold increase in MF despite very high N7-ethylguanine levels. These results indicate that $O^6$-ethylguanine may also be a major premutagenic lesion in bone marrow and confirms the low mutagenicity of N7-ethylguanine. Sequence data showed that 41% of the induced mutations were GC → AT transitions attributable to $O^6$-ethylguanine. Other mutations found were TA → AT (23%) and GC → TA (14%) transversions.

The level of DNA adducts in the brain was determined only in the mice treated with ENU and EMS. Despite the presence of $O^6$-ethylguanine and N7-ethylguanine after exposure to ENU at half the level of that seen in the liver, no increase in MF was observed, not even after 28 d. Very high EMS-induced N7-ethylguanine levels in brain DNA did not lead to a MF increase either.

The difference in the time point at which maximum mutation induction occurred in the liver and bone marrow of mice exposed to ENU can be explained in terms of differences in $O^6$-ethylguanine repair and rate of proliferation. The higher cell turn-over rate in the bone marrow compared to the liver results in more rapid fixation of mutations in the

place in brain tissue.

The mutagenicity data obtained with 40.6 transgenic mice in the present study and those obtained with the *lac* transgenic mice by other investigators agree very well with data acquired with other test systems such as the micronucleus-induction assay and carcinogenicity data. This makes transgenic marker mice an important improvement in the search for short-term *in vivo* test systems suitable for the prediction of the mutagenic potency of genotoxic agents in different organs and tissues.

**Samenvatting**

Om blootstelling aan DNA-beschadigende chemicaliën te beperken en daardoor het ontstaan van mutaties en het optreden van kanker te verminderen, is het belangrijk deze stoffen te identificeren. Dit gebeurt voornamelijk *in vitro*, door gebruik te maken van gekweekte bacteriën en zoogdiercellen. Gekweekte cellen verschillen van die in een intact organisme met betrekking tot bijvoorbeeld metabolisme, DNA-herstel, intercellulair contact en delingssnelheid. Dit maakt het moeilijk *in vitro*-gegevens te extrapoleren naar de *in vivo*-situatie. Als gevolg van chemische modificaties in het DNA kunnen veranderingen op nucleotideniveau optreden, zoals basepaar-substituties en kleine deleties of inserties. Mutaties waarbij grotere stukken DNA zijn betrokken, kunnen leiden tot chromosomale aberraties. Al deze verschillende typen mutaties kunnen gelden als maat voor mutageniteit. Het werk dat in dit proefschrift beschreven staat, beperkt zich tot onderzoek aan relatief kleine gen-mutaties.

De 40.6 transgene marker-muizenstam (Muta$^{TM}$Mouse) is een *in vivo*-diermodel dat geschikt is voor kortdurende mutageniteitstesten, waarin de mutagene werking van stoffen in alle weefsels en organen kan worden bestudeerd. Alle cellen van deze muis bevatten 80 kopieën van bacteriofaag $\lambda$ DNA ($\lambda gt$10-shuttlevectoren). In elk faag $\lambda$-DNA bevindt zich de bacteriële *lac*Z-sequentie die codeert voor het enzym $\beta$-galactosidase. Bij blootstelling van deze muizen aan genotoxische stoffen, zullen beschadigingen optreden in zowel het gastheer- als het *lac*Z-DNA, die vervolgens kunnen leiden tot het ontstaan van mutaties. Een aantal van deze mutaties zal een verandering in de enzymactiviteit veroorzaken. De mate waarin dit voorkomt wordt uitgedrukt als de mutantfrequentie (MF). Deze geeft in relatie tot de blootstellingsdosis een indruk van het mutagene vermogen van een stof.

Na isolatie van genomisch DNA kunnen de individuele shuttlevectoren worden voorzien van een eiwitmantel, d.m.v. "*in vitro*-packaging". Om na te gaan of een mutatie in het *lac*Z-DNA leidt tot een verandering in de enzymactiviteit, worden *E. coli*-cellen geïnfecteerd met de verkregen fagen in aanwezigheid van X-gal, een substraat dat door $\beta$-galactosidase wordt gesplitst in galactose en een blauwe kleurstof. Plaques die gevormd worden na lysis van de bacteriën zijn blauw gekleurd wanneer de infecterende faag een intacte *lac*Z-sequentie bevat en blijven kleurloos wanneer een inaktiverende mutatie in *lac*Z is opgetreden. Door gebruik te maken van dit systeem werd aanvankelijk de MF bepaald, maar deze methode is zeer arbeidsintensief en duur vanwege het feit dat het moeilijk is kleurloze plaques te onderscheiden tussen een zeer groot aantal aan blauw gekleurde plaques.

De introductie van een positief selectiesysteem bleek een grote verbetering te zijn in vergelijking tot de vroegere X-gal-methode. In het nieuwe systeem wordt de vorming van wild-type fagen onderdrukt, waardoor alleen de gemuteerde fagen plaques vormen. In dit [illegible] dat ook als substraat dient voor $\beta$-galactosidase. Door

in vergelijking met de *ca.* 14 000 fagen met de X-gal-methode. Het positieve selectiesysteem (de P-gal-methode) wordt steeds vaker gebruikt door onderzoekers die met de Muta™Mouse werken.

Inherent aan het invoeren van een nieuw systeem is de evaluatie ervan. Een vergelijkende studie werd uitgevoerd tussen de X-gal- en P-gal-methode. Beide werden toegepast om de MF te meten in de lever en hersenen van muizen behandeld met de modelstof *N*-ethyl-*N*-nitrosoureum (ENU). Een andere groep muizen werd behandeld met benzo(a)pyreen, waarbij mutatie-inductie in de lever werd onderzocht. In beide experimenten waren de resultaten verkregen met de X-gal- en P-gal-methode niet significant verschillend. Opvallend was wel dat een aantal mutanten gevonden met de X-gal-methode geen plaques vormde op P-gal-platen. Blijkbaar bestaan er verschillende drempelniveaus van *lac*Z-activiteit waaronder fagen als mutant tevoorschijn komen in aanwezigheid van X-gal of P-gal.

Een belangrijk doel van dit werk was een correlatie te vinden tussen de aanwezigheid van bepaalde DNA-addukten en het ontstaan van mutaties in *lac*Z-DNA in verschillende organen van de transgene muis. De vraag was of de inductie van alkylschade en het herstel daarvan in het transgen-DNA vergelijkbaar waren met die in het gastheer-DNA. Hiervoor is een methode toegepast waarmee specifiek het $O^6$-ethylguanineniveau in *lac*Z bepaald kan worden in het lever- en hersen-DNA van ENU-behandelde muizen. De methode is gebaseerd op het gebruik van monoklonale antilichamen voor de verrijking van $O^6$-ethylguanine-bevattend DNA, gevolgd door quantificering van het *lac*Z-DNA d.m.v. een quantitatieve polymerase-kettingreactie. Ook zijn specifieke antilichamen gebruikt om $O^6$-ethylguanine en N7-ethylguanine te meten in het genomische DNA m.b.v. de immonoslotblot-procedure. De initiële inductie van $O^6$-ethylguanine in het genomische DNA van de lever bleek 1,6x hoger te zijn dan in *lac*Z-DNA. In de hersenen werd geen verschil tussen *lac*Z en totaal genomisch DNA gemeten. Er was ook geen verschil in de lever vanaf 1,5 uur na behandeling. Dit impliceert dat er sprake was van sneller initieel herstel van $O^6$-ethylguanine in genomisch DNA in vergelijking met *lac*Z (een verwijdering van respectievelijk 60% en 28%). Na deze periode van 1,5 uur was er geen verschil wat betreft het transgen- en de gastheer-DNA. Vanwege het feit dat het herstel van een belangrijke pre-mutagene alkyl-lesie, nl. $O^6$-ethylguanine, in lever- en hersen-DNA vergelijkbaar is in het chromosomale-DNA en in de *lac*Z-fractie, met uitzondering van de eerste 1,5 uur, waarin overigens toch weinig fixatie van mutaties wordt verwacht, lijkt het aannemelijk dat de mate waarin $O^6$-ethylguanine-geïnduceerde mutaties worden gevormd in *lac*Z-sequenties vergelijkbaar is met die in totaal genomisch DNA. MF-resultaten verkregen in *lac*Z zijn dus hoogstwaarschijnlijk een goede weerspiegeling van de mutagenese in totaal genomisch DNA.

Het meten van het niveau van DNA-schade en mutatie-inductie maakt het mogelijk een correlatie te vinden tussen de aanwezigheid van bepaalde addukten en de inductie van

mutaties. De bepaling van het type mutaties, d.m.v. DNA-sequencing, geeft meer directe informatie m.b.t. de verantwoordelijke lesie. Om dit te onderzoeken zijn transgene muizen behandeld met alkylerende agentia, nl. ENU, diethyl-nitrosamine (DEN) en ethyl-methaansulfonaat (EMS). Deze agentia veroorzaken voornamelijk $O^6$-ethylguanine en N7-ethylguanine. Addukt- en MF-metingen zijn verricht in lever-, beenmerg- en hersen-DNA, op een aantal tijdstippen na behandeling.

$O^6$-ethylguanine en N7-ethylguanine zijn gemeten in genomisch DNA van de lever na ENU- en DEN-behandeling, maar N7-ethylguanine is alleen gemeten na toediening van EMS, in overeenstemming met de sterke voorkeur van EMS voor N-alkylering. De verhoging van de MF in de lever na ENU- of DEN-behandeling en niet na EMS, suggereert dat $O^6$-ethylguanine een belangrijke premutagene lesie is, in tegenstelling tot N7-ethylguanine. DNA-sequentieanalyse van *lac*Z-mutanten in lever, 28 dagen na ENU-behandeling, laat zien dat 19% van de geïnduceerde mutaties van het GC → AT-type waren, vermoedelijk veroorzaakt door $O^6$-ethylguanine. TA → AT-transversies waren de meest voorkomende mutaties (33%), gevolgd door GC → TA- (29%) en TA → GC-transversiemutaties (14%). Dit suggereert dat $O^2$-ethylthymine, de oorzaak van TA → AT-mutaties, ondanks het lagere niveau, sterker mutageen is in de lever dan $O^6$-ethylguanine.

In beenmerg werden addukten alleen gevormd door ENU en EMS. DEN is een indirect werkend agens dat moet worden geactiveerd om te reageren met DNA. De benodigde P450-enzymen zijn afwezig in het beenmerg. Kort na ENU-behandeling zijn relatief lage niveaus van $O^6$-ethylguanine en N7-ethylguanine gemeten, maar desondanks is er een 23-voudige verhoging van de MF geconstateerd, 3 dagen na behandeling. Op dit tijdstip kon $O^6$-ethylguanine niet meer worden aangetoond. Als gevolg van EMS-behandeling werd er slechts een 2-voudige verhoging gevonden ondanks het hoge N7-ethylguanineniveau. Deze resultaten suggereren dat $O^6$-ethylguanine ook in het beenmerg een belangrijke premutagene lesie is en N7-ethylguanine niet. Uit de DNA-sequentieanalyse van de mutanten is te zien dat 41% van de mutaties van het $O^6$-ethylguanine-geïnduceerde GC → AT-type waren. Ook zijn TA → AT-(23%) en GC → TA (14%)-transversies gevonden.

In de hersenen zijn DNA-addukten alleen gemeten na behandeling met ENU en EMS. Ondanks dat $O^6$-ethylguanine en N7-ethylguanine goed meetbaar waren na ENU en er zeer hoge N7-ethylguanineniveaus werden gemeten na EMS, is er geen verhoging in de MF geconstateerd, zelfs niet na 28 dagen.

Het verschil tussen lever en beenmerg in het tijdstip van maximale mutatie-inductie na toediening van ENU is te wijten aan verschillen tussen de twee weefsels in $O^6$-ethylguanineherstel en delingssnelheid. De hoge mate van celdeling in het beenmerg leidt tot een snelle fixatie van mutaties. De lagere $O^6$-ethylguanine-herstel capaciteit van beenmergcellen in vergelijking met de lever speelt hier ook een rol. De afwezigheid van mutatie-inductie in de hersenen is toe te schrijven aan het lage niveau van DNA-synthese

tijdens de hier beschreven studies met [illegible]
*lac*-muizen door andere onderzoekers, en met andere testsystemen zoals de micronucleus-test en carcinogeniteits-proeven. Een belangrijke conclusie die getrokken kan worden is dat transgene markermuizen een sterke verbetering zijn bij de ontwikkeling van kortdurende *in vivo*-testsystemen die in staat zijn de mutagene potentie van genotoxische stoffen in allerlei organen en weefsels te voorspellen.

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## *Curriculum vitae*

Edwin Johannes Mientjes was born in Rotterdam on December 30, 1964. He attended primary and secondary school at the Ghana International School in Accra, Ghana and completed the British O-Levels in Voorschoten, The Netherlands in 1982. After obtaining his Grade-13 diploma in the summer of 1983 at the Forest Hill Collegiate Institute, Toronto, Canada, he furthered his education by graduating from the University of Toronto with a Bachelors degree in Microbiology and Biochemistry (1987). As part of his Masters degree in Molecular Biology completed at the University of Leiden in May 1991, he participated in two research projects encompassing the processes involved in Bacteriophage Mu transposition carried out at the Department of Molecular Genetics headed by Prof. Dr. P. van de Putte and the regulation of the T-*cyt* promoter from *Agrobacterium tumefaciens,* which took place at MOLBAS headed by Prof. Dr. R.A. Schilperoort. His Ph.D. work commenced on May 1, 1991 at the Department of Genetic Toxicology of TNO Medical Biological Laboratory, Rijswijk, The Netherlands (currently Department of Molecular Toxicology of the TNO Food and Nutrition Research Institute) under supervision of Prof. Dr. P.H.M. Lohman (University of Leiden), Dr. R.A. Baan and Dr. J.H.M. van Delft. The work involved the study of DNA-damage and mutations *in vivo* in *lac*Z transgenic marker mice exposed to several alkylating agents.

## Stellingen

1) Kwantitatieve vergelijking van mutagenese gemeten in een transgen met die in een endogeen gen is alleen toegestaan indien de "mutabiliteit" van beide sequenties bekend is.

*dit proefschrift*

2) De aanwezigheid van het repaireiwit $O^6$-alkyl-DNA-alkyltransferase is geen garantie voor meetbaar herstel van $O^6$-ethylguanine in genomisch DNA.

*dit proefschrift*

3) Mutaties in mutant *λlac*Z fagen afkomstig van DNA uit 40.6 transgene muizen zijn ontstaan in de muis en niet in *E. coli*.

*dit proefschrift*

4) Bij het toepassen van het positieve selectiesysteem (P-gal) bij de Muta™Mouse wordt ten onrechte verwezen naar Gossen *et al.* (1992) *Nucl. Acids Res.* **20**: 3254.

5) De wijze waarop Izzotti *et al.* de gegevens interpreteren van hun $^{32}$P-postlabelinganalyse van DNA-addukten in de lever van al of niet met hepatitis-virus geïnfekteerde bosmarmotten, is aan ernstige bedenkingen onderhevig.

Izzotti *et al.* (1995) *Chem. Biol. Interactions* **97**: 273-285.

6) De benaming "intron" voor "intervening sequences" is niet in overeenstemming met de gevonden functie daarvan.

Tycowski *et al.* (1996) *Nature* **379**: 464-466.

7) Bij onderzoek aan mutageniteit van tequila op beenmergcellen in de muis *in vivo* wordt voorbij gegaan aan de mogelijke effecten van zout en citroen.

Pina-Calva, A. and Madrigal-Bujaider, E. (1993) *Toxicol. Lett.* **66**: 1-5.

8) De grootte van een molecuul staat geenszins in verhouding tot de gevolgen die het voor een individu kan hebben.

9) Het schrijven van het eerste hoofdstuk van een proefschrift dient in een zo vroeg mogelijk stadium van het onderzoek te geschieden.

10) Wie het laatst lacht is traag van begrip.

11) Het snoeien en ontbladeren van de boom der kennis kost menigeen zijn baan.

## Dankwoord

Omdat de U of L geen dankwoord toestaat rest me alleen nog de mogelijkheid om het als een los vel toe te voegen. Er zijn een aantal mensen die heel veel hebben bijgedragen aan mijn onderzoek en nog veel meer mensen die sociaal zeer belangrijk waren. Het probleem is alleen dat ik bang ben om mensen te vergeten (onbewust natuurlijk!). Nou hier gaat 'ie dan.

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