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Influences of Ageing and Diet on Mutational Frequency and Specificity in Big Blue® *lacI* Transgenic Rodents

by

Gregory Roy Stuart
B.Sc., Dalhousie University, 1983
M.Sc., University of British Columbia, 1995

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

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Abstract

Big Blue® *lacI* transgenic mice and rats carry the *E. coli lacI* gene integrated as a tandem array of approximately 40 copies integrated at a single site in chromosome 4. This mutationally well-characterized gene is highly sensitive to base substitution mutations and is readily recovered from virtually any tissue of the transgenic host, facilitating the *in vivo* study of mutation. The Big Blue® assay was used to investigate spontaneous and induced mutation, with an emphasis on dietary influences on mutational frequency and specificity. The effects of ageing and dietary restriction on spontaneous mutation in the *lacI* transgene were determined in mice, permitting evaluation of several well established theories of ageing. Mutation frequencies were found to increase with age in tissues that proliferate (bladder and liver, but not brain), validating a principle tenet of the somatic ageing theory. However, the unexpected lack of a change in mutational specificity in ageing mice suggests that theories of ageing based on oxidative damage, or a reduction in DNA repair efficiency, may not be seminal to the ageing process, at least until more advanced age. Similarly, dietary restriction, which is known to extend lifespan in rodents and was predicted to decrease oxidative DNA damage, had no appreciable effect on either the frequency or the specificity of spontaneous mutation in liver of younger (6 month old) and older (12 month old) mice.

Dietary influences on induced mutation were examined following treatment with aflatoxin B_1 (AFB_1) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), powerful animal carcinogens which demonstrate tissue, species and sex-linked differences in rats and mice. As expected, AFB_1 was found to be potently mutagenic in rat but not mouse liver, in agreement with rodent carcinogenicity studies that found F344 rats to be highly susceptible to AFB_1-induced hepatocarcinogenesis, while C57BL/6 mice are highly resistant. PhIP was found to be potently and equally mutagenic in colon of both male and female rats. The result in female colon was surprising, since PhIP predominantly induces colon cancer in male rats but mammary tumors in female rats. Therefore, the progression of PhIP-induced colon cancer in the rat colon is likely due to factors acting at a later stage in the tumorigenic process, following the damage and

mutation of DNA. Rat prostate tissue, another tumor target tissue in PhIP-treated rats, was also found to be highly susceptible to PhIP-induced mutagenesis. Lastly, the PhIP studies were extended to an additional transgene target located in the shuttle vector construct from Big Blue® rodents, the bacteriophage λ-derived *cII* gene. These studies validated the use of the λ*cII* gene as an alternative mutational target for use in the Big Blue® assay, while the analyses of mutation in the *lacI* and the λ*cII* transgenes serves as a paradigm for mutational studies which compare mutational responses in different genes.

Collectively, these studies demonstrate the utility of the *lacI* (λ*cII*) transgenic mutagenicity assay for the *in vivo* investigation of mutational processes as a function of age, diet, sex, species, and target tissue specificity with respect to sites of mutation and cancer.

Examiners:

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List of Abbreviations

AFB_1, aflatoxin B_1

AFB_1-FAPY, AFB_1-formamidopyrimidine

AP, apurinic

Apc, ademomatous polyposis coli (gene)

Aprt, adenine phosphoribosyltransferase (gene)

B[*a*]P, benzo[*a*]pyrene

B[*a*]PDE, benzo[*a*]pyrenediol-epoxide

bp, base pair(s)

BPDE, benzo[*a*]pyrenediol-epoxide

BBM, Big Blue® mice

BBR, Big Blue® rats

b.s., base substitution (mutation)

cI, bacteriophage λ *cI* repressor (gene)

cII, bacteriophage λ *cII* repressor (gene)

CAS, Chemical Abstracts Registry

CpG, 5′–CpG–3′ dinucleotide sequence

CHO, Chinese hamster ovary

deam, deamination (of 5-methylcytosine)

div, cellular division

dNTP, deoxyribonucleotide

DMSO, dimethyl sulfoxide

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

ENU, *N*-ethyl-*N*-nitrosourea

F344, Fischer 344 rat strain

HCA, heterocyclic amine

Hprt, hypoxanthine-guanine phosphoribosyl transferase (gene)

i.p., intraperitoneal

λLIZ/*lacI*, Big Blue® λ shuttle vector encoding the *lacI* gene

lacI, lactose repressor (*E. coli* gene)

lacZ, β-galactosidase (*E. coli* gene)

LOH, loss of heterozygosity

M13/*lacI*, bacteriophage M13 encoding the *lacI* gene

5MC, 5-methylcytosine

MF, mutant frequency

Mf, mutation frequency

mo, month

MR, mutation rate

MS, mutational spectrum (spectra)

mut, mutation

Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt

8-oxoG, 8-oxo-2′-deoxyguanosine

PCR, polymerase chain reaction

PFU, plaque forming units

PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

P-*gal*, phenyl-β-D-galactoside

RFLP, restriction fragment length polymorphism

SDS, sodium dodecyl sulfate

SM, (bacteriophage) storage medium

SMF, sectored mutant frequency

TD_{50}, dose producing 50% tumor incidence

TE, tris-HCl EDTA (buffer)

tris-HCl, *tris*(hydroxymethyl)aminomethane hydrochloride

X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Acknowledgments

I would like to take this opportunity to acknowledge the assistance I have received during my Ph.D. studies. The nature and scale of most *lacI* transgenic mutagenicity studies (including animal treatments, tissue dissections, DNA isolations, plating and screening for *lacI* mutant plaques, DNA sequencing and data management), as well as collaborations with other laboratories, necessarily involves a "team approach." Therefore, I am especially grateful to the following Glickman laboratory members (past and present) for their excellent technical assistance at various times: Bernadette van der Boom, Nicole Bye, Heather Erfle, Adlane Ferreira, Naheed Haque, Roderick Haesevoets, James Holcroft (also for stimulating discussions), Jana Kangas, Paul Kotturi (also for stimulating discussions), Ken Sojonky, Erika Thorleifson (also for stimulating discussions), Amanda Thornton-Glickman (also for stimulating discussions), David Walsh, Pam Warrington, Haiyan Yang and Shulin Zhang. I would additionally like to acknowledge Glickman lab members Drs. Moyra Brackley, John Curry and Barry Ford and especially Dr. Johan de Boer for stimulating scientific discussions as well as statistical assistance (Johan; Moyra) and computer skills (Johan; John). Finally, I wish to acknowledge the administrative support provided by Centre for Environmental Health administrative assistants Pauline Tymchuk and Veronica Anthony, Biology Stores (Ken Beattie, John Morrison, Sarwan Dillon and staff), Anne Nightingale and Eleanore Floyd (Biology Office), Katy Nelson (Science Librarian) and the University of Victoria Animal Care Unit (Ralph Scheurle and staff, for technical support). The time and dedication contributed by my Ph.D. supervisory and examining committees (Drs. Johan de Boer, Robert Burke, Barry Glickman, Francis Nano, Colleen Nelson, and Miriam Rosin) was also greatly appreciated.

I also wish to acknowledge the assistance (materials, information and discussions) provided by Stratagene employees (past and present) Mark Dycaico, Dr. Patti Kretz, Scott Provost, and Brenda Rogers. I am also grateful to our Japanese collaborators and friends Drs. Minako Nagao, Toshikazu Ushijima, Eriko Okochi, and Hideo Okonogi from the National Cancer Center Research Institute (Tokyo), as well as Dr. Yoshimitsu Oda (Osaka Prefectural Institute of Public Health). Yoshi, in addition to assisting me with

the ageing and dietary restriction studies while on sabbatical leave in our laboratory, became a close personal friend of Susan and I.

My studies were greatly facilitated by the generous financial support provided by various sources. Most significantly, the ageing and dietary restriction studies were funded by the Cancer Research Society, Inc. (Montréal), which additionally provided me with a Graduate Student Stipend throughout my Ph.D. studies. Additional financial support was provided by the University of Victoria Department of Biology (in the form of graduate stipends during two academic years), a travel award from the Environmental Mutagen Society as well as the National Cancer Institute of Canada, and finally, a Foundation for the Promotion of Cancer Research Fellowship for Research in Japan (National Cancer Centre Research Institute, Tokyo, Japan).

Lastly, I am most indebted to my friend and mentor, Dr. Barry W. Glickman, for providing me with the opportunity and resources to execute, explore and complete the various studies described in this Dissertation. Dedicated to the pursuit of excellence both in and out of academia, Barry provides the opportunity for each of his students to discover and realize their potential, to investigate novel scientific projects of fundamental importance, to attend and participate at international conferences, and to acquire the skills which are necessary for publishing and critically reviewing the scientific literature, as well as the preparation of grant proposals. For this and more, I offer my sincerest respect and gratitude – thank you, Barry!

Dedication

Two people were essential for completion of this Dissertation – Dr. Barry Glickman, and my dear wife of over eleven years and closest friend for even longer, Susan Elizabeth Stuart. Susan has been devoted and unwavering in her support of my graduate studies. She has even lent a hand (literally), including the time that we petted and handled newly-arrived Big Blue® rats, in order to tame them for upcoming oral gavage treatments. Although her name does not appear on any of the papers, she contributed significantly to the success of each of the papers which bear my name. For this and more, I lovingly dedicate this Dissertation to you, Susan.

Introduction

A Brief Overview of the Big Blue® *lacI* Transgenic Mutagenicity Assay

The study of mutation *in vivo* has been facilitated during the past decade through the development and use of transgenic rodents that harbor foreign genes (transgenes) either integrated into their chromosomal DNA (*e.g.*, *lacZ* or *lacI*: Gossen *et al.* 1989; Kohler *et al.* 1990, 1991a) or as a plasmid (*e.g.*, *lacZ*: Martus *et al.* 1995). These assays have been the subject of several excellent reviews (Dycaico *et al.* 1994; Mirsalis *et al.* 1994; Mirsalis 1995; de Boer and Glickman 1998; Vijg and van Steeg 1998). The development of transgenic rodent mutagenicity assays allows investigation of mutational processes as a function of age, diet, sex, species, and target tissue specificity with respect to sites of mutation and cancer. Big Blue® *lacI* transgenic mice and rats carry the *E. coli lacI* gene as part of a λLIZ/*lacI* shuttle vector which is integrated as a tandem array of approximately 40 copies at a single site in their chromosomal DNA (Kohler *et al.* 1991a; Provost *et al.* 1993; Dycaico *et al.* 1994).

The well-characterized *lacI* gene is sensitive to base substitution mutations and is easily recovered from virtually any tissue from the transgenic host, facilitating the *in vivo* study of mutation (de Boer and Glickman 1998). The λLIZ_{α}/*lacI* transgene is recovered from purified animal chromosomal DNA by an *in vitro* λ packaging reaction (Rogers *et al.* 1995; Stratagene 1997). The detection of *lacI* mutant λ phage in the Big Blue® assay involves infection of SCS-8 *E. coli* host cells with packaged λ phage containing the *lacI* transgene, and plating on agar media containing the chromogenic substance 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Miller 1978). Phage bearing wild-type or mutant *lacI* genes give rise to colorless or blue plaques, respectively. Cellular β-galactosidase activity is provided by α-complementation of the bacterial-encoded ω-LacZ fragment with the phage encoded α-LacZ fragment (Langley *et al.* 1975; Mirsalis *et al.* 1995; de Boer and Glickman 1998). In cells infected with phage containing a wild-type *lacI* gene, the LacI repressor protein blocks β-galactosidase (*lacZ*) expression. However, cells infected with $lacI^{-}$ phage express β-galactosidase, resulting in hydrolysis of X-gal and blue plaques.

Blue (mutant) plaques are cored from the agar plates using Pasteur pipets and stored indefinitely at 4°C in phage storage medium (SM buffer). The phage are subsequently replated at low density in order to obtain pure plaques. The mutant *lacI* genes are then PCR amplified, then PCR cycle sequenced (Erfle *et al.* 1996). Mutational data are entered into a computer database and managed using custom software (de Boer 1995).

Recently, a variant of the Big Blue® assay was developed which takes advantage of the bacteriophage λ *cII* gene that is also present in the Big Blue® λLIZ/*lacI* shuttle vector (Jakubczak *et al.* 1996). The λ*cII* transgene shows promise as an alternative to the *lacI* transgene as a mutational target. The smaller gene (294 bp, compared to the 1083 bp *lacI* gene) is easily sequenced, yet highly sensitive to base substitution mutations (J.G. de Boer, *pers. comm.*), and mutant λ*cII* phage may be positively selected using a bacterial host strain which permits growth of only the *cII* mutant phage at a restrictive temperature.

A Brief Overview of the Material Presented in This Dissertation

E. coli-based *lacI* mutagenicity assays had been applied intensively for many years in our laboratory to the study of the origin, mechanisms and specificity of spontaneous and induced mutation, including the role of DNA repair processes (*e.g.*, Schaaper *et al.* 1986; Horsfall *et al.* 1990; Glickman *et al.* 1995). These, and other considerations (including the huge body of literature dealing with mutation in the *lacI* gene) made the adoption of the Big Blue® assay by our laboratory the logical choice for the investigation of spontaneous and induced mutation *in vivo*.

The studies described in this Dissertation used the Big Blue® assay to investigate mutational frequency and specificity of spontaneous mutation in the *lacI* transgene as a function of age with and without dietary restriction, as well as mutations induced by the food-borne mutagens and carcinogens, aflatoxin B_1 (AFB_1) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). The following sections briefly introduce the Chapters which make up the main body of this Dissertation.

As noted in the Acknowledgments, the scale and collaborative nature of most Big Blue® assay studies necessarily involves a "team approach," particularly with respect to technical assistance with the plating and screening for mutants, and DNA sequencing. Therefore, while the planning, management, data analyses and writing of manuscripts for

the most of the studies described in this Dissertation were largely done by the Author (except as noted), in the subsections which follow I briefly clarify my role in the each of the studies.

Chapter 1. The genetic analysis of *lacI* mutations in sectored plaques from Big Blue® transgenic mice (Stuart *et al.* (1996) *Environ. Mol. Mutagen.* 28, 385–392)

The Big Blue® assay facilitates *in vivo* studies of mutation, that is, mutations that occur in the in the *lacI* transgene in living mice or rats. However, non-*in vivo*-derived *lacI* mutations occasionally arise "*ex vivo*" due to damaged mouse DNA which is subsequently repaired in the *E. coli* host strain, or "*de novo*" (*in vitro*) due to mutations arising during replication and amplification of the *lacI* DNA in the *E. coli* host cells. Therefore, prior to beginning the various ageing-diet studies, the origin and influence of these mutations on *in vivo* MF and MS was examined.

Disclosure: The Author planned and executed most of this work, including DNA sequencing, and writing the paper. Some of the "sectored" (*ex vivo*; *in vitro*) mutant DNA sequence data (*e.g.*, from mouse skin and M13/*lacI* bacteriophage) originated elsewhere, as indicated in the text.

Chapter 2. Mutation frequency and specificity with age in liver, bladder and brain of *lacI* transgenic mice (Stuart *et al. Genetics*, in press)

This chapter examines MF and MS in liver and brain of *ad libitum*-fed Big Blue® mice aged 6 weeks–25 months, and bladder of mice aged 6 weeks–12 months. The study described had two main functions: (1) to establish spontaneous MS in various tissues at different ages, to establish a baseline for comparison in future studies; and (2) to test several important theories of ageing.

Disclosure: The Author's substantial contribution to the material presented in Chapter 2 involved all aspects of the assay, from the point of frozen tissues (–80°C) onward, with the exception of DNA sequencing. (The animals had been aged, sacrificed and dissected prior to the Author's arrival to the laboratory.) This very large project (*circa* 2 years duration) additionally required technical assistance for the Big Blue® assay plating and screening. The Author wrote this paper.

Chapter 3. No change in spontaneous mutation frequency or specificity in dietary restricted mice (Stuart *et al. Carcinogenesis*, in press)

This chapter describes a study done in parallel with the ageing study (Chapter 2), that examined the effect of 70% dietary restriction (DR) on spontaneous MF and MS in liver of mice aged 6 and 12 months. The purpose of this study was to establish reference MF and MS from DR mice, and also to test theories of ageing which based on observations that DR mice live longer, and are generally healthier, with lower incidence of tumors.

Disclosure: The work described in this chapter was done by the Author, with the exception of the earlier animal work (ageing; tissue dissections) and DNA sequencing. The Author wrote this paper.

Chapter 4. Through a glass, darkly: Reflections of mutation from *lacI* transgenic mice (Stuart *et al.*, *Genetics*, submitted)

This chapter describes the results of reflection and interpretation of the ageing data presented in Chapter 1. Mutation rates (overall, as well as those of deaminations of 5-methylcytosine at CpG dinucleotide sequences) are estimated in liver, bladder and brain using the *Mf* data presented in Chapter 1, plus estimates of cellular proliferation/DNA replication at various developmental stages. Consideration of the origin and contribution of *in vivo* events to observed mutational frequencies led to the development of a semi-quantitative mutational model, which is applied to estimate efficiency of DNA repair *in vivo* of deaminated 5-methylcytosines, and the effect of specific DNA repair defects on overall DNA repair efficiency in Big Blue® mice, and presumably, humans.

Disclosure: The Author contributed and developed the material presented in this chapter, and wrote this paper.

Chapter 5. Species-specific differences in hepatic mutant frequency and mutational spectrum among lambda/*lacI* transgenic rats and mice following exposure to aflatoxin B_1 (Dycaico *et al.* (1996) *Carcinogenesis* 17, 2347–2356)

This chapter describes the first multi-species *in vivo* mutagenicity study using transgenic rodents harboring the same shuttle vector. The effects of aflatoxin B_1 (AFB_1), a potent mutagen and suspected human carcinogen that is present in the human diet, was examined in C57BL/6 Big Blue® mice and F344 Big Blue® rats. As predicted, AFB_1 was potently mutagenic in rat but not mouse liver.

Disclosure: This collaborative study (Stratagene; Glickman laboratory) was initiated and managed by Stratagene. The Author sequenced the *lacI* mutants, analyzed the mutational data and wrote the discussion of the mutagenic effects of AFB_1 in mouse and rat liver, thus securing position as Second Author on this paper.

Chapter 6. Effects of gender and species on Spectra of mutation induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the *lacI* transgene (Okonogi *et al.* (1997) *Mutation Research* 395, 93–99)

This chapter also resulted from a collaborative study, begun by Dr. M. Nagao's laboratory (National Cancer Center Research Institute, Tokyo). In this study, we examined the mutational specificity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a chemical mutagen and suspected human carcinogen which is present in the human diet, in colon tissue of male and female Big Blue® rats. PhIP predominantly induces colon (and prostate) cancer in male rats, but mammary gland tumors in female rats.

Disclosure: The Big Blue® rats used in this study were provided by Dr. Glickman. The Author traveled to Tokyo (January, 1996) assist with this project (Big Blue® assay – DNA isolation, plating and screening for *lacI* mutant phage) in Dr. Nagao's laboratory at the National Cancer Center. Thereafter, the DNA sequencing was split among the two laboratories. The Author supervised the DNA sequencing effort by the Glickman laboratory, and assisted in the analysis of the data and the preparation of the manuscript, thus securing position as Second Author on the paper.

Chapter 7. Prostate mutations in rats induced by the suspected human carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (Stuart *et al. Cancer Res.*, in press)

The work described in this chapter derives from a substantially larger PhIP study (50 Big Blue® rats) initiated by the Author (completed, but not included in this Dissertation as the manuscript is in preparation – please refer to the Author's Vita). In this chapter, we describe the first mutational frequency and spectrum data from prostate tissue, for both spontaneous and PhIP-induced mutations.

Disclosure: The Author planned, executed and managed all aspects this PhIP study. Technical assistance was provided to the Author during the animal treatments and tissue

dissections. DNA isolations, the Big Blue® assay and DNA sequencing were performed by Dr. Glickman's technicians. The Author analyzed the data and wrote the paper.

Chapter 8. Interpretation of mutational Spectra from different genes: Analyses of PhIP-induced mutational specificity in the *lacI* and *cII* transgenes from Big Blue® rats (Stuart *et al.*, *Mutation Research*, submitted)

This study arose directly from the PhIP colon study described in Chapter 6, but was initiated and managed by the Author. The goal of this study was to extend the PhIP colon mutagenicity data from the *lacI* transgene of male and female Big Blue® rats, to the λ*cII* transgene from the same animals. During the analyses of the data, apparent differences in PhIP-induced mutational specificity between the *lacI* and λ*cII* transgenes were resolved by considering the relative numbers of mutations as well as the numbers of mutational "target sequences" available in either gene, thus establishing a paradigm for the comparison of mutagenic effects in different genes.

Disclosure: This work was conducted in Dr. Glickman's laboratory by the Author, with technical assistance provided for the Big Blue® λ*cII* assay, and the DNA sequencing. The analyses of the data and the writing of the paper were done by the Author.

Chapter 9. Transgenic mutagenicity assays: Past, present and future

This chapter contains a concluding discussion which briefly ties the various studies described in this Dissertation to the past validation studies, and looks forward to new developments in the field of transgenic mutagenicity assays.

Appendix A. Benzo[*a*]pyrenediol-epoxide induces loss of heterozygosity in Chinese hamster ovary cells heterozygous at the *Aprt* locus (Mazur-Melnyk *et al.* (1996) *Mutation Research* 358, 89–96)

Benzo[*a*]pyrenediol-epoxide, a potent point mutagen and carcinogen, was demonstrated to induce loss of heterozygosity of the *Aprt* locus in Chinese hamster ovary cells in about one-quarter of all mutants, indicating that similar events involving homologous recombination in somatic cells may have an impact in tumorigenesis.

Disclosure: The Author did not contribute to the experimental work in this paper, but rather updated and revised the manuscript, enabling it's submission for publication.

Appendix B. Construction and characterization of *E. coli* strains for the direct selection of *lacI* bacteriophage recovered from the Big Blue® assay (Stuart, G.R.)

Although an assay for the direct selection of Big Blue® *lacI* mutants is available, the assay is virtually unused due to technical difficulties (particularly, an increase in the number of *lacI* mutant bacterial colonies over time, complicating the determination of mutant frequencies). This Appendix describes the efforts by the Author to understand this phenomenon, as well as the construction of alternative *E. coli* hosts suitable for the direct selection of *lacI* mutant phage. (Work on this project has been temporarily postponed, to allow time for the preparation of various manuscripts including this Dissertation.)

SECTION I. SPONTANEOUS MUTATION

Chapter 1. The Genetic Analysis of *lacI* Mutations in Sectored Plaques from Big Blue® Transgenic Mice

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Environmental and Molecular Mutagenesis (1996) 28, 385–392

Abstract

The Big Blue® *lacI* transgenic rodent assay, which uses the λLIZ/*lacI* gene as the target for mutation, provides a convenient short-term assay for the study of mutation *in vivo* (Kohler *et al.* 1991a; Provost *et al.* 1993). However, the interpretation of data from transgenic animal assays is sometimes complicated by mutants that appear as sectored mutant lambda plaques. These mutants can form a significant fraction of the mutant plaques (Hayward *et al.* 1995). Thus, in order to accurately determine *in vivo* mutant frequencies and mutational specificities, it is necessary to score sectored plaques and partition them from the rest of the data. In this study, the specificity of mutation in sectored plaques recovered from untreated and UVB-treated Big Blue® mouse skin was analyzed and compared to mutations recovered from λLIZ/*lacI* grown on the *Escherichia coli* host. The mutational spectra of sectored plaques from untreated and UVB-treated mice were remarkably similar to each other and resembled those recovered from the λLIZ/*lacI* phage plated directly on *E. coli*. Both the sectored mutants and those recovered in λLIZ/*lacI* phage differed from the spectra of spontaneous mutants in *E. coli* and in Big Blue® mouse skin. While sectored mutants from UVB-treated mouse skin and λLIZ/*lacI* mutants were also different from spontaneous mutants recovered from Big Blue® liver, there was little difference between sectored mutants from untreated mouse skin and spontaneous liver mutants ($P = 0.07$). The mutational spectra of sectored plaques is thus largely consistent with their origin as spontaneous mutations arising *in vitro* during growth of the λLIZ/*lacI* shuttle vector DNA on the *E. coli* host, although the potential contribution from lesions in mouse DNA being expressed *ex vivo* in the *E. coli* host cannot be excluded.

***Keywords*: *ex vivo*; sectored; Big Blue®; *lacI*; spontaneous mutation**

1.1. Introduction

The Big Blue® *lacI* transgenic mouse system provides a convenient short-term assay for the study of mutation *in vivo* (Kohler *et al.* 1991a; Provost *et al.* 1993). The target for mutation is the *lacI* gene, which is integrated into the rodent genome as tandem multiple copies of the λLIZ/*lacI* bacteriophage λ shuttle vector (Dycaico *et al.* 1994). The λLIZ/*lacI* transgene is recovered from purified animal chromosomal DNA by an *in vitro*

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packaging reaction. λ-Phage-containing mutant *lacI* genes are identified as blue plaques when plated with *E. coli* on media supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Miller 1978).

Mutant Big Blue® lambda plaques are typically uniform in color, ranging from faint blue to dark blue, depending on the specific mutation. The intensity of the blue color in mutant plaques depends on the codon change which has occurred in the *lacI* sequence (Rogers *et al.* 1995; de Boer *et al.*, unpublished[2]). Other mutant phenotypes are occasionally observed, including colorless (wild-type) plaques with a pinpoint blue spot, and sectored plaques (Provost *et al.* 1993; Provost and Short 1994), in which a portion of the plaque is blue. Pinpoint plaques likely arise from spontaneous *de novo* mutations arising late during growth on the *E. coli* host cells. Sectored plaques are thought to result from *de novo* mutations that arise during early replication in the host bacteria, or *ex vivo* as a result of replication of unrepaired DNA damage. This damage could remain in DNA from treatment in the mouse or could be due to DNA damage accumulated during DNA isolation and storage (Provost *et al.* 1993; Piegorsch *et al.* 1994); however, the use of standardized procedures (Rogers *et al.* 1995) can alleviate the latter problem.

During Big Blue® experiments, a significant fraction of recovered mutant plaques may be sectored, especially when an inadequate mutation fixation period is allowed for the *in vivo* repair of DNA lesions, or when very low spontaneous background levels are observed (Mirsalis *et al.* 1994; Hayward *et al.* 1995). In the calculation of mutant frequencies (MF), sectored mutants should not be considered (Piegorsch *et al.* 1995). While partitioning sectored mutants from the rest of the mutants may minimize their influence on the data, the origin of sectored mutants needs to be understood better as part of the evaluation of the transgenic system.

In order to interpret the relevance of sectored plaques to the study of mutation using the *lacI* system, the DNA alterations in sectored mutants recovered from the skin of untreated and UVB-treated mice were compared with spontaneous *lacI* mutants recovered from untreated Big Blue® mouse liver and skin, the endogenous *lacI* gene in *E. coli*, M13/*lacI*, and Big Blue® λ phage (packaged λLIZ/*lacI* shuttle vector DNA) plated

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[2] de Boer and Glickman (1998).

on *E. coli*. Based on the unique mutational spectra shared by the λLIZ/*lacI* mutants and the untreated and UVB-treated sectored mutants, we conclude that the sectored mutants examined in this study arose *in vitro* possibly as spontaneous mutation events that are influenced by the λLIZ/*lacI* genetic background.

1.2. Materials and Methods

1.2.1. Isolation of λLIZ/*lacI* mutant phage

E. coli-derived mutant λLIZ/*lacI* phage were isolated by infecting *E. coli* SCS-8 cells with wild-type λLIZ/*lacI* phage from a single, well-isolated plaque, and plating on NZY agar plates containing 1.5 mg/ml X-gal (refer to Stratagene (1992) for general methods and materials). Twenty isolated wild-type 'founder' plaques were picked and individually replated on NZY plates with X-gal at an average density of approximately 17 plaque-forming units (PFU) per square centimeter. Mutant plaques arising from the founder plaques were picked and stored in the dark at 4°C in 0.5 ml SM buffer containing 100 μl/ml chloroform. Then 48 mutant plaque phenotypes were verified and the mutation in *lacI* was determined by DNA sequencing, as previously described (de Boer *et al.*, submitted).[3] To ensure that independent mutations were analyzed, duplicate mutations recovered from two founder plaques were discarded, giving a sample of 46 mutants.

The mutations in an additional set of 29 spontaneous λLIZ/*lacI* mutants isolated at The Procter & Gamble Company (Cincinnati, OH) were also determined. These mutant phage were isolated as described above, except that they were derived from one single, well-isolated wild-type plaque which was plated at a density of approximately 14 PFU/cm^2. Since the mutational spectra in these two datasets were found to be identical (the statistical analysis is described below), the two λLIZ/*lacI* datasets were combined into one dataset of 75 mutants.

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[3] Erfle *et al.* (1996).

1.2.2. DNA sequencing and data management

Template DNA preparation and sequencing was performed as described by de Boer *et al.* (submitted).[4] Mutant sample data and DNA sequence data were managed and analyzed by using custom software (de Boer 1995).

1.2.3. Sources of additional *lacI* spontaneous mutant and sectored datasets

Sequence data for spontaneous *lacI* mutants recovered from *E. coli* (Halliday and Glickman 1991), M13/*lacI* (Yatagai *et al.* 1991), and liver and skin of Big Blue® mice were extracted from the *lacI* database (de Boer *et al.*, submitted).[5] The liver spontaneous mutational spectra consisted of 77 C57BL/6 and 205 B6C3F_1 mutants. There was no significant difference (χ^2, $P = 0.54$) between the C57BL/6 and the B6C3F_1 spontaneous mutational spectra (de Boer *et al.*, submitted).[6] Sectored plaques from untreated and UVB-treated Big Blue® mouse skin were provided by The Procter & Gamble Company, where they were isolated as follows.

For the UVB experiment, female C57BL/6 Big Blue® transgenic mice, nine weeks old, were obtained from Stratagene (La Jolla, CA) and housed individually in Thoren ventilated cages on hardwood chip bedding. All animals were provided with Purina Lab Chow and water *ad libitum*. A 12-hour light:12-hour dark cycle was maintained throughout the study. Animals were acclimated for 1 week before dosing. An area of at least 3 cm wide by 5 cm long on the backs of all the mice was shaved before the first exposure. Any hair regrowth during the week of dosing was reshaved. Animals were restrained individually in 3-in. x 3-in. wire mesh-covered cages without access to food or water during UVB exposure. UVB (290–320 nm) was administered topically under an Oriel solar simulator with the appropriate filters (Schott GG19 and UG5) to permit the transmission of only UVB radiation. The intensity of the bulb was 1.192 mW/cm^2 (0.192 mJ/cm^2/s). UVB treatments consisted of the following dosages: 1.06 J/cm^2/day in 92 minutes, 0.53 J/cm^2/day in 46 minutes, 0.26 J/cm^2/day in 23 minutes, or 0.13 J/cm^2/day in 11.5 minutes for five consecutive days (8 mice/group). UVA exposure at these doses was negligible; for example, the longest exposure resulted in 0.188 J/cm^2/day of UVA. Ten

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[4] *op. cit.*

[5] de Boer (1995).

[6] de Boer *et al.* (1997).

animals served as controls and were not exposed. At 14 days after the last dose, the skins from the backs of all mice were frozen for subsequent DNA isolation and analysis as described elsewhere (Big Blue® Assay Instruction Manual (Stratagene, La Jolla, CA); Gorelick *et al.*, in preparation[7]). The sectored plaques from untreated and UVB-treated mice were collected for sequence analysis, as described in this report. In this experiment, the target plaque density was approximately 14 PFU/cm^2.

1.2.4. Statistical analysis

Statistical comparisons of mutational spectra were made with the method of Adams and Skopek (1987), using 2500 iterations. Tests of significance consisted of pairwise comparisons of mutational spectra, using the numbers of mutations found in 10 mutation classes (Table 1.1[8]): two classes of transition mutations; four classes of transversion mutations; mutations at the 'bacterial hotspot sequence;' other nonbase substitution mutations; and G:C → A:T and G:C → T:A mutations that occurred at 5′–CpG–3′ (CpG) dinucleotide sequences. For the statistical analyses the numbers of G:C → A:T and G:C → T:A mutations were adjusted for the number of mutants which occurred at CpG sequences. For example, if there were 100 G:C →A:T transition mutations with 20% occurring at CpG sequences, the data values for the Adams and Skopek analysis would be 80 and 20, respectively (not 100 and 20). The α-level for significance was set at 0.05.

1.2.5. Definitions

To clarify the discussion which follows, the following terms are defined: *in vivo* indicates a mutation that occurs in the mouse, *ex vivo* indicates a mutation arising in *E. coli* but originating from damaged DNA recovered from the mouse, while *in vitro* refers to a *de novo* mutation that originates in *E. coli*. Sectored (mosaic) plaques are defined as plaques containing less than 50% mutant phage.

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[7] Gorelick *et al.* (1995).

[8] Tables and figures have been placed at the end of each corresponding chapter.

1.3. Results

1.3.1. Comparison of mutational spectra

The mutation data examined in this study, grouped by mutation class, are presented in Table 1.1. Additional data regarding the sectored mutant datasets, including the sectored mutant frequency (SMF) – the proportion of the total number of plaques that are sectored – in untreated and UVB-treated mouse skin, are shown in Table 1.2. Each of the mutational spectra in Table 1.1 were compared to each other, using a statistical test developed specifically to detect differences between mutational spectra (Adams and Skopek 1987). This analysis, which is presented in Table 1.3 and discussed in greater detail below, indicated that the data clustered into four distinct groups. The *E. coli lacI* spontaneous mutational spectrum was significantly different from each of the other spectra. The mutational spectra of the mutants isolated from the liver and skin of untreated mice were not statistically significantly different (columns 3 and 4, Table 1.1) from each other, but they were significantly different from each of the other spectra. The one exception is that the spontaneous liver spectrum and the sectored spectrum from the skin of the untreated mice were marginally similar ($P = 0.07$, Table 1.3). The spontaneous mutational spectra for the λLIZ/*lacI* mutants and the sectored plaques recovered from untreated and UVB-treated mice were identical, but different from the other spectra. In addition, the *lacI* spontaneous mutational spectrum recovered from M13/*lacI* was unique, being significantly different from each of the other spectra.

1.3.2. *E. coli* mutational spectra

Nonbase substitution mutations (89%) predominate in the mutational spectrum observed in spontaneous *lacI* mutants recovered from *E. coli* (Halliday and Glickman 1991). The majority of these mutations (72% of all mutations) involve insertion or deletion of a four base pair repeat (TGGC) at position 621–632 (numbered according to Farabaugh (1978)). The next most common class of mutation are nonhotspot deletions (10%, not explicitly shown in Table 1.1), followed by G:C → A:T transition mutations (6%).

1.3.3. Mouse liver and skin *lacI* mutational spectra

The mutational spectra from spontaneous mutants recovered from liver and skin of Big Blue® mice are identical to each other ($P = 0.18$), yet different from the other

mutational spectra. The spontaneous mutational spectra in the liver and skin are uniquely characterized by the high proportion of G:C → A:T transitions (49% and 54% in liver and skin, respectively), with a large fraction of these transitions occurring at CpG sites (75% in liver; 86% in skin). The next most commonly occurring class of mutations in liver and skin are G:C → T:A transversions (18% and 24%, respectively). The hotspot mutation observed in *E. coli*, either an insertion or deletion of TGGC at base pairs 621–632, was recovered very infrequently in spontaneous Big Blue® mutants (2% and 0% in liver and skin, respectively). The absence of the bacterial hotspot mutation in mutant plaques recovered from Big Blue® tissues is evidence that the mutations have arisen in the mouse and not in *E. coli* (Kohler *et al.* 1991a; Provost *et al.* 1993).

1.3.4. Mutational spectra from sectored and λLIZ/*lacI* mutants

The mutational spectra from sectored plaques recovered from untreated and UVB-treated mouse skin and λLIZ/*lacI* spontaneous mutants were determined to be similar (Table 1.3). Specifically, the mutational spectra for the untreated and UVB-treated sectored mutants appeared to be identical. All three mutational spectra were characterized by an intermediate proportion of G:C → A:T transition mutations (range: 21–26%) (Table 1.1), and an intermediate proportion of G:C → A:T mutations at CpG sequences (range: 18–50%), compared with the bacterial spontaneous *lacI* mutants (6–22% G:C → A:T, with 13% at CpG sites) or the Big Blue® spontaneous mutants (49–54% G:C → A:T, with 75–86% at CpG sites). Also, the λLIZ/*lacI* mutants and the sectored mutants had slightly elevated (range: 4–9%) proportions of insertions or deletions of TGGC at base pairs 620–632, and other nonbase substitution mutations (17–23%), compared with the Big Blue® spontaneous mutants (0–2% TGGC insertions or deletions and 9–14% other nonbase substitutions). One mutant from UVB-treated mouse skin contained a tandem mutation, a G → A transition and a G → T transversion at *lacI* base pairs 586 and 587, respectively.

1.3.5. M13/*lacI* mutational spectra

The M13/*lacI* mutational spectrum was characterized by a large proportion of G:C → A:T transitions (66%), but few of these (12%) occurred at CpG sequences. The M13 mutational spectrum also had the highest proportion of transition mutants (71% of all

mutations), and the proportion of transversion mutations (13%) ranked between those found in *E. coli* (4%) with the hotspot included and the remaining mutational spectra (range: 26–33%). Frameshift mutations at the (*E. coli*) hotspot sequence were not recovered among the M13/*lacI* mutants.

1.4. Discussion

1.4.1. Statistical analysis of mutational spectra

Interpreting data from the Big Blue® *in vivo* mutagenesis assay requires an understanding of the origin of mutants in sectored plaques. To this end, *lacI* mutations recovered from sectored plaques were compared with spontaneous *lacI* mutations obtained in diverse genetic backgrounds (Table 1.1). The *lacI* mutational spectra segregated into four distinct groups, when each spectrum was paired and statistically tested for differences (Table 1.3).

The *E. coli* spontaneous *lacI* mutational spectrum differed substantially ($P < 0.001$) from each of the other spectra, as did the spectrum obtained in bacteriophage M13/*lacI*. The *in vivo* spontaneous *lacI* mutants recovered from Big Blue® liver and skin were similar to each other ($P = 0.18$), but different from all other spectra, with the exception that spontaneous skin mutants were marginally similar ($P = 0.07$) to sectored mutants from untreated skin. The fourth distinct group included the mutants recovered from the sectored plaques and the λLIZ/*lacI* spontaneous mutants recovered from wild-type phage plated on *E. coli*. These observations provide compelling evidence that the genetic context in which the *lacI* gene replicates (mouse transgene; λLIZ/*lacI* phage; bacterial gene; and M13/*lacI* phage) exerts a profound effect on the patterns of spontaneous mutation that are recovered. It has previously been noted in bacteria that the context in which *lacI* replicates has an effect on the observed mutational spectrum (Gordon and Halliday 1994).

The apparent similarity ($P = 0.07$) of the mutational spectra from spontaneous liver and sectored mutants from untreated skin requires comment. Although this *p*-value is close to the α level for significance (0.05), the influence of this comparison on the overall conclusions is small. Greater emphasis should be placed on the comparison between the sectored mutants and Big Blue® spontaneous skin mutants, since this tissue is common to

the three datasets. When this comparison is made, the spontaneous mutants from Big Blue® skin are statistically significantly different from sectored mutants from untreated ($P = 0.01$) and UVB-treated ($P < 0.001$) skin, respectively.

1.4.2. Sectored mutant frequency analysis

It has been previously noted that sectored plaques may arise *de novo* in *E. coli* during phage amplification. The proportion of mutant plaques in untreated animals that are sectored can be substantial (Shane and Tindall 1994; Hayward *et al.* 1995; Piegorsch *et al.* 1995). Data from this study support this observation, since sectored plaques comprised 19.6% of all of the observed spontaneous mutant plaques from untreated mouse skin (Table 1.2). Although sectored and nonsectored plaques are scored separately and MF and SMF are calculated separately, in experiments involving spontaneous mutation or where only modest increases above background are observed, the potential influence of sectored plaques on the MF and mutational spectrum must not be overlooked. Naturally, the influence of sectored plaques that arise due to spontaneous mutation in *lacI* replicating in *E. coli* should diminish when induced MF are substantially higher than background. This conclusion is supported by data in Table 1.2, which show that the sectored mutant fraction decreases from 19.6% in untreated skin to 2.3% in UVB-treated mouse skin. While the SMF remained constant at 2.1×10^{-5} in untreated and UVB-treated animals, the MF increased from $8.7 (\pm 1.1) \times 10^{-5}$ in untreated mouse skin to $88 (\pm 21) \times 10^{-5}$ in UVB-treated mouse skin.

1.4.3. Sectored plaques originate in *E. coli*

The mutational spectra in sectored mutants from untreated and UVB-treated mouse skin were indistinguishable ($P = 0.96$), and both were similar to the λLIZ/*lacI* mutational spectrum ($P = 0.11$ and 0.14, respectively). The similarity of the spectra from untreated and UVB-treated sectored mutants suggests that the mutations recovered from the sectored mutant plaques arose independently of the mutagenic treatment. This is consistent with the identical SMF obtained from untreated and treated mice (Table 1.3). As well, since the sectored mutants are similar to the λLIZ/*lacI* mutants, which originated during replication in *E. coli*, it is concluded that a large fraction of the sectored mutants examined in this study arose due to spontaneous mutation in *lacI* occurring during phage amplification in *E. coli*.

Sectored plaques can also arise due to factors other than spontaneous mutation in λLIZ/*lacI* replicating in *E. coli*. It is thought that some *ex vivo* mutants arise due to DNA adducts or lesions that escape DNA repair *in vivo*. Inadequate mutation fixation times following certain mutagenic treatments may result in incomplete repair of DNA adducts and significant fractions of sectored plaques (Shane and Tindall 1994; Piegorsch *et al.* 1995). The nonsectored MF and mutational spectra reflect the types of DNA lesions produced given a dose and a mutation fixation time in a particular tissue, and it is expected that the SMF and mutational spectra would do the same if sectored plaques are due to the presence of DNA adducts.

1.4.4. Literature reports of sectored plaques

Few, if any, studies have critically examined the nature and origin of sectored plaques in the *lacI* transgenic rodent assay. The data of Provost and Short (1994) are often cited as evidence that DNA adducts in *lacI* DNA can carry over to *E. coli* to generate sectored plaques. In that study seminiferous tubule DNA was isolated 3 days and 90 days after male B6C3F$_1$ Big Blue® mice were treated once with 250 mg/kg or 3 times with 100 mg/kg of ethylnitrosourea (ENU), respectively. It was observed that the fraction of the total plaques that were sectored (mosaic) decreased from 17% to 3% after 3 days and 90 days mutation fixation time, respectively. Thus, it was concluded that the larger fraction of sectored plaques at the earlier time point reflected carryover of unrepaired O^6-ethyldeoxyguanosine adducts in the *lacI* DNA to *E. coli*. However, their data may be reinterpreted, by calculating the SMF. The SMF remained constant at 0.66×10^{-5} and 0.61×10^{-5} after mutation fixation times of 3 days and 90 days, respectively, indicating that the sectored plaques in their study in fact originated due to spontaneous mutation events as the *lacI* DNA was replicating in *E. coli*, and not due to adducted DNA.

In addition to our data (Table 1.2), other studies have reported SMF that remain constant for DNA that has been recovered from untreated and treated animals, suggesting a bacterial origin for sectored plaques. Hayward *et al.* (1995) examined the *in vivo* mutagenicity of the carcinogen/noncarcinogen pair 2,4-diaminotoluene and 2,6-diaminotoluene. They reported a SMF in the liver of untreated male 6-week old B6C3F$_1$ Big Blue® mice of 8.9×10^{-5}, which was 89% of the MF of 10×10^{-5}. The SMF remained unchanged at approximately 9×10^{-5} in the control and diaminotoluene-treated and

dimethylnitrosamine-treated animals at both the 30-day and 90-day time points, indicating that the sectored plaques in that study predominantly originated from spontaneous mutation events in *E. coli*.

In contrast to these findings, other studies have reported that the SMF from DNA recovered from mutagen-treated animals was significantly higher than in untreated controls. For example, Shane and Tindall (1994) report that the SMF in the liver of control and benzo[*a*]pyrene-treated male C57BL/6 Big Blue® mice were 1.4×10^{-5} and 6.1×10^{-5}, respectively, following a 4-day mutation fixation time. These data suggest that bulky DNA adducts remaining in the *lacI* DNA recovered from mouse genomic DNA were packaged and fixed as mutations in *E. coli*, resulting in an increased frequency of sectored mutant plaques relative to control mouse DNA.

Piegorsch *et al.* (1995) also found a higher SMF in mutagen-treated animals compared with untreated control animals. In that study, female C57BL/6 Big Blue® mice were treated with a single dose (250 mg/kg) of ENU, and liver tissue was examined after a 10-day mutation fixation period. The SMF significantly ($P = 0.01$) increased to 4.17×10^{-5} in ENU-exposed mice compared with 2.77×10^{-5} in untreated controls, indicating that DNA adducts formed *in vivo* were being expressed *ex vivo*.

Comparing the data of Provost and Short (1994) and Piegorsch and colleagues (1995), different conclusions are drawn regarding the carryover of ENU adducts in DNA from the mouse to *E. coli*. However, it should be noted that there were many differences in the two study designs, including differences in the dosing, the tissue examined, the mouse strain and sex, and the mutation fixation periods. As well, while Piegorsch and colleagues (1995) found a significant increase ($P = 0.01$) in the SMF, the actual increase in the SMF between control (mean ± standard deviation = $2.77 [\pm 1.39] \times 10^{-5}$) and ENU-treated animals ($4.17 [\pm 1.94] \times 10^{-5}$) was small. Furthermore, when 6-week-old male $B6C3F_1$ mice are given an intraperitoneal injection of 40 mg/kg of ENU, there is no difference in SMF between control (6.2×10^{-5}) and ENU-treated (6.4×10^{-5}) groups in splenic T cells after a 6-week mutation fixation period (Walker *et al.* 1996). The presumed carryover of O^6-ethyldeoxyguanosine adducts in the Piegorsch group's (1995) study may reflect the shorter mutation fixation period (10 days) in this study compared

with those of Provost and Short (1994) and Walker and colleagues (1996) (≥ 4 weeks and 6 weeks, respectively).

It has been noted that the SMF in control animals vary considerably from study to study, as do the SMF:MF ratios (Provost and Short 1994; Shane and Tindall 1994; Hayward *et al.* 1995; Piegorsch *et al.* 1995; this study). In these five studies, the spontaneous SMF ranged from 0.66×10^{-5} to 8.9×10^{-5}, while the SMF:MF ratios ranged from 0.24 to 0.89. Since the rate of spontaneous mutation events in replicating *lacI* DNA *in vitro* is presumed to be relatively constant under standard conditions, these differences are likely simply explained as experimental differences between studies. Spontaneous MF values reported in the literature vary considerably, even when a single tissue type such as liver is examined (Mirsalis *et al.* 1994, and references therein). This is likely a reflection of interlaboratory differences in performing the Big Blue® assay, including the choice of plaque plating densities. Criteria used in individual laboratories to decide whether a plaque is mutant or sectored will also contribute to variation in published data, particularly since it is difficult to identify sectored plaques even under standardized conditions of plating and scoring (≤ 24 PFU/cm^2). Well-isolated plaques can be verified as sectored or mosaic using the mosaic plaque assay (Provost *et al.* 1993).

1.4.5. Complex mutant in sectored dataset

The use of the *recA* host will certainly decrease the mutagenic consequences of those lesions requiring SOS processing for mutation fixation. Nevertheless, one mutation recovered from a sectored plaque after UV treatment of the skin of a Big Blue® mouse was a tandem mutation at a CC dinucleotide (*lacI* position 586–587). It cannot be excluded that this tandem mutation originated from a UV photoproduct formed in the *lacI* DNA *in vivo*, that was subsequently packaged and fixed as a mutation in *E. coli*, generating the sectored mutant plaque. The location of the complex mutant in the *lacI* gene is intriguing, since this GC-rich region of the *lacI* gene has the potential to form a hairpin structure at nucleotides 576–586. The presence of secondary structure at this sequence is indicated by the strong compression that is observed in DNA sequencing gels at nucleotides 585–591. It has previously been proposed that DNA secondary structures can modulate the extent of both error-free and error-prone repair (Todd and Glickman 1982).

1.5. Conclusions

We have examined the mutations in sectored mutant plaques in the Big Blue® *lacI* transgenic mouse assay to investigate their origin. Sectored plaques can arise due to spontaneous mutations that occur in *lacI* DNA as it replicates in *E. coli*, or as the result of damaged *lacI* DNA that has been recovered from Big Blue® tissues, packaged, and replicated in *E. coli*. We have shown that the mutational spectra of sectored mutants from untreated and UVB-treated mouse skin are identical and similar to that obtained from spontaneous λLIZ/*lacI* mutants. The sectored spontaneous λLIZ/*lacI* mutational spectrum is different from the spectra of spontaneous mutants in *E. coli* and Big Blue® liver and skin. Sectored mutants can form a significant fraction (20%) of the mutant plaques in untreated mouse skin. Thus, in order to accurately determine *in vivo* MF and mutational specificities, it is necessary to score sectored plaques and partition them from the rest of the data, particularly when MF and mutational spectra are close to background levels. While the mutational spectra of sectored plaques is largely consistent with their origin as spontaneous mutations arising *in vitro* during growth of the λLIZ/*lacI* shuttle vector DNA on the *E. coli* host, the potential contribution from lesions in mouse DNA that are expressed *ex vivo* in the *E. coli* host cannot be excluded. Therefore, at minimum, it would be prudent to allow sufficient time after the last treatment prior to recovery of tissues for MF analysis to minimize this potential effect. We conclude that until more sequence data are accumulated regarding sectored plaques from treated animals, their origin and impact on MF and spectra will have to be determined for each experiment.

Acknowledgments

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Table 1.1. Comparison of *lacI* mutational spectra from different genetic backgrounds: *E. coli*, M13/*lacI* bacteriophage, untreated Big Blue® mouse liver and skin, λLIZ/*lacI* bacteriophage, and untreated and UVB-treated sectored mutants from Big Blue® mouse skin

Data Set:	Bacteria [a]	Bacteria [b]	Liver	Skin	λLIZ/*lacI*	Skin	Skin	M13/*lacI* [c]
			Spontaneous	Spontaneous	Spontaneous	Sectored	Sectored	Spontaneous
Mutagen:	None	None	None	None	None	None	UVB	None
Strain:	*E. coli*	*E. coli*	B6C3F1; C57BL6	C57BL6 [d]	*E. coli*	C57BL6	C57BL6	*E. coli*
Clonal Correction:			Yes	Yes	Yes	Yes	Yes	
Number of Mutants:	729 (%)	204 (%)	282 (%)	91 (%)	75 (%)	23 (%)	62 (%)	92 (%)
Transitions								
G:C → A:T	45 (6.1)	45 (22.1)	138 (48.9)	49 (53.8)	17 (22.7)	6 (26.1)	13 (21.0)	61 (66.3)
@ *CpG*	*6 (13.3)* [e]	*6 (13.3)*	*103 (74.6)*	*42 (85.7)*	*3 (17.6)*	*3 (50.0)*	*4 (30.8)*	*7 (11.5)*
A:T → G:C	5 (0.7)	5 (2.4)	17 (6.0)	4 (4.4)	14 (18.7)	5 (21.7)	12 (19.4)	4 (4.3)
Total Transitions	50 (6.8)	50 (24.5)	155 (55.0)	53 (58.2)	31 (41.4)	11 (47.8)	25 (40.3)	65 (70.6)
Transversions								
G:C → T:A	13 (1.8)	13 (6.4)	52 (18.4)	22 (24.2)	8 (10.7)	6 (26.1)	12 (19.4)	5 (5.4)
@ *CpG*	*6 (46.2)*	*6 (46.2)*	*20 (38.5)*	*10 (45.5)*	*4 (50.0)*	*4 (66.7)*	*8 (66.7)*	*3 (60.0)*
G:C → C:G	3 (0.4)	3 (1.5)	11 (3.9)	6 (6.6)	0 (0.0)	1 (4.3)	4 (6.4)	6 (6.5)
A:T → T:A	7 (1.0)	7 (3.4)	8 (2.8)	2 (2.2)	3 (4.0)	0 (0.0)	0 (0.0)	1 (1.1)
A:T → C:G	7 (1.0)	7 (3.4)	9 (3.2)	0 (0.0)	9 (12.0)	0 (0.0)	2 (3.2)	0 (0.0)
Total Transversions	30 (4.2)	30 (14.7)	80 (28.4)	30 (33.0)	20 (26.7)	7 (30.4)	18 (29.0)	12 (13.0)
Hotspot Mutation [f]	525 (72.0)	0 (0.0)	6 (2.1)	0 (0.0)	7 (9.3)	1 (4.3)	5 (8.1)	0 (0.0)
Other Mutations								
+1 Frameshifts	7 (1.0)	7 (3.4)	5 (1.8)	1 (1.1)	7 (9.3)	1 (4.3)	3 (4.8)	1 (1.1)
-1 Frameshifts	28 (3.8)	28 (13.7)	20 (7.1)	2 (2.2)	9 (12.0)	1 (4.3)	5 (8.1)	13 (14.1)
Deletions	73 (10.0)	73 (35.8)	6 (2.1)	2 (2.2)	1 (1.3)	0 (0.0)	3 (4.8)	1 (1.1)
Insertions	16 (2.2)	16 (7.8)	2 (0.7)	0 (0.0)	0 (0.0)	1 (4.3)	0 (0.0)	0 (0.0)
Complex Changes	0 (0.0)	0 (0.0)	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.6)	0 (0.0)
Double Mutants	0 (0.0)	0 (0.0)	6 (2.1)	3 (3.3)	0 (0.0)	1 (4.3)	2 (3.2)	0 (0.0)
Total Other Mutations	124 (17.0)	124 (60.7) [g]	41 (14.5)	8 (8.8)	17 (22.7)	4 (17.4)	14 (22.6)	15 (16.3)

[a] Data from Halliday and Glickman (1991).

[b] From Halliday and Glickman (1991), with the TGGC hotspot mutation (105 deletions, 420 insertions) removed.

[c] Data from Yatagai *et al.* (1991).

[d] This dataset includes the spectra from male and female mice; the remaining datasets are from male mice only. The mutants from the untreated male mice were collected in a DMBA experiment and those from the female mice from the UVB experiment described here. The mutational spectra from the control male and female mice were identical.

[e] Mutations occurring at CpG sequences are a subset of the total number of G:C → A:T or G:C → T:A mutations which are reported in the table.

[f] This is the TGGC insertion/deletion hotspot, located at base pairs 621–632 in *lacI*.

[g] The sum of the column percentages may differ from 100% due to rounding errors.

Table 1.2. Analysis of the sectored mutant data

Dataset	Number of plaques	Number of mouse mutants [a]	Mouse mutant frequency ($\times 10^{-5}$) [a]	Number of sectored mutants	Sectored mutant frequency ($\times 10^{-5}$)	Sectored mutant fraction (%) [b]
Untreated Skin	899,805	78	8.7 ± 1.1	19	2.1	19.6
UVB-treated Skin	808,846	717	88.6 ± 21.0	17	2.1	2.3

[a] Excluding sectored mutants.

[b] The sectored mutant fraction is calculated as (sectored mutants)/(sectored mutants + mouse mutants) and is given as a percentage.

Table 1.3. Statistical analyses of the differences between mutational spectra from spontaneous, induced and sectored *lacI* mutants

	Liver spontaneous	Skin spontaneous	λLIZ/*lacI* spontaneous	Sectored spontaneous	Sectored UVB	M13/*lacI* spontaneous
Bacteria	< 0.001 [a]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Liver Spontaneous		0.18	< 0.001	0.07	< 0.001	< 0.001
Skin Spontaneous			< 0.001	0.01	< 0.001	< 0.001
λLIZ/*lacI* Spontaneous				0.11	0.14	< 0.001
Sectored Spontaneous					0.96	< 0.001
Sectored UVB						< 0.001

[a] *P*-values in this table are estimated by generating 2500 random permutations of the corresponding data table. 95% confidence limits may be calculated for each p-value by computing $1.96 \bullet \sqrt{p(1-p)/2500}$, where p is the *P*-value. The null hypothesis (H_0) is that two spectra are the same; a low *P*-value ($P < 0.05$) rejects H_0.

Chapter 2. Mutation Frequency and Specificity With Age in Liver, Bladder and Brain of *lacI* Transgenic Mice

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Abstract

Mutation frequency and specificity were determined as a function of age in nuclear DNA from liver, bladder and brain of Big Blue® *lacI* transgenic mice aged 1.5 to 25 months. Mutations accumulated with age in liver, and accumulated more rapidly in bladder. In the brain a small initial increase in mutation frequency was observed in young animals; however, no further increase was observed in adult mice. To investigate the origin of mutations, the mutational spectra for each tissue and age were determined. DNA sequence analysis of mutant *lacI* transgenes revealed no significant changes in mutational specificity in any tissue at any age. The spectra of mutations found in ageing animals were identical to those in younger animals, suggesting that they originated from a common set of DNA lesions, manifested during DNA replication. The data also indicated that there were no significant age-related mutational changes due to oxidative damage, or errors resulting from either changes in the fidelity of DNA polymerase or the efficiency of DNA repair. Hence, no evidence was found to support hypotheses which predict that oxidative damage or accumulation of errors in nuclear DNA contribute significantly to the ageing process, at least in these three somatic tissues.

2.1. Introduction

Ageing is a complex biological phenomenon, which is reflected by the numerous and diverse theories of ageing that have been proposed (Medvedev 1990; Bernstein and Bernstein 1991; Kowald and Kirkwood 1996). Theories of ageing involve consideration of various forms of damage to cellular organelles and molecules, including DNA. Many of the non-genetic factors have been collectively considered as a "network theory of ageing," integrating the contributions of defective mitochondria, aberrant proteins, and free radicals (Kowald and Kirkwood 1996). Other theories of ageing invoke DNA damage as the primary cause of ageing (Szilard 1959; Curtis 1971; Gensler and Bernstein 1981). For example, the somatic mutation theory predicts that the frequency of mutations should increase with age (Szilard 1959; Alexander 1967; Morley 1995). Interest in mutational theories of ageing reflect the fact that many genetic diseases, like cancer, are more prevalent in older populations.

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The study of mutation *in vivo* is facilitated though the use of transgenic rodents, in which mutational responses can be measured in virtually any tissue as a function of age, sex and diet. The mutational target in Big Blue® transgenic mice and rats (Kohler *et al.* 1990, 1991a; Provost *et al.* 1993; Dycaico *et al.* 1994) is the exceptionally well characterized *lacI* gene from *E. coli*. The *lacI* gene is highly sensitive to base substitution and frameshift mutations, as well as small deletions and insertions, making the transgene an ideal choice for recovery of spontaneous and induced mutations (de Boer and Glickman 1998). As well, spontaneous mutational spectra (MS) have been carefully determined for a variety of tissues, providing a reference or baseline for evaluation of age-related or induced mutational effects (de Boer *et al.* 1997, 1998). Studies from our laboratory, and others, have demonstrated that MS are unique for each chemical and physical agent examined (Glickman *et al.* 1995). All mutagens examined to date induce characteristic mutational spectra in the *lacI* transgene. Indeed, significant changes in mutational specificity have been recovered from treated animals despite changes in mutant frequencies (MF) of less than two-fold, as for example, with *tris*(2,3-dibromopropyl)phosphate (de Boer *et al.* 1996b) and oxazepam (Shane *et al.* 1999).

In this study the Big Blue® mutational assay (Kohler *et al.* 1991a; Provost *et al.* 1993) was used to investigate the frequency and specificity of mutation in the *lacI* transgene *in vivo* as a function of age in liver, bladder and brain of mice. Although the mutation frequencies (M*f*) increased in ageing proliferating tissues, there were no significant age-related differences among the various MS, in nuclear DNA of mice up to 25 months of age. The absence of age-related changes in the MS in these three diverse tissues do not support a significant role for ageing theories which predict that oxidative damage or the accumulation of genetic errors ("error catastrophe") are a major determinant of ageing. Additionally, the relatively modest increases in M*f* (approximately three-fold in bladder of mice 12 months old and in liver of mice aged 25 months) suggest that the contribution of spontaneous mutations to the ageing process is minimal.

2.2. Materials and Methods

2.2.1. Mice

The animals used in this study were male hemizygous λLIZ/*lacI* (Big Blue®) transgenic C57BL/6 mice (Taconic, Germantown, NY). The animals were housed at 20° with a 12 hour

light cycle (6 AM to 6 PM). Purina Mouse Chow 5015 (Ralston Purina Company, St. Louis, MO) and water were provided *ad libitum*. The mice were maintained in the University of Victoria Animal Care Unit, under standards conforming with the NIH Guide for the Care and Use of Laboratory Animals. At the appropriate ages, mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation, and tissues were immediately dissected, flash-frozen in liquid nitrogen, and stored at –80°.

2.2.2. Genomic DNA isolation

High molecular weight mouse genomic DNA from liver and brain tissue was isolated using a dialysis purification method (Suri *et al.* 1996). Bladder tissue, which was refractory to disaggregation using Dounce tissue grinders, was minced using a sterile razor blade and immediately digested with proteinase K at 50°, then dialyzed as previously described.

2.2.3. Big Blue® assay

The Big Blue® assay was performed following the standardized color-screening assay protocol (Stratagene 1997; Rogers *et al.* 1995; Young *et al.* 1995). To facilitate the identification of *ex vivo* and *in vitro* mutants (Stuart *et al.* 1996), which were excluded from the analysis, generally less than 16 plaque-forming units/cm^2 (≤10,000 plaques per 25 cm x 25 cm assay tray) were plated.

2.2.4. DNA sequencing and data management

Mutations in *lacI*-bearing λ phage were determined by DNA sequencing using PCR cycle sequencing and automated DNA sequencers, as previously described (Erfle *et al.* 1995). Only *in vivo* (mouse-derived) mutants were considered for analysis (Stuart *et al.* 1996). DNA sequence data were managed and analyzed using custom software (de Boer 1995). To ensure that independent mutational events were analyzed, the data were corrected for possible clonal expansions (de Boer *et al.* 1996a; de Boer *et al.* 1997) by counting only one mutation for those which were recovered more than once from an individual animal. The ageing frequency data were corrected accordingly and reported as mutation frequencies (M*f*), rather than uncorrected *mutant* frequencies (MF).

2.2.5. Statistical analyses

Statistical comparisons of MS were made using the Monte Carlo method of Adams and Skopek (Adams and Skopek 1987; Cariello *et al.* 1994) with 2500 iterations, using a program provided by the authors. These tests of significance consisted of pairwise

comparisons of MS, using the 12 mutational classes shown in Tables 2.2–2.4, as well as the numbers of G:C → A:T and G:C → T:A mutations which occurred at 5′–CpG–3′ (CpG) dinucleotide sequences (Stuart *et al.* 1996). The α-level for significance was set at 0.05. Trends in the mutation frequency data were analyzed using COCHARM (created by Troy Johnson, Procter & Gamble, Cincinnati, OH), a computer program that executes the Generalized Cochran-Armitage test.

2.3. Results

2.3.1. Mutation frequency *versus* age

As shown in Table 2.1 and in Figure 2.1, there was a statistically significant increase in the M*f* in the *lacI* transgene in liver from mice aged 1.5 to 18 months. The M*f* at 25 months, although higher, was not significantly greater than that observed at 18 months. M*f* in bladder also increased significantly with age, and were significantly higher than liver, at all ages examined (1.5, six and 12 months). In addition, M*f* in the bladder increased faster than those in the liver. Brain M*f* were lower than those observed either in the liver or bladder, at all ages. Following a small, but significant increase in M*f* in mice aged 1.5 to six months, no further change was observed in M*f* in adult brain, even at 25 months. "Sectored" mutant frequencies (the frequencies of *in vitro* and *ex vivo* mutants) (Stuart *et al.* 1996) are also reported in Table 2.1; however, these mutants were partitioned from *in vivo* (mouse-derived) mutants, and were not sequenced. Sectored mutants are believed to arise due to damaged, unrepaired mouse *lacI* DNA which is subsequently repaired *ex vivo* in *E. coli*; they may also arise *de novo* as the *lacI* λ phage replicates *in vitro*.

2.3.2. Mutational specificity *versus* age

A subset of the *lacI* mutant λ phage recovered from each tissue and each age group were randomly selected for DNA sequence analysis (Tables 2.2–2.4). To facilitate direct comparison of the various spectra by the Reader, the data provided in Tables 2.2–2.4 are expressed as percentages. For each tissue and all age groups, the predominant class of mutations was G:C → A:T transitions, comprising 34–56% of all mutations, with the majority (62–92%) of these transitions occurring at CpG sequences. The second most

common class of mutations was G:C → T:A transversions, which comprised 14–31% of all mutations.

Using the Adams-Skopek (Monte Carlo) algorithm, MS from different age groups from each tissue were compared to determine if statistically significant changes in MS occurred with age within a tissue type (data not shown). As well, MS from each tissue and age group were compared with each other, to determine if differences existed in the MS among the three tissues. No obvious differences or interpretable trends were observed among any of the mutational spectra.

2.4. Discussion

The Big Blue® assay provides a versatile and sensitive *in vivo* mutational model. The mutational target in Big Blue® mice is the *lacI* transgene, present in a λ shuttle vector which is (stably) integrated as a tandem array of approximately 40 copies at a single position in chromosome 4 of Big Blue® mice (Dycaico *et al.* 1994). It appears that the *lacI* transgene is fully methylated, with cytosines at CpG sequences present as 5-methylcytosine (Kohler *et al.* 1990; de Boer and Glickman 1998; You *et al.* 1998), and is therefore nontranscribed (Provost and Short 1994). Nevertheless, mutational data determined in the *lacI* transgene are likely to be reasonably accurate estimates of those occurring throughout the mouse genome for several reasons. Firstly, mutations in the *lacI* transgene are thought to be neutral and confer no selective growth advantage or disadvantage to the cell. Also, while sometimes debated, it appears that DNA repair activity is not significantly different in the *lacI* transgene compared with endogenous mammalian genes, as similar mutational responses have been observed in the *lacI* transgene compared to the mouse genes *Dlb-1* (Tao *et al.* 1993) and *Hprt* (Skopek *et al.* 1995; Walker *et al.* 1996). Finally, changes in the *lacI* spontaneous mutational spectrum observed in *Msh2*$^{-/-}$ *lacI* cotransgenic mice indicates that *lacI* transgenes respond as predicted to changes in DNA repair function (Andrew *et al.* 1997).

In the present study, we determined M*f* and MS in liver, bladder and brain of Big Blue® mice aged 1.5–25 months. Age-related increases in M*f* are readily detected using standard statistical methods; in this study, trends in M*f* with age were analyzed using the Cochran-Armitage test. Analyses of MS from *lacI* transgenic animals are routinely compared using a

computer algorithm described by Adams and Skopek (Adams and Skopek 1987; Cariello *et al.* 1994), a Monte Carlo approximation to Fisher's exact test, that is generally regarded (*e.g.*, Piegorsch and Bailer 1994) as one of the most robust methods for statistical comparisons of MS. Therefore, this method was used in this study to evaluate potential changes of MS with age. The application of the Monte Carlo test to analyses of MS is illustrated with selected examples from the literature.

Strong mutagens induce specific mutations at frequencies which result in induced MS that are obviously different from spontaneous MS. For example, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine predominantly induces G:C→T:A transversions and −1 frameshifts in the *lacI* transgene in rat colon (Okonogi *et al.* 1997a).[10] Applying the Monte Carlo test to the data provided in Table 3 in Okonogi *et al.* (1997a), the MS from 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-treated and untreated colon were found to be highly significantly different ($P < 10^{-6}$). However, since the spontaneous MS in mice in the present study arose in the absence of strongly mutagenic agents, it is perhaps more relevant to cite examples where significant differences in MS have been detected following treatment with weakly mutagenic agents. For example, despite changes in MF of less than two-fold in Big Blue® mice treated with *tris*(2,3-dibromopropyl)phosphate (de Boer *et al.* 1996b) or oxazepam (Shane *et al.* 1999), significant changes ($P = 0.02$ and $P < 0.015$, respectively) in MS were detected using the Monte Carlo test. *Tris*(2,3-dibromopropyl)phosphate was found to induce a dose-dependent decrease in the frequency of G:C→A:T transitions (including the frequency of these mutations occurring at CpG sequences) and an increase in the frequency of deletions of G:C basepairs in the tumor target, but not nontarget, tissues. The *tris*(2,3-dibromopropyl)phosphate analyses and conclusions were subsequently confirmed using a log-linear statistical analysis (Brackley *et al.* 1999).

Stuart *et al.* (1996)[11] used the Monte Carlo test to determine the influence of genetic background on *lacI* spontaneous MS recovered from the endogenous gene in *E. coli*, bacteriophage M13/*lacI*, λLIZ/*lacI* phage (*i.e.*, the Big Blue® shuttle vector) propagated *in vitro* in *E. coli*, and *in vivo* and *ex vivo* λLIZ/*lacI* phage recovered from skin and liver of Big

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[10] Chapter 6 of this Dissertation.
[11] Chapter 1 of this Dissertation.

Blue® mice. Despite the fact that all of the *lacI* genes included propagation though *E. coli* at some stage, using the Monte Carlo test we were able to show that the MS segregated into four distinct groups: the *E. coli lacI* gene; M13*lacI*; λLIZ/*lacI* mutations arising *in vitro*/*ex vivo* during passage in *E. coli*; and, λLIZ/*lacI* mutations arising *in vivo* in mouse skin and liver. Lastly, we note that Curry *et al.* (1999) used the Monte Carlo test to examine age-related changes in the human *HPRT* gene. They found that deletions > 1 bp occurred twice as frequently in females as in males, but no other changes in MS with age were observed with the exception of A:T→C:G transversions, which increased in older individuals.

It is our experience (as well as those of other laboratories) that all mutagens, and even spontaneous mutations, exhibit unique MS (reviewed by Glickman *et al.* 1995); therefore, we are confident that age-related differences among MS from various tissues should be readily identified, should they exist. Lastly, we believe that even subtle differences among MS should be apparent upon careful examination of MS. This latter point is illustrated below, during the discussion of increased frequency of TGG→TTT tandem transversion mutations in aging liver.

The data obtained in the present study unambiguously demonstrated that spontaneous *in vivo* M*f* increased in ageing mice in an adult somatic tissue which proliferates (liver) or is capable of proliferating when stimulated (bladder), but not in a nonproliferative tissue (brain) (Figure 2.1; Table 2.1). M*f* increased at a relatively constant rate in liver of ageing mice, and at a significantly higher rate in bladder. Overall, at any age bladder M*f* were higher than in those in liver, and liver M*f* were higher than those in brain. Compared with 1.5 month-old mice, liver M*f* increased two-fold by age six months, and more than three-fold by 25 months of age. In bladder, M*f* in 12 month-old mice had increased almost three-fold, relative to 1.5 month-old animals. A 1.6-fold increase in M*f* was observed in brain in maturing mice (1.5 months old compared with six months); however, after six months of age there was no further significant change in M*f* in adult brain. Collectively, these data suggest a correlation between cellular proliferation (nuclear DNA replication) and an increase in M*f*.

The present study also describes the first detailed analysis of mutational spectra (specificity) as a function of age in selected tissues. As MS may provide insights into the origin of mutation, MS were determined for each tissue at each age (Tables 2.2–2.4).

Interestingly, there were no significant differences in MS in mice of any age, indicating that the age-related increases in *Mf* resulted from the accumulation of the same types of DNA damage by a pathway similar to that occurring earlier in life. This strongly hints that most, if not all of the mutations that accumulate during ageing share a common origin, and are manifested through the process of cell proliferation. Specifically, these data suggest that there are no significant age-related accumulation of mutations that might be attributable to specific ageing mechanisms, such as damage from free radicals, as this would result in changes in the relative proportions of the mutational classes which define the well-characterized spontaneous MS in younger animals (de Boer *et al.* 1997, 1998).

Of the three tissues examined, brain MS from mice of different ages were the most homogeneous, indicating that brain DNA was less affected mutationally by age than liver or bladder. Since *Mf* in brain increased only approximately 1.6-fold (on average) after age 1.5 months (Table 2.1) with no change in MS, it seems probable that the mutations occurred primarily during DNA replication as brain tissue was proliferating, as it does early in life (Korr 1980).

The conclusion that age-related effects on *Mf* and MS in liver and brain observed in the present study accumulate during DNA replication is supported by the known proliferative activity of adult tissues. Liver is regarded as a slowly renewing (proliferating) tissue (Cameron 1970) in which DNA polyploidy levels steadily increase with age (Brodsky and Uryvaeva 1977; Enesco and Samborsky 1983), indicating that DNA replication is maintained in this tissue. Adult brain tissue primarily consists of nonproliferating neuronal cells, plus a much smaller population of glial cells (a fraction of which continue to proliferate in adults) (Cameron 1970; Korr 1980; Korr *et al.* 1983). DNA content is also known to remain diploid in adult brain tissue (Winick *et al.* 1972). It should be noted that we do not suggest that the state of "being polyploid" itself increases *Mf*; since *Mf* are expressed as frequencies (per 10^5 recovered transgenes), a simple doubling of the chromosome number by itself does not affect this ratio. However, the DNA replication which necessarily accompanies polyploidization provides additional opportunity for DNA lesions (or misincorporated nucleotides) to become established as mutations.

The elevated rate of increase in *Mf* with age in bladder, compared with liver was not predicted. Unstimulated urothelium of adult mice is practically mitotically quiescent, based

on the very low mitotic and labeling indices which are observed in this tissue (Clayson and Pringle 1966; Jost and Potten 1986; Jost 1989; Cohen and Ellwein 1991). Although mouse epithelial bladder cells become polyploid, this process is essentially completed by about six to eight weeks of age (Walker 1958; Farsund 1975). Nevertheless, when the *Mf* and MS from bladder are compared to those from liver and brain, it seems possible that DNA synthesis or cellular proliferation rates in the bladder may have been higher than expected, although the factors that may have contributed to such an increase in this study remain unexplained. However, it is noted that normal bladder function is significantly affected by a variety of stimuli, including diet, and bladder retains a capacity for rapid regeneration following mechanical trauma and chemical injury (Hicks 1975; Cohen 1995).

An alternative explanation for the enhanced rate of mutant accumulation in bladder follows from the observation that the frequency of "sectored" (*in vitro, ex vivo*) mutant plaques (Table 2.1) increased dramatically with age in bladder. These mutants, believed to result from expression in *E. coli* of unrepaired, damaged mouse DNA (Stuart *et al.* 1996), indicate that bladder DNA accumulated more damage, compared with liver and brain. This damage would contribute to an elevation in *Mf* when these lesions were expressed as mutations during DNA replication.

DNA replication in adult mouse liver is largely associated with polyploidization and is maintained at a relatively constant rate (Brodsky and Uryvaeva 1977; Enesco and Samborsky 1983). Since liver *Mf* also increased at a similar rate, it seems likely that the increase in *Mf* in adult liver reflected the accumulation of mutations during polyploidizing DNA replication. Assuming that *Mf* double with each round of DNA replication, the 1.95-fold increase in the liver *Mf* of mice aged 1.5 to 12 months (Table 2.1) indicates that approximately 0.96 liver DNA replications occurred during this time (since $2^{0.96} = 1.94$). The 2.93-fold increase in bladder *Mf* from ages 1.5 to 12 months would have corresponded to approximately 1.55 DNA replications ($2^{1.55} = 2.93$). However, since bladder tissue is known generally to proliferate more slowly than liver, and the sectored *Mf* data indicated that bladder accumulated more DNA damage, it seems probable that DNA replicative activity was lower in bladder than in liver, and that decreased DNA repair activity (or possibly, the efficiency of repair) in bladder resulted in elevated *Mf*, compared with liver.

In regard to spontaneous somatic mutations, it has been determined that about half of all spontaneous mutations observed in young mice arise during development, with approximately half of these mutations occurring *in utero* (Zhang *et al.* 1995). Those observations were confirmed in the present study, since the *Mf* increased rapidly, from essentially zero at conception (three weeks before birth), to between 2.9×10^{-5} and 5.6×10^{-5} depending upon the tissue by 1.5 months of age (Table 2.1). These data again demonstrate a relationship between cellular proliferation, the rates of which are maximum during development, and *Mf*. Ames has also noted that "mitogenesis increases mutagenesis" (Ames *et al.* 1993; Shigenaga and Ames 1993).

As indicated earlier, there were no generally interpretable age- or tissue-related differences or trends among the various MS, following pairwise comparisons of MS using the Adams-Skopek (Monte Carlo) algorithm. However, subtle differences in the frequencies of some mutations were nevertheless noted (Tables 2.2–2.4). Among the three tissues, the proportion of G:C → A:T transitions which occurred at CpG sequences was greatest in bladder (82%, average of all age groups), compared with liver (65%, average) and brain (78%, average). Double (tandem) mutations appeared most frequently in liver, compared with bladder and brain. Interestingly, the frequency of TGG/CCA → TTT/AAA (*i.e.*, 5′–TGG–3′ → 5′–TTT–3′ or 5′–CCA–3′ → 5′–AAA–3′ on the opposite strand) tandem mutations increased in liver (at various sites in the *lacI* gene), from approximately 0.054×10^{-5} (on average) in liver ≤ 12 months-old, to 0.43×10^{-5} (8-fold increase) at 18 months and 1.1×10^{-5} (20-fold increase) at 25 months (sequence data not shown). Except for a 5.9-fold increase in the *Mf* for deletions in 25 month-old liver compared with the average *Mf* from liver aged 1.5 to 18 months (1.3×10^{-5} and 0.22×10^{-5}, respectively), no increases in the frequency of deletions were otherwise observed among the three tissues. Examining Tables 2.2 and 2.4, there appeared to be a slight age-related decrease in the proportion (as a percentage) of G:C → T:A transversions which occurred at CpG sequences in liver and brain. However, when *Mf* were calculated, there was only a trivial increase in the frequency of these mutations in liver, and a trivial decrease in brain. Lastly, the frequency of minus-one frameshifts appeared to increase with age in bladder.

The factors which may have contributed to the subtle changes in MS in the oldest tissues remain speculative. The increased frequencies of GG/CC → TT/AA tandem mutations and deletion mutations were specific to liver of the oldest mice, 18 and 25 months old. (An increase in the frequency of GG/CC → TT/AA tandem transversions was also noted by Buettner *et al.* (1999) in the *lacI* transgene from ageing mouse liver.) This tandem transversion is otherwise rarely observed in Big Blue® — excluding the 14 mutants from the present study, and three mutants recovered from dietary-restricted mice aged 6–12 months (Stuart and Glickman, unpublished results) — we have identified GG/CC → TT/AA mutations in only 30/17,016 (0.18%) sequenced spontaneous and induced Big Blue® *lacI* mutants (de Boer 1995; de Boer and Glickman, unpublished results). Among our collection of sequenced *E. coli lacI* mutants, only 2/14,400 (0.01%) GG/CC → TT/AA tandem transversions have been identified (de Boer 1995; de Boer and Glickman, unpublished results).

The observation that 11/14 (79%) of the GG/CC → TT/AA tandem transversions involved TGG/CCA sequences (including 6/6 mutations recovered from 25 month-old mice) suggests that these otherwise infrequent TGG/CCA → TTT/AAA mutations might represent a mutational "signature" of an age-related change in mutational spectrum in older liver. It has been observed that GG/CC → TT/AA tandem transversions result when plasmids treated *in vitro* with acetaldehyde (Matsuda *et al.* 1998), acrolein (Kawanishi *et al.* 1999) or crotonaldehyde (Kawanishi *et al.* 1998) are permitted to replicate in human cells. Interestingly, these and other mutagens can arise endogenously from lipid peroxidation (Nath *et al.* 1996; Chung *et al.* 1999) and normal cellular metabolism (Ostrovsky 1986). Since acrolein-deoxyguanosine but not crotonaldehyde-deoxyguanosine adduct levels increase in liver of older rats (Chung *et al.* 1999), it is possible that the tandem GG/CC → TT/AA transversions observed in liver in the present study were due to acrolein. It is also possible, however, that the tandem mutations and deletions observed in aged liver were attributable to a suspected slight increase in error-prone DNA polymerase activity or template-directed mutagenesis (Taguchi and Ohashi 1997; Hampsey *et al.* 1988), as suggested by the several-fold increase in the sectored M*f* in older liver (Table 2.1).

Ames has proposed that oxidative damage is a major contributor to ageing (Adelman *et al.* 1988; Ames and Shigenaga 1992; Helbock *et al.* 1998). While Ames' predictions of a causal relationship between oxidative damage and ageing (*e.g.*, a decline of mitochondrial function and other physiological changes) are probably valid, our data indicated a negligible effect of oxidative damage on nuclear DNA in liver, bladder and brain of mice aged 1.5 to 25 months. During DNA replication, 8-oxo-2′-deoxyguanosine (8-oxoG) present in the template strand can mispair with adenosine, leading to G:C → T:A transversion mutations, while misincorporation of 8-oxoG as a substrate nucleotide can lead to A:T → C:G transversions (Cheng *et al.* 1992). Our data revealed no age-related increases in the occurrence of either G:C → T:A or A:T → C:G transversions in older mice compared with young mice (Tables 2.2–2.4). Indeed, the proportion of these mutations relative to other changes remained relatively constant in adult liver, bladder and brain, suggesting that oxidative DNA damage is not a major contributor to *Mf* or MS in nuclear DNA. These data also agree with results from a recent study which found no significant age effects for the levels of 10 different oxidatively-induced base lesions in both mitochondrial and nuclear DNA from rat liver (Anson *et al.* 1999). It is possible, however, that 8-oxoG (and hence, 8-oxoG-derived mutations) only accumulate to an appreciable level in animals of advanced age (Hirano *et al.* 1996; Kaneko *et al.* 1997).

Other laboratories have demonstrated significant increases in *Mf* with age in tissues in *lacI* and *lacZ* transgenic mice (*e.g.* Lee *et al.* 1994; Ono *et al.* 1995; Dollé *et al.* 1997); however, none have sequenced sufficient randomly selected mutants to permit evaluation of changes in mutational specificity with age. Lee *et al.* (1994) reported a four-fold increase in *lacI* transgene MF (uncorrected for clonal expansions) in spleen of mice from birth to 25 months-old. Their MS consisted of 14% G:C → A:T transitions (with 33% of these occurring at CpG sequences), 5% G:C → T:A transversions, 18% G:C → C:G transversions, 27% double mutants, and 1.5% "size-change" mutants (determined electrophoretically) in mice aged 1–2 months (increasing to 12–19% size-change mutants in mice age 3–24 months). This MS deviated significantly from spontaneous *lacI* MS from spleen, liver, lung, bone marrow, stomach, skin and kidney of 3–12 week-old Big Blue® mice (de Boer *et al.* 1998), as well as spontaneous *lacI* MS in the endogenous *lacI* gene in *E. coli*, bacteriophage

M13 and "sectored" (*in vitro/ex vivo*) Big Blue® plaques (Stuart *et al.* 1996). Thus, the spleen MS reported by Lee *et al.* (1994) is enigmatic.

Studies using plasmid-based *lacZ* transgenic mice have also demonstrated significant age-related increases in MF in liver and spleen, but not brain (Dollé *et al.* 1997; Vijg *et al.* 1997). However, mutants were simply screened for large size changes on agarose gels, which indicated that approximately 50% of the mutants contained deletions and complex chromosomal changes (Gossen *et al.* 1995; Dollé *et al.* 1997; Vijg *et al.* 1997). Since only eight mutants were sequenced (Dollé *et al.* 1997), a detailed analysis of the effect of age on the mutational spectra was not possible. Although the elevated frequency of deletions/rearrangements observed by Vijg and colleagues might reflect the *in vivo* frequency of these mutations, our transgenic *lacI* data (this study; de Boer *et al.* 1997) as well as a meta-analysis of human *HPRT* mutations (Curry *et al.* 1999) indicate that the frequency of deletions from the *lacZ* plasmid transgenic assay could be overestimated. Similarly, it appears that large deletions, greater than two kilobases in length, are rare in the human factor IX gene (Ketterling *et al.* 1994). Although the Big Blue® assay is likely insensitive to the detection of large deletion events (as well as chromosomal rearrangements), deletions greater than two kilobases have been recovered (Winegar *et al.* 1994; Mirsalis 1995; Buettner *et al.* 1996). Theoretically, *lacI* deletions up to approximately 7.5 kilobases should be detectable (Dycaico *et al.* 1994).

In conclusion, the data presented in the current study demonstrated an age-related increase in the frequency of spontaneous mutations with no significant differences in mutational specificity, in nuclear DNA from three somatic tissues from mice up to 25 months old. It seems probable that the age-related increases in the spontaneous mutation frequencies reflects endogenous DNA damage which was subsequently expressed as mutations following DNA replication. The increases in M*f* with age partly support the somatic mutation theory. However, the absence of significant changes in MS in older animals tends not to support ageing theories which are based primarily on predicted increases of oxidative damage or the accumulation of genetic errors ("error catastrophe") in nuclear DNA. Finally, the relatively small (several-fold) increases in M*f*, combined the absence of significant changes in MS in older animals, indicates that spontaneous mutations are likely to have a modest influence on the ageing process, at least until late middle-age. In

this regard, it should be noted that mice nullizygous for the mismatch repair gene *Pms2* show a 100-fold elevation in mutation frequencies in all tissues examined compared to both wild-type and heterozygous littermates, but develop normally and do not appear to age prematurely (Narayanan *et al.* 1997).

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Table 2.1. Summary of the liver, bladder and brain mutation frequency data

Tissue	Age (months)	No. of mice	No. of mutants [b]	Total PFU ($\times 10^6$)	Mf [a] ($\times 10^{-5}$)	SMF ($\times 10^{-5}$)
Liver	1.5	5	111	2.59	4.29 ± 0.33 [c]	2.47
	6	4	135	2.22	6.08 ± 0.55	1.08
	12	4	167	1.99	8.39 ± 1.22	1.25
	18	2	101	0.795	12.7 ± 0.35	6.92
	25	3	114	0.804	14.2 ± 1.13	6.96
Liver Totals		18	628	8.40		
Bladder	1.5	5	89	1.58	5.63 ± 0.81	3.41
	6	6	127	1.17	10.8 ± 1.72	9.51
	12	4	142	0.861	16.5 ± 2.22	11.5
Bladder Totals		15	358	3.61		
Brain	1.5	5	45	1.56	2.88 ± 0.47	1.92
	6	6	86	1.87	4.60 ± 0.81	3.31
	12	4	62	1.48	4.19 ± 0.56	2.43
	18	2	43	0.874	4.92 ± 0.49	3.09
	25	3	37	0.746	4.96 ± 0.37	2.81
Brain Totals		20	273	6.53		

[a] Mf, mutation frequency; PFU, plaque-forming units; SMF, sectored mutant frequency.
[b] Corrected for possible clonal expansions.
[c] Values represent means ± SE about the mean.

Table 2.2. Spontaneous *lacI* mutations from liver of Big Blue® mice

Age	1.5 Months			6 Months			12 Months			18 Months			25 Months		
	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG
Transitions															
G:C → A:T	28	36.4	71.4	37	33.9	67.6	50	33.6	62.0	22	37.3	63.6	32	41.6	62.5
A:T → G:C	3	3.9		13	11.9		16	10.7		5	8.5		1	1.3	
Transversions															
G:C → T:A	23	29.9	65.2	25	22.9	44.0	34	22.8	50.0	13	22.0	38.5	17	22.1	35.3
G:C → C:G	6	7.8	50.0	5	4.6	80.0	8	5.4	37.5	2	3.4	100.0	1	1.3	0.0
A:T → T:A	4	5.2		3	2.8		7	4.7		2	3.4		5	6.5	
A:T → C:G	1	1.3		0	0.0		6	4.0		4	6.8		2	2.6	
Other Mutations															
+1 Frameshift	2	2.6		6	5.5		6	4.0		1	1.7		0	0.0	
−1 Frameshift	3	3.9		13	11.9		8	5.4		2	3.4		4	5.2	
Deletions	4	5.2		3	2.8		5	3.4		1	1.7		7	9.1	
Insertions	0	0.0		2	1.8		3	2.0		0	0.0		1	1.3	
Complex Changes	0	0.0		0	0.0		3	2.0		0	0.0		0	0.0	
Double Mutants	3	3.9		2	1.8		3	2.0		7	11.9		7	9.1	
Total [a]	77	100		109	100		149	100		59	100		77	100	

[a] The total numbers of mutants after correction for clonality (refer to Materials and Methods). The non-corrected mutant totals were 84 at 1.5 months, 131 at 6 months, 170 at 12 months, 69 at 18 months, and 86 at 25 months.

Table 2.3. Spontaneous *lacI* mutations from bladder of Big Blue® mice

Age	1.5 Months			6 Months			12 Months		
	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG
Transitions									
G:C → A:T	33	49.3	81.8	31	56.4	74.2	29	46.8	89.7
A:T → G:C	3	4.5		3	5.5		9	14.5	
Transversions									
G:C → T:A	18	26.9	61.1	9	16.4	44.4	11	17.7	54.5
G:C → C:G	1	1.5	0.0	0	0.0	0.0	2	3.2	50.0
A:T → T:A	5	7.5		4	7.3		0	0.0	
A:T → C:G	1	1.5		1	1.8		2	3.2	
Other Mutations									
+1 Frameshift	0	0.0		1	1.8		2	3.2	
−1 Frameshift	3	4.5		4	7.3		7	11.3	
Deletions	3	4.5		1	1.8		0	0.0	
Insertions	0	0.0		1	1.8		0	0.0	
Complex Changes	0	0.0		0	0.0		0	0.0	
Double Mutants	0	0.0		0	0.0		0	0.0	
Total [a]	67	100		55	100		62	100	

[a] The total numbers of mutants after correction for clonality (refer to Materials and Methods). The non-corrected mutant totals were 71 at 1.5 months, 64 at 6 months, and 66 at 12 months.

Table 2.4. Spontaneous *lacI* mutations from brain of Big Blue® mice

Age	1.5 Months			6 Months			12 Months			18 Months			25 Months		
	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG	No.	%	%@CpG
Transitions															
G:C → A:T	19	42.2	73.7	28	43.8	64.3	24	39.3	91.7	21	50.0	76.2	18	51.4	83.3
A:T → G:C	3	6.7		3	4.7		3	4.9		2	4.8		3	8.6	
Transversions															
G:C → T:A	8	17.8	62.5	11	17.2	45.5	19	31.1	47.4	8	19.0	25.0	5	14.3	20.0
G:C → C:G	3	6.7	66.7	2	3.1	50.0	3	4.9	66.7	2	4.8	50.0	2	5.7	100.0
A:T → T:A	3	6.7		2	3.1		0	0.0		2	4.8		3	8.6	
A:T → C:G	0	0.0		3	4.7		2	3.3		0	0.0		1	2.9	
Other Mutations															
+1 Frameshift	4	8.9		5	7.8		3	4.9		2	4.8		0	0.0	
−1 Frameshift	2	4.4		4	6.3		2	3.3		3	7.1		2	5.7	
Deletions	1	2.2		4	6.3		3	4.9		1	2.4		0	0.0	
Insertions	0	0.0		1	1.6		2	3.3		1	2.4		0	0.0	
Complex Changes	1	2.2		1	1.6		0	0.0		0	0.0		0	0.0	
Double Mutants	1	2.2		0	0.0		0	0.0		0	0.0		1	2.9	
Total[a]	45	100		64	100		61	100		42	100		35	100	

[a] The total numbers of mutants after correction for clonality (refer to Materials and Methods). The non-corrected mutant totals were 62 at 1.5 months, 74 at 6 months, 70 at 12 months, 46 at 18 months, and 39 at 25 months.

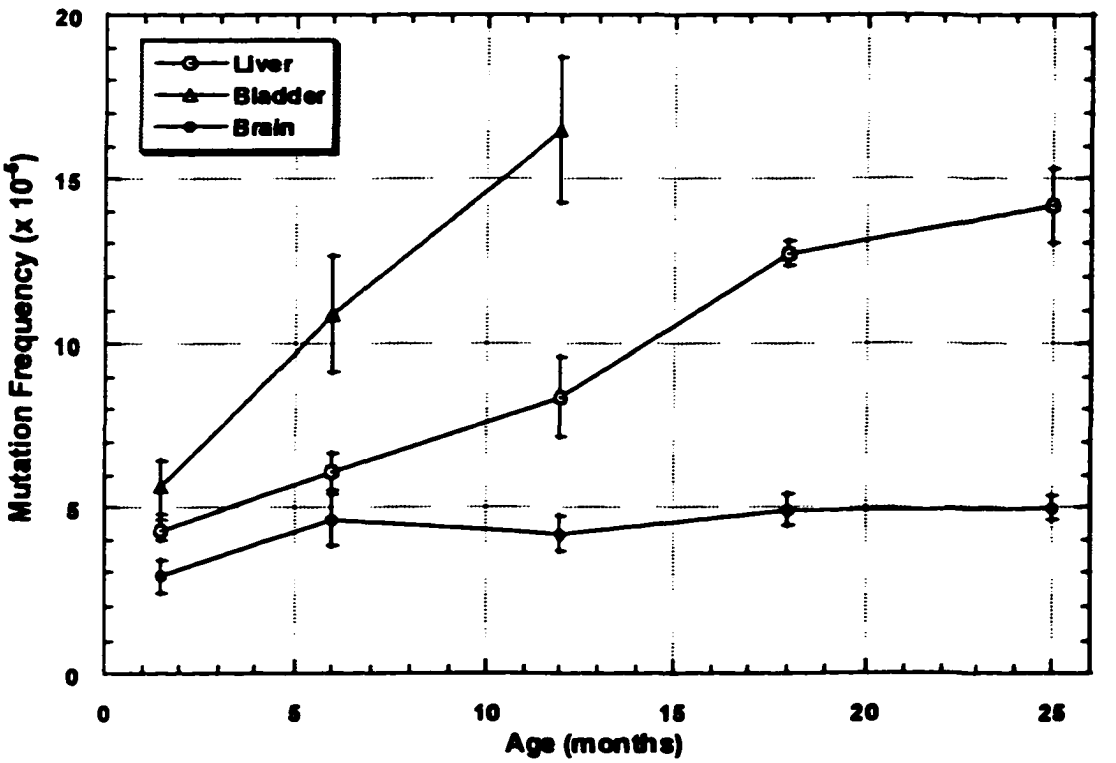

Figure 2.1. Mutation frequency *versus* age in liver, bladder and brain of Big Blue® C57BL/6 *lacI* transgenic mice. Each data point represents the average mutation frequency for that group of animals. The vertical bars indicate the standard error associated with each mutation frequency value.

Chapter 3. No Change in Spontaneous Mutation Frequency or Specificity in Dietary Restricted Mice

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Abstract

It is well-known that dietary restricted rodents live longer and are generally healthier than their *ad libitum* fed counterparts, with fewer tumors. Additionally, while dietary restriction appears to reduce the frequency of chemically induced mutation in laboratory animals, relatively little is known regarding the effect of dietary restriction on spontaneous mutational events. Although spontaneous mutation rates are generally low compared with chemically induced events, spontaneous mutations accumulate in most tissues over the lifetime of the animal, and are therefore expected to contribute significantly to spontaneous neoplasia. It is generally presumed that dietary restriction results in less oxidative damage, and a lowering of the mutation frequency. Here we report the results of dietary restriction on mutation frequency and specificity in *lacI* transgenic mice aged 6 and 12 months. Unexpectedly, no changes were observed in either the frequency or specificity of mutation in dietary restricted mice, compared with *ad libitum* controls. We therefore conclude that dietary restriction appears to have no appreciable effect on spontaneous mutation, at least in chromosomal DNA.

Caloric restriction (a reduction in calories with supplementation to provide essential micronutrients) and dietary restriction (a simple reduction in food intake) are known to dramatically reduce the incidence of spontaneous and chemically induced neoplasias in and to extend the lifespan of rodents (McCay *et al.* 1935; Tannenbaum and Silverstone 1957; Casciano *et al.* 1996). Calorie (dietary) restricted rodents are also believed to experience a reduction in premutagenic DNA lesions and mitotic indices, and an enhancement in the efficiency of DNA repair (Shigenaga and Ames 1993; Youngman 1993). Consequently, it has been generally presumed that the frequency of spontaneous mutations in calorie (dietary) restricted rodents would be depressed, relative to age-matched *ad libitum* fed controls. While there is some indication that dietary restriction has little effect on spontaneous mutant frequencies (MF) (Casciano *et al.* 1996), little if anything is known regarding the effect of dietary restriction on spontaneous mutational spectra (MS). The study of the factors which contribute to the frequency and specificity

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of mutation *in vivo* is facilitated through the use of transgenic rodent mutational assays. We therefore investigated the effect of dietary restriction on spontaneous MF and specificity in liver from Big Blue® *lacI* transgenic mice (Provost *et al.* 1993) aged 6 and 12 months.

Control male C57BL/6 Big Blue® *lacI* transgenic mice were provided with food (Purina Mouse Chow 5015, Ralston Purina Co., St. Louis, MO) and water *ad libitum*, while male dietary restricted mice were provided with ~70% by weight of the diet consumed by the *ad libitum* group, starting at age 11 weeks. The degree of dietary restriction in the present study was slightly less severe than the 60% restriction (of *ad libitum* levels) often used in dietary restriction studies. Nevertheless, the body weights of the dietary restricted mice were 51 and 77% of those of their *ad libitum* controls at ages 6 and 12 months, respectively (Table 3.1), confirming that the mice were truly dietary restricted. Similarly, James and Muskhelishvili (1994) reported that their 60% calorie restricted $B6C3F_1$ mice weighed 69% as much as the *ad libitum* controls at age 12 months. In a review, Tannenbaum and Silverstone (1957) indicated that carcinogenesis is affected by even small degrees of calorie restriction, with the degree of inhibition dependent on the extent of restriction. Similarly, Turturro *et al.* (1993) reported a linear relationship between the body weight of dietary restricted male $B6C3F_1$ mice at 13.5 months of age and the incidence of liver tumors at age 25.5 months. Thus, it is unlikely we would fail to observe any potential effects of 70% dietary restriction on MF and MS, rather than if we had utilized a 60% dietary restriction protocol.

MF and specificity were determined in the *lacI* transgene recovered from chromosomal DNA from liver, as previously described (Suri *et al.* 1996; Stuart *et al.* 2000). Liver was selected for study for several reasons: liver is a slowly proliferating tissue, in which spontaneous MF continue to increase with age in adult mice (Stuart *et al.* 2000); *lacI* spontaneous (and induced) MF and MS are exceptionally well characterized in mouse liver; finally, dietary restriction decreases the incidence of spontaneous and induced liver tumors, as noted above. In *ad libitum* (control) mice, the MF in liver at 12 months of age was significantly increased compared with mice aged 1.5 and 6 months (Table 3.1).

However, we found no significant change in the MF in the *lacI* transgene recovered from mice of the same age when *ad libitum* fed mice were compared to dietary restricted animals. The question might be raised as to the time at which effects of dietary restriction on MF can first be observed. In this regard, Casciano *et al.* (1996) began dietary restriction of their F344 rats at age 10 weeks, followed by aflatoxin B_1 treatment at age 16 weeks. This brief period of dietary restriction was sufficient to significantly decrease aflatoxin-induced MF in splenic lymphocytes. Based in part on these observations, we believe that we would have detected a significant change in spontaneous MF in our dietary restricted mice relative to control mice, noting that dietary restriction began at age 11 weeks, and had therefore continued for ~15 and ~41 weeks by ages 6 and 12 months, respectively.

As we have previously demonstrated that mutational spectra can be sensitive indicators of changes in mutational outcome despite inconsequential changes in MF (de Boer *et al.* 1996b), we sequenced mutant *lacI* genes recovered from *ad libitum* fed and dietary restricted mice (Table 3.2). Only slight differences were noted in the mutational spectra from *ad libitum* fed and dietary restricted mice; for example, the frequency of A:T→G:C transition mutations appeared to be lower in dietary restricted mice compared with *ad libitum* fed mice, though not significantly. Indeed, when complete mutational spectra (Table 3.2) from *ad libitum* fed and dietary restricted mice were compared using the Adams-Skopek test (Adams and Skopek 1987) (data not shown), overall there were no significant changes ($P > 0.05$) in mutational spectra, suggesting that dietary restriction does not alter the distribution of spontaneous mutagenesis. Furthermore, we noted no differences in the contribution of G:C→T:A or A:T→C:G transversions (Cheng *et al.* 1992) to the spectra of either *ad libitum* fed or dietary restricted animals or reference mutational spectra (Stuart *et al.* 2000; de Boer *et al.* 1997), indicating that oxidative DNA damage is unlikely to be a major contributor to the observed MF. This contradicts specific predictions arising from aging and diet models (Ames and Shigenaga 1992). Collectively, our data indicate that dietary restriction has a minimal influence on spontaneous mutational processes in nuclear DNA of somatic cells. We tentatively conclude that the well-established effects of dietary restriction on the physiology,

longevity and neoplastic status of rodents are likely to be attributable to factors other than modulation of spontaneous mutation in chromosomal DNA, with the following caveats. Firstly, although the Big Blue® assay is exceptionally sensitive to base substitution mutations and small deletions, larger deletions and chromosomal aberrations are likely to remain undetected. Secondly, although the effects of dietary restriction on spontaneous mutation were negligible in this study, the effects of dietary restriction on end points such as cancer incidence might be manifested due to uncharacterized effects on non-genetic factors and cellular organelles, including mitochondria, and cellular macromolecules, including proteins and RNA (Kowald and Kirkwood 1996).

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Table 3.1. Mutation frequencies in liver of *ad libitum* fed and dietary restricted mice

Study group	Age (months)	No. of mice	No. of mutants [b]	Total PFU	MF [a] ($\times 10^{-5}$)	Body weight (g)
Ad libitum	1.5	5	111	2 590 000	4.29 ± 0.55 [c]	N.D.
	6	4	135	2 220 000	6.08 ± 0.55	46.6 ± 3.2
	12	4	167	1 990 000	8.39 ± 1.22	50.6 ± 6.7
Dietary restricted	6	5	185	2 380 000	7.77 ± 1.24	23.9 ± 1.0
	12	4	98	1 370 000	7.15 ± 0.35	39.0 ± 2.2

[a] MF, mutation frequency; N.D., not determined; PFU, plaque-forming units.

[b] Independent mutations, after correction for clonality (see de Boer *et al.* 1997a).

[c] MF and body weight values represent means ± SE or SD, respectively. *Ad libitum* fed mice approached adult weights by age 6 months while dietary restricted mice were much smaller at both 6 and 12 months.

Table 3.2. Spontaneous *lacI* mutational spectra from liver of *ad libitum* fed and dietary restricted Big Blue® mice

	Ad libitum				Dietary restricted			
	6 Months		12 Months		6 Months		12 Months	
	No.	%	No.	%	No.	%	No.	%
Transitions								
G:C→A:T	37	33.9 (67.6) [a]	50	33.6 (62.0)	35	43.2 (74.3)	33	35.1 (78.8)
A:T→G:C	13	11.9 [b]	16	10.7	2	2.5	2	2.1
Transversions								
G:C→T:A	25	22.9 (44.0)	34	22.8 (50.0)	20	24.7 (50.0)	22	23.4 (40.9)
G:C→C:G	5	4.6	8	5.4	2	2.5	2	2.1
A:T→T:A	3	2.8	7	4.7	6	7.4	7	7.4
A:T→C:G	0	0.0	6	4.0	3	3.7	5	5.3
Other mutations								
+1 Frameshift	6	5.5	6	4.0	1	1.2	6	6.4
−1 Frameshift	13	11.9	8	5.4	5	6.2	11	11.7
Deletions	3	2.8	5	3.4	3	3.7	2	2.1
Insertions	2	1.8	3	2.0	1	1.2	1	1.1
Complex changes	0	0.0	3	2.0	1	1.2	0	0.0
Double mutants	2	1.8	3	2.0	2	2.5	3	3.2
Total [c]	109	100	149	100	81	100	94	100

[a] Numbers in parentheses represent the proportion of these mutations which occurred at 5′–CpG–3′ dinucleotide sequences.

[b] While we observed an ~9% decrease in A:T→G:C transitions in dietary restricted mice compared with *ad libitum* fed mice, variations in individual mutational classes, particularly in the absence of xenobiotic mutagenic treatments which preferentially induce specific classes of mutations, are likely to reflect random statistical variation in the data (de Boer and Glickman, unpublished data).

[c] The total numbers of mutants after correction for clonality (see de Boer *et al.* 1997). The non-corrected mutant totals were: *ad libitum*, 131 at 6 months and 170 at 12 months; dietary restricted, 97 at 6 months and 164 at 12 months.

Chapter 4. Through A Glass, Darkly*: Reflections of Mutation From *lacI* Transgenic Mice

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Genetics (Submitted)

Abstract

The study of mutational frequency (M*f*) and specificity in ageing Big Blue® *lacI* transgenic mice provides a unique opportunity to determine mutation rates (MR) *in vivo* in different tissues. We found that MR are not static, but rather, vary with the age or developmental stage of the tissue. Although M*f* increase more rapidly early in life, MR are actually lower in younger animals than in older animals. For example, we estimate that the change in M*f* are 4.9×10^{-8} and 1.1×10^{-8} mutations/base pair/month in the liver of younger mice (< 1.5 months old) and older mice (≥ 1.5 months old), respectively (a 4-fold decrease), and that the MR are 3.9×10^{-9} and 1.3×10^{-7} mutations/base pair/cell division, respectively (~30-fold increase). These data also permit an estimate of the MR of G:C→A:T transitions occurring at CpG dinucleotide sequences. Subsequently, the contribution of these transitions to age-related demethylation of genomic DNA can be evaluated. Finally, in order to better understand the origin of observed M*f*, we consider the contribution of various factors including DNA damage and repair by constructing a descriptive mutational model. We then apply this model to estimate the efficiency of repair of deaminated 5-methylcytosine nucleosides occurring at CpG sequences, as well as the effect of the *Msh2*$^{-/-}$ DNA repair defect on overall DNA repair efficiency in Big Blue® mice. We conclude that slight even changes in DNA repair efficiency could lead to significant increases in mutation frequencies, potentially contributing significantly to human pathogenesis, including cancer.

4.1. Introduction

The use of transgenic rodents has greatly facilitated *in vivo* studies of the mechanisms of mutation, DNA repair and carcinogenesis (Kohler *et al.* 1991b; Mirsalis *et al.* 1994; Mirsalis 1995; de Boer and Glickman 1998). While transgenic rodent mutagenicity assays provide a practical approach to the study of genotoxicity, they offer the additional advantage of providing novel insights into mechanisms of mutation. These observations often include unexplained or unpredicted responses that reflect the true biological complexity inherent in mammalian systems, and challenge our current understanding of these systems. Some recent examples of unpredicted results arising from transgenic

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* *For now we see through a glass, darkly; but then face to face: now I know in part; but then shall I know even as also I am known.* 1 Corinthians 13:12.

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Abbreviations: 5MC, 5-methylcytosine; bp, base pair; CpG, 5'–CpG–3' dinucleotide sequence; deam, deamination; div, cellular division; MF, mutation frequency; mo, month; MS, mutational spectrum; MR, mutation rate; mut, mutation.

rodent mutational assays include: (1) an apparent lack of correlation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine- or 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline-induced tissue adduct levels and mutagenicity with carcinogenicity in target tissues (Okonogi *et al.* 1997a; Ochiai *et al.* 1998); (2) a higher-than-expected spontaneous mutation frequency (M*f*) in *mrkII* transgenic mice encoding a *lacI* gene with reduced CpG content, due to increased frequency of G:C → A:T transition mutations at the few remaining CpG sequences (Skopek *et al.* 1998); (3) a decline in *lacI* M*f* during spermatogenesis in younger but not older mice (Walter *et al.* 1998); and, (4) chemoprotection by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin against aflatoxin B_1-induced mutation in female, but not male, *lacI* transgenic rats (Glickman *et al.*, in preparation).

Mutations accumulate in a tissue-specific manner during the lifespan of an organism, contributing significantly to the risk of diseases including cancer. The study of the origin, frequency and especially the specificity of mutation is a necessary first step towards understanding the fundamental molecular mechanisms responsible for mutation. Recently, we reported the changes in spontaneous M*f* and mutational spectra (MS) with age in the *lacI* transgene recovered from liver, bladder and brain of Big Blue® mice (Stuart *et al.* 2000).[14] Those data enable us to combine M*f* data from ageing mice with estimates of cellular turnover to calculate here, for the first time, mutation rates (MR) in young and adult animals. The data show that MR are not static, as might be inferred based on numerous literature reports which quote a single value, but rather appear to vary as a function of developmental age or level of proliferative activity of the tissue.

The validity of these estimates of MR are strengthened by the use of DNA sequencing to correct for nonindependent mutational events (*i.e.*, clonal expansions), and the use of the well-characterized *lacI* transgene as the mutational target. DNA sequence analysis from this laboratory of nearly 20,000 *lacI* mutants recovered from Big Blue® rodents indicates that approximately 410 of the 1080 (38%) nucleotides which encode the *lacI* gene may be recovered as mutations (http://darwin.ceh.uvic.ca/bigblue/sites.htm; B.W. Glickman and J.G. de Boer, unpublished data). These data enable the calculation of M*f* per mutable nucleotide.

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[14] Chapter 2 of this Dissertation.

4.2. Materials and Methods

4.2.1. Experimental data

Please refer to Stuart *et al.* (2000) for detailed descriptions of the experimental treatments and sources of the M*f* data. For convenience, the relevant M*f* data for liver and brain are provided in a footnote to Table 4.1.

4.2.2. Definitions

Mutation frequency (M*f*) is defined as the prevalence of mutation in a gene at a given age, corrected by DNA sequence analysis for possible nonindependent mutational events due to clonal expansion (de Boer *et al.* 1996; de Boer *et al.* 1997). (Note that uncorrected M*f* data provide a *mutant* frequency, MF.) Mutation rates (MR) are best described as the change in M*f* (ΔM*f*) per cell division, although at times (*e.g.*, for convenience of comparison to literature values when discussing MR of deaminations of 5-methylcytosine), MR are expressed as the ΔM*f* per unit time. Accordingly, increases in *lacI* M*f* with *time* (as commonly reported for data from the Big Blue® assay) are better described as ΔM*f* rather than MR. A mutational spectrum (MS) describes the nature, nucleotide position and frequency of mutations which have occurred within a gene (or a defined DNA sequence). Demethylation is used generically to describe the genome-wide loss of 5-methylcytosine (5MC), which can occur by spontaneous hydrolytic deamination of 5MC, or enzymatically by DNA (cytosine-5)-methyltransferase or DNA demethylase (refer to Results and Discussion).

4.3. Results and Discussion

4.3.1. Liver growth and development

Before MR can be calculated, it is necessary to estimate the numbers of cell divisions (in some cases, more specifically the number of rounds of DNA replication) that have occurred. Two week-old CBA/C57BL mouse liver contains about 0.8×10^8 hepatocytes, increasing 1.5-fold to about 1.2×10^8 cells in three month-old mice (Brodsky and Uryvaeva 1985). Noting that the liver of young adult mice weighs approximately 1.5 grams, these numbers agree reasonably well with those provided by Buetow (1985), who reported that two-month old male mouse liver contains approximately 1.16×10^8

cells/cm^3, with this number decreasing slightly to 0.98 x 10^8 and 0.88 x 10^8 cells/cm^3 at ages 12 months and 24 months, respectively. Cell proliferation decreases significantly, approximately 3.3-fold, in the liver of male mice from ages 10 to 13 weeks (2.5 – 3.2 months) (Eldridge and Goldsworthy 1996). Although the number of cells in the post-natal liver reach a plateau, DNA synthesis continues at a reduced rate throughout adulthood, resulting in an age-related increase in mean polyploidy (Carriere 1969; Brodsky and Uryvaeva 1977; Brodsky and Uryvaeva 1985). Thus, mean DNA ploidy levels in mouse liver double from ages one week to one month, and thereafter increase steadily, doubling again by age 24 months (Brodsky and Uryvaeva 1977). This increase in liver polyploidy is accompanied by increase in liver weight, but not cell number.

Calculations of MR should therefore consider DNA replication resulting from polyploidization in addition to that contributed by cellular proliferation, since most mutations are established ("fixed," as in fixation of mutations) during DNA replication (Stuart *et al.* 2000). From the age of 2–3 weeks, it has been reported that each liver cell in the mouse enters the mitotic cycle from 1–6 times (three on the average), resulting in an 8–10-fold increase in liver mass and about a three-fold increase in the number of cells. Mature hepatocytes are fully differentiated, self-maintaining cells with low proliferative rate and low, if any, rate of cell elimination from the population during the life of the mouse. The liver cells in newborn mice are diploid, but polyploidy levels increase in young animals (Uryvaeva 1981). In adult mice most, if not all, mitoses are polyploid.

The relatively small increases in the post-natal number of liver cells is reflected by the slow growth rate of this tissue. During normal growth, hepatocytes rarely divide – even in young rapidly growing animals, two to 12 days pass between successive mitoses, and several months in adults (Schultze *et al.* 1978; Uryvaeva 1981). Mouse hepatocytes are generally regarded as having a turnover time of 480–620 days (Cameron 1971).

4.3.2. Brain growth and development

In the mouse, proliferative activity associated with brain development appears to be largely completed by three to four weeks after birth (Korr 1980). Accordingly, the number of cells in the whole brain of the mouse stabilizes at 0.85 x 10^8 nuclei (cells) by one month of age, with no significant change in this value up to 36 months of age (Franks *et al.* 1974; Buetow 1985). Adult mouse brain is practically mitotically quiescent, except

for a small population of glial cells (Korr 1980; Bowman 1985). DNA polyploidy levels are also known to be remain low, mostly diploid, in adult brain (Winick *et al.* 1972).

4.3.3. Liver mutation frequency and rate

The *lacI* M*f* at conception (0.7 months before birth) is zero, since an inherited mutation in any one of the estimated 40 *lacI* transgenes present on mouse chromosome 4 (Dycaico *et al.* 1994) would result in a spontaneous M*f* of at least 2.5×10^{-2}, more than 500-fold higher than the spontaneous M*f* of approximately $4–5 \times 10^{-5}$ normally observed at age 1.5 months (Heddle 1998). Therefore, we were able to calculate that the increase in M*f* (ΔM*f*) during the 2.2 month period from conception (M*f* of zero) to age 1.5 months postnatal (4.3×10^{-5}; M*f* are provided in a footnote to Table 4.1) was 2.0×10^{-5} mut/*lacI* transgene/mo. Since approximately 410 *lacI* nucleotide positions result in mutants recoverable in the Big Blue® assay, the ΔM*f* in animals less than 1.5 months old was therefore 4.9×10^{-8} mut/bp/mo.

It has previously been noted that spontaneous mutations in somatic cells appear to accumulate steadily throughout adult life (Curtis 1971). This result was confirmed in our mutational studies of M*f* in liver of mice which were greater than 1.5 months old (Stuart *et al.* 2000). The least squares plot for the ΔM*f* in liver of mice greater than 1.5 months old (Figure 1 of Stuart *et al.* 2000) gave a slope of 0.45×10^{-5} mut/*lacI* transgene/mo ($R = 0.987$). Dividing by the 410 *lacI* nucleotide positions recoverable as mutants in the Big Blue® assay, the ΔM*f* in animals greater than 1.5 months old was therefore 1.1×10^{-8} mut/bp/mo. Based on these values, the ΔM*f* in liver in younger mice (less than 1.5 months old) increased approximately 4-fold faster than in mice greater than 1.5 months old.

We also calculated the MR (the ΔM*f* per cell division), using the estimates of cellular proliferation and DNA replication provided above. Since adult liver proliferates slowly but mean polyploidy levels increase, the ΔM*f* with age in this tissue primarily results from DNA replication in nondividing cells, resulting in fixation of DNA lesions or DNA mispairs as mutations (Stuart *et al.* 2000). Therefore, to facilitate both the calculation of MR in adult liver and also to simplify the discussion which follows, polyploidizing DNA replications were considered to be functionally equivalent to cellular divisions.

Since the liver of younger mice, aged 1.5–2 months, contains approximately 1.3×10^8 cells, we estimated that approximately 27 cell divisions (DNA replications) had occurred ($2^{27} = 1.3 \times 10^8$) during the period from conception to age 1.5 months. Dividing the Mf of 4.3×10^{-5} at age 1.5 months by 410 mutable *lacI* nucleotide positions and 27 cell divisions gave a MR of approximately 3.9×10^{-9} mut/bp/div, in mice aged up to 1.5 months. The estimate of the number of cell divisions assumed that cell death (for example, due to apoptosis) was negligible. However, it may be noted that the magnitude of this MR estimate is rather modestly affected over a wide range of cell divisions; for example, using half as many (13) and twice as many (54) cell divisions only resulted in approximately a two-fold change upward or downward (respectively) in the calculated MR.

We estimated the extent of cellular proliferation (DNA replication) in liver greater than 1.5 months old in two ways. Firstly, the relatively steady increase in Mf (ΔMf) with age in adult liver suggested that the balance between DNA replication (fixation of DNA mispairs or DNA lesions as mutations) and DNA repair was maintained throughout this period. Secondly, Brodsky and Uryvaeva (1977) found that mean polyploidy levels in mouse liver increased only about two-fold from ages one to 24 months. Thirdly, the turnover time for hepatocytes is 480–600 days (1.3–1.6 years) (Cameron 1971), indicating that the population of liver cells are replaced about 1.5 times over two years. Collectively, these observations suggest that from ages 1.5–25 months there are probably less than two cell divisions (or DNA replications), on average per cell.

In liver from mice aged 1.5–25 months, by assuming that two cell divisions (DNA replications) had occurred, we calculated the MR to be approximately 1.3×10^{-7} mut/bp/div (= $(1.1 \times 10^{-8}$ mut/bp/mo)(23.5 mo)/(2 div)). Assuming that three cell divisions had occurred reduced the MR only 1.5-fold, to 8.5×10^{-8} mut/bp/div.

For convenience, the liver ΔMf and MR values calculated above are summarized in Table 4.1.

4.3.4. Brain mutation frequency and rate

Since cellular proliferation in the mouse brain is essentially completed by one month of age resulting in 0.85×10^8 cells, it can be estimated that approximately 27 cell

divisions have occurred ($2^{26} = 0.67 \times 10^8$; $2^{27} = 1.3 \times 10^8$). Using this value and the brain M*f* at 1.5 months of 2.9×10^{-5} mut/*lacI*/mo, we calculated that the MR rate in mouse brain from conception (–0.7 months) to age 1.5 months was 1.1×10^{-6} mut/*lacI*/div, or 2.6×10^{-9} mut/bp/div. This compares with the ΔM*f* during this period of 3.2×10^{-8} mut/bp/mo.

The small, but statistically significant increase in brain M*f* between ages 1.5 and 6 months (Table 4.1) is consistent with the known low proliferative capacity of brain tissue. The small increase of brain M*f* of 1.6-fold suggests that some DNA replication had occurred; thus, it was assumed that the brain cell population probably underwent less than one doubling during this period. Therefore, using the M*f* at 6 months of 4.6×10^{-5} and assuming one cell division, we calculated that in brain aged 1.5 to 6 months the ΔM*f* was 9.2×10^{-9} mut/bp/mo, and the MR during this period was approximately 1.7×10^{-5} mut/*lacI*/div (or 4.2×10^{-8} mut/bp/div). Finally, since there was no significant ΔM*f* in brain greater than six months old, the ΔM*f* and the MR in brain older than 6 months was practically zero (not detectable). This result is consistent with the fact that adult brain is essentially mitotically quiescent, and thus the contribution to MR from proliferative mechanisms is negligible.

These brain ΔM*f* and MR data are also summarized in Table 4.1.

4.3.5. Distinguishing between M*f* and MR

Why should we distinguish between M*f* and MR? Most data from mutagenicity assays report M*f* (the prevalence of mutations) at a specified point in time, while most studies which discuss mutation with regard to human health or evolution report MR (change in prevalence over time). Therefore, we feel that in order to better understand data from model systems, including the Big Blue® assay, we should also understand the nuances between M*f* (the accumulated mutational burden) and MR (the rate of increase in M*f* per cell division, or less preferably, unit of time).

Inspecting the data in Table 4.1, the increase in M*f* (ΔM*f*) in liver of younger mice (< 1.5 months old) occurred more than 4-fold faster than in older (≥ 1.5 mo) mice. Conversely, the MR in younger mice (< 1.5 mo) was approximately 33-fold slower than in older mice (≥ 1.5 mo). The simplest explanation for this seemingly paradoxical

relationship between ΔMf and MR is that in developing tissues DNA replication probably contributes more to increases in Mf (for example, through generation of replication-dependent DNA mismatches) than does DNA damage (non-replication-dependent premutagenic lesions; *e.g.* hydrolytic deamination of 5MC). This is supported by the observation that the Mf which had accumulated during the first 2.2 months of life (*i.e.*, from conception to 1.5 months postnatal) required an additional 10.5 months to double in frequency. Thus, in younger animals the ΔMf is greater than in older animals. Conversely, during the period of rapid cellular proliferation in developing mouse liver, the spontaneous mutations which occurred were relatively quickly partitioned among progeny daughter cells, resulting in a lower MR in younger animals.

Another way of understanding the changes in ΔMf and MR with age is to note that: (1) ΔMf appear to be proportional to cellular proliferation ($\Delta Mf \propto$ div); (2) MR appear to be inversely proportional to cellular proliferation (MR $1/\propto$ div) (such a relationship was previously speculated by Drost and Lee 1995 in their interesting paper on germline MR); and (3) cellular proliferation varies inversely with age (div $1/\propto$ age). By substituting expression (3) into expressions (1) and (2), we see that as age increases, ΔMf decrease and MR increase, consistent with observed values.

The data in Table 4.1 clearly indicate that MR in a tissue are not constant throughout the lifespan of an animal. Interestingly, however, for comparable developmental periods of growth the values for the ΔMf as well as the MR in liver and brain are remarkably similar. Whether or not this similarity is coincidental remains to be determined.

4.3.6. Deamination of 5-methylcytosine

The most prevalent spontaneous mutation, greater than 25% of all mutations in mammalian tissues, are G:C → A:T transitions occurring at 5′–CpG–3′ dinucleotide sequences (CpG sequences) (Stuart *et al.* 2000). These mutations are generally attributed to hydrolytic deamination of 5-methylcytosine (5MC) bases which are present at CpG sequences (Coulondre *et al.* 1978; Cooper and Krawczak 1989). The *lacI* gene contains 190 CpG sequences, considering both DNA strands (Farabaugh 1978); of these sequences, 84/190 (44%) have been recovered as mutants in the Big Blue® *lacI* assay (B.W. Glickman and J.G. de Boer, unpublished data;

http://darwin.ceh.uvic.ca/bigblue/sites.htm). Using the M*f* data from our ageing study (Stuart *et al.* 2000) we calculated the ΔMf of G:C → A:T mutations at CpG sequences in liver of mice aged 1.5–25 months to be 0.19×10^{-5} mut/*lacI*/mo, corresponding (after dividing by 84 recoverable CpG mutations per *lacI* transgene) to 2.3×10^{-8} deaminations/5MC/mo.

The 5MC deamination MR determined in mouse liver was compared to those determined *in vitro* and *in vivo* in double-stranded DNA from different taxonomic groups (Table 4.2). Several observations are immediately apparent. Firstly, the rate of spontaneous hydrolytic deamination of 5MC measured *in vitro* is amply sufficient to account for the MR observed *in vivo*. The very low *in vivo* deamination rates indicate that repair of G:T mispairs must be highly efficient, perhaps greater than 99% (Yang *et al.* 1996). Secondly, the significant lowering of MR from *E. coli* to mice, apes and humans, respectively, indicates that MR are not uniform among different taxonomic groups or evolutionary time (Wilson and Jones 1983; Li *et al.* 1996; Li and Tanimura 1987; Wilson *et al.* 1987; Matsuo *et al.* 1993). While these generalizations are not new, the reasonably accurate determination of this rate in transgenic rodents strengthens the validity of these observations.

Caveats associated with the MR determined for deamination of 5MC in the *lacI* transgene include the belief that the bacterial-derived *lacI* transgene is fully methylated (Kohler *et al.* 1990; Scrable and Stambrook 1997; de Boer and Glickman 1998; You *et al.* 1998), and is therefore nontranscribed (Provost and Short 1994). The transgenes are stably integrated into the mouse genome as a tandem array of approximately 40 copies, at a single locus on chromosome 4 (Dycaico *et al.* 1994). Also, the density of CpG sequences in the *lacI* transgene is higher than the average density of these sequences in the mammalian genome (de Boer and Glickman 1998). Nevertheless, MR determined in the *lacI* transgene are likely to be reasonably accurate estimates of the average rate occurring throughout the mouse genome for several reasons. Firstly, mutations in the *lacI* transgene are thought to be neutral and therefore confer no selective growth advantage or disadvantage to the cell. Secondly, the consideration of MR per mutable site (base pair) effectively normalizes the data, with respect to under- or over-representation of CpG sequences throughout the genome. Finally, although sometimes debated, it appears that

DNA repair activity is not significantly different in the *lacI* transgene compared with endogenous mammalian genes, as similar mutational responses have been observed in the *lacI* transgene compared to the endogenous mouse genes *Dlb-1* (Tao *et al.* 1993) and *Hprt* (Skopek *et al.* 1995; Walker *et al.* 1996). As well, the change in the *lacI* spontaneous mutational spectrum observed in *Msh2*$^{-/-}$ *lacI* cotransgenic mice indicates that *lacI* transgenes respond as predicted to changes in DNA repair function (Andrew *et al.* 1997).

4.3.7. Demethylation of DNA

5MC residues in mammalian genomes occur predominantly at CpG sequences (Bird 1980; Cooper and Youssoufian 1988). Demethylation of genomic DNA, principally involving the loss of 5MC nucleotides, has been reported to occur at high frequency in ageing mammalian cells (Gama-Sosa *et al.* 1983; Wilson and Jones 1983; Hoal-van Helden and van Helden 1989; Mazin 1994). Demethylation (we use the term generically, to describe any loss of 5MC) can potentially occur *via* three different mechanisms. (1) As previously mentioned, hydrolytic deamination of 5MC yields thymine directly, resulting in premutagenic G:T mispairs (Coulondre *et al.* 1978; Cooper and Krawczak 1989). (2) Immediately following DNA replication, cytosines present in the nascent DNA strands are unmethylated. During the methylation of hemimethylated CpG sequences, DNA (cytosine-5)-methyltransferase covalently bonds at the C6–position of cytosines, greatly labilizing the amino group, resulting in deamination events prior to methylation at the C5–position (Shen *et al.* 1992; Steinberg and Gorman 1992; Laird and Jaenisch 1996). Thus, cytosines at CpG sequences may be converted directly to thymines, again resulting in premutagenic G:T mispairs. (3) DNA demethylase, a novel enzyme which specifically recognizes 5MC residues at CpG sequences in mammalian DNA, has recently been described (Bhattacharya *et al.* 1999; Ramchandani *et al.* 1999). The product of this enzymatic reaction is a normal G:C base pair.

Published estimates of the rate of demethylation of mouse and rat genomes vary greatly and are at times contradictory, even for a single species and tissue such as mouse liver (Gama-Sosa *et al.* 1983; Wilson and Jones 1983; Singhal *et al.* 1987; Wilson *et al.* 1987; Hoal-van Helden and van Helden 1989; Tawa *et al.* 1990; Kanungo and Saran 1992; Mazin 1994; Mazin 1995). The reasons for these discrepancies are not obvious, but

might involve one or more of the following: differences in species, age, strain, and tissues; dietary factors affecting DNA methylation levels; or, choice of analytical method (Drahovsky and Boehm 1980; Rein *et al.* 1998). Nevertheless, the estimates of the demethylation rate are very large; for example, Wilson *et al.* (1987) determined that the rate of loss of 5MC from the genome of C56BL/6J mouse liver was 0.012% per month. This corresponds to the loss of approximately 2.2×10^3 5MC per diploid mouse nucleus per month, a rate which is approximately 5.5×10^3-fold greater than that occurring due to deamination of 5MC determined using the *lacI* transgene. In other words, if the demethylation rate determined by Wilson *et al.* (1987) was due solely to hydrolytic deamination of 5MC to thymine (or possibly, DNA (cytosine-5)-methyltransferase-mediated conversion of cytosine to thymine during methylation of hemimethylated CpG sequences), the M*f* observed in the *lacI* transgene would be approximately 1.3×10^{-4} deam/5MC/mo (= 1.1×10^{-2} deam/*lacI* transgene/mo), an extraordinarily high MF. The demethylation rate reported by Mazin (1994) in mice (unspecified tissue) of 0.033% per cell per day (or 0.99 %/cell/mo) is ~50-fold greater than that reported by Wilson *et al.* (1987), which again would be an unrealistically high mutation rate in the *lacI* transgene. Similar demethylation rates may be inferred from the data described by Hoal-van Helden and van Helden (1989), who reported a 46% decrease in the percentage of 5MC in liver of rats from one day before birth, to 6 months of age.

As the global demethylation rates reported previously are much higher than the rates of deamination of 5MC in *lacI* determined in this study, we needed to reconcile this apparent discrepancy. Since 5MC residues in mammalian genomes occur most frequently at CpG sequences, we can use the mutational data from *lacI* transgenic rodents to evaluate the relative contribution of the aforementioned three routes of demethylation to previously reported rates of demethylation of the mouse genome. G:C → A:T transitions occurring at CpG sequences presumably arise due to spontaneous hydrolytic deamination of 5MC (Coulondre *et al.* 1978; Cooper and Krawczak 1989), and possibly due to deamination of nonmethylated cytosines, present at hemimethylated CpG sequences, during methylation by DNA (cytosine-5)-methyltransferase (Shen *et al.* 1992; Steinberg and Gorman 1992; Laird and Jaenisch 1996). Since these G:T mispairs, however they arise, are recognized and repaired with high but not absolute efficiency by thymine DNA

glycosylase (Brown and Jiricny 1987; Brooks *et al.* 1996; Marietta *et al.* 1998), G:C → A:T transitions inevitably arise at a low, but finite, frequency with each round of DNA replication.

If demethylation events are solely attributable to deamination of 5MC at the rate determined in mouse liver, we can estimate the frequency of demethylation as follows. The mouse genome contains approximately 7×10^9 DNA base pairs per diploid nucleus, with approximately one mole percent of the cytosines in the liver of C57BL/6 mice methylated as 5MC (Gama-Sosa *et al.* 1983; Tawa *et al.* 1990). (It should be noted that some laboratories report somewhat higher, possibly age-related, values for the 5MC content in young mouse liver; for example, Wilson *et al.* (1987) reported that 3% of the cytosines in four week-old C57BL/6J mouse liver were present as 5MC.) Therefore, assuming that cytosines comprise about one-quarter of the nucleotides in the mouse genome with one mole percent of these present as 5MC, there are approximately 1.8×10^7 5MC per diploid mouse nucleus. Multiplying this value by the 5MC deamination rate determined in the *lacI* transgene of adult mice (2.3×10^{-8} deam/5MC/mo; Table 4.1) indicates that overall, approximately 0.4 deaminations of 5MC are expected to occur per month per diploid mouse genome.

Based on these calculations, demethylation of genomic DNA at the levels described by Gama-Sosa, Wilson and others cannot be attributable to spontaneous hydrolytic or enzyme-mediated deamination events. The recent discovery of a mammalian DNA demethylase specific for 5MC (Bhattacharya *et al.* 1999; Ramchandani *et al.* 1999) therefore provides the most reasonable explanation, at present, for the nonmutagenic hypomethylation of DNA. As mentioned, this enzyme directly converts 5MC to cytosine, resulting in G:C base pairs.

4.3.8. A descriptive mutational model

As noted previously (Stuart *et al.* 2000; this Discussion), mutations occur at different rates in mouse liver and brain tissue, especially in adult tissues. Furthermore, when M*f* for the most commonly-occurring mutation, deamination of 5MC occurring at CpG sequences, closely parallel the larger, overall spontaneous M*f* shown in Figure 1 of Stuart *et al.* (2000). Since these transition mutations are presumably of specific origin, hydrolytic deamination of 5MC, the spontaneous rate of deamination of 5MC is expected

(based on thermodynamic considerations, *i.e.*, Arrhenius kinetics) to be constant at a given temperature. Thus, deviations from this rate observed *in vivo* in different tissues must reflect biological influences, including DNA repair.

Observed MR (M*f*) likely reflect a balance between the formation of premutagenic DNA lesions, DNA replication (required for fixation of mutations), DNA repair, and cell death. Consideration of the effects of these processes on mutation lead to the description of mutation as a function

$$MF_{observed} = f(\text{damage, replication, repair, death}) \quad (1)$$

which can be expressed as an equation

$$MF_{observed} = (\text{Mutation Fixation})(1-\text{DNA Repair})(1-\text{Cell Death}). \quad (2)$$

(It is noted that an analogous expression has been proposed by Burkhart and Malling (1993), and perhaps, others.) Also, it follows that

$$Mf_{observed} = (MR_{observed})(\text{elapsed time}). \quad (3)$$

This basic model (Equation 2) can be amended or refined as required. The "Mutation Fixation" term (a rate) incorporates the rate of DNA damage, as well as the effect of DNA replication including the miscoding efficiency of DNA lesions or missing bases (*i.e.*, mutation fixation); the "DNA Repair" term (a proportion) includes various forms of repair including direct alkyl transfer, base and nucleotide excision repair, mismatch repair, and DNA recombination; while the "Cell Death" term (a proportion) includes necrotic and apoptotic cell death, if these are affected by mutation or treatments.

Interestingly, DNA replication contributes a linear increase to observed M*f*, according to the formula

$$Mf_i = Mf_{initial} + ((MR)/2)(i), \quad (4)$$

where i = the number of cell divisions (or DNA replications) that have occurred. For example, assuming an initial population of 10^6 largely wild type cells, but containing 100 *lacI* mutants and having a MR of 10^{-5} new mutants per cell division (or DNA replication), after 5 cell divisions the initial M*f* of 1.00×10^{-4} will have increased to 1.25×10^{-4}. (The M*f* increases are 1.05×10^{-4}, 1.10×10^{-4}, 1.15×10^{-4}, 1.20×10^{-4}, and 1.25×10^{-4} after 1, 2, 3, 4, and 5 divisions, respectively.)

In time, sufficient data might be accumulated for each of the factors inherent in Equation 2 to permit the quantitative evaluation of mutation *a priori*. Nevertheless, in the interim this function might serve as a useful model for qualitatively or semi-quantitatively evaluating the relative contributions of various factors to M*f* observed *in vivo* or *in vitro*.

To illustrate, we applied our descriptive mutational model to the calculation of the efficiency of repair of G:T DNA mispairs in mouse liver DNA arising due to deamination of 5MC. Since mouse hepatocytes are relatively long-lived, and apoptotic indices in adult mouse liver are typically about 0.007% (James *et al.* 1998], we may ignore the Cell Death term. Therefore, substituting the rate of hydrolytic deamination of 5MC determined *in vitro* (Table 4.2) and the rate of ΔMf due to G:C → A:T at CpG sequences (Table 4.1) into Equation 2

2.3×10^{-8} deam/5MC/mo = (1.5×10^{-6} deam/5MC/mo)(1–DNA Repair)

we calculate that DNA repair of G:T mispairs in mouse liver must was approximately 98.5–99.9% efficient, depending on the *in vitro* MR chosen. (Note that in this instance, we needed to use the ΔMf value from Table 1 rather than the MR, for the units to cancel.). Similarly, we predict that since the human 5MC deamination rate is slower than in mice, that the efficiency of repair of G:T mispairs is higher, approximately 99.99–99.9996%, based on the data from Table 4.2.

In a second application of our mutational model, the recent report of spontaneous mutations in the *lacI* transgene of DNA repair-proficient and $Msh2^{-/-}$ mice (Andrew *et al.* 1997) provides the opportunity to estimate the contribution of this DNA repair pathway to observed M*f*. In the three tissues examined (small intestine, thymus and brain), MF were elevated approximately 11, 15 and 5-fold, respectively, in $Msh2^{-/-}$ mice compared to control animals. Using thymus as an example, and assuming that the differences in the observed MF in control and $Msh2^{-/-}$ mice were solely attributable to the DNA repair defect, Equation 2 may be simplified to as a pair of equations

$3.1 \times 10^{-5} = (\text{constant})(1-\text{DNA Repair}_{\text{control thymus}})$

$47 \times 10^{-5} = (\text{constant})(1-\text{DNA Repair}_{Msh2\ \text{thymus}})$

that can be solved simultaneously to give

$\text{DNA Repair}_{Msh2\ \text{thymus}} = (15.16)(\text{DNA Repair}_{\text{control thymus}}) - 14.16.$

Thus, if we make the assumption that DNA repair in the control thymus was 99.99% efficient, then the equation suggests that DNA repair in the *Msh2*$^{-/-}$ thymus would have been 99.848% efficient, a very small change in overall DNA repair. Whether or not this conclusion is correct remains to be validated. As well, it cannot be excluded that small differences in the extent of DNA replication or cell death in *Msh2*$^{-/-}$ mice relative to control animals also contributes significantly to the differences in spontaneous M*f* observed in these animals. Nevertheless, the model predicts that subtle differences in the efficiency of DNA repair might profoundly influence observed M*f*. If this is true, one implication is that mutator phenotypes (Loeb 1991) could be attributable to small perturbations in the efficiency of DNA repair.

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Table 4.1. Summary of mutation frequencies and mutation rates in ageing mouse liver and brain

Tissue	Age (months)	No. of Divisions	ΔMf [a, b] (mut/bp/mo)	MR (mut/bp/div)	Ratio of ΔMf/MR
All Mutations:					
Liver	(–0.7)–1.5	27	4.9×10^{-8}	3.9×10^{-9}	13
	1.5–25	2	1.1×10^{-8}	1.3×10^{-7}	0.085
	(Ratio)		(4.5)	(0.03)	
Brain	(–0.7)–1.5	27	3.2×10^{-8}	2.6×10^{-9}	12
	1.5–6	1	9.2×10^{-9}	4.2×10^{-8}	0.22
	(Ratio)		(3.5)	(0.06)	
	6–25	~zero	~zero	~zero	
Deaminations:			(deam/5MC/mo)	(deam/5MC/div)	
Liver	(–0.7)–1.5	27	6.0×10^{-8}	4.9×10^{-9}	12
	1.5–25	2	2.3×10^{-8}	2.7×10^{-7}	0.085
	(Ratio)		(2.6)	(0.02)	

[a] ΔMf is the change (increase) in Mf during the indicated period of time. The Mf were: liver: 4.3×10^{-5} at age 1.5 mo, 14×10^{-5} at 25 months; brain: 2.9×10^{-5} at age 1.5 months, 4.6×10^{-5} at 6 months, and 5.0×10^{-5} at 25 months (taken from Stuart *et al.* 2000).

[b] Abbreviations: bp, base pair; deam, deaminations (of 5-methylcytosine); 5MC, 5-methylcytosine; mo, month; mut, mutations.

Table 4.2. Rates of deamination of 5-methylcytosine in double-stranded DNA

Species	Target	MR [a] (deam/5MC/mo)	References
in vitro	M13mp2SV DNA (scored in *E. coli*)	3.9×10^{-5}	Zhang and Matthews (1994)
in vitro	pSV2-*neo* plasmid reversion assay (scored in *E. coli*)	1.5×10^{-6}	Shen *et al.* (1994)
Mouse	*lacI* transgene (mature liver)	2.3×10^{-8}	This article
Primates	*p53 Alu* sequences (germline)	$\sim 1.4 \times 10^{-9}$ [b]	Yang *et al.* (1996)
Human	factor IX gene	1.5×10^{-10} [c]	Koeberl *et al.* (1990)

[a] Abbreviations: deam, deaminations (of 5-methylcytosine); 5MC, 5-methylcytosine; mo, month; MR, mutation rate. To convert MR cited in the literature from seconds^{-1} to months^{-1}, we assumed that 1 month = 30 days.

[b] This value was estimated by subtracting the MR from humans (1.5×10^{-10} deam/5MC/mo) from the rate constant (1.5×10^{-9} deam/5MC/mo) based on G:C $\rightarrow$ A:T transitions from humans and Old World monkeys reported by Yang *et al.* (1996). Alternatively, the average rate constants for the primates provided in Table 1 of Yang *et al.* (1994) are $\sim(1-2) \times 10^{-8}$ deam/5MC/year, which is $\sim(0.83-1.6) \times 10^{-9}$ deam/5MC/mo.

[c] Koeberl *et al.* (1990) estimated a MR of 3.7×10^{-8} deam/5MC/generation; assuming a generation time of 20 years (suggested by Shen *et al.* 1994), this corresponds to a MR of about 1.5×10^{-10} deam/5MC/mo.

SECTION II. INDUCED MUTATION

Chapter 5. Species-specific Differences in Hepatic Mutant Frequency and Mutational Spectrum Among Lambda/*lacI* Transgenic Rats and Mice Following Exposure to Aflatoxin B_1

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Abstract

In vivo mutations were studied in lambda/*lacI* (Big Blue®) transgenic C57BL/6 mice and F344 rats following exposure to either AFB_1 (aflatoxin B_1) or DMSO vehicle. Fourteen days after exposure, livers were removed for DNA extraction and subsequent mutational analysis of the *lacI* gene. Mice injected with a single i.p. (intraperitoneal) dose of AFB_1 at 2.5 mg/kg did not show a significant increase in liver mutant frequency relative to vehicle-treated controls. DNA sequence analysis of *lacI* mutations collected from the AFB_1-treated mice showed a pattern of mutation similar to that of the previously observed spontaneous mouse liver mutational spectrum. In contrast, rats subjected to one-tenth the mouse AFB_1 dosage responded with an approximate 20-fold induction in liver mutant frequency over background. Sequencing of *lacI* mutations also revealed spectral differences between vehicle- and AFB_1-treated rats. A large increase in G:C → T:A transversions was observed among *lacI* mutations isolated from the AFB_1-treated rats. This work is among the first multi-species *in vivo* mutagenicity studies using transgenic rodents harboring the same shuttle vector. Such multi-species *in vivo* assays may prove to be valuable in the areas of mechanistic analysis and risk assessment.

5.1. Introduction

The study of genetic damage sustained *in vivo* in mammals has been greatly facilitated in recent years by the development of a lambda/*lacI* shuttle vector that is readily recovered from transgenic C57BL/6 mice (Kohler *et al.* 1991a). These mice, along with their $B6C3F_1$ hybrids, permit the study of mutation fixation in diverse tissues, and allow the observation of treatment-induced changes in mutational spectra within these tissues (Kohler *et al.* 1991b; Provost *et al.* 1993; Gorelick 1995). While transgenic mice provide a way to study the effects of mutagens in several tissues, the recent development of an analogous transgenic F344 (Fischer 344) rat harboring the same

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lambda/*lacI* shuttle vector makes it possible to perform multiple-species mutation testing *in vivo* in rodents (Dycaico *et al.* 1994).

AFB_1 (aflatoxin B_1) is a classic example of a substance for which toxic and carcinogenic potential varies widely between species (Newberne and Butler 1969). This well-characterized mycotoxin is produced by the food spoilage fungus *Aspergillus flavus* (Wogan 1973) and demonstrates species-specific carcinogenicity and mutagenicity through differences in metabolic activation and detoxification (recent reviews include Eaton and Gallagher 1994; Eaton and Groopman 1994; Massey *et al.* 1995). AFB_1 is an effective liver carcinogen in some species, including rats and humans (Wogan 1973; Ross *et al.* 1992; Coursaget *et al.* 1993; IARC 1993; Aguilar *et al.* 1994; NTP 1994). Numerous comparative studies using rodents have shown that rats are highly susceptible to the hepatocarcinogenic effects of AFB_1, while mice are relatively resistant (Busby and Wogan 1984). Rat hepatocytes have also been shown to be approximately 10-fold more effective than mouse hepatocytes at generating mutagenic metabolites of AFB_1 (Hsu *et al.* 1987). The observation that AFB_1-induced mutagenicity and carcinogenicity is more pronounced in rats over mice is thought to be due mainly to the higher levels of glutathione-*S*-transferase activity in mice (Eaton and Gallagher 1994; Van Ness *et al.* 1994).

To examine whether species-specific differences can be observed using an *in vivo* transgenic mutation assay, mutant frequencies were measured in the livers of lambda/*lacI* F344 rats and C57BL/6 mice following vehicle or AFB_1 exposure. As expected based on earlier studies, rats demonstrated high susceptibility to AFB_1-induced *lacI* mutation within the liver, whereas mice were refractory to the mutagenic effects of this mycotoxin. DNA sequence analysis of *lacI* mutations recovered from AFB_1-treated lambda/*lacI* mouse and rat liver also revealed species-dependent differences. Rats treated with AFB_1 demonstrated a large increase in G:C $\rightarrow$ T:A transversions compared with the vehicle-treated controls. In AFB_1-treated mice, however, the pattern of mutation in AFB_1-treated animals was very similar to the spontaneous mutational spectrum previously observed in liver.

5.2. Materials and Methods

5.2.1. Husbandry

Hemizygous Fischer 344 rats from the transgenic lineage QX (Big Blue®, Stratagene, La Jolla, CA), 13–19 weeks of age, were bred at Stratagene in La Jolla, CA. Hemizygous C57BL/6 mice from the transgenic lineage A1 (Big Blue®), 13 weeks of age, were bred at Taconic (Germantown, NY) for Stratagene. All animals were housed at 20°C and maintained on a 12 h light cycle (5 a.m. to 5 p.m.). Diet consisted of 4% fat rodent blocks (Harlan Teklad, Madison, WI) and water, both administered *ad libitum*. All husbandry procedures were established according to the standards set within the NIH Guide for the Care and Use of Laboratory Animals.

5.2.2. Aflatoxin B_1 administration

AFB_1, CAS (Chemical Abstracts Registry) no. 1162-65-8, catalog no. A-6636 and DMSO (dimethyl sulfoxide), CAS no. 67-68-5, catalog no. D-8779 were obtained from Sigma Chemical Company (St. Louis, MO). Each experimental group consisted of six animals. AFB_1 was dissolved in DMSO and administered in a single i.p. injection at 2.5 mg/kg in both mice and rats. After observing acute toxicity at this dosage in the AFB_1-treated rats, an additional group of male rats was administered a single dose of AFB_1 at 0.25 mg/kg. The dosing volume for both species remained constant at 1 ml/kg. The control groups for both species were given a single i.p. injection of DMSO at 1 ml/kg. An expression period of 14 days was carried out before animals were killed for tissue harvest. After quick removal, the livers were flash-frozen in liquid nitrogen and stored at −80°C until DNA isolation. Compound dosing and tissue harvesting were performed at Microbiological Associates, Inc. (Rockville, MD).

5.2.3. Genomic DNA isolation

For each individual, approximately 100 mg of liver was homogenized in 3 ml of Dounce buffer (6.5 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4; 10 mM Na_2EDTA, pH 8.0) supplemented with 20 µl/ml RNAce-It™ ribonuclease cocktail (Stratagene, La Jolla) using a Wheaton 7 ml dounce with pestle size B. The homogenate was then gently combined with 3 ml of 2X protease solution (2 mg/ml proteinase K; 2% SDS; 100 mM Na_2EDTA, pH 7.5) and allowed to digest at 50°C for 3 h, followed by two extractions with phenol:chloroform (pH 8.0), one chloroform extraction, and ethanol

precipitation. All extractions were performed gently to encourage the isolation of high molecular weight DNA. After removing the residual ethanol, all DNA precipitates were allowed to dissolve in 200–500 µl of TE solution (10mM Tris-HCl; 1mM Na_2EDTA, pH 8.0) at 4°C for at least 2 days. All DNA samples were coded and randomized in order to perform a blind study.

5.2.4. Big Blue® color-screening assay

All assay reagents were obtained from Stratagene (La Jolla, CA). Mutant frequency was evaluated for each DNA sample in a blind study using a block analysis of vehicle- and AFB_1-treated individuals. Mutant frequencies were measured according to the standardized Big Blue® color-screening assay protocol (Stratagene, La Jolla; Rogers *et al.* 1995; Young *et al.* 1995). Briefly, shuttle vector was recovered from the genomic DNA using Transpack® *in vitro* packaging extract. In each packaging reaction, 8 µl of genomic DNA was combined with 10 µl of Transpack and incubated at 30°C for 3 h, with the addition of another 10 µl of Transpack midway through the reaction period. Approximately 1 ml of SM medium was added to each packaging reaction. If multiple packaging reactions were performed for a particular DNA sample, each sample's reactions were pooled together. After addition of chloroform (50 µl per ml) to each packaged phage solution, the phage suspensions were gently vortexed and stored at 4°C. The initial titer of each phage solution was estimated by infecting 200 µl of *Escherichia coli* SCS-8 host bacteria (*recA1*, *endA1*, *mcrA*, Δ(*mcrBC-hsdRMS-mrr*), Δ(*argF-lac*)*U169*, ϕ80*dlacZ*Δ*M15*, Tn*10*(tet^r)) at 0.5 OD_{600nm} with 1 µl of packaged phage and plating on 100 mm NZY agar plates, in duplicate. The initial titers were used to ensure that phage were plated onto 25 cm x 25 cm Big Blue® assay trays at plating densities of approximately 15,000–20,000 PFU (plaque forming units) per tray. For mutant screening, packaged phage were first adsorbed to *E. coli* SCS-8 host cells at 0.5 OD_{600nm} for 15 min at 37°C. After removing a small aliquot for dilution titering (see below), the host-adsorbed phage were plated onto NZY assay trays using 35 ml top agarose containing 1.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). After an appropriate venting period required to remove excess moisture introduced by the top agarose, all assay and titer trays were incubated at 37°C

for 16 hours. The total number of PFU plated was determined by counting two dilution titer trays per sample. Mutations in the *lacI* gene were scored over white fluorescent light boxes using a red Big Blue® plaque reading enhancer screen. Mutant frequency was determined as the ratio of the number of blue plaques to the total number of PFU plated. Statistical tests of differences in mutant frequency between animal groups were performed with a two-tailed Student's *t*-test using the analysis tools in Microsoft® Excel software.

5.2.5. Recovery and analysis of *lacI* mutants

Mutant plaques were recovered according to the standardized Big Blue® color-screening assay protocol (Rogers *et al.* 1995; Young *et al.* 1995), and were purified and sequenced as described by de Boer *et al.* (1995). Briefly, mutant plaques were confirmed and purified by infecting *E. coli* SCS-8 cells with the mutant phage and replating on medium containing X-gal. The *lacI* gene and flanking regions were amplified by PCR (polymerase chain reaction), and the products were purified on Wizard™ PCR Prep DNA purification columns (Promega, Madison, WI). Thermal cycle sequencing of the *lacI* gene was performed using A.L.F. automated DNA sequencers (Pharmacia, Piscataway, NJ). DNA sequence data were managed and analyzed using custom software (de Boer 1995). To ensure that independent mutational events were scored, the data were corrected for possible clonal expansions (de Boer *et al.* 1996a; de Boer *et al.*, in preparation[16]) by counting only one mutation for those which were recovered more than once from a single animal. Statistical tests of differences between mutational spectra were done using the Monte Carlo method (with 2500 iterations) of Adams and Skopek (1987), using a computer program provided by the authors.

5.3. Results

Four groups of six animals each were subjected to a single i.p. injection of either AFB_1 at 2.5 mg/kg or DMSO vehicle. While all six of the AFB_1-treated mice survived through day 14, five out of the six AFB_1-treated rats died by the second day. (The liver of the surviving rat was harvested on the fourteenth day, post-injection, and stored at –80°C but not subjected to either mutant frequency or spectral analysis.) Approximately one

month later, another group of six rats was dosed with AFB_1 at 0.25 mg/kg, resulting in 100% survival through day 14. The vehicle-treated rats from the first set of injections were used as the control group for rats in this study. Consequently, the vehicle-treated rats were approximately six weeks older than their AFB_1-treated counterparts.

The mutant frequency data collected using a block analysis treatment of vehicle- and AFB_1-treated samples are summarized in Table 5.1. Transgenic C57BL/6 mice subjected to a single dose of AFB_1 at 2.5 mg/kg showed no induction in mutant frequency over the vehicle-treated mouse group ($P = 0.25$). Transgenic F344 rats subjected to one-tenth the mouse dosage, however, were highly susceptible to the mutagenic effects of AFB_1. At a mean mutant frequency of 49×10^{-5} for the AFB_1-treated group, induction in rat liver was nearly 20 fold over background ($P = 0.001$).

Table 5.2 summarizes the *lacI* sequence data for liver mutations induced by AFB_1 in lambda/*lacI* mice and rats (a list of all sequenced mutants is included in the Appendix).[17] The vehicle-treated rat liver *lacI* data from this study are also presented, as well as historical data from untreated C57BL/6 mouse liver (*lacI* database; de Boer 1995). All mutation data were corrected for possible clonal expansions (de Boer *et al.* 1996a; de Boer *et al.*, in preparation[18]), resulting in the elimination of seven out of 84 mutants from the historical dataset for untreated mice, five out of 47 mutants from AFB_1-treated mice, five out of 49 mutants from vehicle-treated control rats, and three out of 48 mutants from AFB_1-treated rats.

In 45 (corrected) mutants from AFB_1-treated rats, 78% of the mutations were G:C → T:A transversions (with 71% of these transversions occurring at CpG sites (5′–CpG–3′ dinucleotide sequences), followed by 11% G:C → C:G transversions (80% at CpG sites) and only 4% G:C → A:T transitions (50% at CpG sites). In 44 (corrected) mutants from vehicle-treated rat liver, we observed 27% transversion (11% as G:C → T:A) and 48 % G:C → A:T transition mutations (76% at CpG sites). In the mouse, sequence analysis of 42 (corrected) mutants from AFB_1-treated animals revealed 19% G:C → T:A

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[16] de Boer *et al.* (1997).

[17] This Appendix is located in Section 5.5 at the end of this Chapter.

[18] de Boer *et al.* (1997).

transversions (38% at CpG sites) and 57% G:C → A:T transitions (71% at CpG sites). This spectrum is similar to the C57BL/6 spontaneous liver mutational spectrum.

5.4. Discussion

The transgenic A1 mouse and QX rat lineages used in this study are currently commercially available as the Big Blue® mouse and rat, respectively. They each harbor the lambda-based shuttle vector transgene (λLIZ), which employs the *lacI* gene from *E. coli* as a mutational target (Kohler *et al.* 1991a, 1991b; Provost *et al.* 1993; Dycaico *et al.* 1994; Gorelick 1995). The copy number of the lambda/*lacI* shuttle vector in the hemizygous Big Blue® mouse is approximately 40 per cell, while that of the hemizygous Big Blue® rat is approximately 15–20 per cell. Although homozygous versions of Big Blue® animals are available, all animals used in this study were hemizygous.

The existence of a multi-species mutation assay using transgenic rodents that share the same target gene/shuttle vector should afford the opportunity to perform tissue-specific, inter-species mutagenesis testing in mammals. The study described here was designed to test the ability of a transgenic-based assay to show an *in vivo* species-specific differential response to mutagen exposure.

AFB_1 was chosen for this study because its biological effects are relatively well-characterized (McLean and Dutton 1995). The observation that AFB_1 was highly mutagenic in the livers of transgenic F344 rats and not in those of transgenic C57BL/6 mice is consistent with what is presently known about the metabolism of the toxin in rodents. AFB_1 is converted by cytochrome P450 pathways into the highly reactive AFB_1-8,9-epoxide, which reacts with DNA mainly forming an adduct at the N7-position of guanine (Essigmann *et al.* 1977; IARC 1993; Raney *et al.* 1993; Eaton and Gallagher 1994; Bailey *et al.* 1996). A major pathway for the detoxification of the epoxide is through conjugation with glutathione (Hayes *et al.* 1991; Buetler *et al.* 1992; Hayes *et al.* 1994; Van Ness *et al.* 1994). The balance between activation and detoxification is believed to influence the reaction of AFB_1 with genomic DNA in different species (Gorelick 1990). The fact that rats are more efficient than mice in bioactivation of AFB_1 into the mutagenic AFB_1-8,9-epoxide, and less efficient in the conjugation of this active form is reflected by the high sensitivity of rats to the mutagenic effects of AFB_1 while

mice that were given ten times the rat dose showed no increase in mutant frequency in this study.

To a lesser extent than species-specific factors, the effects of sex and strain are likely to have had an influence on the results of this study. Sex- and strain-specific differences in carcinogenicity induced by AFB_1 have previously been reported (Wogan 1992). AFB_1-induced TD_{50} values for rat strains range from 1.3 μg/kg/day for male Fischer rats to 12.5 μg/kg/day for female Porton rats (TD_{50} defined as the dose that produces a 50% incidence in tumors). Those for mouse strains range from >70 μg/kg/day for male C3H and C57BL mice to >5300 μg/kg/day for male Swiss mice. This study was performed on male F344 rats and C57BL/6 mice, which are among the more AFB_1-sensitive inbred representatives reported for each species.

Another factor that could conceivably contribute to the differential response between AFB_1-treated rats and mice observed in this study is the effect of transgene integration site. It is possible that the QX rat lineage harbors its 15-20 copies of *lacI* in a region of the genome more accessible to mutagen exposure. Previous studies measuring spontaneous mutant frequencies among different transgenic mouse and rat lineages have supported the assumption that transgene insertion site in the QX rat and A1 mouse lineages has little effect on the outcome of the mutagenicity assay (Dycaico *et al.* 1994). Nevertheless, there is evidence that factors such as DNA conformation and chromatin structure can influence intragenomic localization of carcinogen-DNA binding and repair (Wogan 1992). Thus any mutagenicity assay utilizing a transgenic target is potentially influenced by the integration site of a particular transgenic lineage. A study wherein the response to a known mouse-specific mutagen is measured in both the A1 and QX lineages may help to further address this issue with respect to the lambda/*lacI* rodent assay that was used in our experiments.

It is unlikely that the six-weeks difference in age between the AFB_1-treated rats and the older vehicle-treated control rats in this study had a significant effect on the increase in mutant frequency measured in the livers of AFB_1-exposed rats. Previous work in lambda/*lacI* mice has shown that spontaneous mutant frequency increases approximately four fold during the first 24 months of life (Lee *et al.* 1994) suggesting that age might have a similar effect on mutant frequency in the rat. Regardless, six weeks is a relatively

small percentage of the average rat lifespan of 2.5–3 years (Baker *et al.* 1979) and is probably not large enough to result in a significant increase in mutant frequency in the control rats relative to the nearly 20 fold induction by AFB_1. Age-related sensitivity to AFB_1 is also not suspected to be a significant factor in this study. Sensitivity to AFB_1 toxicity in F344 rats increases during the first weeks of post-natal development and reaches a plateau at approximately 65 days of age (Croy and Wogan 1981). The AFB_1-treated and vehicle-treated rats used in this study were approximately 80 and 117 days old, respectively, at the time of i.p. injection.

The spectrum of mutations in the *lacI* gene in the liver of AFB_1-treated lambda/*lacI* mice and rats differed considerably (Table 5.2), probably due to species differences in the metabolic activation and detoxification of AFB_1 (Hsu *et al.* 1987). In the rat, the mutational spectrum induced by AFB_1 was clearly different ($P < 0.001$) from that observed for vehicle-treated controls, where a large proportion of the mutations are G:C → A:T transitions at CpG sites (this study; de Boer *et al.*, in preparation[19]). After treatment of rats with AFB_1, a dramatic increase was seen in the proportion of G:C → T:A transversions which then accounts for 78% of all mutations compared to only 11% in the vehicle-treated spectrum. This was accompanied by a large decrease in the proportion of G:C → A:T transitions, from 48% to only 4%.

The recovery of a high proportion of AFB_1-induced mutations as G:C → T:A transversions in our study is largely in accordance with the reported mutational specificity of AFB_1 in other systems. For example, in the endogenous *lacI* gene in *E. coli*, metabolically activated AFB_1 predominantly (93%) induced the formation of G:C → T:A transversion mutations (Foster *et al.* 1983). In addition, a study of the mutational properties of the primary AFB_1-N7-DNA adduct inserted into bacteriophage M13 DNA by site-directed mutagenesis and replicated in *E. coli* found that 75% of all mutations were G → T transversions (Bailey *et al.* 1996), similar to our determination of 78% G:C → T:A transversions in AFB_1-treated rat liver. However, it should be noted that several other studies have found somewhat lower contributions of G:C → T:A transversion mutations, with a higher fraction of other base substitution mutations. For example, in a

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[19] de Boer *et al.* (1996a).

study in which shuttle vector DNA was treated *in vitro* with AFB_1 and replicated in human cells, 90% of the recovered mutations were base substitutions, but only about one-half of these were G:C → T:A transversions (Levy *et al.* 1992). Transfection of *in vitro*-modified phage M13 DNA into DNA repair-deficient *E. coli* cells resulted in approximately equal numbers of G → T transversion and G → A transition mutations (Sambamurti *et al.* 1988; Sahasrabudhe *et al.* 1989).

The variation observed in AFB_1-induced mutational spectra in various studies likely reflects unique characteristics of the test systems, including whether the DNA was reacted with activated AFB_1 *in vitro* or *in vivo*. Conceivably, differences in mutational spectra might reflect differences in the relative formation and repair of the three principal AFB_1-DNA adducts: AFB_1-N7-guanine, the AP (apurinic) site formed by depurination of this principal N7-adduct, and the AFB_1-FAPY (AFB_1-formamidopyrimidine) adduct formed by scission of the imidazole ring of AFB_1-N7-guanine (Tudek *et al.* 1992; Bailey *et al.* 1996). Bailey *et al.* (1996) have shown that the AFB_1-N7-guanine adduct and not the AP site best explains mutations in AFB_1-treated cells. However, in studies where formation of the AP adduct is enhanced, changes in the AFB_1 mutational spectrum might be expected depending on repair of the AP site or the preference for nucleotide insertion opposite unrepaired AP sites during DNA replication (Sagher and Strauss 1983; Neto *et al.* 1992). It should be noted that the AFB_1-FAPY adduct is believed to block DNA replication, and is thus primarily a lethal lesion unless repaired (Tudek *et al.* 1992).

In our study, AFB_1-induced G → T transversion events (*i.e.* G:C → T:A) in rat liver occurred at guanine residues that were flanked 97% of the time (34 of 35 events) on the 5′-side by a guanine or a cytosine, and 57% of the time (20 of 35 events) on the 3′ side by a cytosine (Table 5.3). Forty percent (14 of 35) of these mutations occurred at the sequence 5′–C<u>G</u>C–3′ (underlining denotes the mutated nucleotide throughout this discussion). Noting that it is difficult to identify simple rules which predict AFB_1 reactivity toward a particular guanine (Marien *et al.* 1987), our data agree reasonably well with the specificity of AFB_1 adduct formation in DNA described by Benasutti *et al.* (1988), who determined that guanines and cytosines on the 5′side and guanines and thymines on the 3′-side are the most influential in determining guanine reactivity with AFB_1. The differences between their data and our data might reflect the *in vitro versus*

the *in vivo* nature of the experiments, respectively. Our data also agree well with that of Misra *et al.* (1983), who found that guanines flanked by A:T sequences were poor targets for AFB_1-induced lesions.

Seventy-one percent (25 of 35) of the G:C → T:A transversion mutations recovered from AFB_1-treated rats occurred at CpG sites. This is substantially higher than the approximately 30% that is expected statistically (de Boer *et al.* 1996a). Inspection of the flanking DNA sequences revealed that 44% of G → T transversions at CpG sites (11 of 25) occurred at the sequences 5′–GCGG–3′ or 5′–CCGC–3′ (Table 5.3).

Since 5′–GCGG–3′ and 5′–CCGC–3′ are complementary, it appeared that CpG sites flanked by G:C basepairs (specifically, 5′–GCGG–3′ / 5′–CCGC–3′ sequences) might be hotspots for AFB_1-induced mutation. While there are 95 CpG sites per strand in *lacI*, only 39 are known to generate a mutant phenotype when mutated by a G:C → T:A transversion (*lacI* database; de Boer 1995). Of these 39 CpG sites, five occur at 5'-GCGG-3' / 5'-CCGC-3' sequences and 34 occur at other CpG sites. If the 25 recovered G:C → T:A mutations at CpG sites (from AFB_1-treated rats) were distributed randomly among the 39 recoverable mutable sites, we would expect to recover 3.2 mutations at 5′–GCGG–3′ / 5′–CCGC–3′ sequences and 21.8 mutations at other CpG sites. However, we observed 11 such mutations at 5′–GCGG–3′ / 5′–CCGC–3′ sequences, and 14 mutations at other CpG sites. Therefore, AFB_1-induced G → T mutations at 5′–GCGG–3′ or 5′–CCGC–3′ occurred significantly more frequently ($P < 0.0076$, Fisher exact test) than at other CpG sites, suggesting that these sequences are indeed hotspots for mutation induced by AFB_1. It may be noted that 100% of the recoverable G → T mutations at 5′–GCGG–3′ or 5′–CCGC–3′ were observed (with 11/5 or 2.2-fold 'saturation'), whereas only 41% (14 of 34) of recoverable G → T mutations at other CpG sites were observed.

The five 5′–GCGG–3′ / 5′–CCGC–3′ regions in *lacI* containing CpG sites at which G → T transversions are recoverable were also examined to determine if a strand bias existed for such mutations induced by AFB_1 in rat liver. Eight of the 11 recovered G → T transversions at 5′–GCGG–3′ or 5′–CCGC–3′ occurred in the noncoding strand (Table 5.3). However, such transversions are known to generate a mutant phenotype at all of the

five above mentioned regions when occurring in the noncoding strand but only at two of these five regions when occurring in the coding strand. The ratio of observed coding:noncoding strand mutations (3:8, Table 5.3) is very close to the ratio of recoverable coding:noncoding strand mutations (2:5), suggesting that there is no strand bias for AFB_1-induced G → T mutation at 5′–GC<u>G</u>G–3′ / 5′–CC<u>G</u>C–3′ sequences in the *lacI* gene.

Activated AFB_1 is known to react almost exclusively with guanines, at the N7-position (Essigmann *et al.* 1977). It has previously been suggested that AFB_1 may preferentially induce mutations at certain sites, including 5′–GpG–3′, 5′–CpC–3′ and 5′–CpG–3′ sequences (Misra *et al.* 1983). In a bacterial forward mutagenesis assay using a *lacZ* segment in M13, the sequence 5′–TG<u>G</u>CG–3′ was found to be an AFB_1 hotspot, with the underlined guanine having the most mutations (Sambamurti *et al.* 1988). Other AFB_1-induced hotspots have been reported. For example, in the human *HPRT* gene, activated AFB_1 produced a G:C → T:A transversion at basepair 209 in exon 3 in 10–17% of all mutants (Cariello *et al.* 1994). This hotspot occurs in a GG<u>G</u>GGG sequence. These observations, and our data (5′–GC<u>G</u>G–3′ / 5′–CC<u>G</u>C–3′ hotspot), correlate reasonably well with data from a study which indicated that 5′–(G/C)<u>G</u>(G/T)–3′ sequences in DNA react preferentially with activated AFB_1 (Benasutti *et al.* 1988).

The induction of G:C → T:A transversions by AFB_1, particularly at 5′–GpG–3′ dinucleotide sequences, is of particular relevance in regard to activation of oncogenes. In AFB_1-induced hepatocarcinomas in rats and trout, mutations at codons 12 and 13 of the *ras* gene, a 5′–GpG–3′ target, were primarily (83%) G:C → T:A transversions (McMahon *et al.* 1990; Chang *et al.* 1991). In addition, G:C → T:A transversions are frequently found (77%) at 5′–AG<u>G</u>–3′ in codon 249 of the *p53* gene in human hepatocellular carcinomas from humans exposed to dietary aflatoxins (Bressac *et al.* 1991; Hsu *et al.* 1991).

In contrast to the dramatic changes in the pattern of mutation observed in the rat, the liver mutational spectrum of AFB_1-treated mice was very similar to the pattern of spontaneous mutations, except that G:C → T:A transversions occurred at CpG sites almost twice as frequently in AFB_1-treated mice as in untreated mice (Table 5.2). In

addition, there was a modest increase in the proportion of G:C → A:T transitions (from 48% to 57%) in treated mice. It was determined that there was no significant difference ($P = 0.74 \pm 0.02$) in the mutational spectra between AFB_1-treated and untreated mice.

An intriguing observation in the mouse AFB_1 data was the recovery of a G:C → T:A transversion at position 791 which was found once in each of three animals. Mutation at this position has previously been observed only twice among more than 5800 sequenced *lacI* mutations (unpublished: *lacI* database; de Boer *et al.* 1995). The rarity of the mutation, and the sequence at which this mutation occurs (5'–TGCGC–3') tends to support the idea that this mutation was induced by AFB_1. Position 791 may thus be an AFB_1 hotspot in the mouse liver environment, although this event was not recovered in the rat collection.

In this study, the lambda/*lacI* transgenic mutagenesis assay was used to demonstrate a species-specific *in vivo* response to the mycotoxin AFB_1. The results described here are in general concordance with what is known about AFB_1 and its mechanism of action on rodent liver DNA *in vivo*. The sensitivity of the F344 rat to the mutagenic effects of AFB_1 in the liver was evidenced by an increase in *lacI* mutant frequency and a shift in mutational spectrum to predominantly G:C → T:A transversions. The C57BL/6 mouse was relatively resistant to AFB_1-induced liver mutations. This work illustrates the utility of having the same shuttle vector system in both mice and rats for comparative mutation testing. The lambda/*lacI* assay confers the potential to measure species-specific differences in mutant frequency and mutational spectra in essentially any tissue. Moreover, the lambda/*lacI* transgenic assay uses the same model species that have been established for the two-year rodent bioassay. Thus, carcinogenicity studies can be augmented by transgenic mutational studies, providing detailed sequence-level analysis of *in vivo* mutational events. The information that is now achievable from the two-species lambda/*lacI* transgenic assay may have beneficial applications in many fields, including mechanistic analysis and risk assessment.

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Table 5.1. *lacI* mutation data from the livers of male lambda/*lacI* mice and rats exposed to AFB_1

Species	Age at tissue harvest (wks)	Treatment [a]	Expression period [b]	Animal ID	Mutant PFU [c]	Total PFU [d]	Mutant frequency [e]	Mean ± SD [f]
C57 mouse	13	vehicle-treated	14	26	8	311,376	2.6	3.1 ± 0.88 (28%)
				27	7	323,722	2.2	
				28	21	668,417	3.1	
				29	16	340,720	4.7	
				30	8	279,856	2.9	
				31	14	407,728	3.4	
C57 mouse	13	2.5 mg/kg AFB_1	14	32	11	331,038	3.3	4.1 ± 1.6 (39%)
				33	21	342,696	6.1	
				34	10	330,634	3.0	
				35	9	326,598	2.8	
				36	30	495,288	6.1	
				37	10	331,049	3.0	
F344 rat	19	vehicle-treated	14	76	7	272,840	2.6	2.7 ± 0.69 (26%)
				77	12	401,720	3.0	
				78	11	372,020	3.0	
				79	5	326,918	1.5	
				80	11	304,980	3.6	
				81	8	313,901	2.5	
F344 rat	13	0.25 mg/kg AFB_1	14	166	100	283,506	35.3	49 ± 17 (35%)
				167	195	304,594	64.0	
				168	234	363,240	64.4	
				169	144	296,272	48.6	
				170	201	356,520	56.4	
				171	72	317,232	22.7	

[a] Single dose, i.p. injection, DMSO solvent, 1 ml/kg dosing volume.
[b] Number of days between injection and euthanasia/tissue collection.
[c] Number of blue PFU.
[d] Total number of PFU screened.
[e] Mutant frequency is expressed as the number of blue (*lacI* mutated) plaques per 100,000 PFU screened.
[f] Numbers in parentheses are standard deviations expressed as a percentage of the mean.

Table 5.2. Spectra of spontaneous and AFB_1-induced mutations in the *lacI* gene in male C57BL/6 mouse and F344 rat liver [a]

	C57BL/6 Mouse Spontaneous [b]			C57BL/6 Mouse 2.5 mg/kg AFB_1			F344 Rat vehicle-treated			F344 Rat 0.25 mg/kg AFB_1		
	#	%	% @ CpG	#	%	% @ CpG	#	%	% @ CpG	#	%	% @ CpG
Transitions												
G:C → A:T	37	48.0	75.7	24	57.1	70.8	21	47.7	76	2	4.4	50
A:T → G:C	3	3.9		0	0		1	2.3		0	0	
Transversions												
G:C → T:A	17	22.1	23.5	8	19.0	37.5	5	11.4	100	35	77.8	71
G:C → C:G	3	3.9	33.3	1	2.4	100	4	9.1	50	5	11.1	80
A:T → T:A	2	2.6		1	2.4		2	4.5		1	2.2	
A:T → C:G	4	5.2		1	2.4		1	2.3		0	0	
Other Mutations												
+1 Frameshifts	3	3.9		0	0		0	0		0	0	
−1 Frameshifts	4	5.2		1	2.4		2	4.5		0	0	
Deletions	1	1.3		2	4.8		3	6.8		0	0	
Insertions	0	0		1	2.4		2	4.5		0	0	
Complex changes	1	1.3		1	2.4		3	6.8		1	2.2	
Double mutants	2	2.6		2	4.8		0	0		1	2.2	
Total others	11	14.3		7	16.7		10	22.8		2	4.4	
Totals	77	100		42	100.1 [c]		44	99.9		45	99.9	

[a] These data were automatically corrected for clonal expansions, using the *lacI* database software (de Boer 1995).

[b] The C57BL/6 mouse spontaneous (untreated) liver mutational spectrum is virtually indistinguishable ($P = 0.72 \pm 0.02$) from that observed in a larger dataset (205 mutants) of $B6C3F_1$ mouse spontaneous liver mutants (*lacI* database; de Boer 1995). There were no statistically significant differences between the mutational spectra from untreated and AFB_1-treated C57BL/6 mice ($P = 0.74 \pm 0.02$), or between untreated C57BL/6 mice and vehicle-treated F344 rats ($P = 0.14 \pm 0.01$). The vehicle-treated and AFB_1-treated F344 rat mutational spectra were significantly different ($P < 0.001$).

[c] Totals may not be 100%, due to rounding errors.

Table 5.3. Sequence analysis of G:C → T:A transversion mutations observed in the *lacI* gene from liver tissue of AFB_1-treated male lambda/*lacI* transgenic mice and rats (corrected for clonal expansions).

Species	5′– Sequence	Mutated Nucleotide [a]	3′– Sequence	Mutation Position [b]	Strand	CpG Site [c]	Occurrences
C57BL/6	CACCG	G	CATAC	66	non-coding	–	1
(Mouse)	TGGCG	G	AGCTG	158	coding	–	1
	ACGCG	G	TTGGG	178	non-coding	–	1
	TGAAT	G	AGGGC	731	coding	–	1
	GGCGC	G	CATTG	791	non-coding	+	3
	CATGA	G	CTGTC	865	non-coding	–	1
F344	CACCG	G	CATAC	66	non-coding	–	1
(Rat)	TGCCG	G	TGTCT	69	coding	–	1
	GATAA	G	AGACA	75	non-coding	–	1
	CACGC	G	GGAAA	92	non-coding	+, *	2
	TTCCC	G	CGTGG	93	coding	+, *	3
	GCCAC	G	TTTCT	116	coding	+	1
	CGCGG	G	AAAAA	134	coding	–	1
	TGGCG	G	AGCTG	158	coding	–	1
	CACGC	G	GTTGG	179	non-coding	+, *	1
	TGCCC	G	CCAGT	198	non-coding	+, *	2
	GATTG	G	CGTTG	222	coding	–	1
	TCGCC	G	CGACA	270	non-coding	+, *	1
	TAATC	G	CCGCG	273	non-coding	+	1
	CGCTG	G	CACCC	303	non-coding	–	1
	GCACC	G	CCGCT	357	non-coding	+, *	2
	GACGC	G	TTGCG	380	non-coding	+	1
	ACAGC	G	CGATT	576	non-coding	+	1
	GCGCC	G	AGACA	606	non-coding	+	3
	ACATG	G	CACTC	693	non-coding	–	2
	GCATC	G	CAGTG	750	non-coding	+	1
	TTACC	G	AGTCC	803	coding	+	1
	TCGTC	G	TATCC	847	non-coding	+	1
	ATACC	G	AAGAC	857	coding	+	1
	AGCAG	G	CGAAA	910	non-coding	–	1
	TCCAC	G	CTGGT	928	non-coding	+	3

[a] Sequenced C → A transversion mutations were interpreted as originating from G → T mutations in the non-coding strand, and are presented as such in the table.

[b] Nucleotide positions are numbered as described by Farabaugh (1978).

[c] A (+) sign indicates the mutation occurred at a CpG site; a (–) sign indicates that the mutation did not occur at a CpG site. An asterisk (*) indicates that a G:C → T:A transversion occurred at a CpG site within the sequence 5′–GCGG–3′ or its complement.

5.5. Appendix: List of Mutants

Table 5A.1. AFB_1-treated mouse liver mutants, uncorrected

Mutant ID #	Mutation Type	CpG Site	Mutated Base #1	Mutated Base #2	Amino Acid Change	Mutagen	Animal ID #	Other Changes, Comments
31C361	substitution	yes	C 42 T	none	Thr 5 Met	AFB1	35	
31A014	substitution	yes	C 42 T	none	Thr 5 Met	AFB1	36	
31E008	substitution	yes	G 56 A	none	Ala 10 Thr	AFB1	34	
31F001	substitution	yes	G 56 A	none	Ala 10 Thr	AFB1	34	clonal
31A006	substitution	yes	G 56 A	none	Ala 10 Thr	AFB1	36	
31C320	substitution	no	C 66 A	none	Ala 13 Asp	AFB1	35	
31G011	substitution	yes	C 92 T	none	Arg 22 Cys	AFB1	32	
31G006	substitution	yes	G 93 A	none	Arg 22 His	AFB1	32	
31G009	substitution	yes	G 93 A	none	Arg 22 His	AFB1	32	clonal
31F003	substitution	yes	G 93 A	none	Arg 22 His	AFB1	34	
31C270	substitution	yes	G 95 A	none	Val 23 Met	AFB1	33	
31A013	substitution	no	G 158 T	none	Glu 44 amber	AFB1	36	
31G019	substitution	no	C 178 A	none	Asn 50 Lys	AFB1	37	
31A009	substitution	yes	C 179 T	none	Arg 51 Cys	AFB1	36	
31C268	substitution	yes	G 180 A	none	Arg 51 His	AFB1	33	
31C269	substitution	yes	G 180 A	none	Arg 51 His	AFB1	33	clonal
31E001	multiple	no	G 184 T	C 186 T	Val 52 Val	AFB1	34	also Ala(53) → Val
31C264	substitution	yes	C 198 T	none	Ala 57 Val	AFB1	33	
31C267	substitution	yes	C 198 T	none	Ala 57 Val	AFB1	33	clonal
31E003	substitution	yes	C 198 T	none	Ala 57 Val	AFB1	34	
31G005	substitution	no	C 206 T	none	Gln 60 amber	AFB1	32	
31E002	substitution	no	G 222 A	none	Gly 65 Asp	AFB1	34	
31C323	substitution	no	G 222 A	none	Gly 65 Asp	AFB1	35	
31G015	insertion	no	620	none		AFB1	37	+TGGC @ 620/631
31G016	complex	no	T 246 A	none		AFB1	37	also -9 bp @ 234/242
31C326	substitution	no	C 260 T	none	Gln 78 ochre	AFB1	35	
31C266	substitution	yes	G 269 A	none	Ala 81 Thr	AFB1	33	
31G017	substitution	yes	G 269 A	none	Ala 81 Thr	AFB1	37	
31F002	substitution	yes	C 270 T	none	Ala 81 Val	AFB1	34	
31G002	substitution	yes	C 329 T	none	Arg 101 opal	AFB1	32	
31G020	substitution	yes	C 329 T	none	Arg 101 opal	AFB1	36	

Table 5A.1. (AFB_1 mouse liver mutants, *continued*)

Mutant ID #	Mutation Type	CpG Site	Mutated Base #1	Mutated Base #2	Amino Acid Change	Mutagen	Animal ID #	Other Changes, Comments
31A008	substitution	no	T 369 G	none	Leu 114 Arg	AFB1	36	
31D005	-1 frameshift	no	429	none		AFB1	35	-T @ 429/430
31G014	substitution	no	C 513 T	none	Ser 162 Phe	AFB1	37	
31C325	deletion	no	637	none		AFB1	35	deletion @ 637/638
31G007	multiple	no	A 689 T	G 690 A		AFB1	32	AGT(Ser) → TAT(Tyr)
31A011	substitution	no	C 719 T	none	Gln 231 ochre	AFB1	36	
31G001	substitution	no	G 731 T	none	Glu 235 amber	AFB1	32	
31G004	substitution	no	G 731 T	none	Glu 235 amber	AFB1	32	clonal
31H001	deletion	no	777	none		AFB1	37	8 bp deletion @ 777-784
31G012	substitution	yes	C 791 A	none	Arg 255 Ser	AFB1	32	
31C265	substitution	yes	C 791 A	none	Arg 255 Ser	AFB1	33	
31C360	substitution	yes	C 791 A	none	Arg 255 Ser	AFB1	35	
31C324	substitution	yes	G 794 C	none	Ala 256 Pro	AFB1	35	
31G010	substitution	no	C 865 A	none	Ser 279 Arg	AFB1	32	
31A012	substitution	no	C 944 T	none	Gln 306 ochre	AFB1	36	
31A010	substitution	no	A1004 T	none	Arg 326 opal	AFB1	36	

Table 5A.2. Vehicle-treated control rat liver mutants, uncorrected

Mutant ID #	Mutation Type	CpG Site	Mutated Base #1	Mutated Base #2	Amino Acid Change	Mutagen	Animal ID #	Other Changes, Comments
31J002	complex	no	none	none		none	77	T(704) → A; C(762) → A
31C365	complex	no	none	none		none	79	AC(1010/1011) → G
31E051	complex	no	none	none		none	81	30–43 replaced with GT
31E050	substitution	yes	G 29 T	none	Val 30 Phe	none	81	
31G134	-1 frameshift	no	61	none		none	80	-G @ 61
31B001	substitution	no	T 64 G	none	Tyr 12 amber	none	78	
31E164	deletion	no	68	none		none	77	GGTGCTCTTA del @ 68/78
31E006	substitution	no	A 81 T	none	Gln 18 Leu	none	77	
31B003	substitution	yes	C 92 T	none	Arg 22 Cys	none	78	
31G130	substitution	no	C 104 T	none	Gln 26 amber	none	80	
31E043	substitution	no	G 140 A	none	Val 38 Met	none	81	
31B007	substitution	no	C 174 G	none	Pro 49 Arg	none	78	
31B004	substitution	yes	G 180 A	none	Arg 51 His	none	78	
31C362	substitution	no	G 185 A	none	Ala 53 Thr	none	79	
31E166	substitution	no	T 195 C	none	Leu 56 Pro	none	77	
31B005	substitution	yes	C 198 A	none	Ala 57 Glu	none	78	
31B006	substitution	yes	C 198 A	none	Ala 57 Glu	none	78	clonal
31H008	substitution	yes	C 198 T	none	Ala 57 Val	none	76	
31A004	substitution	yes	C 198 T	none	Ala 57 Val	none	78	
31D002	substitution	yes	C 198 T	none	Ala 57 Val	none	79	
31E049	substitution	yes	C 198 T	none	Ala 57 Val	none	81	
31B008	substitution	no	T 209 A	none	Ser 61 Thr	none	78	
31E160	substitution	yes	G 269 A	none	Ala 81 Thr	none	77	
31E044	substitution	yes	G 269 A	none	Ala 81 Thr	none	81	
31E165	substitution	yes	C 329 T	none	Arg 101 opal	none	77	
31A001	substitution	yes	C 329 T	none	Arg 101 opal	none	78	
31G131	substitution	yes	C 329 T	none	Arg 101 opal	none	80	
31G137	substitution	yes	C 329 T	none	Arg 101 opal	none	80	clonal
31G138	substitution	yes	C 329 T	none	Arg 101 opal	none	80	clonal
31H006	substitution	yes	C 380 A	none	Arg 118 Ser	none	76	
31A003	substitution	yes	G 381 A	none	Arg 118 His	none	78	
31G129	substitution	yes	G 381 A	none	Arg 118 His	none	80	

Table 5A.2. (control rat liver mutants, *continued*)

Mutant ID #	Mutation Type	CpG Site	Mutated Base #1	Mutated Base #2	Amino Acid Change	Mutagen	Animal ID #	Other Changes, Comments
31E046	substitution	yes	G 381 A	none	Arg 118 His	none	81	
31E162	substitution	yes	G 383 T	none	Val 119 Phe	none	77	
31G141	substitution	yes	C 530 T	none	Arg 168 opal	none	80	
31G142	substitution	yes	C 530 T	none	Arg 168 opal	none	80	clonal
31H007	substitution	yes	G 575 C	none	Ala 183 Pro	none	76	
31G139	substitution	yes	G 575 C	none	Ala 183 Pro	none	80	
31E045	deletion	no	620	none		none	81	–TGGC @ 620
31H005	insertion	no	620	none		none	76	+TGGC @ 620/632
31E158	insertion	no	620	none		none	77	+CTGG @ 620
31E163	insertion	no	620	none		none	77	+TGGC @ 620; clonal
31J001	substitution	no	G 687 A	none	Trp 220 amber	none	77	
31G132	substitution	yes	G 803 A	none	Glu 259 Lys	none	80	
31E159	substitution	yes	C 834 A	none	Ser 269 amber	none	77	
31D001	substitution	no	C 953 T	none	Gln 309 amber	none	79	
31E007	substitution	no	G 962 C	none	Ala 312 Pro	none	77	
31A002	deletion	no	996	none		none	78	17 bp del. @ 996-1012
31C363	-1 frameshift	no	1010	none		none	79	–G @ 778

Table 5A.3. AFB_1-treated rat liver mutants, uncorrected

Mutant ID #	Mutation Type	CpG Site	Mutated Base #1	Mutated Base #2	Amino Acid Change	Mutagen	Animal ID #	Other Changes, Comments
31A056	substitution	no	G 31 C	none	Val 1 Val	AFB1	171	Initiation codon
31A041	substitution	yes	C 42 T	none	Thr 5 Met	AFB1	171	
31H125	substitution	yes	G 56 C	none	Ala 10 Pro	AFB1	166	
31A040	substitution	no	T 64 A	none	Tyr 12 ochre	AFB1	171	
31H122	substitution	no	C 66 A	none	Ala 13 Asp	AFB1	166	
31C037	substitution	no	G 69 T	none	Gly 14 Val	AFB1	170	
31H123	substitution	no	C 75 A	none	Ser 16 Tyr	AFB1	166	
31E059	substitution	no	C 80 T	none	Gln 18 amber	AFB1	168	
31H130	substitution	yes	C 92 A	none	Arg 22 Ser	AFB1	166	
31C002	substitution	yes	C 92 A	none	Arg 22 Ser	AFB1	169	
31C003	substitution	yes	C 92 A	none	Arg 22 Ser	AFB1	169	clonal
31G021	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	167	
31E053	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	168	
31E054	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	168	clonal
31C010	substitution	yes	G 93 T	none	Arg 22 Leu	AFB1	169	
31A036	substitution	yes	G 116 T	none	Val 30 Phe	AFB1	171	
31C007	substitution	yes	G 132 C	none	Arg 35 Pro	AFB1	169	
31H127	substitution	no	G 134 T	none	Glu 36 ochre	AFB1	166	
31C038	substitution	no	G 158 T	none	Glu 44 amber	AFB1	170	
31G026	substitution	yes	C 179 A	none	Arg 51 Ser	AFB1	167	
31G028	substitution	yes	G 180 C	none	Arg 51 Pro	AFB1	167	
31C034	substitution	yes	C 198 A	none	Ala 57 Glu	AFB1	170	
31A038	substitution	yes	C 198 A	none	Ala 57 Glu	AFB1	171	
31C008	substitution	no	G 222 T	none	Gly 65 Val	AFB1	169	
31E060	multiple	no	C 270 A	C 273 A	Ala 81 Glu	AFB1	168	also Ala(82) → Glu
31C035	substitution	yes	C 270 A	none	Ala 81 Glu	AFB1	170	
31C040	substitution	yes	C 270 A	none	Ala 81 Glu	AFB1	170	clonal
31E052	substitution	yes	C 273 A	none	Ala 82 Glu	AFB1	168	
31H121	substitution	no	C 303 A	none	Ala 92 Asp	AFB1	166	
31G024	substitution	yes	C 357 A	none	Ala 110 Glu	AFB1	167	
31E055	substitution	yes	C 357 A	none	Ala 110 Glu	AFB1	168	
31A039	substitution	yes	C 380 A	none	Arg 118 Ser	AFB1	171	
31A043	substitution	yes	C 576 A	none	Ala 183 Glu	AFB1	171	

Table 5A.3. (AFB_1 rat liver mutants, *continued*)

Mutant ID #	Mutation Type	CpG Site	Mutated Base #1	Mutated Base #2	Amino Acid Change	Mutagen	Animal ID #	Other Changes, Comments
31H124	substitution	yes	C 606 A	none	Ser 193 amber	AFB1	166	
31G025	substitution	yes	C 606 A	none	Ser 193 amber	AFB1	167	
31C001	substitution	yes	C 606 A	none	Ser 193 amber	AFB1	169	
31G023	substitution	no	C 693 A	none	Ala 222 Asp	AFB1	167	
31A037	substitution	no	C 693 A	none	Ala 222 Asp	AFB1	171	
31G027	substitution	yes	C 750 A	none	Ala 241 Glu	AFB1	167	
31C041	complex	yes	C 750 A	none		AFB1	170	also +T @ 703/706
31H128	substitution	yes	G 785 C	none	Ala 253 Pro	AFB1	166	
31H126	substitution	yes	G 803 T	none	Glu 259 amber	AFB1	166	
31E057	substitution	yes	C 847 A	none	Tyr 273 ochre	AFB1	168	
31A042	substitution	yes	G 857 T	none	Glu 277 ochre	AFB1	171	
31C006	substitution	no	C 910 A	none	Arg 294 Arg	AFB1	169	
31H129	substitution	yes	C 928 A	none	Ser 300 Arg	AFB1	166	
31E056	substitution	yes	C 928 A	none	Ser 300 Arg	AFB1	168	
31C036	substitution	yes	C 928 A	none	Ser 300 Arg	AFB1	170	

Chapter 6. Effects of Gender and Species on Spectra of Mutation Induced by 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the *lacI* Transgene

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Abstract

Feeding of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) to F344 rats induces colon tumors specifically in male rats. Mutant frequencies and mutational spectra of the *lacI* transgene were studied in male and female Big Blue® transgenic rats after feeding 400 ppm of PhIP in the diet for 60 days. Mutant frequencies in the colon mucosa were increased 20-25 times compared with those of the control rats, being $661.4 \pm 33.3 \times 10^{-6}$ and $718.2 \pm 16.9 \times 10^{-6}$ in males and females, respectively. No significant differences in types and distribution of the mutations were detected between males and females. One-base deletions were the most frequent mutation, including the characteristic guanine deletion at 5′–GGGA–3′ which is also seen in the *Apc* gene of rat colon cancers induced by PhIP. Comparison of the *lacI* mutations in the rat colon with those previously identified in the mouse colon showed that the rate of G to T transversions was significantly higher in the mouse. This is the first report stating that there exist differences in the mutation specificity on the same gene, among mammalian species. However, the characteristic guanine deletion was recovered in both the mouse and the rat. These findings do not offer a mechanistic explanation of the gender specificity of PhIP-induced colon cancer in rats, though the universality of the guanine deletion suggests that this alteration may prove a useful indicator of human exposure. © 1997 Elsevier Science B.V.

Keywords: **2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Gender effect; *lacI* gene; Mutation, spectrum, frequency; Species Specificity**

6.1. Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a food borne carcinogen to which humans are exposed on a daily basis. PhIP induces colon and prostate cancers in male Fischer 344 rats (Ito *et al.* 1991; Shirai *et al.* 1997) and mammary cancer, but not colon cancer, in female rats (Ito *et al.* 1991). However, similar levels of PhIP-DNA adducts are produced in the colon of male and female rats exposed to PhIP in the diet (Ochiai *et al.* 1996). DNA adduct formation is not a sole factor for induction of a mutation which is an important process of carcinogenesis (Ames *et al.* 1993). Mutation frequencies are the result of combination of the level of DNA adducts and the rate of cell proliferation, although it would, to some extent, be influenced by the nature of repair.

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We have previously found that colon tumors of rats induced by PhIP possess a characteristic mutation in the *Apc* (*ademomatous polyposis coli*) gene (Kakiuchi *et al.* 1995). All five mutations detected in four colon tumors included a guanine deletion at a 5′–GGGA–3′ sequence, and four of the five mutations shared the same seven nucleotides surrounding the deleted guanine, 5′–GTGGGAT–3′. The same type of guanine deletion was preferentially induced by PhIP in the *Hprt* gene of hamster (Yadollahi-Farsani *et al.* 1996) and in the episomal *supF* gene transfected into a human fibroblast (Endo *et al.* 1994). The frameshift mutation observed is consistent with the covalent binding of PhIP to guanine base to form N^2-(guanin-8-yl)-PhIP (Lin *et al.* 1992; Nagaoka *et al.* 1992; Fukutome *et al.* 1994). Other food-related heterocyclic amines (HCAs), such as 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-9*H*-pyrido[2,3-*b*]indole (A α C) also form DNA adducts by covalently binding to the C8 position of guanine (King and Kadlubar 1996; Tada *et al.* 1994). We have examined the mutational spectra of PhIP, MeIQ and A α C in the *lacI* gene of Big Blue® mice (BBM), and found that each chemical leaves a unique mutational signature. The deletion of guanine from the 5′–GGGA–3′ sequence is specific to PhIP. This mutation, being unique to PhIP-treated animals, makes it a good candidate for detecting PhIP exposure similar to how codon 249 mutation of *p53* gene is taken as an indicator of aflatoxin B_1-associated human hepatocellular carcinomas (Vogelstein and Kinzler 1992).

Since PhIP induces colon tumors specifically in male rats, we have examined the effect of gender on the mutant frequencies and mutational spectra in Big Blue® rats (BBRs). Furthermore, we analyzed species differences in PhIP-induced mutational spectra, comparing the results obtained in this study using the colon mucosa of BBR with those obtained previously using the same tissue of BBM.

6.2. Materials and Methods

6.2.1. Animals and chemical

Five-week-old male and female Big Blue® F344 rats were purchased from Taconic Laboratories (New York, NY). After acclimatization to the housing and the basal diet (CE-2) from CLEA Japan (Tokyo, Japan) for a week, the rats were fed the CE-2 diet supplemented with 400 ppm of PhIP for 60 days followed by CE-2 for another seven

days. Control rats were given CE-2 for 67 days. Each experimental group consisted of 5 rats.

PhIP-HCl was obtained from the Nard Institute (Osaka, Japan). Its purity was previously confirmed by HPLC analysis (Shirai *et al.* 1997).

6.2.2. DNA extraction and packaging

After killing the rat colon was removed, opened along the longitudinal median axis, and flushed with phosphate buffered saline. Then the mucous membrane of the colon was recovered using the back of a razor, and kept frozen at –80°C for analysis. High molecular weight genomic DNA was extracted from the mucous membrane by the phenol/chloroform extraction method and precipitated with ethanol, and λ phages were recovered with the packaging extract (Transpack®; Stratagene, La Jolla, CA). *LacI* mutant frequencies were analyzed by the method recommended by Stratagene and modified as previously described (Suzuki *et al.* 1996). Each full-blue plaque was stored in 0.5 ml of SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, and 0.01% gelatin) containing 50 μl of chloroform at 4°C for analysis of mutational spectra.

6.2.3. PCR-restriction-SSCP analysis and fluoro-cycle sequencing

Mutations in the *lacI* gene were determined by the cycle sequencing method (Erfle *et al.* 1996), or a combination of the PCR-single strand conformation polymorphism (SSCP) analysis (Ushijima *et al.* 1995) and cycle sequencing method as described below.

The region covering the *lacI* coding region and its promoter was amplified by PCR with two primers (5′–GACACCATCGAATGGTGCAA–3′ (Y1) and 5′–TTCCACACAACATACGAGCC–3′ (Y2)) in the presence of [α-^{32}P]dCTP (Amersham International plc, Buckinghamshire) and the PCR product was digested with *Hae*III, *Eco*RV and *Bcl*I (Toyobo, Osaka). The resulting eight fragments (fragments A–H, from the 5′ to 3′ end) were then subjected to SSCP analysis under three conditions (20°C with or without 5% glycerol, and 10°C with 5% glycerol) to identify the fragment(s) with a mutation(s) (Ushijima *et al.* 1995).

Mutations in the *lacI* gene were identified by the following fluoro-cycle sequencing method. The region covering the *lacI* coding region and its promoter was amplified by PCR with two primers (Y1 and Y2). The PCR mixture (10 μl) consisted of 0.2 mM of each dNTP, 0.8 mM of each primer, 1 μl of 10X PCR buffer (100 mM Tris-HCl (pH 8.3),

500 mM KCl, 15 mM $MgCl_2$, and 0.01% (w/v) gelatin), 0.2 U of Taq DNA polymerase (USB) and 0.4 μl of the phage suspended in SM buffer. PCR was performed for 50 cycles, each consisting of 0.5 min at 94°C and 2 min at 60°C. After the PCR, 2 μl (20 U) of *E. coli* exonuclease I (USB) and 2 μl (4 U) of shrimp alkaline phosphatase (USB) were added to each PCR reaction mixture to remove the excess dNTPs and primers from PCR products. After incubation at 37°C for 15 min, the mixture was inactivated at 80°C for another 15 min (heat-inactivated solution). Fluoro-cycle sequencing was then performed for both strands of each mutant with the appropriate Cy5™-labeled forward and reverse primers (Table 6.1) and thermo Sequenase (Amersham). The reaction mixture (8 μl) consisted of 0.25 mM fluoro-primer (Table 6.1), 2 μl of A, C, G or T reagent (Amersham) and 2 μl of the heat-inactivated solution. Cycle sequencing reactions were performed for 30 cycles, each consisting of 0.5 min at 94°C and 2 min at 65°C. After the reaction, 4 μl of formamide loading dye was added to each reaction mixture, then 6 μl of this solution was loaded on the 6% Long Ranger™ gel (FMC) and sequenced automatically using and ALF express DNA sequencer (Pharmacia). Mutations in the *lacI* gene were determined using GENETYX-Mac software (Software Development) by comparing processed sequence data with the known *lacI* sequence.

Mutations were classified into following types: one base substitutions (six different types), one base deletions, and other mutations. 'Other mutations' included deletion of two or more bases, insertions and other mutations. When a base deletion mutation occurred in a consecutive run of the same nucleotide, the nucleotide number of the most 5′ site was assigned as a mutation site.

6.2.4. Statistics

Results were statistically examined, using *t*-tests for mutant frequencies and chi-square tests for mutational spectra.

6.3. Results and Discussion

6.3.1. Mutant frequencies

Mutant frequencies (MFs) are summarized in Table 6.2. PhIP caused 20- and 25-fold increases in MFs in the colon of male and female BBRs, respectively. No significant difference in the MF was observed between male and female BBRs fed PhIP ($P = 0.50$).

These values were in the same range as those reported for (C57BL/6 x SWR) F_1 mice fed a diet containing 400 ppm of PhIP, under a protocol similar to that used in this study (Zhang *et al.* 1996). These results exclude the possibility that the gender specificity for colon carcinogenicity of PhIP in rats is caused by differences in PhIP-induced mutant frequencies in the colon mucosa.

6.3.2. Mutational spectra and gender specificity

The analysis of 113, 118, 15 and 15 mutants from five rats each of PhIP-fed male, PhIP-fed female, control male and control female animals resulted in the detection of 113, 114, 14 and 15 mutations, respectively. G:C to A:T transitions were the most frequently observed, accounting for about 50% of all mutations in both male and female control groups (Table 6.3). In contrast, G:C base pair deletions were most frequently observed in the PhIP-treated male and female groups, followed by G:C to T:A, G:C to C:G and G:C to A:T substitutions in descending order of frequency in both male and female animals (Table 6.3).

Mutational hot spots, defined here as mutations recovered 3 or more times in 2 or more rats are indicated in Figure 6.1. The initial analysis of 113 and 114 mutants from PhIP-treated male and female rats, respectively, indicated that G:C base-pair deletions at nucleotide positions (Farabaugh 1978) 90–92 were specifically induced in male rats. Six mutants were recovered from 3 male rats and none from female rats ($P = 0.014$, chi-square test). Subsequently, 93 more mutants from 5 male and 47 more mutants from 5 female rats were analyzed. After initial SSCP analysis, only mutants with a mutation in the 'A' fragment (nucleotides 50–107) were sequenced. The G:C base-pair deletion at nucleotides 90–92 was detected in 2 of the additional 93 mutants from male rats and 5 of the additional 47 mutants from female rats, adding up to 8 mutants (3.9%) in a total of 206 mutants from males, and 5 of 161 mutants (3.1%) from females. These 8 and 5 mutants were derived from 5 male and 2 female rats, respectively. There was no significant difference between male and female rats in the number of G:C base-pair deletions at 90–92 ($P = 0.69$). There was also no significant difference in other nucleotide positions between male and female rats. Considering the equivalent MF in male and female rats, the different colon carcinogenicity of PhIP between males and females is not attributable to differences in MF or mutational specificity induced by PhIP in the colonic

mucosa but may probably be due to difference in the later step than initiation of carcinogenesis.

6.3.3. Species difference

It is crucially important to know if the mutational spectrum of a carcinogen is conserved in different species of animals in order to extrapolate animal data to humans. The PhIP mutational spectrum obtained in the rat was compared with that in the mouse, identified previously (Okonogi *et al.* 1997b) (Figure 6.2). In the mouse study, 115 mutations were identified (56 from males and 59 from females). The distribution by class (Figure 6.2) reveals G:C to T:A transversions are observed about twice more often in the mouse compared with the rat ($P < 0.01$). The frequency of the G:C deletion does not appear to be higher in the rat than in the mouse, but this difference was not statistically significant. Other types of mutations (*e.g.*, the deletion of two or more bases, insertions or other mutations) were more frequent in the rat than in the mouse ($P < 0.05$ for females). The base-change mutation at nucleotide 56 was detected exclusively in the mouse ($P < 0.05$), while the G:C deletion at nucleotide 90 was specifically detected in the rat ($P < 0.05$) (Figure 6.3). As far as we know, this is the first report stating that a difference exists in the mutation specificity on the same gene, among mammalian species. It is important to clarify the mechanisms implicated in this species difference.

The rat and mouse shared two hot spots at nucleotides 92 and 877. In both cases, the target sequence involves guanine in a 5′–GGGA–3′ sequence, indicating the potency of PhIP to induce mutations at this sequence in both the mouse and the rat. In the *lacI* gene, there are ten 5′–GGGA–3′ sequences, but the common deletion hot spot was limited to the nucleotide 877. While this might reflect sample size, it is interesting to note that the surrounding sequence, 5′–GCGGGAT–3′, has high homology with the PhIP-induced hot spot sequence in the *Apc* gene in rat colon tumors, 5′–GTGGGAT–3′. We note however that the guanine deletion at nucleotide 843 is contained within a 5′–GTGGGAT–3′ sequence, but was less frequently recovered than at nucleotide 877. This may reflect that mutational hot spots produced by a chemical are determined by a combination of the primary DNA sequence and other factors, such as secondary or tertiary structure of DNA and the efficiency of DNA repair.

The fact that the major mutational event, the loss of G:C in a 5′–GGGA–3′ sequence, was seen in both male and female rats indicates that this change may be taken as an indicator of human exposure to PhIP.

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Table 6.1. Sequences of the primers used for fluoro-cycle sequencing of the *lacI* gene

Name	Direction	Position [a]	Sequence
H-1	Forward	(–50) – (–31)	5′–GACACCATCGAATGGTGCAA–3′
H-2	Forward	540 – 563	5′–TGGAGCATCTGGTCGCATTGGGTC–3′
H-3	Reverse	208 – 185	5′–CTGTTTGCCCGCCAGTTGTTGTGC–3′
H-4	Forward	140 – 163	5′–GTGGAAGCGGCGATGGCGGAGCTG–3′
H-8	Forward	348 – 371	5′–GTAAAGCGGCGGTGCACAATCTTC–3′
H-9	Reverse	634 – 611	5′–ATGCCAGCCAGCCAGACGCAGACG–3′
H-11	Reverse	871 – 848	5′–ACATGAGCTGTCTTCGGTATCGTC–3′
H-12	Forward	785 – 808	5′–GCAATGCGCGCCATTACCGAGTCC–3′
H-22	Reverse	1201 – 1178	5′–ACAATTCCACACAACATACGAGCC–3′

[a] Nucleotide numbers of the *lacI* gene (Farabaugh 1978).

Table 6.2. Mutant frequencies in the colon mucosa of Big Blue® rats

	Rat		No. of plaques		Average mutant
Treatment	Sex	Number	Total	Mutant	frequency ± SE [a] (x 10^{-6})
PhIP	Male	5	582 402	382	661.4 ± 33.3
	Female	5	505 519	375	718.2 ± 16.9
None	Male	5	661 128	16	32.2 ± 5.93
	Female	5	599 539	17	28.7 ± 1.7

[a] Standard error of the individual animal.

Table 6.3. Types of *lacI* mutations in the colon mucosa of Big Blue® rats

		One base substitutions										
		G:C to			A:T to			One base deletions		Two or three base deletions	Other mutations	Total no. of mutations
Treatment	Sex	T:A	C:G	A:T	C:G	T:A	G:C	G:C	A:T			
PhIP	Male	26	16	12	1	3	1	44	1	6	3	113
	Female	31	17	15	1	1	0	38	1	6	4	114
None	Male	2	3	7	0	1	0	0	0	0	1	14
	Female	2	2	6	0	3	0	1	0	0	1	15

Sequence context around the mutation	Nucleotide number	Male	Female	Male	Female
TCC CGC GTG	90	⊘●●●●●●	●	●●	●●●●●
TCC CGC GTG	92	●●⊘	●●⊘⊘⊘⊘●	⊘⊘●	●●⊘⊘⊘●
ACG CGG GAA	132	⊘⊘●	⊘		
GCA ATG CGC	790–791	OOOO	⊘●OOOO	Not determined	
TAT ATC CCG	877	●●●●●●	●●●●●		
Number of mutations		113	114	93	47
Number of mutations clustered		23 (20 %)	20 (18 %)		

● G:C to T:A transversion; ⊘G:C to C:G transversion; ●G:C to A:T transition; ●G:C base-pair deletion; O two-base pair deletion

Figure 6.1. Mutational hot spots for PhIP in the colon of BBRs. The nucleotide numbers show hot spots where three or more mutations from two or more rats were observed in any of the experimental groups. The sequences around the hot spots are shown and the mutated bases are underlined. A total of 113 mutants from male and 114 mutants from female rats were determined by sequencing the *lacI* promoter and coding region, while another 93 mutants from male and 47 from female rats were analyzed for mutations in the A region (ntd-50 ~ 107).

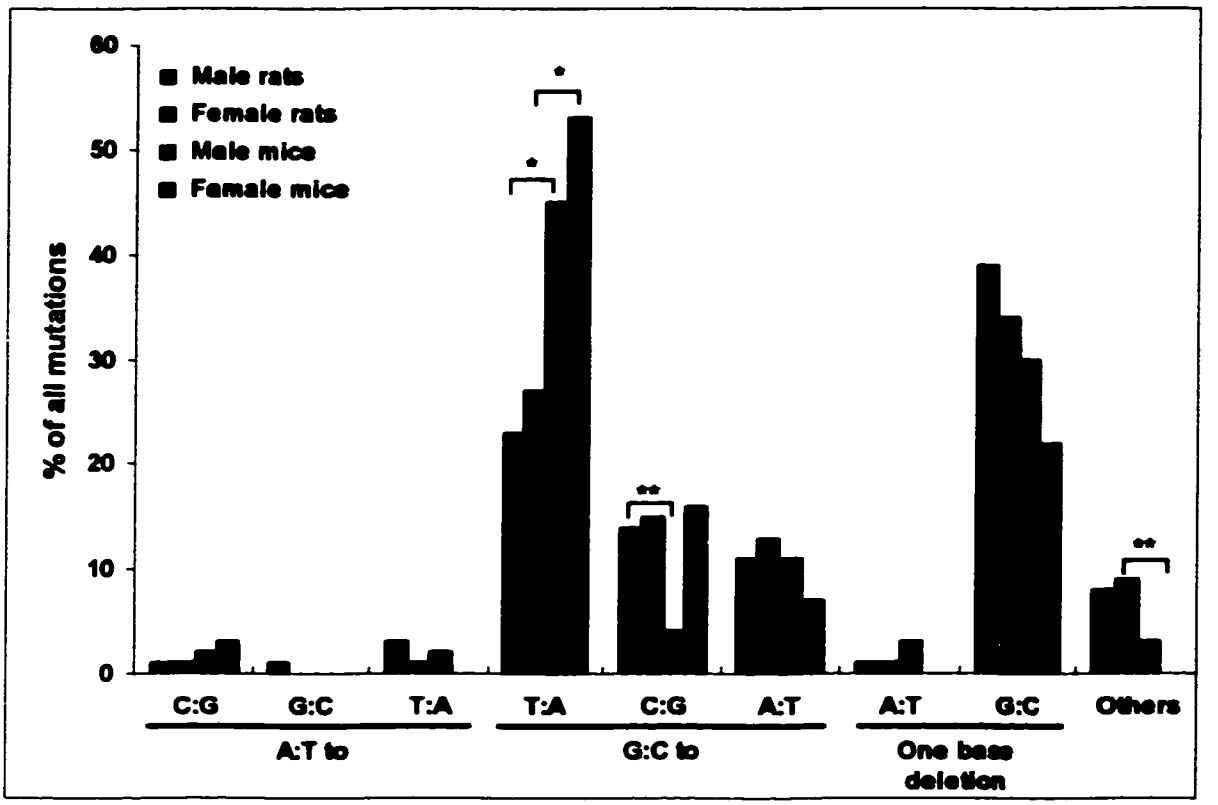

Figure 6.2. Mutational types observed in the colon of male and female BBRs and BBMs. The frequencies of G to T transversions in males and females were significantly different between rats and mice ($^{*}P < 0.01$). G to C transversions in males and 'others' in females were also significantly different between rats and mice ($^{**}P < 0.05$).

Sequence context around the mutation	Nucleotide number	Rat	Mouse
GTC GCA GAG	56		⊘⊘⊘
TCC CGC GTG	90	●●●●●●⊘●	
TCC CGC GTG	92	⊘⊘⊘⊘●●●●●	●●●●●
ACG CGG GAA	132	●⊘⊘⊘	
AAA GTG GAA	140	⊘	●●●
CCC AAC CGC	178	⊘⊘●●	●●●●●
AAA GCG GCG	355	●	●●●
CTG GGC GCA	782	●●	●●●
GCA ATG CGC	790–791	○○○○○○○⊘●	○
GTG GGA TAC	843	●●	●●●
TAT ATC CCG	877	●●●●●●●●●●	●●●
Number of mutations		227	115
Number of mutations clustered		43 (19 %)	25 (22 %)

●G:C to T:A transversion; ⊘G:C to C:G transversion; ●G:C to A:T transition; ●G:C base-pair deletion; ○wo-base pair deletion

Figure 6.3. Comparison of mutational hot spots between rats and mice. G to T transversions at nucleotide 56 were detected specifically in the mouse ($P < 0.05$), and deletion mutations at nucleotide 90 were specifically detected in the rat ($P < 0.05$). Two hot spots, at nucleotides 92 and 877, were found in both rats and mice.

Chapter 7. Prostate Mutations in Rats Induced By the Suspected Human Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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Abstract

Male *lacI* transgenic rats were fed a diet containing 200 ppm of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP),[2] a heterocyclic amine present in cooked meats. PhIP was found to be a powerful prostate mutagen. After 61 days of treatment, the *lacI* mutant frequency was 71×10^{-5}, >20-fold higher than the spontaneous mutant frequency of 3.2×10^{-5}. The predominant PhIP-induced mutations were G:C→T:A transversions and deletions of G:C base pairs. The results directly link PhIP-induced mutations with the earlier observation of PhIP-induced prostate cancer in rats, and suggest that exposure to dietary PhIP could be a risk factor in the incidence of human prostate cancer.

7.1. Introduction

Prostate cancer is the most prevalent cancer affecting U.S. males, and after lung cancer, the most prevalent cause of cancer mortality in males (Wingo *et al.* 1999). While the specific factors contributing to the initiation and progression of prostate cancer remain undefined, genetic and environmental factors are likely to be involved. Epidemiological studies indicate that populations that consume more saturated fat and meats have greater incidence of prostate cancer (Kolonel *et al.* 1999). Of particular interest are the heterocyclic amines, an important class of food mutagens and animal carcinogens (Wakabayashi *et al.* 1992; Schut and Snyderwine 1999). One of the more prevalent heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Felton *et al.* 1986), is a carcinogen in rat colon, mammary gland and prostate (Ito *et al.* 1991; Shirai *et al.* 1997); interestingly, these three organs are among the sites of the most prevalent human cancers (Wingo *et al.* 1999). Using the Big Blue® *lacI* mutational assay (Provost *et al.* 1993), we show for the first time that PhIP can induce a large increase in

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The abbreviations used are: MF, mutant frequency; MS, mutational spectrum; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

mutant frequency (MF) in the rat prostate. The results suggest that PhIP could be a potential factor in the incidence of human prostate cancer.

7.2. Materials and Methods

7.2.1. Animal treatments

Big Blue® F344 *lacI* transgenic rats (Taconic, Germantown, NY) were individually housed and treated following guidelines conforming with the Guide for the Care and Use of Laboratory Animals (Laboratory Animal Resources, National Research Council, Washington DC 1996). Food and water were provided *ad libitum*. The basal (control) diet consisted of powdered AIN-93G (Dyets Inc., Bethlehem, PA) minus the *tert*-butylhydroquinone antioxidant, supplemented with 2% (w/w) tocopherol-stripped corn oil (ICN Biomedicals, Aurora, OH). Diets with PhIP (Toronto Research Chemicals, Toronto, ON, Canada) consisted of the basal diet supplemented with 200 ppm (w/w) of PhIP. Diets were prepared twice weekly and stored at 4°C under argon. The control and PhIP study groups each consisted of 5 male rats. The control rats were 4–6 weeks old, while the rats in the PhIP treatment group were 5 weeks old. After acclimatization to the housing and basal diet for 7 days, rats received either basal diet (control animals) or basal diets supplemented with PhIP for 61 days, after which all of the animals were returned to the basal diet for 7 days. The rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Tissues, including prostate, were immediately dissected, flash-frozen in liquid nitrogen, and then stored at –80°C.

7.2.2. *lacI* mutational assay

High molecular weight DNA was recovered from prostate tissue by a dialysis purification procedure previously described (Suri *et al.* 1996). *lacI* transgenes were recovered from purified rat chromosomal DNA by an *in vitro* λ packaging reaction, and packaged phage were plated on the *E. coli* host strain following recommended methods (Rogers *et al.* 1995). MF were calculated by dividing the total number of *lacI* mutant plaques by the total number of mutant plus wild-type plaques.

7.2.3. DNA sequencing and data analyses

Mutations in the *lacI* transgene were determined by the PCR cycle sequencing method previously described (Erfle *et al.* 1996). All 12 spontaneous prostate mutants

were sequenced, as were 178 mutants from PhIP-treated rats. The *lacI* gene is numbered according to Farabaugh (1978).

7.3. Results and Discussion

Previous studies using the Big Blue® *lacI* transgenic rodent assay have shown that PhIP causes mutations in rat colon (Okonogi *et al.* 1997a) and mammary gland (Okochi *et al.* 1999), which are also sites for PhIP-induced carcinogenesis (Ito *et al.* 1991). In the present study we examined the frequency and specificity of PhIP-induced mutation in the prostate from Big Blue® rats. We found PhIP to be highly mutagenic in rat prostatic tissue, inducing a MF of $71 \pm 4 \times 10^{-5}$, >20-fold higher than the spontaneous MF of $3.2 \pm 1.5 \times 10^{-5}$ in control animals (Table 7.1).

PhIP is unique among heterocyclic amines in that rat liver is neither a target for PhIP-induced DNA adduct formation (Takayama *et al.* 1989) nor carcinogenesis (Wakabayashi *et al.* 1992). We therefore assessed MF from the liver of PhIP-treated male and female Big Blue® rats (data not shown). The MF of 4×10^{-5} in either PhIP-treated male or female liver, respectively, was not significantly different from the spontaneous MF of 3×10^{-5} previously observed in male rat liver (Dycaico *et al.* 1996). These data indicate that PhIP-induced MF are elevated in tissues that are actual targets for PhIP-induced carcinogenesis.

We then determined the mutational specificity of spontaneous and PhIP-induced mutations in rat prostate tissue. Twelve mutants were recovered and sequenced from the prostates of the untreated animals and 11 independent mutations were identified (Tables 7.1, 7.2). Despite the small sample size, the prostate spontaneous mutational spectrum (MS) was consistent with those previously determined in *lacI* transgenes recovered from a variety of tissues (Dycaico *et al.* 1996; de Boer *et al.* 1998). Specifically, the spontaneous prostate MS was characterized by 55% G:C→A:T transition mutations (all of which occurred at 5′–CpG–3′ dinucleotide sites), 18% transversions, and 27% non-base substitution mutations (Table 7.2).

One hundred and seventy-eight PhIP prostate mutants were sequenced yielding 155 independent mutations. The PhIP MS consisted of 12% transitions, 42% transversions, 46% other mutations (mostly deletions of G:C base pairs). The predominant PhIP-

induced mutations included 39% minus one (−1) frameshifts (of which 59/60 (98%) involved deletions of G:C base pairs), 32% G:C→T:A transversions, and 10% each of G:C→A:T transitions and G:C→T:A transversions. (In the following sentence, the numbers in parentheses indicate the numbers of independent mutations.) With regard to sequence specificity, the PhIP mutations included five (four) −1 frameshifts of G:C at *lacI* nucleotide positions 90-92, five (four) base substitution mutations at position 92, and 11 (four) −1 frameshifts of G:C at position 877. Of the six independent deletion mutations, five were dinucleotide deletions involving cytosine and guanine: CC, CG, CG, GC and GC.

The PhIP-induced MS from prostate was consistent with those determined previously in rat colon (Okonogi *et al.* 1997a) and mammary gland (Okochi *et al.* 1999). Particularly, the large increase in the proportion of −1 frameshifts involving G:C base pairs and the increase in the proportion of G:C→T:A transversions in PhIP-treated prostate (Table 7.2) are characteristic of PhIP mutational spectra recovered from colon and mammary gland.

To our knowledge, this is the first report of spontaneous and induced mutation frequencies and spectra from prostate. The PhIP mutational data from prostate, combined with the previous observation that PhIP (mixed into the diet at a dose of 400 ppm for 52 weeks) causes prostate tumors in the rat (Shirai *et al.* 1997), provides convincing evidence that PhIP is a genotoxic carcinogen in the case of rat prostate cancer.

The rats in our mutagenicity study received 200 ppm of PhIP mixed into the diet for 61 days, an exposure sufficient to dramatically elevate the mutation frequency in this organ. During this period, the average daily food consumption and bodyweights were approximately 13 grams and 180 grams, respectively, giving an estimated daily consumption of PhIP of 14 μg of PhIP per gram of bodyweight (*i.e.*, 14 ppm PhIP, adjusted for bodyweight). It has been estimated that daily human dietary intake of heterocyclic amines approaches microgram quantities (Wakabayashi *et al.* 1992; Layton *et al.* 1995). Therefore, assuming that the average human consumes 1 μg of PhIP daily at a bodyweight of 70 kg (calculated using the data provided in Table III by Layton *et al.* (1995), the estimated daily human consumption is approximately 1.4×10^{-5} μg of PhIP

per gram of bodyweight (*i.e.*, 1.4×10^{-5} ppm, adjusted for bodyweight), a difference of approximately 10^6-fold, compared with the rats. However, this fold-difference in exposures is approximately 10^3-fold, once the durations of exposures are considered (61 days for the rat mutagenicity study; 60 years, assumed, for humans).

Wakabayashi *et al.* (1992) have noted that the carcinogenic effects of various heterocyclic amines appear to be additive or synergistic, while Felton *et al.* (1986) have found that the binding of heterocyclic amines to DNA is linear at doses well in excess of the average daily intakes of heterocyclic amines (Layton *et al.* 1995). Felton and colleagues have estimated the overall risk of cancer due to dietary exposure to heterocyclic amines is approximately 1×10^{-4}, with the incremental risk of cancer due to exposure to PhIP being approximately 5×10^{-5} (Layton *et al.* 1995).

Thus, the mutagenicity data presented here, combined with the previous demonstration that PhIP is a rat prostate carcinogen, provide additional evidence that humans who consume excessive amounts of PhIP may risk developing prostate cancer. This conclusion is also consistent with the recent demonstration that PhIP appears to be a substrate for *N*-acetyltransferase activity present in human prostate epithelial cells, *in vitro* (Wang *et al.* 1999) and in human prostate tissue implanted into nude mice (Shirai *et al.* 1998).

The transgenic rodent model provides a practical opportunity to investigate the mechanisms contributing to cancer in this organ. Studies including the role of diet, as well as the efficacy of potential chemopreventive therapies can be undertaken using the approach described.

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Table 7.1. Mutant frequencies in the *lacI* transgene from prostate of Big Blue® rats

Male PhIP-treated Big Blue® *lacI* transgenic rats received 200 ppm of PhIP mixed into the diet for 61 days. Following isolation of chromosomal DNA from prostate glands, *lacI* transgenes were recovered by an *in vitro* λ packaging reaction and plating on the *E. coli* host strain on media containing the chromogenic substrate X-gal. Mutant frequencies were calculated by dividing the number of blue (*lacI* mutant) plaques by the total number of plaques. Data for each animal are shown, as well as the average mutant frequency for each group.

Treatment	Animal	No. of plaques		Mutant frequency
		Total	Mutant	
Control	MC1	14,000	0	$<7 \times 10^{-5}$
	MC2	136,000	9	7×10^{-5}
	MC3	164,000	2	1×10^{-5}
	MC4	30,000	0	$<3 \times 10^{-5}$
	MC5	31,000	1	3×10^{-5}
	Total	375,000	12	$(3.2 \pm 1.5) \times 10^{-5}$ [a]
PhIP	MP1	14,000	11	79×10^{-5}
	MP2	47,000	26	55×10^{-5}
	MP3	134,000	117	87×10^{-5}
	MP4	222,000	140	63×10^{-5}
	MP5	283,000	206	73×10^{-5}
	Total	700,000	500	$(71 \pm 4) \times 10^{-5}$ [a]

[a] Average mutant frequency ± standard error of the mean.

Table 7.2. PhIP-induced *lacI* mutations from rat prostate

Blue (*lacI* mutant) plaques from the Big Blue® assay were purified, and the mutations in the *lacI* transgene were identified by PCR cycle DNA sequencing. To ensure that only independent (non-clonal) mutations were analyzed, identical mutations recovered more than once from the same animal were counted as one mutation.

	Untreated		PhIP-treated	
	No.	%	No.	%
Transitions				
G:C → A:T	6	55	16	10
(at CpG sequences)	(6)	(55)	(4)	(3)
A:T → G:C	0	0	2	1
Transversions				
G:C → T:A	1	9	49	32
(at CpG sequences)	(1)	(9)	(12)	(8)
G:C → C:G	0	0	16	10
A:T → T:A	0	0	0	0
A:T → C:G	1	9	1	<1
Other mutations				
+1 frameshift	0	0	1	<1
−1 frameshift	1	9	60	39
Deletions	1	9	6	4
Insertions	0	0	0	0
Complex changes	0	0	1	<1
Tandem mutants	1	9	3	2
Total independent [a]	11	100 %	155	100 %
Clonal mutations	0		19	
lacI non-mutant [b]	1		4	

[a] The total numbers of mutants after correction for clonal expansions.
[b] *lacI* non-mutants were blue mutant plaques which did not contain mutations in the *lacI* gene.

Chapter 8. Interpretation of Mutational Spectra From Different Genes: Analyses of PhIP-induced Mutational Specificity in the *lacI* and *cII* Transgenes From Big Blue® Rats

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Mutation Research (Submitted)

Abstract

The *cII* assay provides an alternative choice to the *lacI* transgene for mutational studies involving Big Blue® transgenic mice and rats, or permits the evaluation of mutational responses in both genes. Here, we compare the mutational response of the *cII* gene from colon of Big Blue® F344 rats treated with a dietary mutagen and animal carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), to those previously determined in the *lacI* transgene from the same group of animals. A cursory inspection of PhIP-induced mutational spectra (MS) in *cII* and *lacI* suggests that the two transgenes respond differently to PhIP-induced mutation. However, a more thorough analysis of the MS in the two transgenes, including consideration of the number of mutational target sequences in each gene and nearest neighbor analyses of mutated nucleotides, indicates that PhIP-induced mutational specificity is similar in both genes. The evaluation of PhIP-induced mutational responses in these two transgenes serves as a model for intergenic mutational analyses. Additionally, a possible explanation for the sex-linked preference for PhIP-induced cancer in colon of male, but not female, rats is hypothesized, based on the spontaneous mutational data from the *cII* transgene.

Keywords: **2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Big Blue®; *cII*; colon; *lacI*; mutation frequency; mutation spectrum; PhIP; transgenic rat**

8.1. Introduction

We recently reported the mutational specificity of the food mutagen and carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the *lacI* transgene in colon of male and female Big Blue® rats (Okonogi *et al.* 1997a). In order to determine the mutational specificity of PhIP in another gene, we extended our previous *lacI* PhIP study (Okonogi *et al.* 1997a) to include the *cII* transgene (Jakubczak *et al.* 1996), using DNA from the same PhIP-treated animals. The cost of assaying the mutational properties of this PhIP, including the effects of potential chemoprotective agents, could be mitigated through the use of an *in vivo* mutational assay in which mutants are directly selected,

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such as the *cII* assay. The *cII* assay has the additional advantage of a small (294 bp) and therefore easily sequenced gene.

At first glance, PhIP-induced mutational responses in the *cII* and *lacI* transgenes appeared to be different. For example, the proportion of PhIP-induced mutations occurring as minus one (−1) frameshifts was ~3-fold higher in *lacI* compared with *cII* (Tables 2, 3). Furthermore, compared with the spontaneous mutational spectra (MS) from colon of untreated control animals, the proportion of PhIP-induced −1 frameshifts was elevated ~10-fold in *lacI*, but not in *cII*. However, once the mutational data are normalized (facilitating the comparison of responses in either gene) and the numbers of "mutable sites" (target sequences) are considered, the PhIP-induced mutational responses appear to be similar in either transgene. These results validate the use of the *cII* transgene as a sensitive mutational target for mutational studies involving PhIP, and the intergenic analyses establish a paradigm for the comparison of mutational responses in two different genes. Finally, a possible sex-linked preference for spontaneous frameshift mutations at polynucleotide repeat sequences in the *cII* transgene from colon of male rats might offer a clue as to why PhIP-treated male, but not female rats develop colon cancer (Ito *et al.* 1991).

8.2. Materials and Methods

8.2.1. Animal treatments and tissue isolations

The treatment and tissue isolations from control and PhIP-treated rats were previously described (Okonogi *et al.* 1997a). In that study, male and female Big Blue® F344 rats aged 6 weeks were fed a diet containing 400 ppm of PhIP (CAS 105650-23-5) for 60 days. High molecular weight genomic DNA from PhIP-treated rats was extracted from the mucous membrane by the phenol/chloroform extraction method and precipitated with ethanol (Okonogi *et al.* 1997a). DNA from these rats was used in the *lacI* assay (controls; PhIP-treated), and the *cII* assay (PhIP-treated).

Control male and female rats (aged 4–6 weeks) from a separate PhIP study (Stuart *et al.*, manuscript in preparation)[23] were maintained on standard laboratory chow. At the completion of the treatments, colon tissue mucosa was isolated by a dialysis purification

method (Suri *et al.* 1996) and stored at –80°C. DNA from these control animals was used in the *cII* assay.

8.2.2. *lacI* and *cII* mutational assays

lacI transgenes were recovered by an *in vitro* λ packaging reaction and *lacI* mutant frequencies were analyzed following recommended methods (Rogers *et al.* 1995; Stuart *et al.* 1996[24]; Stratagene 1997). Mutant plaques were stored at 4°C in 0.5 ml of SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin) containing 50 µl of chloroform.

The *cII* mutational assay was conducted essentially as described by Jakubczak *et al.* (1996), using the bacterial strains G1217 for determination of λ phage titers and G1225 for the positive selection of *cII* mutant plaques. *cII* transgenes were recovered by an *in vitro* λ packaging reaction using Transpack® packaging extract (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The assay was modified as follows. Phage titers were determined in triplicate by mixing 200 µl of G1217 cells, suspended at an OD_{600nm} of 0.5 in 10 mM $MgSO_4$, with 2 µl of the phage packaging reaction, incubating at 37°C for 15–20 minutes, plating and incubating the plates at 37°C overnight. For mutant phage plating and screening, it was necessary to maintain a ≥ 2:1 (v/v) ratio of cells to packaged phage, to minimize adverse effects of the phage storage medium (SM buffer) on the bacterial cell growth. G1225 cells and the remaining phage packaging reaction were mixed, incubated at 37°C for 15–20 minutes, plated and incubated at 24 ± 0.5°C in a temperature-controlled incubator. *cII* mutant plaques were cored with sterile Pasteur pipets and were stored at 4°C in 0.5 ml of SM buffer containing 50 µl of chloroform. For both titering and plating of packaged phage, 0.75X LM medium (per liter: 7.5 g bacto tryptone, 3.75 g yeast extract, 3.75 g NaCl, 1.5 g $MgSO_4$, 15 g granulated agar) agar plates and 0.75X LM top agar (7 g/liter of granulated agar) were used.

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[23] Refer to Vita, at the end of this Dissertation.

[24] Chapter 1 of this Dissertation.

8.2.3. PCR amplification and DNA sequencing

DNA sequencing of the *lacI* gene is described in Okonogi *et al.* (1997a). Mutations in the *cII* transgene were determined by the cycle sequencing method as described previously (Erfle *et al.* 1996), with the following modifications. The *cI-cro-cII* gene region was PCR-amplified using the primer pair 5′–CTTGCTCAATTGTTATCAGC–3′ (*cII*-PCR-1) and 5′–GTCATAATGACTCCTGTTGA–3′ (*cII*-PCR-2), then the *cII* gene region was PCR cycle sequenced using fluorescent-labeled primers 5′–ACCACACCTATGGTGTATGCA–3′ (*cII*-FSP) and 5′–CCGAAGTTGAGTATTTTTGCTGT–3′ (*cII*-RSP). DNA sequencing was performed using LI-COR automated DNA sequencers. Mutational data were managed and analyzed using custom software (de Boer 1995) which was modified by the author of the program for use with the *cII* assay. To ensure that independent mutational events were analyzed, the data were corrected for possible clonal expansions (de Boer *et al.* 1996a; de Boer *et al.* 1997) by counting only one mutation for those which were recovered more than once from an individual animal.

8.2.4. Lists of mutations

Independent (nonclonal) spontaneous and PhIP-induced mutations recovered from colon of male and female Big Blue® rats, along with the 5′– and 3′–flanking sequences, are listed in Appendix Tables 8A.1 (*lacI*) and 8A.2 (*cII*).[25] We believe that the proper numbering for the *cII* gene is such that the first nucleotide of the *cII* start codon corresponds with position (+)338 of the mRNA transcript (Schwarz *et al.* 1978; Hobom *et al.* 1979); nevertheless, for convenience we shall refer to the first nucleotide of the start codon of the *cII* gene as position number (+)1. The *lacI* gene is numbered according to Farabaugh (1978).

For reference, the *lacI* gene consists of 1083 bp (encoding 360 amino acids plus the stop codon), including 240 deoxyadenosines, 299 deoxycytidines, 311 deoxyguanosines, and 233 thymidines; 56% of the gene are deoxyguanosines and deoxycytidines, including 95 5′–CpG–3′ dinucleotide sequences (CpG sequences; occupying 17.5% of the gene). The *cII* gene consists of 294 bp (encoding 97 amino acids plus the stop codon), including

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[25] These Appendix tables are located the end of this chapter.

81 deoxyadenosines, 63 deoxycytidines, 87 deoxyguanosines, and 63 thymidines; 51% of the gene are deoxyguanosines and deoxycytidines, including 22 CpG sequences (15.0% of the gene).

8.2.5. Mutation frequencies

Uncorrected *mutant* frequencies (MF) were calculated by dividing the total number of mutants by the total number of mutant plus nonmutant plaques. Following DNA sequence analyses, the numbers of independent (nonclonal) mutations were used to calculate *mutation* frequencies (M*f*). M*f* for each type of mutation (*e.g.*, G:C→A:T transitions) were also calculated, by multiplying the fraction of mutations contributed by a type of mutation by the M*f* for the appropriate treatment group (*e.g.*, male control rats). Fold-increases of M*f* were determined by subtracting spontaneous M*f* from induced M*f*, and dividing the result by the spontaneous M*f*.

8.2.6. Statistical analyses

Statistical comparisons of MS were made using the Monte Carlo algorithm of Adams and Skopek (Adams and Skopek 1987; Cariello *et al.* 1994), with 2500 iterations, using a program provided by the authors. These tests of significance consisted of pairwise comparisons of MS, using the numbers of the 12 types of mutations shown in Tables 8.2 and 8.3, as well as the numbers of G:C → A:T mutations which occurred at CpG dinucleotide sequences (Stuart *et al.* 1996). The α-level for significance was set at 0.05. Trends in the mutation frequency data (standard errors about the mean) were determined using COCHARM (created by Troy Johnson, Procter & Gamble, Cincinnati, OH), a computer program that executes the Generalized Cochran-Armitage test.

8.2.7. Nearest-neighbor analyses of mutational specificity

To determine if sex- or transgene-linked differences exist in PhIP-induced mutational specificity, nucleotide sequences flanking base substitution mutations and −1 frameshift mutations of G:C base pairs were inspected. In separate analyses, attention was directed at sequences flanking base substitution mutations occurring at CpG sequences, CC/GG dinucleotide sequences (*i.e.*, 5′–CC–3′ and its complementary sequence 5′–GG–3′ on the opposite strand), G:C→T:A and G:C→C:G transversions, and –(G:C) frameshifts. The occurrence of –(G:C) frameshifts at CC/GG, CCC/GGG and CpG sequences was also examined. Since the numbers of PhIP-induced mutations varied among the various

groups (male; female; *lacI*; *cII*), and the number of available targets for mutation at specific sequences also differs among the *cII* and *lacI* transgenes, the data were first normalized. This was accomplished by dividing the number of mutations occurring at a particular nucleotide sequence ("target sequence") by the total number of mutations for the class of mutation being examined, and dividing the result by the number of available sites. For example, in the analysis of base substitutions occurring at CpG sequences (Table 8A.3), the two male *lacI* mutations which occurred at ACGA/TCGT (mutated nucleotides are underlined) were divided first by the total number of male *lacI* base substitutions (58) and then by the number of these sequences (eight sites) in *lacI*. (Please refer to Tables 8A.1 and 8A.2 for the listings of the spontaneous and PhIP-induced *lacI* and *cII* mutations, Tables 8A.3–8A.5 for the nearest-neighbor analyses.)

8.3. Results

8.3.1. Mutation frequencies

Spontaneous (control) and PhIP-induced MF and M*f* in the *cII* and *lacI* transgenes recovered from colon of male and female Big Blue® rats are summarized in Table 8.1. In control rats, *lacI* M*f* were 2.3×10^{-5} and 2.6×10^{-5} respectively in males and females, while PhIP-induced *lacI* M*f* were 60×10^{-5} and 67×10^{-5}, respectively. On average, *lacI* M*f* were elevated ~25-fold in PhIP-treated versus control rats. M*f* determined in the *cII* transgene were higher than those determined in the *lacI* gene for both control and PhIP-treated rats (Table 8.1). The M*f* in the *cII* gene from colon of male and female control animals were 7.8×10^{-5} and 3.8×10^{-5} for males and females, respectively, while PhIP-induced M*f* were 131×10^{-5} in males and 199×10^{-5} in females. On average, *cII* M*f* were elevated ~27-fold in PhIP-treated versus control rats.

8.3.2. Mutational spectra

Mutational spectra for control and PhIP-treated rats are provided in Tables 8.2 (*lacI*) and 8.3 (*cII*). As there was no significant difference ($P = 0.30$) between male and female spontaneous *lacI* MS, these MS were combined giving a total of 28 independent *lacI* spontaneous mutants (14 each from male and female colon). The spontaneous *lacI* mutational spectrum consisted predominantly of 46% G:C→A:T transitions (with 69% of these occurring at CpG sequences), followed by 18% G:C→C:G, 14% G:C→T:A and

11% A:T→T:A transversions. The *lacI* spontaneous mutational spectrum included a single minus-one (−1) frameshift mutation, of a G:C base pair.

As there were no significant differences ($P = 0.15$) between the male and female spontaneous *cII* MS, these MS were also combined (Table 8.3), giving a total of 61 independent *cII* spontaneous mutants (36 male; 25 female). This MS mainly consisted of 28% G:C→A:T (with 65% of these occurring at CpG sequences) and 12% G:C→C:G transitions, 26% G:C→T:A and 8% G:C→C:G transversions, and 8% plus one (+1) and 10% −1 frameshift mutations (comprising two −(G:C) and four −(A:T) frameshifts, all from male rats).

PhIP-induced *lacI* MS from colon of male and female rats (Table 8.2) appeared to be identical ($P = 0.82$), as did PhIP-induced *cII* MS (Table 8.3) from male and female rats ($P = 0.76$). However, because of the interest in understanding the basis for the sex-linked differences in PhIP-induced carcinogenicity in this organ (Ito *et al.* 1991; Okonogi *et al.* 1997a), male and female PhIP MS were each tabulated separately in Tables 8.2 and 8.3. In *lacI*, PhIP induced 11–14% G:C→A:T transitions (with 25–33% of these occurring at CpG sequences), 24–30% G:C→T:A and 13–15% G:C→C:G transversions, and 33–37% −1 frameshifts. These −1 frameshifts were almost exclusively (38/39 in males and 34/35 in females) deletions of G:C base pairs.

The *cII* male and female PhIP-induced MS (Table 8.3) consisted primarily of 16% G:C→A:T transitions (with 38–53% of these occurring at CpG sequences), 37–51% G:C→T:A and 16–27% G:C→C:G transversions, and 12% −1 frameshifts (almost exclusively deletions of G:C base pairs, 10/11 in males and 4/6 in females).

8.4. Discussion

8.4.1. Mutation frequencies

M*f* in the *cII* gene were higher than those in the *lacI* gene, for both controls and PhIP-treated male and female rats. Nevertheless, the fold-increases in M*f* in PhIP-treated rats compared with untreated controls were nearly identical in either transgene. Thus, when male and female data were combined and averaged, *cII* M*f* were ~27-fold higher and *lacI* M*f* were ~25-fold higher in PhIP-treated *versus* control rats.

The spontaneous *lacI* MS was unremarkable when compared with other Big Blue® spontaneous MS with the exception that G:C→C:G transversions, comprising 18% of the mutants, were higher than the 2–5% normally seen in various tissues in the mouse (de Boer *et al.* 1998). This difference possibly reflects the rather small sample size of 28 independent (non-clonal) spontaneous *lacI* mutations, since each mutation contributed 3.8% of the MS. Otherwise, the proportions of the remaining predominant mutations, G:C→A:T transitions (46%, with 69% of these occurring at CpG sequences), and G:C→T:A (14%) and A:T→T:A (11%) transversions fell with the expected ranges.

The spontaneous *cII* mutational spectrum predominantly consisted of 28% G:C→A:T (with 65% of these occurring at CpG sequences) and 12% A:T→G:C transitions, 26% G:C→T:A and 8% G:C→C:G transversions, and 8% +1 and 10% −1 frameshifts. Compared with *lacI*, the proportion of all spontaneous *cII* mutants that were G:C→A:T transitions and G:C→C:G transversions were reduced, while A:T→G:C transitions, G:C→T:A transversions and ±1 frameshifts were increased. Thus, the *cII* spontaneous mutational spectrum was marginally but significantly ($P = 0.042$) different than the *lacI* spontaneous spectrum. This slight difference possibly reflects *in vivo* (*e.g.*, transgene-specific DNA sequences) or *in vitro* (*e.g.*, bacterial host strain) assay influences, or random statistical variation in the data. The similarity of the two transgenes with regard to overall nucleotide composition and density of CpG sequences (refer to section 8.2.4) tends to support either of the latter two possibilities; that is, *in vitro* effects or random variation in the data.

In the *lacI* transgene, the proportion of G:C→T:A transversions increased from 14% in controls to 24–30% in PhIP-treated male and female rats, while the proportion of G:C→C:G transversions remained relatively unchanged at 18% in controls *versus* 13–15% in PhIP-treated males and females (Table 8.2). In the *cII* transgene, the PhIP treatment increased the proportion of G:C→T:A (from 26% to 37–51%) and G:C→C:G (from 8% to 16–27%) transversions (Table 8.3). Notably, the proportion of −1 frameshifts in the *cII* transgene remained relatively constant in control (10%) and PhIP-treated (12%) rats, whereas in *lacI* these mutations increased from 4% in controls to 33–37% in PhIP-treated rats.

Thus, compared with *lacI*, PhIP appeared to induce more G:C→T:A and G:C→C:G transversions, and substantially fewer −1 frameshifts in the *cII* transgene, as a fraction of all mutants in either transgene. Accordingly, the PhIP-induced *cII* and *lacI* MS were significantly different in both males ($P = 0.0012$) and females ($P = 0.020$). Interestingly, when −1 frameshifts were partitioned from the PhIP-induced MS, the *lacI* and *cII* MS were similar to one another (males, $P = 0.49$; females, $P = 0.32$).

Considering only the relative proportions of specific mutations, and the results of the Adams-Skopek statistical comparisons of *lacI* and *cII* MS, it is tempting to conclude that the mutational specificity of PhIP is significantly different in the two transgenes. For example, the proportion of PhIP-induced mutations which were −1 frameshifts was ~3-fold higher in *lacI* compared with *cII* (Tables 8.2 and 8.3), suggesting that −1 frameshifts could be over-represented in the *lacI* transgene. This preliminary conclusion appears to be supported when the proportions −1 frameshifts in control and PhIP-treated animals are compared. Compared with untreated controls, the proportion of PhIP-induced −1 frameshifts was elevated ~10-fold in the *lacI* transgene, but was essentially unchanged (1.2-fold increase) in the *cII* transgene. To better understand why the two induced MS appeared to differ, we began a more rigorous analysis of the data.

8.4.2. Comparing mutational spectra in different genes

Potential biases in interpreting mutational data are minimized when mutation-specific fold increases in M*f*, rather than proportions, are considered (Tables 8.2 and 8.3). As well, in order to directly compare MS in different genes, it is necessary to consider the frequencies of mutations at known or suspected target sequences, following normalization of the data. The data are normalized by dividing the numbers of mutations at the particular target sequence by the total number of mutations, and then dividing this result by the number of target sequences present in the gene (refer to Tables 8A.3–8A.5).

Comparing the mutation-specific increases in M*f* from PhIP-treated male and female rats relative to controls (Tables 8.2 and 8.3), in *lacI* G:C→T:A transversions increase ~30–50-fold (with those occurring at CpG sequences increasing ~60–100-fold), G:C→C:G transversions increase ~20-fold, and −1 frameshifts increase ~240-fold. In *cII*, G:C→T:A transversions increase ~30–65-fold (and ~10–30-fold at CpG sequences),

G:C→C:G transversions increase ~60–70-fold, and –1 frameshifts increase ~20–40-fold. Significantly, although the proportion of mutations which are frameshifts do not increase in *cII* (1.2-fold, comparing PhIP-treated animals with controls), the *Mf* for these events increases substantially (> 20-fold). Furthermore, *Mf* of same classes of mutations are induced in both *cII* and *lacI*, indicating that the two transgenes are in fact behaving similarly upon treatment with PhIP.

The mutational classes with the largest fold-increases in *Mf* (Tables 8.2 and 8.3), along with previous reports of PhIP-induced mutational specificity (*e.g.*, Okonogi et al. 1997a, 1997b), aided in the identification of nucleotide sequences on which to focus attention for closer analyses of possible gene-specific mutational specificity. These included G:C-rich DNA sequences, CpG dinucleotide sequences, and sites of G:C→T:A and G:C→C:G transversions. To determine the possible effects of local sequence context, 5′– and 3′–flanking nucleotides were also considered.

To illustrate the various nearest-neighbor analyses which were made, consider base substitution mutations involving CpG sequences (Table 8A.3). The sequence ACGA/TCGT occurs eight times in *lacI* and six times in *cII*. In the *lacI* gene from colon of male rats, mutations at this sequence were recovered twice among a total of 58 base substitution mutations. Normalizing this data [(2/58)/8], these mutations occurred with a frequency of 0.0043 (mutations per base substitution mutation) per ACGA/TCGT target sequence. Similar calculations for female *lacI* mutations and male and female *cII* mutations at this sequence gave normalized values of 0.0020, 0.0238 and 0.0278, respectively. The *lacI*/*cII* ratio (using male- plus female-averaged data) for this particular sequence was 0.123, indicating that mutations at this sequence occurred ~8-fold more frequently in *cII* as in *lacI*. However, in order to draw generalized conclusions for each transgene, similar calculations should be made for each tetranucleotide sequence involving mutations at CpG dinucleotides, and the overall averages should be considered. The results (Table 8A.3) indicated that base substitution mutations at CpG sequences occurred with identical frequency in males and females in the same transgene, but that these mutations occurred approximately three times as frequently in *cII* as in *lacI*, since the *lacI*:*cII* ratio was 0.33.

Likewise, when other PhIP-induced base substitution mutations were similarly analyzed, base substitution mutations involving CC/GG dinucleotide sequences, G:C→T:A transversions, and G:C→C:G transversions gave average *lacI*:*cII* ratios of 0.22–0.30 (Table 8A.4; due to limitations of space, Tables 8A.4 and 8A.5 summarize the remaining analyses).[26] These data indicate that the *cII* transgene, despite being 3.7-fold smaller than the *lacI* transgene, is actually ~3–5 three times as sensitive as the *lacI* transgene for the recovery of PhIP-induced base substitution mutations. Interestingly, the exception to these (0.22–0.33):1 ratios involved −1 frameshift mutations involving G:C base pairs (Table 8A.5). For example, the *lacI*:*cII* ratio for −(G:C) (frameshifts/all mutations)/all sites was 0.91, indicating that these mutations were recovered with almost equal efficiency from either transgene. The weighted average of the base substitution data and the −1 frameshift data indicates that the ~3-fold higher PhIP-induced M*f* in the *cII* transgene compared with the *lacI* transgene is probably real. Although the basis for the enhanced sensitivity of the *cII* transgene for detecting PhIP-induced mutations remains unexplained, differences in the relative sensitivity of the CII and LacI proteins to base substitution and frameshift mutations cannot be discounted.

These analyses therefore offer a possible explanation of the disparity of MF in various studies which report *cII* MF from treated animals that are higher than or lower than the *lacI* MF from the same animals (*e.g.*, Jakubczak *et al.* 1996; Monroe *et al.* 1998; Zimmer *et al.* 1999). In each case, normalization of the data with respect to nucleotide sequence (target) specificity, the availability of target sequences, and the numbers of recovered mutations might account for the relative differences in the MF observed in each transgene.

As previously mentioned, PhIP-induced G:C→T:A transversions at A<u>CG</u>A/T<u>CG</u>T sequences occurred ~8-fold more frequently in *cII* than in *lacI* (Table 8A.3). In contrast, PhIP-induced G:C→T:A transversions at A<u>CG</u>C/G<u>CG</u>T occurred ~3-fold more frequently in *lacI* than in *cII*. These, and other, variations in the frequency of PhIP-induced mutation at particular target sequences indicate that transgene-specific

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[26] Due to limitations of space, Tables A8.4 and A8.5 in the submitted manuscript replace the (unpublished) material contained in Tables B8.1–B8.6, found at the end of this Chapter.

differences in mutational specificity do exist at particular sequences. However, when data from all target sequences for a particular type of mutation were averaged (Tables 8A.3–8A.5), there were essentially no differences among males and females for neither the *lacI* transgene data, nor for the *cII* transgene data. Furthermore, extensions of the target sequences by considering additional flanking nucleotides revealed no obvious transgene-specific sequences greater than four nucleotides, in colon of either male or female rats (data not shown). Thus, these data agree with the comparisons of male and female PhIP mutational spectra (Tables 8.2 and 8.3), which found no statistically significant sex-linked differences (refer to section 8.3.2).

8.4.3. Additional comments regarding PhIP mutational specificity

A potential limitation of relying on mutational data from a single gene is that mutational specificities can be overlooked if specific target sequences are under-represented or absent. PhIP-induced frameshift deletions of G:C base pairs in the sequence GGGA/TCCC have been reported in the *lacI* transgene from colon of male and female rats (Okonogi *et al.* 1997a), the *lacI* transgene from rat mammary gland (Okochi *et al.* 1999), the *Apc* gene from PhIP-induced rat colon tumors (Kakiuchi *et al.* 1995), and the *Hprt* gene from a Chinese hamster cell line (Yadollahi-Farsani *et al.* 1996). The *lacI* gene contains 10 of these PhIP "signature mutation" nucleotide target sequences, although not all are mutational "hotspots" (Okonogi *et al.* 1997a; Okochi *et al.* 1999; Table 8A.1), while *cII* contains only one GGGA sequence, which was not a PhIP mutational hotspot (Table 8A.2).

Interestingly, one *cII* complex mutation, consisting of a $C_{247} \rightarrow G$ transversion and a deletion of deoxycytidine at *lacI* position 249–251, was recovered once each from male and female colon. Also in the *cII* transgene, one male PhIP mutant had a mutation which changed the *opal* stop codon to a leucine, extending the CII protein by 12 amino acids, presumably resulting in a nonfunctional CII protein.

8.4.4. PhIP-induced mutations at *cII* polynucleotide repeat sequences

The $G_{179-184}$ sequence in *cII* appears to be a hotspot for PhIP-induced −1 frameshifts and G:C→T:A transversions, as well as spontaneous frameshift mutations (Table 8A.2). As well, one deletion of an A:T base pair was recovered in the *cII* transgene from colon of a PhIP-treated female rat, at the $A_{241-246}$ deoxyadenosine hexanucleotide sequence.

The question which immediately arises is: "How frequently might PhIP-induced mutations be expected to occur at these or other repeat sequences in the human genome?"

This question cannot be addressed using data from the *lacI* gene, due a paucity of repeat sequences greater than four nucleotides in length in *lacI*. There are no pentanucleotide deoxyguanosine sequences in *lacI*. Compared with the *cII* sequence $TGGGGGGT_{178-185}$, the closest matching sequences in *lacI* are $TGGGGC_{915-920}$ and $TCCCCG_{1042-1047}$. With the exception of the $G_{179-184}$ sequence, there are no other tetra- or pentanucleotide deoxyguanosine sequences in *cII*. At either of the two deoxyguanosine tetranucleotide sequences in *lacI*, no −1 frameshift mutations were recovered either in control animals (28 independent mutations) or PhIP-treated animals (210 independent mutations). The reasons for the absence of −1 mutations at these sequences in *lacI* possibly include DNA sequence context, effects due to the shorter length of the deoxyguanosine polynucleotide sequence, or position within the gene. With regard to the last possibility, only three –(G:C) frameshifts were recovered after *lacI* nucleotide position 896 in each of male and female colon, and that the most distal *lacI* nucleotide position recovered as a PhIP-induced –(G:C) frameshift occurred at position 986 (Table 8A.1). On this basis, −1 frameshift mutations at $GGGG_{916-919}$ and $CCCC_{1043-1046}$ might therefore be expected to be recovered infrequently. In comparison, the most distal nucleotide position in *cII* recovered as PhIP-induced –(G:C) mutation occurred at position 252, well downstream of the $G_{179-184}$ sequence.

8.4.5. Sex-specific PhIP-induced colon tumors: A hypothesis

PhIP predominantly induces colon tumors in male rats, and mammary gland tumors in female rats (Ito *et al.* 1991); however, the PhIP-induced *lacI* MS from male and female colon (Okonogi *et al.*, 1997a) and mammary gland (Okochi *et al.*, 1999) offer no explanation as to why PhIP-treated male, but not female, rats predominantly develop colon cancer. In the *cII* transgene, it was noted that spontaneous −1 frameshift mutations occurred exclusively in male colon (six independent occurrences, Table 8A.2) with a Mf of 0.6×10^{-5} (Table 8.3). Among these frameshifts, two mutations occurred at each of the $G_{179-184}$ and the $A_{241-246}$ hexanucleotide repeat sequences. Spontaneous and induced frameshifts at homonucleotide sequences in *lacI* cannot be similarly evaluated, since *lacI*

contains only two deoxyadenosine pentanucleotide sequences, and no homonucleotide sequences of six or more nucleotides. Based on the *cII* spontaneous mutational data, we hypothesize that *spontaneous* −1 frameshift mutations in repeat sequences in colon accumulate preferentially in male rats, and that these mutations either act as initiating events in the tumorigenic process, or predispose male rat colon tissue to chemically-induced carcinogenesis. PhIP, although well-known for inducing −1 frameshifts and G:C→T:A transversions, is also known to stimulate cellular proliferation preferentially in male rat colon (Ochiai *et al.* 1996) and to promote microsatellite instability (Canzian *et al.* 1996). Carcinogenic doses of PhIP were also recently shown to increase proliferation of the epithelial cells of the terminal end buds in the rat mammary gland (Snyderwine 1999). Therefore, we speculate that PhIP could be acting predominantly as a tumor promoting agent in the progression of colon cancer in male, but not female, rats. This scenario would also be consistent with the absence of sex-linked differences in PhIP-induced mutational specificity in DNA recovered from colon from male and female rats (this study; Okonogi *et al.* 1997a]. The hypothesis would also be consistent with the observation that sporadic and hereditary nonpolyposis colon cancers often involve frameshift mutations in repeat sequences in genes affecting apoptosis, growth regulation and DNA mismatch repair genes (*e.g.*, Yamamoto *et al.* 1997, 1998; Duval *et al.* 1999; Schwartz *et al.* 1999). Finally, the observation that in the *cII* transgene, +1 frameshifts also appeared to occur preferentially in male colon at the $G_{179\text{-}184}$ hexanucleotide sequence (eight total and three independent occurrences in males; one occurrence in females), suggests that colon chromosomal DNA may be more susceptible to ±1 frameshift mutations in male rats than in female rats.

Although highly speculative at present, this hypothesis is testable. Firstly, increasing the size of the database of spontaneous *cII* mutations from colon of male and female rats would confirm the sex-linked preference for frameshift mutations at repeat sequences in male rats. Secondly, it would be desirable to verify these results in a third gene (other than *lacI* or *cII*) containing repeat sequences, in male and female colon tissue. Lastly, studies of the sex-specific frequency of −1 frameshift mutations in tissues other than colon could be informative. For example, −1 frameshift mutations occur almost twice as frequently in the *HPRT* gene (which contains a G_6 hexanucleotide sequence) of

lymphocytes in human males, compared with females, despite the fact that deletions (> 1 base pair) are recovered twice as frequently in females than in males (Curry *et al.* 1999).

8.5. Conclusions

We have extended the study of PhIP-induced mutational specificity from the *lacI* transgene to the *cII* transgene, using DNA isolated from colon tissue from the same group of rats. To compare mutational responses in different transgenes, it was necessary to consider the availability of specific mutational target sequences, and to normalize the data from each transgene, to facilitate meaningful comparison of the data. While transgene-specific differences in PhIP-induced mutational specificity were found, overall, the mutational responses in both transgenes were remarkably uniform. The previous observation of the absence of a sex-linked difference in PhIP-induced mutational specificity in *lacI* transgenic DNA from male and female rat colon was confirmed in the *cII* transgene. Nevertheless, the observation of an increased frequency of spontaneous frameshift mutations in repeat sequences in the *cII* transgene from male colon offers a possible explanation for the predominance of PhIP-induced carcinogenesis in the colon of male, but not female, rats. Finally, the strategy used in this paper to compare mutational responses in two different genes serves as a model for intergenic comparisons of mutational responses, including the Big Blue® (*lacI*, *cII* transgenes) and Muta™Mouse (*lacZ*, *cII* transgenes; Swiger *et al.* 1999) mutational assays.

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Table 8.1. Mutant frequencies in the colon mucosa of Big Blue® rats

Treatment	Rat Sex	Rat Number	Number of Plaques Total	Number of Plaques Mutant	MF [a] ± SE ($\times 10^{-5}$)	M*f* [b] ($\times 10^{-5}$)
lacI Transgene [c]						
Control	Male	5	661,000	16	2.4 ± 5.9	2.3
	Female	5	600,000	17	2.8 ± 1.7	2.6
PhIP	Male	5	582,000	382	66 ± 33	60
	Female	5	506,000	375	74 ± 17	67
cII Transgene						
Control [d]	Male	3	606,000	77	13 ± 3.8	7.8
	Female	3	846,000	59	7.0 ± 1.6	3.8
PhIP [e]	Male	5	307,000	591	192 ± 14	131
	Female	3	115,000	283	246 ± 42	199

[a] MF, mutant frequency; M*f*, mutation frequency; SE, standard error about the mean.

[b] The correction factors (the ratio of independent mutants/total number of mutants sequenced) used to calculate M*f* were: *lacI* controls: male 14/15, female 14/15; *lacI* PhIP: male 105/114, female 105/117; *cII* controls: male 36/59; female 25/46; *cII* PhIP: 94/138; female 51/63.

[c] These data are adapted from Okonogi *et al.* (1997a).

[d] Untreated rat colon DNA samples used for the *cII* experiments were from control rats from a separate experiment (Glickman, unpublished data).

[e] PhIP-treated rat colon DNA samples used for the *cII* experiments were the same as those used in the earlier *lacI* study (Okonogi *et al.* 1997a)

Table 8.2. Spontaneous and PhIP-induced mutational spectra in the *lacI* transgene from Big Blue® rat colon [a]

	Male + Female Spontaneous			Male PhIP				Female PhIP			
	No.	%	Mf [b,c] (x 10^{-5})	No.	%	Mf (x 10^{-5})	Fold-induction [d]	No.	%	Mf (x 10^{-5})	Fold-induction
Transitions											
G:C → A:T	13	46	1.1	12	11	6.9	5.3	15	14	9.5	7.6
(@ CpG) [e]	(9)	(32)	(0.8)	(3)	(3)	(2)	(2)	(5)	(5)	(3)	(3)
A:T → G:C	0	0	0	1	1	0.6	n.c.	0	0	0	n.c.
Transversions											
G:C → T:A	4	14	0.4	25	24	14	30	31	30	20	50
(@ CpG) [f]	(1)	(4)	(0.09)	(10)	(9.5)	(5.8)	(60)	(14)	(13)	(8.9)	(100)
G:C → C:G	5	18	0.4	16	15	9.2	20	14	13	8.9	20
A:T → T:A	3	11	0.3	3	3	2	6	1	1	0.6	1
A:T → C:G	0	0	0	1	1	0.6	n.c.	1	1	0.6	n.c.
Other Mutations											
+1 Frameshift	0	0	0	0	0	0	n.c.	0	0	0	n.c.
−1 Frameshift	1	4	0.09	39	37	22	240	35	33	22	240
Deletions	1	4	0.09	5	5	3	30	5	5	3	30
Insertions	0	0	0	0	0	0	n.c.	0	0	0	n.c.
Complex Changes	0	0	0	1	1	0	n.c.	3	3	2	n.c.
Tandem Mutants	1	4	0.09	2	2	1	10	0	0	0	n.c.
Total Independent [g]	28			105				105			
Clonal Mutants	1 (female)			8				8			
lacI Nonmutant [h]	1 (male)			1				4			

[a] Mf, mutation frequency; n.c., not calculated.

[b] These data are adapted from Okonogi *et al.* (1997a).

[c] Mf were calculated by multiplying the proportions from this table by the MF from Table 8.1. For male and female Spontaneous, the average Mf (2.45 x 10^{-5}) was used. Values in the table are rounded to the nearest significant figure.

[d] Fold-induction = (PhIP-induced Mf − spontaneous Mf)/(spontaneous Mf).

Table 8.2. (*continued*)

[e] The proportion of G:C→A:T mutations occurring at CpG sequences were: male and female spontaneous, 69%; male PhIP, 25%; and female PhIP, 33%.

[f] The proportion of G:C→T:A mutations occurring at CpG sequences were: male and female spontaneous, 25%; male PhIP, 40%; and female PhIP, 45%.

[g] The total numbers of mutants after correction for clonality (refer to sections 8.2.3 and 8.2.5). Numbers in parentheses are not included in column totals. There were 14 independent mutants in each of the male and female spontaneous MS.

[h] *lacI* nonmutants were blue (mutant) plaques which did not contain mutations in the *lacI* gene or promoter region. The percent of all mutants sequenced which were *lacI* nonmutants were 3.3% (male and female spontaneous), 0.9% (male PhIP) and 3.4% (female PhIP).

Table 8.3. Spontaneous and PhIP-induced mutational spectra in the *cII* transgene from Big Blue® rat colon

	Male + Female Spontaneous			Male PhIP				Female PhIP			
	No.	%	Mf [a,b] ($\times 10^{-5}$)	No.	%	Mf ($\times 10^{-5}$)	Fold-induction [c]	No.	%	Mf ($\times 10^{-5}$)	Fold-induction
Transitions											
G:C → A:T	17	28	1.6	15	16	21	12	8	16	31	18
(@ CpG) [d]	(11)	(18)	(1.0)	(8)	(8)	(10)	(9)	(3)	(6)	(10)	(9)
A:T → G:C	7	12	0.7	1	1	1	0	0	0	0	n.c.
Transversions											
G:C → T:A	16	26	1.5	35	37	49	30	26	51	100	65
(@ CpG) [e]	(7)	(12)	(0.7)	(8)	(8)	(10)	(10)	(5)	(10)	(20)	(30)
G:C → C:G	5	8	0.5	25	27	35	70	8	16	31	60
A:T → T:A	3	5	0.3	0	0	0	n.c.	0	0	0	n.c.
A:T → C:G	2	3	0.2	1	1	1	4	0	0	0	n.c.
Other Mutations											
+1 Frameshift	5	8	0.5	0	0	0	n.c.	0	0	0	n.c.
−1 Frameshift	6	10	0.6	11	12	15	20	6	12	23	40
Deletions	0	0	0	2	2	3	n.c.	0	0	0	n.c.
Insertions	0	0	0	0	0	0	n.c.	0	0	0	n.c.
Complex Changes	0	0	0	2	2	3	n.c.	3	6	10	n.c.
Tandem Mutants	0	0	0	2	2	3	n.c.	0	0	0	n.c.
Total Independent [f]	61			94				51			
Clonal Mutants	9 (8 male; 1 female)			7				6			
cII Nonmutant [g]	35 (15 male; 20 female)			37				6			

[a] Mf, mutation frequency; n.c., not calculated.

[b] Mf were calculated by multiplying the proportions from this table by the Mf from Table 8.1. For male and female spontaneous, the average Mf (5.77×10^{-5}) was used. Values in the table are rounded to the nearest significant figure.

[c] Fold-induction = (PhIP-induced Mf − spontaneous Mf)/(spontaneous Mf).

[d] The proportion of G:C→A:T mutations occurring at CpG sequences were: male and female spontaneous, 65%; male PhIP, 53%; and female PhIP, 38%.

Table 8.3. (*continued*)

[e] The proportion of G:C→T:A mutations occurring at CpG sequences were: male and female spontaneous, 44%; male PhIP, 23%; and female PhIP, 19%.

[f] The total numbers of mutants after correction for clonality (refer to sections 8.2.3 and 8.2.5). Numbers in parentheses are not included in column totals. There were 36 male and 25 female independent spontaneous mutations.

[g] *cII* nonmutants were plaques which did not contain mutations in the *cII* gene or promoter region. These plaques presumably arise due to mutations in the *cI* gene (Harbach *et al.* 1999), or elsewhere. The percent of all mutants sequenced which were *cII* nonmutants were 33.3% (male and female spontaneous), 26.8% (male PhIP) and 9.5% (female PhIP). Among the 105 sequenced spontaneous mutants, 15/59 (25.4%) and 20/46 (43.5%) were *cII* nonmutants in males and females, respectively.

8.6. Appendix A: Supplementary Tables

Table 8A.1. Independent spontaneous and PhIP-induced *lacI* mutations from colon of male and female Big Blue® rats

Mutated Nucleotide	Type of Mutation	*lacI* Flanking Sequences	Spontaneous		PhIP	
			Male	Female	Male	Female
A. Base Substitutions						
−15	G:C→A:T	CGCGGTATGGCATGATA	0	0	0	1
−15	G:C→T:A	CGCGGTATGGCATGATA	0	0	0	1
−8	A:T→T:A	TGGCATGATAGCGCCCG	0	0	2	0
31	G:C→C:G	GTGAATGTGAAACCAGT	0	0	1	0
35	G:C→A:T	ATGTGAAACCAGTAACG	0	0	1	0
39	A:T→C:G	GAAACCAGTAACGTTAT	0	0	0	1
42	G:C→A:T	ACCAGTAACGTTATACG	0	1	1	0
42	G:C→T:A	ACCAGTAACGTTATACG	0	0	1	0
56	G:C→A:T	ACGATGTCGCAGAGTAT	0	1	0	0
57	G:C→T:A	CGATGTCGCAGAGTATG	0	0	0	1
59	G:C→T:A	ATGTCGCAGAGTATGCC	0	0	1	0
66	G:C→C:G	AGAGTATGCCGGTGTCT	1	0	0	0
66	G:C→T:A	AGAGTATGCCGGTGTCT	0	0	1	0
69	G:C→T:A	GTATGCCGGTGTCTCTT	0	0	1	0
75	G:C→A:T	GCCGGTGTCTCTTATCA	0	0	0	1
81	A:T→T:A	CTCTTATCAGACCGTTT	1	0	0	0
84	G:C→A:T	TTATCAGACCGTTTCCC	0	0	1	1
86	G:C→T:A	ATCAGACCGTTTCCCGC	0	0	0	1
90	G:C→C:G	GACCGTTTCCCGCGTGG	1	0	1	0
90	G:C→T:A	GACCGTTTCCCGCGTGG	0	0	0	1
92	G:C→A:T	CCGTTTCCCGCGTGGTG	1	1	0	1
92	G:C→C:G	CCGTTTCCCGCGTGGTG	0	0	1	2
92	G:C→T:A	CCGTTTCCCGCGTGGTG	0	0	2	2
93	G:C→A:T	CGTTTCCCGCGTGGTGA	0	1	0	0
93	G:C→T:A	CGTTTCCCGCGTGGTGA	0	0	1	0
94	G:C→T:A	GTTTCCCGCGTGGTGAA	0	0	0	1
95	G:C→A:T	TTTCCCGCGTGGTGAAC	0	1	0	1
95	G:C→C:G	TTTCCCGCGTGGTGAAC	0	0	1	0
96	A:T→T:A	TTCCCGCGTGGTGAACC	0	1	0	1
116	G:C→T:A	CCAGCCACGTTTCTGCG	0	0	0	1
120	G:C→C:G	CCACGTTTCTGCGAAAA	0	0	0	1
129	G:C→C:G	TGCGAAAACGCGGGAAA	0	0	0	1
131	G:C→C:G	CGAAAACGCGGGAAAAA	0	0	0	2
132	G:C→C:G	GAAAACGCGGGAAAAAG	0	0	2	1
140	G:C→C:G	GGGAAAAAGTGGAAGCG	0	0	1	0
143	G:C→T:A	AAAAAGTGGAAGCGGCG	0	0	1	1
150	G:C→T:A	GGAAGCGGCGATGGCGG	0	0	1	2
155	G:C→C:G	CGGCGATGGCGGAGCTG	0	0	0	1
158	G:C→T:A	CGATGGCGGAGCTGAAT	0	0	0	2
173	G:C→A:T	ATTACATTCCCAACCGC	0	0	1	0
174	G:C→A:T	TTACATTCCCAACCGCG	0	0	0	1
178	G:C→A:T	ATTCCCAACCGCGTGGC	0	0	0	1
178	G:C→C:G	ATTCCCAACCGCGTGGC	0	0	1	1
178	G:C→T:A	ATTCCCAACCGCGTGGC	0	0	1	0

Table 8A.1. (*lacI* mutants, *continued*)

Mutated Nucleotide	Type of Mutation	*lacI* Flanking Sequences	Spontaneous		PhIP	
			Male	Female	Male	Female
179	G:C→C:G	TTCCCAACCGCGTGGCA	0	0	1	0
179	G:C→T:A	TTCCCAACCGCGTGGCA	0	0	0	1
185	G:C→T:A	ACCGCGTGGCACAACAA	0	0	1	0
188	G:C→A:T	GCGTGGCACAACAACTG	1	0	0	0
194	G:C→C:G	CACAACAACTGGCGGGC	1	0	0	0
197	G:C→C:G	AACAACTGGCGGGCAAA	0	1	1	0
198	G:C→T:A	ACAACTGGCGGGCAAAC	0	0	0	2
200	G:C→T:A	AACTGGCGGGCAAACAG	0	0	0	1
210	G:C→T:A	CAAACAGTCGTTGCTGA	0	0	1	0
221	G:C→A:T	TGCTGATTGGCGTTGCC	0	0	1	1
221	G:C→T:A	TGCTGATTGGCGTTGCC	1	0	0	0
222	G:C→A:T	GCTGATTGGCGTTGCCA	0	0	1	0
222	G:C→T:A	GCTGATTGGCGTTGCCA	0	1	0	0
237	G:C→T:A	CACCTCCAGTCTGGCCC	0	0	1	0
248	G:C→A:T	TGGCCCTGCACGCGCCG	0	0	1	0
269	G:C→A:T	AAATTGTCGCGGCGATT	0	0	0	1
273	G:C→T:A	TGTCGCGGCGATTAAAT	1	0	0	1
283	A:T→G:C	ATTAAATCTCGCGCCGA	0	0	1	0
285	G:C→C:G	TAAATCTCGCGCCGATC	0	0	1	1
303	G:C→T:A	ACTGGGTGCCAGCGTGG	0	0	1	1
329	G:C→A:T	TGGTAGAACGAAGCGGC	2	0	1	0
380	G:C→T:A	TCGCGCAACGCGTCAGT	0	0	1	0
381	G:C→A:T	CGCGCAACGCGTCAGTG	0	1	1	1
381	G:C→C:G	CGCGCAACGCGTCAGTG	0	0	1	0
411	A:T→C:G	CTATCCGCTGGATGACC	0	0	1	0
437	G:C→T:A	TTGCTGTGGAAGCTGCC	0	0	1	0
448	G:C→T:A	GCTGCCTGCACTAATGT	0	0	0	1
484	G:C→T:A	GTCTCTGACCAGACACC	0	0	0	1
524	G:C→C:G	ATGAAGACGGTACGCGA	0	0	1	0
530	G:C→A:T	ACGGTACGCGACTGGGC	0	0	0	1
536	G:C→C:G	CGCGACTGGGCGTGGAG	0	0	1	0
542	G:C→T:A	TGGGCGTGGAGCATCTG	0	0	1	0
545	G:C→C:G	GCGTGGAGCATCTGGTC	0	0	0	1
569	G:C→A:T	GTCACCAGCAAATCGCG	1	0	1	0
575	G:C→C:G	AGCAAATCGCGCTGTTA	0	0	2	0
576	G:C→T:A	GCAAATCGCGCTGTTAG	0	0	1	0
587	G:C→A:T	TGTTAGCGGGCCCATTA	0	0	0	1
588	G:C→T:A	GTTAGCGGGCCCATTAA	0	0	0	1
629	A:T→T:A	TGGCTGGCTGGCATAAA	0	0	1	0
630	G:C→A:T	GGCTGGCTGGCATAAAT	0	0	0	1
653	G:C→A:T	CTCGCAATCAAATTCAG	0	0	1	0
671	G:C→T:A	CGATAGCGGAACGGGAA	0	0	1	0
677	G:C→T:A	CGGAACGGGAAGGCGAC	0	0	1	0
692	G:C→A:T	ACTGGAGTGCCATGTCC	0	0	0	1
702	G:C→T:A	CATGTCCGGTTTTCAAC	0	0	0	2
777	G:C→T:A	TCAGATGGCGCTGGGCG	0	0	1	0
782	G:C→T:A	TGGCGCTGGGCGCAATG	0	0	1	1
790	G:C→C:G	GGCGCAATGCGCGCCAT	0	0	0	1
791	G:C→T:A	GCGCAATGCGCGCCATT	0	0	1	0

Table 8A.1. (*lacI* mutants, *continued*)

Mutated Nucleotide	Type of Mutation	*lacI* Flanking Sequences	Spontaneous Male	Spontaneous Female	PhIP Male	PhIP Female
795	G:C→T:A	AATGCGCGCCATTACCG	0	0	0	1
818	G:C→T:A	GGCTGCGCGTTGGTGCG	0	0	0	1
834	G:C→T:A	GGATATCTCGGTAGTGG	0	0	0	1
842	G:C→C:G	CGGTAGTGGGATACGAC	0	0	0	1
843	G:C→T:A	GGTAGTGGGATACGACG	0	0	1	1
847	G:C→T:A	GTGGGATACGACGATAC	0	0	0	1
867	G:C→T:A	AGACAGCTCATGTTATA	0	0	1	0
888	G:C→A:T	GCCGTTAACCACCATCA	0	0	0	1
917	G:C→C:G	GCCTGCTGGGGCAAACC	0	0	0	1
918	G:C→A:T	CCTGCTGGGGCAAACCA	1	0	0	0
920	G:C→A:T	TGCTGGGGCAAACCAGC	1	0	0	0
990	A:T→T:A	GTTGCCCGTCTCACTGG	0	1	0	0
959	G:C→A:T	CTCAGGGCCAGGCGGTG	0	0	1	0
993	G:C→T:A	GCCCGTCTCACTGGTGA	0	0	0	1
1005	G:C→C:G	GGTGAAAAGAAAAACCA	0	1	0	0
1005	G:C→T:A	GGTGAAAAGAAAAACCA	0	1	0	0
B. −1 Frameshifts						
35	−(G:C)	ATGTGAAACCAGTAACG	0	0	0	1
90	−(G:C)	GACCGTTTCCCGCGTGG	0	0	3	0
94	−(G:C)	GTTTCCCGCGTGGTGAA	0	0	0	1
123	−(G:C)	CGTTTCTGCGAAAACGC	0	0	1	0
130	−(G:C)	GCGAAAACGCGGGAAAA	0	0	0	1
132	−(G:C)	GAAAACGCGGGAAAAAG	0	0	1	0
146	−(G:C)	AAGTGGAAGCGGCGATG	0	0	1	0
173	−(G:C)	ATTACATTCCCAACCGC	0	0	1	0
180	−(G:C)	TCCCAACCGCGTGGCAC	0	0	1	0
199	−(G:C)	CAACTGGCGGGCAAACA	0	0	0	1
224	−(G:C)	TGATTGGCGTTGCCACC	0	0	1	0
228	−(G:C)	TGGCGTTGCCACCTCCA	0	0	0	1
251	−(G:C)	CCCTGCACGCGCCGTCG	0	0	1	0
252	−(G:C)	CCTGCACGCGCCGTCGC	0	0	0	1
261	−(A:T)	GCCGTCGCAAATTGTCG	0	0	1	0
303	−(G:C)	ACTGGGTGCCAGCGTGG	0	0	0	1
334	−(G:C)	GAACGAAGCGGCGTCGA	0	1	0	0
353	−(G:C)	CCTGTAAAGCGGCGGTG	0	0	1	0
355	−(G:C)	TGTAAAGCGGCGGTGCA	0	0	1	0
358	−(G:C)	AAAGCGGCGGTGCACAA	0	0	1	0
371	−(G:C)	ACAATCTTCTCGCGCAA	0	0	1	0
373	−(G:C)	AATCTTCTCGCGCAACG	0	0	0	1
374	−(G:C)	ATCTTCTCGCGCAACGC	0	0	0	2
375	−(G:C)	TCTTCTCGCGCAACGCG	0	0	0	2
383	−(G:C)	CGCAACGCGTCAGTGGG	0	0	0	1
389	−(G:C)	GCGTCAGTGGGCTGATC	0	0	1	0
407	−(G:C)	TTAACTATCCGCTGGAT	0	0	1	0
412	−(G:C)	ATCCGCTGGATGACCAG	0	0	1	0
444	−(G:C)	GGAAGCTGCCTGCACTA	0	0	1	0
460	−(G:C)	AATGTTCCGGCGTTATT	0	0	0	1
496	−(G:C)	ACACCCATCAACAGTAT	0	0	0	1
513	−(G:C)	TATTTTCTCCCATGAAG	0	0	1	0

Table 8A.1. (*lacI* mutants, *continued*)

Mutated Nucleotide	Type of Mutation	*lacI* Flanking Sequences	Spontaneous Male	Spontaneous Female	PhIP Male	PhIP Female
521	–(G:C)	CCCATGAAGACGGTACG	0	0	0	2
541	–(G:C)	CTGGGCGTGGAGCATCT	0	0	1	0
559	–(G:C)	GTCGCATTGGGTCACCA	0	0	0	1
576	–(G:C)	GCAAATCGCGCTGTTAG	0	0	1	0
586	–(G:C)	CTGTTAGCGGGCCCATT	0	0	1	1
589	–(G:C)	TTAGCGGGCCCATTAAG	0	0	1	0
607	–(G:C)	TCTGTCTCGGCGCGTCT	0	0	1	0
610	–(G:C)	GTCTCGGCGCGTCTGCG	0	0	1	0
670	–(G:C)	CCGATAGCGGAACGGGA	0	0	0	1
675	–(G:C)	AGCGGAACGGGAAGGCG	0	0	0	1
699	–(G:C)	TGCCATGTCCGGTTTTC	0	0	1	0
701	–(G:C)	CCATGTCCGGTTTTCAA	0	0	0	1
733	–(G:C)	CTGAATGAGGGCATCGT	0	0	0	1
743	–(G:C)	GCATCGTTCCCACTGCG	0	0	0	1
779	–(G:C)	AGATGGCGCTGGGCGCA	0	0	0	1
784	–(G:C)	GCGCTGGGCGCAATGCG	0	0	1	0
790	–(G:C)	GGCGCAATGCGCGCCAT	0	0	0	1
792	–(G:C)	CGCAATGCGCGCCATTA	0	0	1	0
809	–(G:C)	CCGAGTCCGGGCTGCGC	0	0	1	0
818	–(G:C)	GGCTGCGCGTTGGTGCG	0	0	2	0
826	–(G:C)	GTTGGTGCGGATATCTC	0	0	0	1
877	–(G:C)	TGTTATATCCCGCCGTT	0	0	3	3
881	–(G:C)	ATATCCCGCCGTTAACC	0	0	1	0
891	–(G:C)	GTTAACCACCATCAAAC	0	0	0	1
896	–(A:T)	CCACCATCAAACAGGAT	0	0	0	1
920	–(G:C)	TGCTGGGGCAAACCAGC	0	0	1	0
934	–(G:C)	AGCGTGGACCGCTTGCT	0	0	0	1
955	–(G:C)	CTCTCTCAGGGCCAGGC	0	0	1	0
964	–(G:C)	GGCCAGGCGGTGAAGGG	0	0	0	1
986	–(G:C)	AGCTGTTGCCCGTCTCA	0	0	1	1
C. Other Mutations						
35,39	complex: –$(G:C)_{35}$ & T_{39}→G	GTGAAACCAGTAACGTT	0	0	1	0
221–222	tandem: GG→CC	GCTGATTGGCGTTGCCA	1	0	0	0
265–280	16 bp deletion: TGT...AAA	ATTGTCGCGGCGATTAAATC	0	1	0	0
373–374	CG deletion	ATCTTCTCGCGCAACGC	0	0	1	0
523–524	tandem: CG→AC	ATGAAGACGGTACGCGA	0	0	1	0
740–742	GTT deletion	GGGCATCGTTCCCACTG	0	0	0	1
779–780	tandem: CT→TA	GATGGCGCTGGGCGCAA	0	0	1	0
784–785	complex: GC→T	CGCTGGGCGCAATGCGC	0	0	0	1
791–792	CG deletion	GCGCAATGCGCGCCATT	0	0	3	3
937–938	complex: CT→G	GTGGACCGCTTGCTGCA	0	0	0	1
948–949	TC deletion	CTGCAACTCTCTCAGGG	0	0	1	1
1009–1010	complex: AA→T	AAAGAAAAACCACCCTG	0	0	0	1
Totals			14	14	105	105

Table 8A.2. Independent spontaneous and PhIP-induced *cII* mutations from colon of male and female Big Blue® rats

Mutated Nucleotide	Type of Mutation	*cII* Flanking Sequences	Spontaneous		PhIP	
			Male	Female	Male	Female
A. Base Substitutions						
-3	G:C→A:T	AATACTTACATATGGTT	0	0	1	0
3	G:C→A:T	TTACATATGGTTCGTGC	0	1	0	0
3	G:C→T:A	TTACATATGGTTCGTGC	0	0	2	0
11	G:C→A:T	GGTTCGTGCAAACAAAC	0	0	1	1
15	G:C→T:A	CGTGCAAACAAACGCAA	0	0	0	1
19	G:C→A:T	CAAACAAACGCAACGAG	0	1	0	0
19	G:C→C:G	CAAACAAACGCAACGAG	0	0	1	0
24	G:C→C:G	AAACGCAACGAGGCTCT	0	0	1	1
25	G:C→A:T	AACGCAACGAGGCTCTA	0	0	2	1
25	G:C→T:A	AACGCAACGAGGCTCTA	1	0	1	2
28	G:C→A:T	GCAACGAGGCTCTACGA	1	0	0	1
29	G:C→C:G	CAACGAGGCTCTACGAA	0	0	1	0
29	G:C→T:A	CAACGAGGCTCTACGAA	1	0	0	0
31	G:C→C:G	ACGAGGCTCTACGAATC	0	0	0	1
31	G:C→T:A	ACGAGGCTCTACGAATC	0	0	1	0
34	G:C→A:T	AGGCTCTACGAATCGAG	1	0	1	0
34	G:C→C:G	AGGCTCTACGAATCGAG	0	1	0	1
35	G:C→A:T	GGCTCTACGAATCGAGA	0	0	2	1
35	G:C→T:A	GGCTCTACGAATCGAGA	0	1	0	0
39	G:C→C:G	CTACGAATCGAGAGTGC	0	0	0	1
40	G:C→T:A	TACGAATCGAGAGTGCG	0	1	0	0
42	G:C→C:G	CGAATCGAGAGTGCGTT	0	0	1	0
42	G:C→T:A	CGAATCGAGAGTGCGTT	0	1	1	1
46	G:C→C:G	TCGAGAGTGCGTTGCTT	0	0	0	1
50	A:T→G:C	GAGTGCGTTGCTTAACA	1	0	0	0
51	G:C→T:A	AGTGCGTTGCTTAACAA	0	0	0	1
52	G:C→T:A	GTGCGTTGCTTAACAAA	1	0	0	0
57	G:C→C:G	TTGCTTAACAAAATCGC	0	0	1	0
62	A:T→G:C	TAACAAAATCGCAATGC	1	0	0	0
64	G:C→C:G	ACAAAATCGCAATGCTT	0	0	2	0
64	G:C→T:A	ACAAAATCGCAATGCTT	0	0	0	1
71	A:T→T:A	CGCAATGCTTGGAACTG	1	0	0	0
73	G:C→A:T	CAATGCTTGGAACTGAG	0	0	1	0
73	G:C→T:A	CAATGCTTGGAACTGAG	0	0	1	2
74	G:C→T:A	AATGCTTGGAACTGAGA	1	0	0	1
79	G:C→T:A	TTGGAACTGAGAAGACA	0	0	1	0
83	A:T→C:G	AACTGAGAAGACAGCGG	1	0	0	0
88	G:C→C:G	AGAAGACAGCGGAAGCT	1	0	0	0
89	G:C→T:A	GAAGACAGCGGAAGCTG	0	1	1	1
94	G:C→A:T	CAGCGGAAGCTGTGGGC	0	0	1	0
95	G:C→A:T	AGCGGAAGCTGTGGGCG	1	0	0	0
99	G:C→C:G	GAAGCTGTGGGCGTTGA	0	0	1	0
100	G:C→T:A	AAGCTGTGGGCGTTGAT	0	0	0	1
101	G:C→C:G	AGCTGTGGGCGTTGATA	0	0	1	0
101	G:C→T:A	AGCTGTGGGCGTTGATA	0	0	0	1
103	G:C→A:T	CTGTGGGCGTTGATAAG	0	1	0	0
103	G:C→T:A	CTGTGGGCGTTGATAAG	1	0	0	0

Table 8A.2. (*cII* mutants, *continued*)

Mutated Nucleotide	Type of Mutation	*cII* Flanking Sequences	Spontaneous Male	Spontaneous Female	PhIP Male	PhIP Female
106	G:C→T:A	TGGGCGTTGATAAGTCG	0	1	0	0
107	A:T→C:G	GGGCGTTGATAAGTCGC	0	0	1	0
111	G:C→T:A	GTTGATAAGTCGCAGAT	0	0	1	0
113	G:C→A:T	TGATAAGTCGCAGATCA	1	0	0	0
113	G:C→C:G	TGATAAGTCGCAGATCA	0	0	0	1
113	G:C→T:A	TGATAAGTCGCAGATCA	0	0	1	0
115	G:C→T:A	ATAAGTCGCAGATCAGC	0	0	0	1
117	G:C→C:G	AAGTCGCAGATCAGCAG	0	0	1	1
117	G:C→T:A	AAGTCGCAGATCAGCAG	0	0	0	1
120	G:C→C:G	TCGCAGATCAGCAGGTG	0	0	1	0
125	G:C→A:T	GATCAGCAGGTGGAAGA	0	0	0	1
125	G:C→C:G	GATCAGCAGGTGGAAGA	0	0	2	0
125	G:C→T:A	GATCAGCAGGTGGAAGA	0	0	1	0
126	G:C→C:G	ATCAGCAGGTGGAAGAG	0	0	1	0
129	G:C→C:G	AGCAGGTGGAAGAGGGA	0	0	0	1
129	G:C→T:A	AGCAGGTGGAAGAGGGA	0	0	0	1
130	A:T→G:C	GCAGGTGGAAGAGGGAC	0	1	0	0
132	G:C→T:A	AGGTGGAAGAGGGACTG	0	0	0	1
134	G:C→T:A	GTGGAAGAGGGACTGGA	0	0	1	0
136	G:C→T:A	GGAAGAGGGACTGGATT	1	0	0	1
140	G:C→T:A	GAGGGACTGGATTCCAA	0	0	1	0
141	G:C→C:G	AGGGACTGGATTCCAAA	0	0	1	0
141	G:C→T:A	AGGGACTGGATTCCAAA	0	0	1	1
142	A:T→G:C	GGGACTGGATTCCAAAG	1	0	0	0
145	G:C→C:G	ACTGGATTCCAAAGTTC	0	0	1	0
148	A:T→T:A	GGATTCCAAAGTTCTCA	0	1	0	0
155	G:C→A:T	AAAGTTCTCAATGCTGC	1	0	0	0
159	G:C→T:A	TTCTCAATGCTGCTTGC	0	0	1	0
163	G:C→C:G	CAATGCTGCTTGCTGTT	1	0	0	0
163	G:C→T:A	CAATGCTGCTTGCTGTT	0	0	0	1
164	A:T→T:A	AATGCTGCTTGCTGTTC	0	1	0	0
166	G:C→T:A	TGCTGCTTGCTGTTCTT	0	1	0	0
167	G:C→C:G	GCTGCTTGCTGTTCTTG	0	0	1	0
167	G:C→T:A	GCTGCTTGCTGTTCTTG	0	0	1	0
169	G:C→T:A	TGCTTGCTGTTCTTGAA	1	0	0	0
172	G:C→A:T	TTGCTGTTCTTGAATGG	0	0	0	1
172	G:C→C:G	TTGCTGTTCTTGAATGG	0	0	1	0
172	G:C→T:A	TTGCTGTTCTTGAATGG	0	0	0	1
175	G:C→T:A	CTGTTCTTGAATGGGGG	0	0	1	0
179	G:C→A:T	TCTTGAATGGGGGGTCG	0	0	2	0
179	G:C→T:A	TCTTGAATGGGGGGTCG	0	0	2	0
180	G:C→A:T	CTTGAATGGGGGGTCGT	0	0	0	1
180	G:C→C:G	CTTGAATGGGGGGTCGT	0	0	1	0
180	G:C→T:A	CTTGAATGGGGGGTCGT	0	0	1	0
181	G:C→T:A	TTGAATGGGGGGTCGTT	0	0	2	1
182	G:C→C:G	TGAATGGGGGGTCGTTG	1	0	0	0
182	G:C→T:A	TGAATGGGGGGTCGTTG	0	0	4	2
187	G:C→T:A	GGGGGGTCGTTGACGAC	0	1	1	0
190	G:C→A:T	GGGTCGTTGACGACGAC	0	1	0	0

Table 8A.2. (*cII* mutants, *continued*)

Mutated Nucleotide	Type of Mutation	*cII* Flanking Sequences	Spontaneous Male	Spontaneous Female	PhIP Male	PhIP Female
191	A:T→C:G	GGTCGTTGACGACGACA	1	0	0	0
193	G:C→A:T	TCGTTGACGACGACATG	0	1	1	0
193	G:C→T:A	TCGTTGACGACGACATG	0	1	3	0
196	G:C→A:T	TTGACGACGACATGGCT	0	2	0	1
196	G:C→C:G	TTGACGACGACATGGCT	0	1	0	0
202	G:C→C:G	ACGACATGGCTCGATTG	0	0	1	0
202	G:C→T:A	ACGACATGGCTCGATTG	0	0	0	1
205	G:C→A:T	ACATGGCTCGATTGGCG	1	0	0	0
206	G:C→A:T	CATGGCTCGATTGGCGC	0	0	1	0
211	G:C→C:G	CTCGATTGGCGCGACAA	0	0	1	0
211	G:C→T:A	CTCGATTGGCGCGACAA	0	0	1	0
212	G:C→A:T	TCGATTGGCGCGACAAG	0	2	0	0
212	G:C→T:A	TCGATTGGCGCGACAAG	0	0	1	0
214	G:C→A:T	GATTGGCGCGACAAGTT	1	0	1	0
214	G:C→C:G	GATTGGCGCGACAAGTT	0	0	1	0
215	G:C→C:G	ATTGGCGCGACAAGTTG	0	0	2	0
215	G:C→T:A	ATTGGCGCGACAAGTTG	0	0	0	1
220	G:C→T:A	CGCGACAAGTTGCTGCG	0	0	1	0
221	A:T→G:C	GCGACAAGTTGCTGCGA	0	1	0	0
224	G:C→A:T	ACAAGTTGCTGCGATTC	1	0	0	0
226	G:C→C:G	AAGTTGCTGCGATTCTC	0	0	1	0
229	A:T→G:C	TGCTGCGATTCTCACCA	1	0	0	0
232	G:C→A:T	CTGCGATTCTCACCAAT	0	0	1	0
232	G:C→T:A	CTGCGATTCTCACCAAT	0	0	0	1
233	A:T→G:C	TGCGATTCTCACCAATA	0	0	1	0
236	G:C→T:A	GATTCTCACCAATAAAA	0	0	1	0
274	G:C→T:A	GTTCTGAACAAATCCAG	0	1	0	0
292	A:T→G:C	TGGAGTTCTGAGGTCAT	1	0	0	0
293	G:C→T:A	GGAGTTCTGAGGTCATT	0	0	1	0
B. −1 Frameshifts						
7	−(G:C)	ATATGGTTCGTGCAAAC	0	0	1	0
53	−(A:T)	TGCGTTGCTTAACAAAA	0	0	1	0
58	−(A:T)	TGCTTAACAAAATCGCA	0	0	0	1
73	−(G:C)	CAATGCTTGGAACTGAG	0	0	1	0
90	−(G:C)	AAGACAGCGGAAGCTGT	0	0	1	0
103	−(G:C)	CTGTGGGCGTTGATAAG	0	0	0	1
123	−(G:C)	CAGATCAGCAGGTGGAA	0	0	1	0
134	−(G:C)	GTGGAAGAGGGACTGGA	0	0	0	1
179	−(G:C)	TCTTGAATGGGGGGTCG	2	0	3	1
185	−(A:T)	ATGGGGGGTCGTTGACG	1	0	0	0
206	−(G:C)	CATGGCTCGATTGGCGC	0	0	1	0
215	−(G:C)	ATTGGCGCGACAAGTTG	0	0	0	1
221	−(A:T)	GCGACAAGTTGCTGCGA	1	0	0	0
241	−(A:T)	TCACCAATAAAAAACGC	2	0	0	1
252	−(G:C)	AAACGCCCGGCGGCAAC	0	0	2	0

Table 8A.2. (*cII* mutants, *continued*)

Mutated Nucleotide	Type of Mutation	*cII* Flanking Sequences	Spontaneous Male	Spontaneous Female	PhIP Male	PhIP Female
C. Other Mutations						
13,73	complex:					
	$(A:T)_{13}\rightarrow C:G$	TTCGTGCAAACAAACGC				
	& $(G:C)_{73}\rightarrow T:A$	CAATGCTTGGAACTGAG	0	0	0	1
56–57	AC deletion	TTGCTTAACAAAATCGC	0	0	1	0
73–74	tandem: GG→CC	AATGCTTGGAACTGAGA	0	0	1	0
98–99	tandem: TG→GT	GAAGCTGTGGGCGTTGA	0	0	1	0
99,103	complex:					
	$-(G:C)_{99}$ &					
	$(G:C)_{103}\rightarrow C:G$	AGCTGTGGGCGTTGATA	0	0	1	0
150,286	complex:					
	$(G:C)_{150}\rightarrow T:A$	ATTCCAAAGTTCTCAAT				
	& $(G:C)_{286}\rightarrow T:A$	TCCAGATGGAGTTCTGA	0	0	0	1
179	+(G:C)	TCTTGAATGGGGGGTCG	0	1	0	0
179	+(G:C)	TCTTGAATGGGGGGTCG	3	0	0	0
190–192	GAC deletion	GGTCGTTGACGACGACA	0	0	1	0
234–235	+(A:T)	CGATTCTCACCAATAAA	1	0	0	0
247,249	complex:					
	$(C:G)_{247}\rightarrow G:C$					
	& $-(G:C)_{249}$	TAAAAAACGCCCGGCGG	0	0	1	1
Totals			36	25	94	51

Table 8A.3. PhIP-induced base substitution mutations involving 5′–CpG–3′ dinucleotide sequences

A. Frequency of mutations

	Occurrences		Total base substitution mutations				CpG mutations per target site			
			lacI		*cII*		*lacI*		*cII*	
Target site	*lacI*	*cII*	Male	Female	Male	Female	Male	Female	Male	Female
ACGA/TCGT [a]	8	6	58	62	77	42	2	1	11	7
ACGC/GCGT	18	5	58	62	77	42	4	5	1	0
ACGG/CCGT	6	0	58	62	77	42	1	1	0	0
ACGT/ACGT	2	0	58	62	77	42	2	1	0	0
CCGA/TCGG	8	1	58	62	77	42	0	1	0	0
CCGC/GCGG	20	2	58	62	77	42	7	11	1	1
GCGA/TCGC	15	4	58	62	77	42	4	6	7	3
GCGC/GCGC	12	1	58	62	77	42	3	0	1	0
TCGA/TCGA	2	2	58	62	77	42	0	0	1	1
Totals, all CpG	91	21	n.a. [b]	n.a.	n.a.	n.a.	23	26	22	12

B. Frequency of mutations normalized per target sequence

	(CpG mutations/base substitution)/site						
	lacI		*cII*		Average: M + F		Ratio
Target site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
ACGA/TCGT	0.0043 [c]	0.0020	0.0238	0.0278	0.0032	0.0258	0.12
ACGC/GCGT	0.0038	0.0045	0.0026	0.0000	0.0042	0.0013	3.20
ACGG/CCGT	0.0029	0.0027	(div 0)	(div 0)	0.0028	(div 0)	(div 0)
ACGT/ACGT	0.0172	0.0081	(div 0)	(div 0)	0.0127	(div 0)	(div 0)
CCGA/TCGG	0.0000	0.0020	0.0000	0.0000	0.0010	0.0000	(div 0)
CCGC/GCGG	0.0060	0.0089	0.0065	0.0119	0.0075	0.0092	0.81
GCGA/TCGC	0.0046	0.0065	0.0227	0.0179	0.0055	0.0203	0.27
GCGC/GCGC	0.0043	0.0000	0.0130	0.0000	0.0022	0.0065	0.33
TCGA/TCGA	0.0000	0.0000	0.0065	0.0119	0.0000	0.0092	0.00
Averages	0.0044	0.0046	0.0136	0.0136	0.0045	0.0136	0.33

[a] Underlined nucleotides indicate mutated positions.

[b] div 0, division by zero; n.a., not applicable.

[c] Sample calculation: Using the data from Part A of this table, 0.0043 (male *lacI* CpG mutations per base substitution) per ACGA/TCGT target site = [(2 male *lacI* CpG mutations at ACGA/TCGT)/(58 total male *lacI* base substitution mutations)]/(8 ACGA/TCGT target sites in *lacI*).

Table 8A.4. Summary of the remaining nearest-neighbor analyses: PhIP-induced base substitution mutations occurring at specified target sequences

A. Frequency of mutations

Class of mutation (target sequence)	Occurrences		Total base substitution mutations				"Class of mutation," from first column, all target sites [a]			
			lacI		*cII*		*lacI*		*cII*	
	lacI	*cII*	Male	Female	Male	Female	Male	Female	Male	Female
1. Base substitutions at CC/GG	143	26	58	62	77	42	28	33	31	16
2. Base substitutions at CC/GG, $-(G_{179-184})$	143	26	58	62	65	38	28	33	19	12
3. G:C→T:A transversions (all sites)	600	150	58	62	77	42	25	31	35	26
4. G:C→C:G transversions (all sites)	600	150	58	62	77	42	16	14	25	8

B. Frequency of mutations normalized per target sequence

Class of mutation (target sequence)	(Class of mutation/base substitution)/site						
	lacI		*cII*		Average: M + F		Ratio
	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
1. Base substitutions at CC/GG	0.0034 [b]	0.0037	0.0155	0.0147	0.0035	0.0151	0.24
2. Base substitutions at CC/GG, $-(G_{179-184})$	0.0034	0.0037	0.0112	0.0121	0.0035	0.0117	0.30
3. G:C→T:A transversions (all sites)	0.0007	0.0008	0.0030	0.0041	0.0008	0.0036	0.22
4. G:C→C:G transversions (all sites)	0.0005	0.0004	0.0022	0.0013	0.0004	0.0017	0.24

[a] These values are the total occurrences of the class of mutation specified in the first column. The first row includes all base substitutions involving CC/GG dinucleotides (ACCA/TGGT, ACCG/CGGT, *etc.*). The second row is the same as the first, except that mutations which occurred at the $G_{179-184}$ sequence, a hotspot for spontaneous and PhIP-induced mutation in *cII* and which potentially bias the data, were not considered. The third row and fourth rows include all G:C→T:A and G:C→C:G transversions (ACA/TGT; ACC/GGT; *etc.*), respectively. Underlined nucleotides indicate mutated positions.

[b] Refer to Table 8A.3 for a sample calculation.

Table 8A.5. Summary of the remaining nearest-neighbor analyses: PhIP-induced –1 frameshifts occurring at specified target sequences

A. Frequency of mutations

Class of mutation (target sequence)	Occurrences		All mutations				"Class of mutation," from first column, all target sites [a]			
			lacI		*cII*		*lacI*		*cII*	
	lacI	*cII*	Male	Female	Male	Female	Male	Female	Male	Female
1. –1 frameshifts of G:C base pairs (all sites)	522	128	105	105	94	51	38	34	10	4
2. –1 frameshifts of G:C base pairs at CC/GG	149	27	105	105	94	51	24	20	7	2
3. –1 frameshifts of G:C base pairs at CCC/GGG	31	7	105	105	94	51	15	10	3	2
4. –1 frameshifts of G:C base pairs at 5′–CpG–3′	190	44	105	105	94	51	16	17	5	2

B. Frequency of mutations normalized per target sequence

Class of mutation (target sequence)	(Class of mutation/all mutations)/site				Average: M + F		Ratio
	lacI		*cII*				
	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
1. –1 frameshifts of G:C base pairs (all sites)	0.0007 [b]	0.0006	0.0008	0.0006	0.0007	0.0007	0.91
2. –1 frameshifts of G:C base pairs at CC/GG	0.0015	0.0013	0.0028	0.0015	0.0014	0.0021	0.67
3. –1 frameshifts of G:C base pairs at CCC/GGG	0.0046	0.0031	0.0046	0.0056	0.0038	0.0051	0.76
4. –1 frameshifts of G:C base pairs at 5′–CpG–3′	0.0008	0.0009	0.0012	0.0009	0.0008	0.0011	0.79

[a] These values are the total occurrences of the class of mutation specified in the first column. The first row includes all –1 frameshift mutations involving any G:C base pair (ACC/GGT; ACG/CGT; *etc.*). The second and third rows include all –1 frameshifts involving G:C base pairs which occurred at CC/GG dinucleotide or CCC/GGG trinucleotide sequences, respectively. The fourth row includes all –1 frameshifts involving G:C base pairs which occurred at a CpG dinucleotide sequence (ACG/CGT; CCG/CGG; CGA/TCG; and CGC/GCG). Underlined nucleotides indicate mutated positions.

[b] Refer to Table 8A.3 for a sample calculation.

8.7. Appendix B: Supplementary, Unpublished Tables

Table 8B.1. PhIP-induced base substitution mutations involving CC/GG dinucleotide sequences

A. Frequency of mutations

	Occurrences		Total Base Substitution Mutations				CC/GG Mutations per Target Site					
			lacI		*cII*		*lacI*		*cII*, Male [a]		*cII*, Female	
Target Site	*lacI*	*cII*	Male	Female	Male	Female	Male	Female	All b.s.	$-G_{179-184}$	All b.s. [b]	$-G_{179-184}$
ACCA/TGGT	18	2	58	62	77	42	1	2	3	3	0	0
ACCG/CGGT	12	1	58	62	77	42	6	6	0	0	0	0
ACCT/AGGT	2	1	58	62	77	42	0	0	4	4	1	1
CCCA/TGGG	13	2	58	62	77	42	2	3	7	1	2	1
CCCC/GGGG	2	3	58	62	77	42	0	0	6	0	3	0
CCCG/CGGG	11	1	58	62	77	42	5	8	0	0	0	0
CCCT/AGGG	5	1	58	62	77	42	0	0	1	1	0	0
GCCA/TGGC	21	2	58	62	77	42	5	7	3	3	1	1
GCCC/GGGC	15	2	58	62	77	42	0	1	1	1	1	1
GCCG/CGGC	12	2	58	62	77	42	1	0	0	0	0	0
GCCT/AGGC	7	1	58	62	77	42	0	0	0	0	1	1
TCCA/TGGA	8	6	58	62	77	42	3	1	6	6	6	6
TCCC/GGGA	10	1	58	62	77	42	4	3	0	0	1	1
TCCG/CGGA	7	1	58	62	77	42	1	2	0	0	0	0
Totals	143	26	n.a.	n.a.	n.a.	n.a.	28	33	31	19	16	12
$-G_{179-184}$					(65)	(38)						

B. Frequency of mutations normalized per target sequence

	(CC/GG Mutations/Base Substitution)/Site [c]						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI*/*cII*
ACCA/TGGT	0.0010 [d]	0.0018	0.0231	0.0000	0.0014	0.0115	0.12
ACCG/CGGT	0.0086	0.0081	0.0000	0.0000	0.0083	0.0000	(div 0)
ACCT/AGGT	0.0000	0.0000	0.0615	0.0263	0.0000	0.0439	0
CCCA/TGGG	0.0027	0.0037	0.0077	0.0132	0.0032	0.0104	0.31
CCCC/GGGG	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	(div 0)
CCCG/CGGG	0.0078	0.0117	0.0000	0.0000	0.0098	0.0000	(div 0)

Table 8B.1. (*continued*)

B. Frequency of mutations normalized per target sequence (*continued*)

	(CC/GG Mutations/Base Substitution)/Site [c]						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
CCCT/AGGG	0.0000	0.0000	0.0154	0.0000	0.0000	0.0077	0
GCCA/TGGC	0.0041	0.0054	0.0231	0.0132	0.0047	0.0181	0.26
GCCC/GGGC	0.0000	0.0011	0.0077	0.0132	0.0005	0.0104	0.05
GCCG/CGGC	0.0014	0.0000	0.0000	0.0000	0.0007	0.0000	(div 0)
GCCT/AGGC	0.0000	0.0000	0.0000	0.0263	0.0000	0.0132	0
TCCA/TGGA	0.0065	0.0020	0.0154	0.0263	0.0042	0.0209	0.20
TCCC/GGGA	0.0069	0.0048	0.0000	0.0263	0.0059	0.0132	0.45
TCCG/CGGA	0.0025	0.0046	0.0000	0.0000	0.0035	0.0000	(div0)
Averages	0.0034	0.0037	0.0112	0.0121	0.0035	0.0117	0.30

[a] *cII* mutants are listed with all base substitutions considered (All b.s.), and minus those occurring at the hexanucleotide guanine run at $G_{179-184}$ ($-G_{179-184}$).

[b] b.s., base substitutions; div 0, division by zero; n.a., not applicable.

[c] The values in Part B of this table were calculated using *cII* data *minus* the mutations occurring at $G_{179-184}$.

[d] Refer to Table 8A.3 for a sample calculation.

Table 8B.2. PhIP-induced G:C→T:A transversion mutations

A. Frequency of mutations

Target Site	Occurrences *lacI*	Occurrences *cII*	Total Base Substitution Mutations *lacI* Male	*lacI* Female	*cII* Male	*cII* Female	G:C→T:A Mutations per Target Site *lacI* Male	*lacI* Female	*cII* Male	*cII* Female
ACA/TGT	24	8	58	62	77	42	0	0	0	1
ACC/GGT	36	5	58	62	77	42	2	3	2	0
ACG/CGT	36	11	58	62	77	42	2	4	1	0
ACT/AGT	23	7	58	62	77	42	1	0	2	0
AGA/TCT	30	12	58	62	77	42	1	0	2	5
AGC/GCT	37	13	58	62	77	42	0	0	1	1
AGG/CCT	16	3	58	62	77	42	0	0	1	0
CCA/TGG	50	12	58	62	77	42	0	1	6	2
CCC/GGG	31	7	58	62	77	42	1	2	7	4
CCG/CGG	42	5	58	62	77	42	2	3	0	0
CGA/TCG	35	15	58	62	77	42	1	1	5	3
CGC/GCG	77	13	58	62	77	42	5	6	2	2
GCA/TGC	49	15	58	62	77	42	0	2	1	2
GCC/GGC	55	7	58	62	77	42	3	3	1	2
GGA/TCC	27	8	58	62	77	42	6	5	1	4
TCA/TGA	32	9	58	62	77	42	1	1	3	0
Totals	600	150	n.a. [a]	n.a.	n.a.	n.a.	25	31	35	26

B. Frequency of mutations normalized per target sequence

Target Site	(G:C→T:A Mutations/Base Substitution)/Site *lacI* Male	*lacI* Female	*cII* Male	*cII* Female	Average: M + F *lacI*	Average: M + F *cII*	Ratio *lacI*/*cII*
ACA/TGT	0.0000 [b]	0.0000	0.0000	0.0030	0.0000	0.0015	0
ACC/GGT	0.0010	0.0013	0.0052	0.0000	0.0012	0.0026	0.44
ACG/CGT	0.0010	0.0018	0.0012	0.0000	0.0014	0.0006	2.33
ACT/AGT	0.0007	0.0000	0.0037	0.0000	0.0004	0.0019	0.20
AGA/TCT	0.0006	0.0000	0.0022	0.0099	0.0003	0.0060	0.05
AGC/GCT	0.0000	0.0000	0.0010	0.0018	0.0000	0.0014	0
AGG/CCT	0.0000	0.0000	0.0043	0.0000	0.0000	0.0022	0
CCA/TGG	0.0000	0.0003	0.0065	0.0040	0.0002	0.0052	0.03

Table 8B.2. (*continued*)

B. Frequency of mutations normalized per target sequence (*continued*)

	(G:C→T:A Mutations/Base Substitution)/Site						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
CCC/GGG	0.0006	0.0010	0.0130	0.0136	0.0008	0.0133	0.06
CCG/CGG	0.0008	0.0012	0.0000	0.0000	0.0010	0.0000	(div0)
CGA/TCG	0.0005	0.0005	0.0043	0.0048	0.0005	0.0045	0.10
CGC/GCG	0.0011	0.0013	0.0020	0.0037	0.0012	0.0028	0.42
GCA/TGC	0.0000	0.0007	0.0009	0.0032	0.0003	0.0020	0.16
GCC/GGC	0.0009	0.0009	0.0019	0.0068	0.0009	0.0043	0.21
GGA/TCC	0.0038	0.0030	0.0016	0.0119	0.0034	0.0068	0.50
TCA/TGA	0.0005	0.0005	0.0043	0.0000	0.0005	0.0022	0.24
Averages	0.0007	0.0008	0.0030	0.0041	0.0008	0.0036	0.22

[a] div 0, division by zero; n.a., not applicable.

[b] Refer to Table 8A.3 for a sample calculation.

Table 8B.3. PhIP-induced G:C→C:G transversion mutations

A. Frequency of mutations

Target Site	Occurrences *lacI*	Occurrences *cII*	Total Base Substitution Mutations *lacI* Male	Total Base Substitution Mutations *lacI* Female	Total Base Substitution Mutations *cII* Male	Total Base Substitution Mutations *cII* Female	G:C→C:G Mutations per Target Site *lacI* Male	G:C→C:G Mutations per Target Site *lacI* Female	G:C→C:G Mutations per Target Site *cII* Male	G:C→C:G Mutations per Target Site *cII* Female
ACA/TGT	24	8	58	62	77	42	0	0	1	0
ACC/GGT	36	5	58	62	77	42	1	1	1	0
ACG/CGT	36	11	58	62	77	42	1	1	2	2
ACT/AGT	23	7	58	62	77	42	1	0	0	0
AGA/TCT	30	12	58	62	77	42	0	1	3	2
AGC/GCT	37	13	58	62	77	42	0	0	2	0
AGG/CCT	16	3	58	62	77	42	0	0	2	0
CCA/TGG	50	12	58	62	77	42	0	0	2	0
CCC/GGG	31	7	58	62	77	42	1	2	1	0
CCG/CGG	42	5	58	62	77	42	5	3	0	0
CGA/TCG	35	15	58	62	77	42	0	0	1	2
CGC/GCG	77	13	58	62	77	42	4	3	3	0
GCA/TGC	49	15	58	62	77	42	0	2	1	1
GCC/GGC	55	7	58	62	77	42	1	1	3	0
GGA/TCC	27	8	58	62	77	42	1	0	2	1
TCA/TGA	32	9	58	62	77	42	1	0	1	0
Totals	600	150	n.a. [a]	n.a.	n.a.	n.a.	16	14	25	8

B. Frequency of mutations normalized per target sequence

Target Site	(G:C→C:G Mutations/Base Substitution)/Site *lacI* Male	*lacI* Female	*cII* Male	*cII* Female	Average: M + F *lacI*	Average: M + F *cII*	Ratio *lacI*/*cII*
ACA/TGT	0.0000 [b]	0.0000	0.0016	0.0000	0.0000	0.0008	0
ACC/GGT	0.0005	0.0004	0.0026	0.0000	0.0005	0.0013	0.36
ACG/CGT	0.0005	0.0004	0.0024	0.0043	0.0005	0.0033	0.14
ACT/AGT	0.0007	0.0000	0.0000	0.0000	0.0004	0.0000	(div0)
AGA/TCT	0.0000	0.0005	0.0032	0.0040	0.0003	0.0036	0.08
AGC/GCT	0.0000	0.0000	0.0020	0.0000	0.0000	0.0010	0
AGG/CCT	0.0000	0.0000	0.0087	0.0000	0.0000	0.0043	0
CCA/TGG	0.0000	0.0000	0.0022	0.0000	0.0000	0.0011	0
CCC/GGG	0.0006	0.0010	0.0019	0.0000	0.0008	0.0009	0.86

Table 8B.3. (*continued*)

B. Frequency of mutations normalized per target sequence (*continued*)

	(G:C→C:G Mutations/Base Substitution)/Site						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
CCG/CGG	0.0021	0.0012	0.0000	0.0000	0.0016	0.0000	(div0)
CGA/TCG	0.0000	0.0000	0.0009	0.0032	0.0000	0.0020	0
CGC/GCG	0.0009	0.0006	0.0030	0.0000	0.0008	0.0015	0.51
GCA/TGC	0.0000	0.0007	0.0009	0.0016	0.0003	0.0012	0.27
GCC/GGC	0.0003	0.0003	0.0056	0.0000	0.0003	0.0028	0.11
GGA/TCC	0.0006	0.0000	0.0032	0.0030	0.0003	0.0031	0.10
TCA/TGA	0.0005	0.0000	0.0014	0.0000	0.0003	0.0007	0.37
Averages	0.0005	0.0004	0.0022	0.0013	0.0004	0.0017	0.24

[a] div 0, division by zero; n.a., not applicable.

[b] Refer to Table 8A.3 for a sample calculation.

Table 8B.4. PhIP-induced −1 frameshift mutations involving G:C base pairs

A. Frequency of mutations

	Occurrences		All Mutations				−(G:C) Mutations per Target Site			
			lacI		*cII*		*lacI*		*cII*	
Target Site	*lacI*	*cII*	Male	Female	Male	Female	Male	Female	Male	Female
ACC/GGT	36	5	105	105	94	51	0	3	0	0
ACG/CGT	36	11	105	105	94	51	3	1	0	1
AGA/TCT	30	12	105	105	94	51	1	2	0	0
AGC/GCT	37	13	105	105	94	51	2	1	0	0
AGG/CCT	16	3	105	105	94	51	1	1	0	1
CCA/TGG	50	12	105	105	94	51	2	1	4	1
CCG/CGG	42	5	105	105	94	51	6	8	3	0
CGA/TCG	35	15	105	105	94	51	0	1	2	1
CGC/GCG	77	13	105	105	94	51	7	7	0	0
GCA/TGC	49	15	105	105	94	51	1	1	1	0
GCC/GGC	55	7	105	105	94	51	4	3	0	0
GGA/TCC	27	8	105	105	94	51	11	4	0	0
TCA/TGA	32	9	105	105	94	51	0	1	0	0
Totals	522	128	n.a.	n.a.	n.a.	n.a.	38	34	10	4

B. Frequency of mutations normalized per target sequence

	(−(G:C) Mutations/All Mutations)/Site						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI*/*cII*
ACC/GGT	0.0000	0.0008	0.0000	0.0000	0.0004	0.0000	(div0)
ACG/CGT	0.0008	0.0003	0.0000	0.0018	0.0005	0.0009	0.59
AGA/TCT	0.0003	0.0006	0.0000	0.0000	0.0005	0.0000	(div0)
AGC/GCT	0.0005	0.0003	0.0000	0.0000	0.0004	0.0000	(div0)
AGG/CCT	0.0006	0.0006	0.0000	0.0065	0.0006	0.0033	0.18
CCA/TGG	0.0004	0.0002	0.0035	0.0016	0.0003	0.0026	0.11
CCG/CGG	0.0014	0.0018	0.0064	0.0000	0.0016	0.0032	0.50
CGA/TCG	0.0000	0.0003	0.0014	0.0013	0.0001	0.0014	0.10
CGC/GCG	0.0009	0.0009	0.0000	0.0000	0.0009	0.0000	(div0)
GCA/TGC	0.0002	0.0002	0.0007	0.0000	0.0002	0.0004	0.55
GCC/GGC	0.0007	0.0005	0.0000	0.0000	0.0006	0.0000	(div0)
GGA/TCC	0.0039	0.0014	0.0000	0.0000	0.0026	0.0000	(div0)
TCA/TGA	0.0000	0.0003	0.0000	0.0000	0.0001	0.0000	(div0)
Averages	0.0007	0.0006	0.0008	0.0006	0.0007	0.0007	0.91

Table 8B.5. PhIP-induced –1 frameshift mutations involving G:C base pairs at CC/GG and CCC/GGG

A. Frequency of mutations

	Occurrences		All Mutations				–(G:C) Mutations per Target Site			
			lacI		*cII*		*lacI*		*cII*	
Target Site	*lacI*	*cII*	Male	Female	Male	Female	Male	Female	Male	Female
CC/GG	149	27	105	105	94	51	24	20	7	2
CCC/GGG	31	7	105	105	94	51	15	10	3	2

B. Frequency of mutations normalized per target sequence (all mutations considered as a denominator)

	(–(G:C) Mutations/All Mutations)/Site						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
CC/GG	0.0015[a]	0.0013	0.0028	0.0015	0.0014	0.0021	0.67
CCC/GGG	0.0046	0.0031	0.0046	0.0056	0.0038	0.0051	0.76

C. Frequency of mutations normalized per target sequence (only –(G:C) mutations considered as a denominator)

	(–(G:C) Mutations at CC(C)/GG(G)/All –(G:C) Mutations)/Site						
	lacI		*cII*		Average: M + F		Ratio
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
CC/GG	0.0042	0.0039	0.0259	0.0185	0.0041	0.0222	0.18
CCC/GGG	0.0127	0.0095	0.0429	0.0714	0.0111	0.0571	0.19

[a] Refer to Table 8A.3 for a sample calculation.

Table 8B.6. PhIP-induced −1 frameshift mutations involving G:C base pairs at 5′–CpG–3′ dinucleotide sequences

A. Frequency of mutations

	Occurrences		All Mutations				−(G:C) Mutations per Target Site			
			lacI		*cII*		*lacI*		*cII*	
Target Site	*lacI*	*cII*	Male	Female	Male	Female	Male	Female	Male	Female
ACG/CGT	36	11	105	105	94	51	3	1	0	1
CCG/CGG	42	5	105	105	94	51	6	8	3	0
CGA/TCG	35	15	105	105	94	51	0	1	2	1
CGC/GCG	77	13	105	105	94	51	7	7	0	0
Totals	190	44	n.a. [a]	n.a.	n.a.	n.a.	16	17	5	2

B. Frequency of mutations normalized per target sequence (all mutations considered as a denominator)

	(−(G:C) Mutations at CpG/All Mutations)/Site				Average: M + F		Ratio
	lacI		*cII*				
Target Site	Male	Female	Male	Female	*lacI*	*cII*	*lacI/cII*
ACG/CGT	0.0008 [b]	0.0003	0.0000	0.0018	0.0005	0.0009	0.59
CCG/CGG	0.0014	0.0018	0.0064	0.0000	0.0016	0.0032	0.50
CGA/TCG	0.0000	0.0003	0.0014	0.0013	0.0001	0.0014	0.10
CGC/GCG	0.0009	0.0009	0.0000	0.0000	0.0009	0.0000	(div0)
Averages	0.0008	0.0009	0.0012	0.0009	0.0008	0.0011	0.79

[a] div 0, division by zero; n.a., not applicable.

[b] Refer to Table 8A.3 for a sample calculation.

SECTION III. GENERAL DISCUSSION AND CONCLUSIONS

Chapter 9. Transgenic Mutagenicity Assays: Past, Present and Future

It is perhaps useful first to reflect briefly on the development of the Big Blue® assay, before discussing the present period of activity (arbitrarily defined as the period spanning the studies described in this Dissertation) Finally, we speculate briefly on the need for a novel, *in situ*-based transgenic mutagenicity assay.

9.1. Early studies: Validation and Standardization

The study of the fundamental mechanisms of mutagenesis was revolutionized in the past decade by the development of the *lacZ* (Muta™Mouse: Gossen *et al.* 1989) and *lacI* (Big Blue®: Kohler *et al.* 1990) transgenic mouse mutagenicity assays. Early studies using these assays were generally devoted to determining spontaneous MF and MS in various tissues, as well as mutations induced by various agents (*e.g.*, 2-acetylaminofluorene (AAF); aflatoxin B_1 (AFB_1); dimethylbenzanthracene (DMBA); dimethyl nitrosamine (DMN); *N*-ethyl-*N*-nitrosourea (ENU); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG); γ-rays) that had been previously well-characterized in established mutagenicity and cytogenetic assays. These studies established that Big Blue® spontaneous *lacI* MS differed significantly from the spontaneous *lacI* MS obtained from the endogenous *lacI* gene in *E. coli*, confirming that *lacI* MS determined in the Big Blue® assay were due to mutations occurring *in vivo* in the *lacI* transgene, and not artifacts arising during passage of recovered transgenes in *E. coli* (*e.g.*, Stuart *et al.* 1996[27], Hill *et al.* 1999; and references therein). Additionally, *lacI* MS determined *in vivo* following treatment with known mutagens generally agreed with the mutagenic properties of those agents determined *in vitro* (*e.g.*, AFB_1: Dycaico *et al.* 1996),[28] indicating that the assay was behaving as predicted with these agents. An equally important goal of the early Big Blue® experiments was the subsequent development of standardized assay protocols (Rogers *et al.* 1995; Young *et al.* 1995) and recommendations for the statistical design of experiments (*e.g.*, Piegorsch *et al.* 1995; Carr and Gorelick 1995) and statistical analyses of the mutational data (Carr and Gorelick 1996, and references therein).

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[27] Chapter 1 of this Dissertation.

[28] Chapter 5 of this Dissertation.

Many of the early Big Blue® studies additionally yielded novel and important information regarding the mechanism of mutagenesis that could only have been obtained in whole animals, as illustrated by DMN, a mouse hepatocarcinogen. In 3 week old mice, DMN (2 mg/kg/day) produced 10- to 20-fold elevations in mutant frequency that increased with the number of treatments (Mirsalis *et al.* 1993). In 6 week old mice, DMN failed to induce mutations at 2 mg/kg/day, but 4 mg/kg/day yielded significant elevations in hepatic mutations. As DMN is known to significantly induce hepatic cellular proliferation, this observation demonstrated a threshold effect in 6 week old (developmentally mature) liver tissue, as well as the role of cellular proliferation in establishing DNA damage as mutations.[29]

9.2. Contemporary Studies: Applied Studies of the Mechanisms of Mutagenesis and Carcinogenesis

The experimental work described in the various Chapters of this Dissertation span the period towards the end of the validation stage of the Big Blue® assay to the present day. Indeed, the first Chapter of this Dissertation (Stuart *et al.* 1996) describes the characterization of mutants arising *ex vivo* (due to damaged DNA recovered from the mouse and repaired in the *E. coli* host strain) and *in vitro* (arising *de novo* during replication and amplification of the *lacI* transgene in the *E. coli* host) and their influence on Big Blue® *lacI* mutations which occurred *in vivo*. This study established that the *lacI* MS derived from these so-called "sectored" (*ex vivo*; *in vitro*) mutants differed significantly from the *in vivo* spontaneous *lacI* MS, and occurred at a frequency (perhaps a fifth to half or more of that of the spontaneous MF) high enough that such that they should be identified and partitioned from the *in vivo* mutants. The simple solution to this problem was to plate the phage-infected *E. coli* cells such that the density of plaques on the agar media plates did not exceed approximately 16 PFU/cm^2, allowing visual identification of the sectored mutant plaques. Subsequently, all the spontaneous *lacI* mutational data, and most of the chemically-induced data,[30] was collected following the recommendations of Stuart *et al.* (1996), and was therefore substantially free of contamination from these sectored mutants.

The Big Blue® assay permitted us to evaluate spontaneous *lacI* MF and MS as a function of age in liver, bladder and brain of Big Blue® mice from the age of 6 weeks to 12 months (bladder) or 25

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[29] This theme, *i.e.* the relationship between cellular proliferation and MF, is reiterated in Chapters 2 and 4.

[30] The influence of sectored plaques on the *in vivo* data diminishes as induced MF increase (Stuart *et al.* 1996) (Chapter 1), and is believed to be essentially negligible at very high induced MF, as obtained in the PhIP prostate study (Chapter 8).

months (brain; liver). This study (Chapter 2) contributed to our database of spontaneous mutations (de Boer, 1995), particularly in older mice, providing a valuable reference for comparison in subsequent studies with older mice. More importantly, however, this data enabled us to critically evaluate the potential contribution of spontaneous mutation in chromosomal DNA to the ageing process.

As expected and predicted by the somatic mutation theory, spontaneous MF were found to increase significantly with age in tissues which proliferate (liver) or have proliferative capacity (bladder), but not in a largely mitotically quiescent tissue (brain). MF in brain increased significantly only during the period in which brain tissue was still proliferating (≤ 6 months of age); brain MF did not increase in adult mice. These data demonstrated that cellular proliferation is required for increases in MF. The absence of a change in MS with age was not expected, based on several long-standing theories of ageing. For example, if free radical damage accumulated with age, or accumulated more rapidly in older mice, we should have observed increases in the frequencies of G:C→T:A and G:C→C:G transversion mutations (due to 8-oxoG) relative to the remaining mutational classes; if the fidelity of DNA polymerases decreased substantially in older mice ("error catastrophe"), we might have expected to see an increase in the relative frequencies frameshift or complex mutations, or deletions. The ageing data, including the relatively small (three-fold, in liver) increases in MF with no significant change in MS, argued that spontaneous mutations are likely to play a relatively small role in the ageing process, at least in nuclear DNA of animals up to late middle age. The possibility that MF increase exponentially or that MS change significantly in animals of extreme old age cannot be discounted, however. Nevertheless, the Big Blue® ageing data suggests that a genetic basis for ageing might lie elsewhere in the cell, for example, the mitochondria (*e.g.*, Adelman *et al.* 1988; Ames and Shigenaga 1992; Kowald and Kirkwood 1996), or due to non-genetic damage involving cellular macromolecules (*e.g.*, Kowald and Kirkwood 1996; Lee *et al.* 1999).

A study done in parallel with the ageing study examined the effect of 70% dietary restriction on spontaneous *lacI* mutation in liver of ageing mice aged 6 and 12 months (Chapter 3). The data were equally as unexpected as those from the larger ageing study of mice maintained on an *ad libitum* diet, described in Chapter 2. Specifically, we found neither a decrease in spontaneous MF nor a change in spontaneous MS in liver of DR mice, compared with *ad libitum* fed controls. We had predicted that MF would be lower in DR mice, since 60-70% DR (or calorie restricted) animals live 30-50% longer than their *ad libitum* fed counterparts, and are generally healthier, with lower

incidences of diseases including cancer. Combined with the observation that DR mice have lower bodyweights and slower metabolism, it was believed for example that there would be less oxidative DNA damage, and therefore, MF would be lower in DR *versus ad libitum* fed mice. The data from DR mice, along with the observation that spontaneous MF only increased modestly in aging *ad libitum* fed mice (Chapter 2), indicated that DR was unlikely to have a significant impact on spontaneous MF and MS in ageing mice.[31]

The data from the ageing-diet studies described in Chapters 2 and 3 permitted us to determine, for the first time, reasonably accurate estimates of the rates of mutation in various tissues (liver, bladder, and brain), including those of the most prevalent spontaneous mutation, G:C→A:T transitions occurring at CpG dinucleotide sequences (Chapter 4). These rates were found to occur at different rates dependent upon the developmental stage of the tissue; furthermore, the MR in mice were slower than those in bacteria, but faster than those occurring in apes and humans (Table 4.2), in agreement with previous literature reports (*e.g.*, Britten 1986; Li *et al.* 1996; Li and Tanimura 1987). When the mouse, ape and human MR were plotted versus the age at sexual maturity (*i.e.*, the age at which the genes can be passed onto the next generation), a linear plot was obtained (Figure 9.1), suggesting that different MR have evolved among various taxonomic groups (Britten 1986). Additionally, Wilson and Jones (1983) and Wilson *et al.* (1987) noted that genome demethylation rates were inversely proportional to species lifespan. The implications of perturbations of MR, arising for example due to defective DNA repair pathways, with regard to mutagenic and carcinogenic processes remains to be evaluated.

Genomic DNA in mammalian cells undergoes demethylation, a progressive loss of 5-methylcytosine (5MC), as a function of age. Rates of demethylation reported in the literature (*e.g.*, Wilson *et al.* 1987; Hoal-van Helden and van Helden 1989; Mazin 1993) are several orders of magnitude larger than would be predicted based on hydrolytic loss of 5MC measured mutationally in Big Blue® mice as G:C→A:T transitions at CpG dinucleotide sequences (Chapters 2, 4). These paradoxical observations were resolved with the recent report of mammalian demethylases, which directly (non-mutationally) convert 5MC` to cytosine (Bhattacharya *et al.* 1999; Ramchandani *et al.* 1999).

Additional inferences (Chapter 4) were drawn upon consideration of the ageing data presented in Chapter 2. For example, it should be possible, eventually, to measure rates of DNA damage and repair, cellular proliferation and cellular death, to order to calculate MF *a priori*, for comparison

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[31] As noted in Chapter 3, however, DR is likely to significantly reduce the frequency of chemically-induced MF.

with observed MF. In Chapter 4, a semi-quantitative mutational model was described, which enables predictions of what would happen to MF if one or more of the factors is perturbed. Even in the semi-quantitative form, we can use Big Blue® mutational data to simultaneously solve pairs of equations for an unknown, allowing specific predictions to be made. Two examples were illustrated: (1) that the repair of G:T mispairs in DNA due to hydrolytic deamination of 5MC must be highly (~98.5–99.9%) efficient in adult mouse liver; and (2) that functional elimination of a mismatch DNA repair gene, *Msh2*, leads to a trivial change (~0.14% decrease) in overall DNA repair but a relatively large increase (about an order of magnitude) in observed spontaneous MF. The implications regarding mutation and cancer are obvious: for example, "mutator" phenotypes (Loeb 1991) might be attributable to small perturbations in the efficiency of DNA repair.

The theme of the influence of age and diet on spontaneous mutation in Chapters 2–4 was extended to include studies of mutations induced by mutagens that are naturally present in the human diet, and which were additionally known to be tissue, species and/or sex-specific animal carcinogens and suspected human carcinogens. The agents selected for study were AFB_1 (Chapter 5) and PhIP (Chapters 6–8). AFB_1, a fungal mycotoxin, causes liver tumors in rats and other species (but not mice) and is present as a food contaminant, particularly in regions of Africa and China. PhIP, present in cooked meats and fish, causes lymphomas in mice, and colon and prostate cancer in male rats, and mammary gland tumors (but much less frequently, colon cancer) in female rats.

The AFB_1 study (Chapter 6) was the first direct comparison of mutagenic specificity in an identical genetic construct in two different species (C57BL/6 mouse; F344 rat). As such, the study served as a validation study for the newly developed *lacI* transgenic rat, but also provided novel information regarding the specificity of AFB_1-induced mutation in the *lacI* transgene. For example, slightly fewer than half of AFB_1-induced mutations occurred at GCGG/CCGC sequences (Table 5.3; mutated nucleotides are underlined).

The study described in Chapter 6 was conducted to determine the mutational effects of PhIP in colon tissue from treated male and female rats. Due to the prevalence of PhIP-induced tumors in male but not female colon, it was predicted that female colon tissue would be relatively refractory to the mutagenic effects induced By PhIP. We were therefore surprised to find that MF were strongly induced and identical in both male *and* female colon, regardless of the previous observation (Ochiai *et al.* 1996) that colon PhIP-DNA adduct levels were identical in either sex. More surprising, however, was that following DNA sequence analysis that there was no significant difference in PhIP-induced MS in male and female rat colon. We therefore tentatively concluded, at least in

colon, that the sex-linked tumorigenicity induced by PhIP likely results from a stage in the tumorigenic progression to cancer sometime after the DNA damage/mutation stage.[32]

An important and novel discovery with regard to PhIP, a known rat prostate carcinogen, was the demonstration that PhIP is potently mutagenic in the rat prostate gland (Chapter 7). Indeed, these data represent the report of MF and MS from this organ. Although prostate cancer is the most prevalent cancer in men and the leading cause of death from cancer, very little is known about what causes prostate cancer. The data thus directly link PhIP-induced mutation and cancer in this organ, indicating that more attention needs to be directed towards understanding the importance of chemically-induced prostate cancer in humans.

The final chapter (Chapter 8) of the main body of this Dissertation described the extension of the PhIP *lacI* mutagenicity (Chapter 6) to include the *λcII* transgene from the same animals. This study served to validate the suitability of this transgene as a mutational target for PhIP-induced mutation, by comparing the data with that obtained from the *lacI* transgene. Additionally, since the DNA sequences are different in each gene, potentially important target sequences which were absent in the *lacI* transgene (*e.g.*, homonucleotide repeat sequences > 5 bp) could be evaluated. At first, it appeared that the specificity of PhIP-induced mutations differed considerably among the two transgenes; however, detailed analyses of the data led to the conclusion that PhIP was inducing similar patterns of mutation in either gene. The considerations (analyses) leading to this conclusion offer an paradigm for comparing mutational responses in different genes. Lastly, the observation (with the caveat that small numbers of mutants were involved) that frameshift mutations at homonucleotide repeat sequences appear to be more prevalent in male rat colon lead me to speculate that the sex-linked specificity of PhIP-induced colon cancer in the rat might be due to predisposition of male colon tissue to induced colon cancer, due to accumulation of spontaneous frameshift mutations at critical genes throughout the rat genome.

The various studies described above have at times resulted in unexpected data and conclusions (*e.g.*, no change in MS in ageing mice; no effect of DR on spontaneous MF or MS; no difference in PhIP-induced MS in colon of PhIP-treated male and female rats). Other recent studies have also resulted surprising results. For example, Dr. T. Skopek created a novel *lacI* transgenic mouse, *mrkII*, in which the CpG content in the *lacI* gene was reduced (Skopek *et al.* 1998). It was believed that this would result in a lower background of spontaneous *lacI* MF, by reducing the number of

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[32] Please note that in Chapter 8 the Author subsequently proposed a mutation-based explanation for the sex-linked difference of PhIP-induced colon cancer in rats.

G:C→A:T transitions at CpG dinucleotide sequences. Surprisingly, however, spontaneous MF the *mrkII* transgene remained the same as in the Big Blue® mice; MF increased at the remaining CpG sequences; furthermore, mutations began appearing at the CpG sequences which remained but which had heretofore not been observed to mutate in Big Blue® mice. In a different study, pretreatment of rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to treatment with AFB_1 resulted in chemoprotection by TCDD against aflatoxin B_1-induced mutation in female, but not male, Big Blue® rats (Thornton *et al.* 1999; Glickman *et al.*, manuscript in preparation[33]). Collectively, these data reflect the true biological complexity inherent in mammalian systems and demonstrate the power and utility of the Big Blue® assay to reveal gaps in our understanding of the mechanisms of mutagenesis and carcinogenesis, and suggest new avenues of investigation. This is perhaps one of the best indications that transgenic mutagenicity assays (as illustrated using the Big Blue® assay) have matured as useful tools for the *in vivo* investigation of fundamental mechanisms of mutagenesis.

9.3. Transgenic Mutagenicity Assays: Perspectives

During the past half dozen or so years, transgenic mutagenicity assays including the Big Blue® *lacI* transgenic assay have been remarkably successful, in terms of new and useful data generated. However, any method is subject to improvement, evolution, or replacement with newer methodology. One of the more serious limitations of current transgenic mutagenicity assays has been the considerable costs associated with materials and labor required to assay each chemical or mutagenic treatment. Unfortunately, these costs effectively limit these assays to well-funded laboratories, restricting their general availability. A second limitation of the current models is that considerable quantities of purified, high-molecular weight DNA is required for recovery of the transgene. Thus, entire smaller organs must be used (*e.g.*, prostate; germ cells) in order to obtain sufficient quantities of DNA. With larger organs (*e.g.*, liver) the reverse problem might apply - although not believed to be a problem generally, the possibility exists that examining mutations from pieces of a tissue might lead to a non-representative sampling of the mutations present within the tissue. Lastly, the homogenization of tissues prior to analysis effectively averages the mutational data, by virtue of the fact that mutated cells (as recovered λ phage) must be identified from a much larger pool of non-mutated cells (phage).

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[33] The Author assisted with the animal treatments and tissue dissections for this TCDD-AFB_1 study. Refer to Vita, at the end of this Dissertation.

Previously, it has been shown that mutations induced by *tris*(2,3-dibromopropyl)phosphate occur preferentially in a certain regions of mouse kidney tissue, which might account in part for the specific sites for tumor formation in this organ caused by this chemical (de Boer *et al.* 1998). Although this limited success was hampered by difficulties in obtaining enough material to complete the Big Blue® assay, the results demonstrate the need to consider subpopulations of cells within organs which might be more susceptible to mutation and cancer.

We, and others, anticipate the development of the "next generation" of transgenic mutagenicity assays, the ability to identify and recover mutations *in situ*, at modest cost. Ideally, this assay would permit the facile identification of mutated cells directly in place in whole or sectioned tissues, perhaps due to de-repression of genes which serve as the mutational target and which also function to regulate the expression of a gene leading to chromogenic changes in mutated cells (*e.g.*, β-galactosidase green fluorescent protein expression). Ideally, the assay would permit the detection not only of base substitutions, but also large deletions and chromosomal translocations.

The benefits of *in situ* mutagenicity assays are immediately obvious, including improvements in sensitivity and specificity for the detection of mutation. The ability to detect mutations *in situ* would also greatly improve the capability for identification of subpopulations of cells within organs which are more potentially susceptible to spontaneous or induced mutation (or genetic aberrations). *In situ* assays also have the potential to identify cell types which are directly involved in diseases, including cancer. *In situ* assays, by virtue of increased sensitivity, might also address in part the disparity between the acute exposures applied to the experimental animals used in the Big Blue® and other genotoxicity assays, *versus* the chronic occupational or environmental exposures (including those present in the diet) generally encountered by humans. Such a need is indicated by the discussion near the end of Chapter 7, of PhIP exposures in treated rats *versus* estimated average daily human exposures in the human diet.

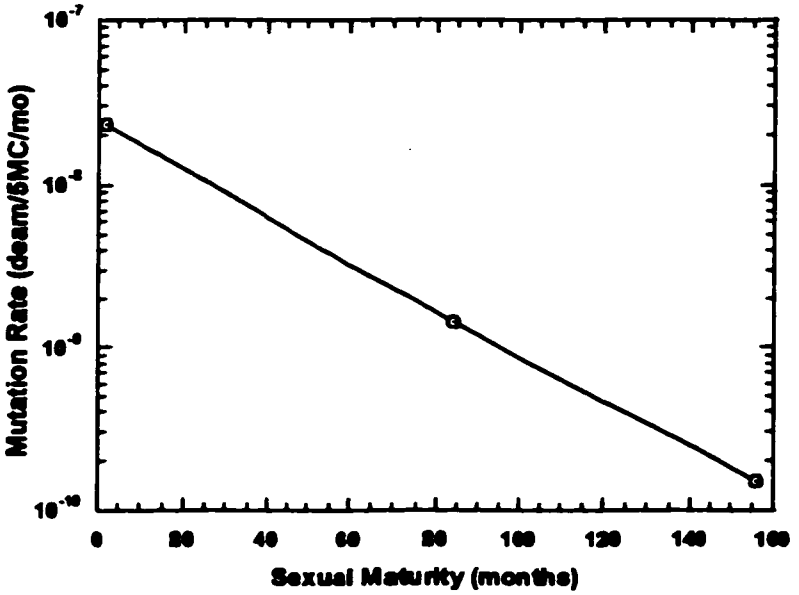

Figure 9.1. Mutation rates for G:C→A:T transition mutations occurring at CpG dinucleotide sequences (presumably, due to hydrolytic deamination of 5-methylcytosine) occurring in mice, apes and humans are plotted *versus* the age at sexual maturity (assumed to be approximately 2, 84 and 156 months in mice, chimpanzees and humans, respectively).

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APPENDICES

Appendix A. Benzo[*a*]pyrenediol-epoxide Induces Loss of Heterozygosity in Chinese Hamster Ovary Cells Heterozygous at the *Aprt* Locus

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Abstract

Benzo[*a*]pyrenediol-epoxide (BPDE), a metabolite of the ubiquitous environmental carcinogen benzo[*a*]pyrene (B[*a*]P), has been implicated as a point mutagen. However, as mutational events other than point mutations are also often associated with cancer, we have investigated whether BPDE can induce other classes of mutation. This was done by analyzing mutation at the *Aprt* and *Hprt* loci, both in hemizygous (D422) and heterozygous (D423) Chinese hamster ovary (CHO) cell strains. Southern blotting analysis indicated that BPDE is not an effective producer of either deletions or insertions in the hemizygous environment. The analysis of mutation in the *Aprt* heterozygote was done to investigate the frequency of loss of heterozygosity (LOH) events following BPDE treatment. Using PCR to produce an artificial restriction fragment length polymorphism in the functional *Aprt* allele, BPDE was found to induce LOH in about one-quarter of the mutants recovered. While the precise mechanism of this phenomenon remains obscure, it is likely to have important implications, since similar events involving homologous recombination in somatic cells may have an impact in tumorigenesis.

***Keywords*: *Aprt*; Benzo[*a*]pyrenediol-epoxide; *Hprt*; Loss of heterozygosity**

A.1. Introduction

There is substantial evidence that genome rearrangements are associated with cancer (Cairns 1981; Klein 1981; Pall 1981; Marx 1982; Wintersberger 1982). Furthermore, homologous mitotic recombination has been implicated as one component in the multistep process of tumorigenesis (Hansen and Cavanee 1988; Ponder 1988). For example, it has been demonstrated that mitotic recombination between genes on the human chromosome 13 results in reduction to homozygosity in retinoblastoma tumors. This was the first documented case to suggest that conversion of the normal allele was a critical event in oncogenesis (Cavanee *et al.* 1983). Numerous studies (Rommelaere and

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Miller-Faurrès 1975; Moore and Holliday 1976; Latt 1981; Wang *et al.* 1988) have shown that DNA damage can increase levels of homologous recombination. In particular, carcinogens have been shown to induce intrachromosomal recombination in yeast (Kunz and Haynes 1981; Schiestl *et al.* 1989), and *E. coli* (Quinto and Radman 1987), the frequency of which is dependent on the repair ability of the cell. It is thus reasonable to suppose that carcinogens might enhance recombination in mammalian cells. Using specifically designed systems of gene transfer (Liskay and Stachelek 1983; Lin and Sternberg 1984; Liskay *et al.* 1984; Rubnitz and Subramani 1986), it has been shown that carcinogens indeed induce homologous recombination in mouse L cells (Liskay *et al.* 1984; Wang *et al.* 1988).

The major goal of this study was to characterize events other than point mutations which can be induced by (+/–)-anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE). This kind of study has been greatly facilitated by the advent of the polymerase chain reaction (PCR) (Saiki *et al.* 1985), which permits rapid detection of DNA sequence variations. Here we report the analysis of mutation at the adenine phosphoribosyltransferase (*Aprt*) locus of Chinese hamster ovary (CHO) cells in a heterozygous cell strain (D423; Simon *et al.* 1982; Simon *et al.* 1983). The mutation in the non-functional allele of D423 has recently been identified as a G:C → T:A transversion at position 52 (aspartic acid to tyrosine amino acid substitution) (Ward *et al.* 1990). Taking advantage of this information, PCR primers were constructed to produce an artificial restriction fragment length polymorphism (RFLP) in the functional allele (creating a novel *SalI* site), and not in the non-functional allele. Consequently, restriction enzyme digestion of PCR-amplified mutant alleles, followed by analysis on acrylamide/urea gels, permit the quantification of LOH events at the *Aprt* locus in CHO cells (Ward *et al.* 1990).

A.2. Materials and Methods

A.2.1. Cell strains

The cell strains used in this study, D423 and D422, were isolated from CHO pro^- (Bradley and Letanovec 1982). D422 is hemizygous (+/0) for the *Aprt* gene, whereas D423 is heterozygous (+/–) (Simon *et al.* 1982 1983). Cells were grown in 25-cm^2 tissue culture flasks (Nunc), in alpha minimal essential medium (MEM) (Gibco) supplemented

with 5% fetal bovine serum, 5% inactivated horse serum (Gibco), and 0.01% penicillin/streptomycin (Sigma). Prior to mutagenic treatment, cells were passaged through medium containing aminopterin, adenine, and thymidine (AAT), in order to eliminate any pre-existing spontaneous mutants.

A.2.2. Mutagenesis

Independent exponentially growing cell cultures (3 x 10^6 cells), seeded from 1000 cell inocula, were exposed for 1 h to 0.7 μM of the racemic mixture of anti-BPDE (National Cancer Institute repository, lot # CSL-85-008-09) in alpha MEM containing dialysed fetal calf/inactivated horse serum. BPDE was dissolved in anhydrous ethanol (Aldrich). The final concentration of alcohol to which cultures were exposed was 0.1%. The length of exposure was one hour at 37°C in the dark. After exposure, the cells were washed with PBS, trypsinized, and plated either for survival, or subsequent mutant selection.

A.2.3. Mutant isolation

Molecular analysis was carried out on mutants selected from the heterozygous cell strain. We have already examined the nature of mutations produced by BPDE in the hemizygous D422 strain (Mazur and Glickman 1988). Each independent D423 culture was split into two plates immediately following treatment. After a 5 day phenotypic expression period, the cells were challenged with medium containing 0.4 mM 8-azadenine (8-AA), which is toxic only to cells containing a functional *Aprt* gene. Two sets of *Aprt*$^-$ mutants were collected, corresponding to 10 and 21 day growth periods in 8-AA. Mutation induction curves derived from data obtained for each set revealed that the different growth periods had little effect on mutation frequency (data not shown). To ensure the independence of individual mutants, only one mutant per plate was selected for subsequent molecular analysis.

A.2.4. Southern blotting analysis

The entire *Aprt* gene is flanked by *Bgl*II and *Hind*III restriction sites separated by 4.3 kb (Lowy *et al.* 1980). Therefore, approximately 10 μg of genomic DNA was cut with *Bgl*II/*Hind*III, and Southern analysis performed as described previously (Grosovsky *et al.* 1986). The probe (0.5 μg), a 3.8 kb genomic *Bam*HI fragment (Lowy *et al.* 1980), was

labeled by random priming (Boehringer Mannheim) with ^{32}P (Amersham) (the specific activity of the probe was > 10^8 cpm/μg).

A.2.5. PCR amplification of D423 mutants and LOH analysis

One μg of genomic DNA was used to amplify mutant *Aprt* alleles. The reaction volume (100 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM dNTP's (Pharmacia), 10 pmol of each primer, and 1.0 unit of ampliTaq polymerase (Cetus). Thirty amplification cycles were performed at 94°C for 2 min (denaturation), 55°C for 2 min (annealing), and 72°C for 5 min (extension), using a Perkin-Elmer Cetus thermocycler. The two primers used for amplification were R31, 5′-CAGCGCATCCGCAGTTTCGTC–3′ and P232, 5′–ACCCCGCCTCCTTCCGAGCT–3′ which anneal at the 5′ and 3′ end of the *Aprt* gene, respectively (Figure A.1). Primer R31 contains two mismatches and results in the creation of a *SalI* restriction site in the functional *Aprt* allele following amplification. Alternatively, no artificial RFLP is formed following amplification of the non-functional allele (containing a G:C → T:A transversion at position 52).

Following PCR amplification, 10 μl of reaction product was cut with six units of *SalI* (Pharmacia) in a total reaction volume of 40 μl (containing 200 mM potassium glutamate, 50 mM Tris-acetate (pH 7.6), 20 mM magnesium acetate, 100 μg/ml bovine serum albumin, 1 mM 2-mercaptoethanol, and 200 μM of each dNTP), and incubated at 37°C for 2 h. In order to label the amplified alleles, 2 pmol of [α-^{32}P]dATP (spec. act. > 400 Ci/mmol; Amersham) and 2 units of Klenow fragment of *E. coli* DNA polymerase I (Pharmacia) were added directly to the reaction tube and incubated at 37°C for 30 min. Sequencing dye (99% formamide, 5 mM EDTA, and 0.1% bromophenol blue) was then added to stop the reaction, and samples were denatured and loaded on an 8% acrylamide/7M urea sequencing gel. The observation of only one band on the resulting autoradiograph provides evidence for a LOH event, while two bands indicate retention of the heterozygous state.

A.3. Results

Figure A.2 illustrates the survival of the hemizygous CHO D422 and the heterozygous CHO D423 cell lines following exposure to BPDE. The heterozygous strain

D423 was much more sensitive than the hemizygous strain D422. Mutation induction data (Figure A.3) is expressed as the frequency of induced mutation as a function of cell killing. This method of comparing induced mutation has been previously suggested to be the most appropriate when comparing two different cell lines (Smith and Martignoni 1976). The X-linked gene, *Hprt*, was used as an internal control, since only one functional allele is present. Mutation induction rates at the *Hprt* locus were similar in both the *Aprt* hemizygous (D422) and heterozygous (D423) *Aprt* cell lines. At the same survival, the heterozygous strain yielded more mutations than the hemizygous strain.

Eighty independent BPDE-induced *Aprt*$^-$ mutants were isolated from the heterozygous D423 cell line, as described in the Materials and methods. Forty of these were selected after 10 days growth in medium containing 8-AA, while the rest were selected after 21 days growth in 8-AA. It was hypothesized that the longer growth period would allow for recovery of putative slow growth mutants. However, we observed no differences in the growth rates on 20 randomly picked *Aprt*$^-$ mutants.

Genomic DNA was isolated from all 80 *Aprt*$^-$ D423 derivatives. Southern analysis was performed in order to detect major alterations such as deletions or insertions. Some typical results are illustrated in Figure 4. No major genomic alterations were found in any of the 80 mutants recovered in the heterozygous D423 cell line. This result is similar to that obtained by the analysis of 57 *Aprt*$^-$ mutants recovered following BPDE treatment in the hemizygous D422 cell line (Mazur and Glickman 1988). Mutation by BPDE presumably occurred as a result of point mutation or microdeletion/insertion. However, complete deletion of the functional *Aprt* allele could not be ruled out by this analysis.

PCR technology permits the analysis of the mutations at the heterozygous *Aprt* locus. Using an artificial RFLP generated by PCR we were able to study the frequency of LOH induced by BPDE. Figure A.5 illustrates the analysis of 40 of the BPDE-induced mutants recovered in the D423 cell line. We found that BPDE is not only capable of inducing point mutations (Mazur and Glickman 1988), as nine of the 40 mutant strains manifested autoradiographic patterns consistent with LOH events at the *Aprt* locus. We note (this study) that mutations induced by BPDE have not been recovered at position 52 in the hemizygous D422 cell line, which corresponds to the G:T polymorphic site in the heterozygous D423 cell line (Figure A.1; Ward *et al.* 1990).

A.4. Discussion

Our earlier studies on BPDE mutagenesis were conducted at the endogenous *Aprt* locus in the hemizygous CHO strain D422 (Mazur and Glickman 1988). This limited the classes of rearrangements that could be recovered, because large deletions and some rearrangements may not be viable due to the possible presence of essential genes within the region of hemizygosity (Bradley *et al.* 1988; Harwood and Meuth 1995; Hutchinson 1995). In this study, we have analyzed BPDE mutagenesis in an *Aprt* heterozygote. This should make it possible to recover virtually all classes of mutation. Interestingly, 22.5% (nine of 40) of the mutants induced following BPDE treatment in this strain showed LOH, although the precise mechanism of this event remains unknown. Loss of heterozygosity often accompanies the loss of known tumor suppressor genes that are associated with human malignancies including breast cancer (Kirchweger *et al.* 1994; Zhuang *et al.* 1995), gliomas (Coleman *et al.* 1994), leukemias (Coleman *et al.* 1994), melanomas (Fountain *et al.* 1992), lung cancer (Olopade *et al.* 1993), mesotheliomas (Cheng *et al.* 1993), and bladder cancer (Stadler *et al.* 1994). Therefore, it is significant that the potent carcinogen BPDE not only induces point mutations, but also acts *via* an alternative mutational pathway to yield LOH.

The heterozygous strain D423 was much more sensitive than the hemizygous strain D422 to the cytotoxic effects of BPDE (Figure A.2). While we have no information on why that might be, it appears that the two CHO cell lines differ in their response to BPDE. Inspection of the survival curves in Figure A.2 show that the curve for the D422 strain has a shoulder at lower doses of BPDE, suggesting perhaps an initial repair capacity not found in the D423 cells. As well, the slopes of the two curves differ, suggesting differences in survivability upon exposure to BPDE.

Mutation induction by BPDE was analyzed both in hemizygous (D422) and heterozygous (D423) cell lines, at the *Aprt* and *Hprt* loci. The *Hprt* gene served as an internal control for the *Aprt* experiment. As illustrated in Figure A.3, the kinetics of *Hprt* mutation induction, when plotted as a function of cell viability, was nearly identical in both strains. *Hprt* is X-linked, and thus functionally hemizygous. Consequently, gross deletions or non-disjunction may be lethal, because of the elimination of adjacent

essential genes (Evans *et al.* 1986; Klinedinst and Drinkwater 1991). One observation consistent with this model is the report that mutation induction by X-rays is considerably lower at the *Hprt* than at the heterozygous *Aprt* locus, despite the fact that *Hprt* offers a much larger potential target size (Bradley *et al.* 1988). In the case of BPDE, however, the opposite has been observed. Mutation frequencies were considerably higher at the *Hprt* rather than the *Aprt* locus. This was true in both the hemizygous and heterozygous strains. This difference suggests that factors other than the size of the gene, such as the sequence specificity of the target, and the accessibility of the target to DNA damage and/or repair, may also play an important role in mutation fixation.

At the same survival, the heterozygous strain D423 yielded slightly more *Aprt* mutations than the hemizygous strain D422 upon treatment with BPDE (Figure A.3). This may reflect the nature of mutations occurring at the heterozygous locus *versus* the hemizygous locus, as described above (Evans *et al.* 1986; Klinedinst and Drinkwater 1991).

In a previous study, we found that 43% (16 of 37) of the spontaneous *Aprt*$^-$ mutants arising from the D423 cell line had undergone a LOH event (Ward *et al.* 1990). However, when D423 cells are treated with 0.7 μM BPDE, the frequency of *Aprt*$^-$ mutants arising by LOH drops to 22.5% (nine of 40 mutants), as shown in this study. A similar observation was made by Kubo *et al.* (1994), who observed that in the rat, 60% (six of 10) of spontaneous renal carcinoma mutants showed loss of the wild-type allele, whereas none (zero of 9) of *N*-ethyl-*N*-nitrosourea-induced renal carcinomas had allelic loss. Thus, chemically-induced mutations in genes might reflect a shift toward lower frequencies of LOH at endogenous alleles, with an accompanying increase in base substitution and other (frameshift; small insertions/deletions) mutations.

While it has been demonstrated that many carcinogens can induce recombination in yeast and bacteria (Kunz and Haynes 1981; Quinto and Radman 1987; Schiestl *et al.* 1989), less is known about this response in mammalian cells. We report here, that BDPE is not only capable of inducing point mutations, but may also act *via* an alternative mechanism, possibly mitotic recombination or multilocus deletion, to produce LOH events.

Several mechanisms have been postulated to explain how DNA damaging agents might stimulate recombination. The simplest model suggests that bulky adducts perturb the normal nucleosomal structure and produce short stretches of non-protected DNA. These regions are then susceptible to nucleases, which then stimulate recombination (Lieberman *et al.* 1979; Cleaver 1985). In addition, the bulk of BPDE damage undergoes DNA repair, a process which involves single-strand breaks.

Other factors might contribute to LOH, including sequence effects. It is suspected that a large spontaneous deletion found in a CHO cell line heterozygous for the *Aprt* locus resulted from an illegitimate recombination event involving an overlapping tetranucleotide sequence (Dewyse and Bradley 1991). Comparisons with sequences of other deletions at various loci revealed a number of similarities, including a motif found within 6 bp of the upstream breakpoint of the *Aprt* deletion and breakpoints at eight of 21 previously described short deletions at the CHO *Aprt* locus. Homology also existed between the downstream breakpoint and breakpoints of retinoblastoma gene deletions (three of six cases) and also a 20-bp stretch of an Alu sequence in which breakpoints at the low-density lipoprotein receptor locus have been shown to cluster.

One might also speculate that some of the LOH events observed in the *Aprt* gene may involve repeat sequences, which are often associated with small deletions, insertions, and frameshifts (Skandalis *et al.* 1992). Direct repeats are a feature of a number of recombination, replication, or repair-based models of deletion mutagenesis. A putative consensus sequence (TGA/GA/GG/TA/C) for deletion hotspots in genes has been proposed (Krawczak and Cooper 1991). This sequence, which is frequently found at sites of spontaneous deletion in the hamster *Aprt* gene and many larger human gene deletions and translocations, acts as an arrest site for human polymerase α during DNA replication.

In conclusion, we have found the chemical carcinogen, BPDE, to be capable of stimulating mutational events other than base substitutions or frameshifts. While the precise mechanism by which D423 undergoes conversion to homozygosity remains to be resolved, the ability of BPDE to stimulate this response may contribute to its high carcinogenic potency.

Acknowledgments

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A.5. References

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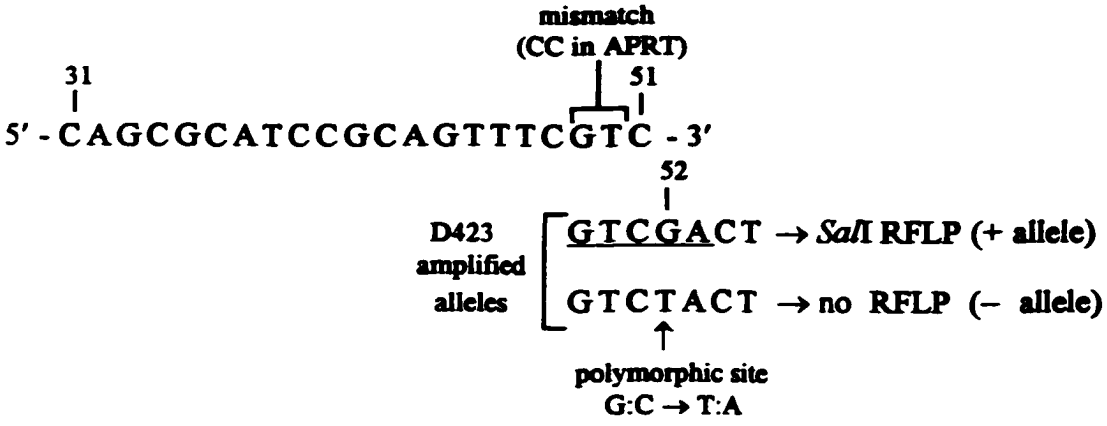
Primer R31



Primer 232

Figure A.1. PCR primers used to create an artificial RFLP in the functional *Aprt* allele in the heterozygous CHO cell line D423.

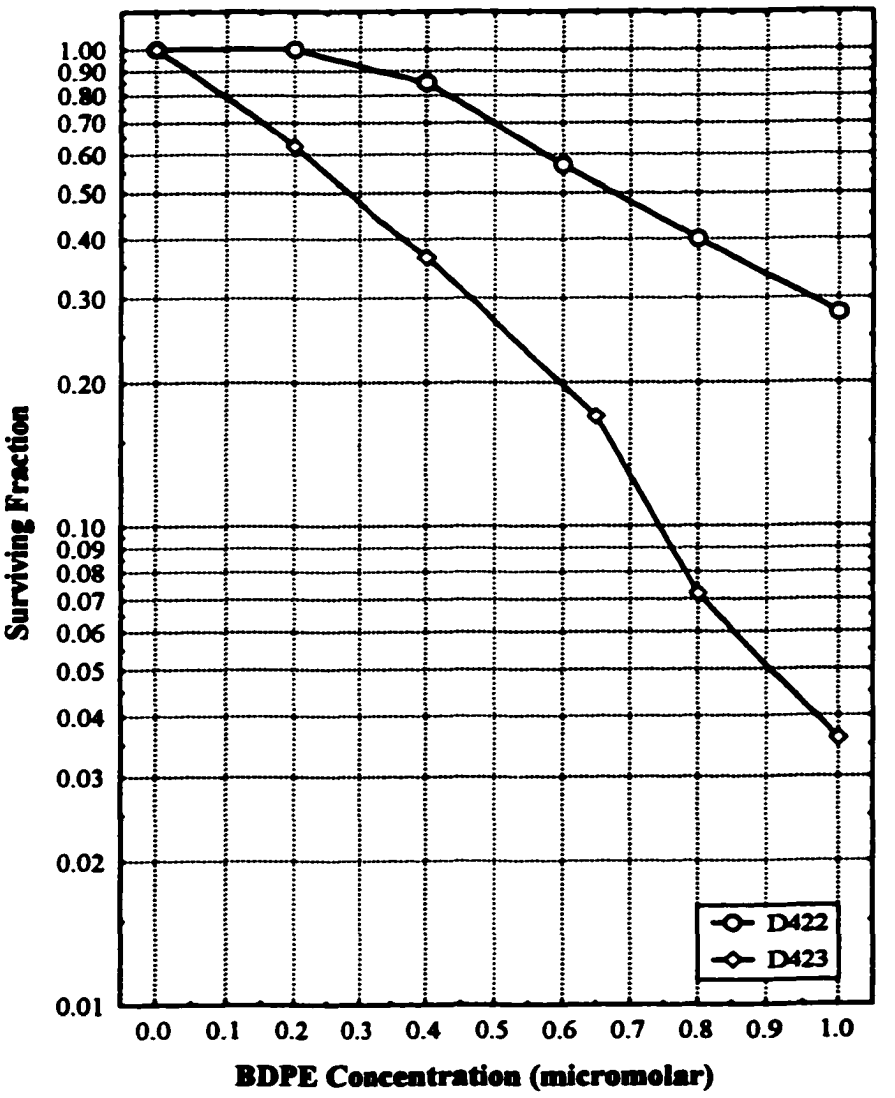


Figure A.2. Survival of the hemizygous (D422) and heterozygous (D423) *aprt* cell lines following exposure to BPDE. Each point represents the mean of at least two independent experiments.

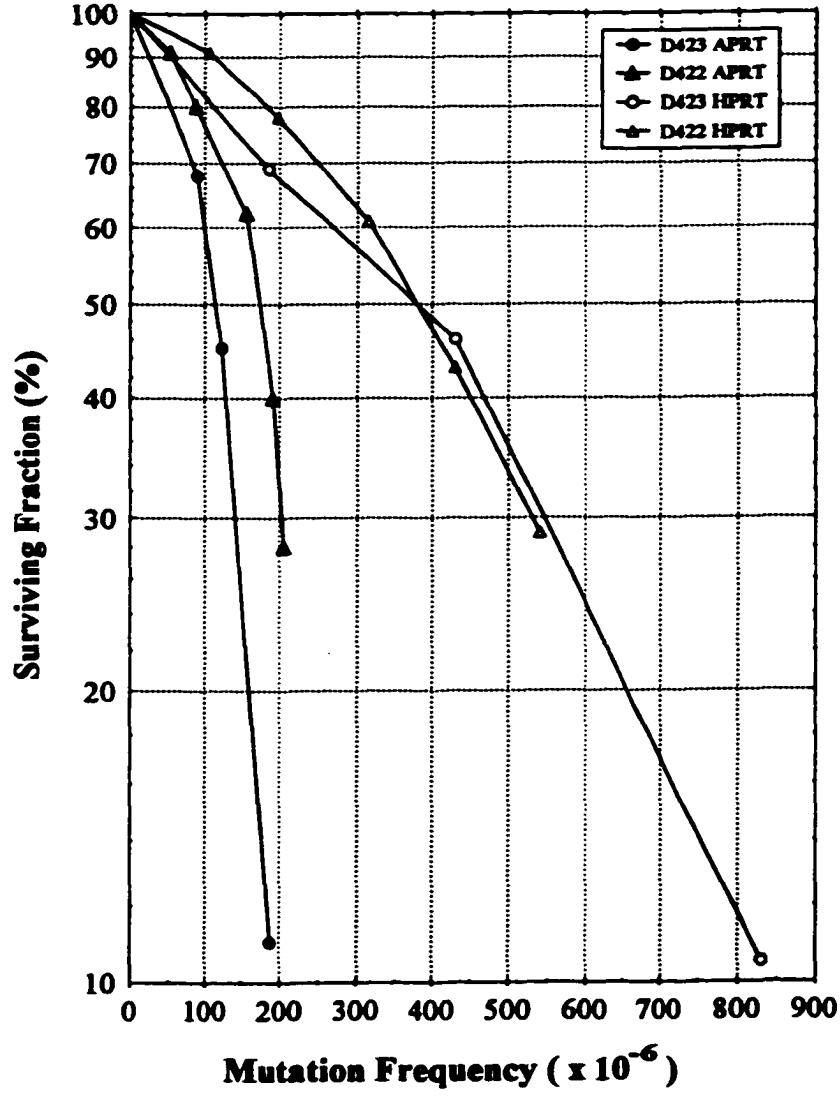


Figure A.3. The frequency of induced mutation at the *Hprt* and *Aprt* loci by BPDE as a function of cell killing in the hemizygous (D422) and heterozygous (D423) cell lines.

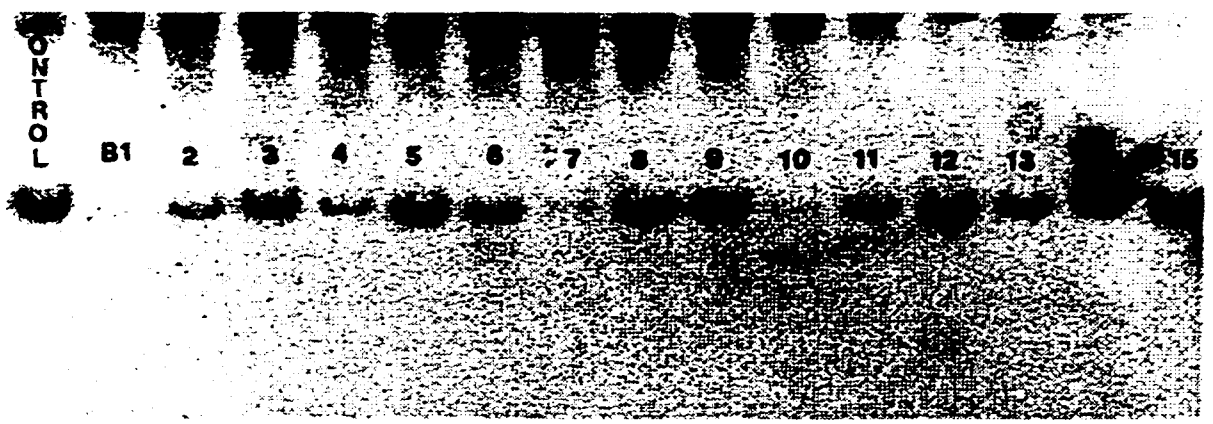


Figure A.4. Southern analysis of representative BPDE-induced *Aprt* mutations recovered in the heterozygous *Aprt* D423 cell line. 10 μg of genomic DNA was digested with *Bgl*II/*Hind*III, and Southern blotted. The probe used was a 3.8 kb *Bam*HI fragment of pH*Aprt* (Lowy *et al.*, 1980).

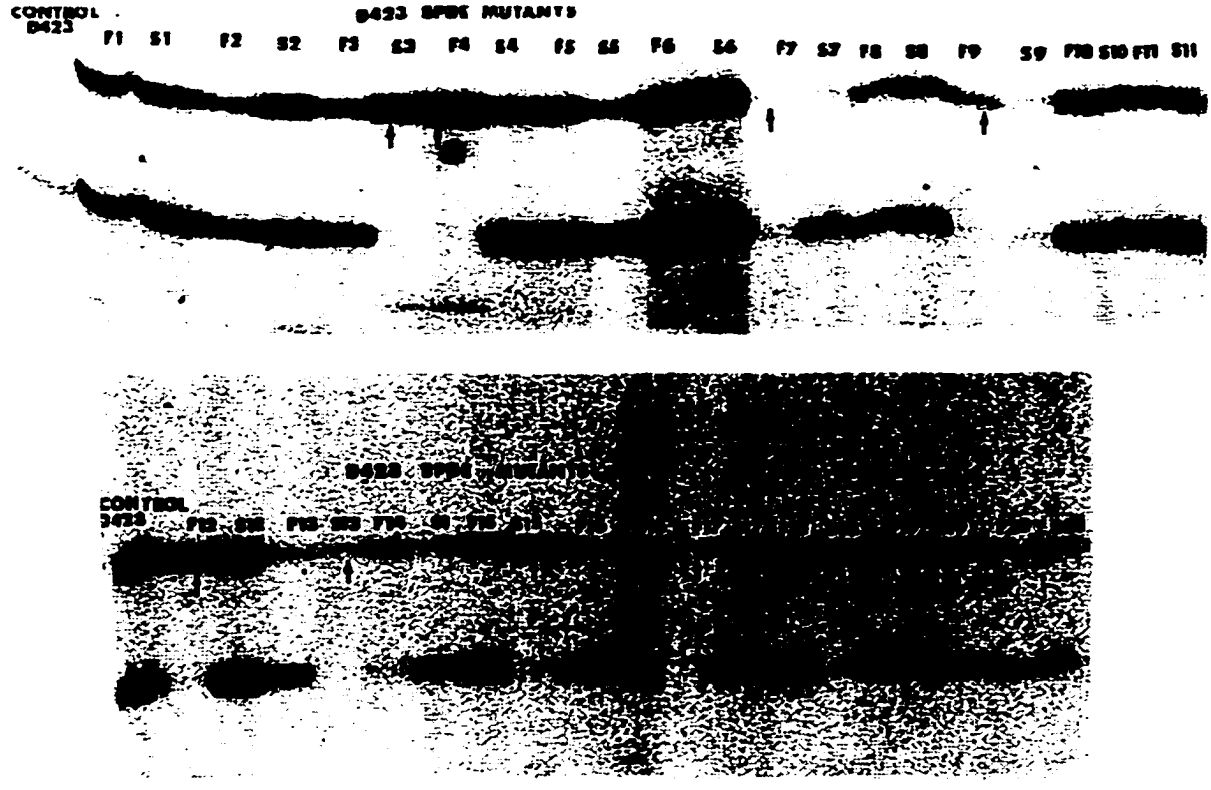


Figure A.5. Analysis of 40 of the BPDE-induced mutant D423 cell lines. 'F' denotes mutants with a 10 day expression period, and 'S' denotes mutants with a 21 day expression period. Nine mutants, which have undergone conversion to homozygosity of the mutant *Aprt* allele, are indicated by one fragment (the arrows on the photograph), since both alleles cut with *Sal*I. All analyses were repeated at least once, in order to positively confirm homozygosity.

Appendix B. Construction and Characterization of *E. coli* Strains for the Direct Selection of *lacI* Bacteriophage Recovered From the Big Blue® Assay

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B.1. Introduction and Background

A method for direct selection of *E. coli lacI* mutants has been described (Lundberg *et al.* 1993) for use with the Big Blue® *lacI* mutational assay (Kohler *et al.* 1991a). Briefly, packaged λLIZ/*lacI* phage particles rescued from transgenic rodent chromosomal DNA are used to infect *E. coli* host cells encoding the λ cI^+ repressor gene, resulting in lysogenic cells. Functional β-galactosidase[36] is produced in cells infected with *lacI* mutant phage, by selectively allowing bacterial growth and colony formation on lactose minimal media. Cells infected with wildtype *lacI*-bearing phage will not grow under these conditions.

The Big Blue® direct selection assay offers the potential of considerable savings in labor and material costs, compared with the non-selective plaque color assay. However, the direct selection assay is currently compromised by the steady accumulation of mutant plaques, which results in an increase in mutant frequency (MF) with increasing time of incubation of the agar plates. The choice of a defined plating time is not a solution to this problem, due to the variability in the rate of appearance of mutant plaques, particularly during the early stages of the assay. Furthermore, exploratory investigations of the unmodified direct selection assay in our laboratory revealed that extended incubation times (days) were required before bacterial colonies became visible on the plates, and that the rate of appearance of these colonies increased dramatically as the plate incubation times were extended (G. Stuart, unpublished data). The direct selection mutant frequency (MF) curves were similar in appearance to plots of the reversion frequencies of (+)1 frameshift *lacZ* plasmids in adaptive mutation studies in stationary (non-growing) cells

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[36] β-galactosidase (LacZ) activity results from α-complementation of the phage-encoded $LacZ_\omega$ and bacterial-encoded $lacZ_\Delta$ protein fragments (Langley *et al.*, 1975).

(*e.g.* Cairns and Foster 1991), suggesting a possible adaptive mutation effect in the *lacI* direct selection assay. A second mechanism which could more likely account for the dramatic increase in $LacZ^+$ mutant colonies with extended plate incubation time is the appearance of *ebg* (evolved β-galactosidase) cells. The wildtype *ebgA* gene of *E. coli* encodes a virtually inactive β-galactosidase, but that gene has the potential to evolve sufficient activity to replace the *lacZ* gene for growth on the β-galactoside sugars lactose (Hartl and Hall 1974; note also Figure 1 in Hall 1997).

Attempts to modulate the appearance of late-occurring mutants in the assay by biochemical additions (*e.g.*, α-methylglucoside, a competitive inhibitor of glucose uptake; Ambrose and MacPhee 1998) had limited success. Introduction of the *dnaE915* antimutator gene (Schaaper 1993) into the SCS-8cI^+ host strain also had no significant effect on the direct selection assay.[37] Finally, the assay was found to be complicated by the presence of an apparently wildtype *lacI* gene in the SCS-8cI^+ host strain (Gu *et al.* 1994). The presence of a *lacI* gene in the SCS-8cI^+ host strain was confirmed by PCR analysis (G. Stuart, unpublished data).

To circumvent the difficulties encountered with the SCS-8cI^+ strain, we ("Glickman laboratory:" G. Stuart) initiated the construction of a novel *E. coli* direct selection strain, for use with the assay described by Lundberg *et al.* (1993).

B.2. Strain Construction

The starting strain for the construction of the new direct selection strain was XL1-Blue MR (Figure 1; Table1; Bullock *et al.* 1987; Jerpseth *et al.* 1992). The presence of the *lacI* gene in this strain was confirmed by PCR analysis (G. Stuart, unpublished data); as well, the strain was chloramphenicol sensitive and UV^s. To facilitate genetic manipulations, XL1-Blue MR was transformed with plasmid pYG630, which encodes the $recA^+$ and chloramphenicol resistance genes.[38] The resulting cam^r, UV^r strain was named CEH1. The entire *lac* operon in CEH1 was deleted by an interrupted mating with Hfr strain SH210 (Δ(*argF-lac*)U169::Tn*10*(tet^r)) (Schweizer and Boos 1983), generating the

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[37] Refer to Table B.1 for a list of strains and genotypes.

[38] pYG630 was generously provided by Dr. T. Nohmi, who indicated that he obtained the plasmid from Dr. W.D. Rupp.

tetracycline-resistant strain CEH2. Both SH210 and CEH2 were Lacl$^-$ by PCR analysis, and Lacl$^-$ (white colonies) on Lactose-MacConkey plates. CEH2 was also characterized as being λ^s.[39] The wildtype λ *cI*$^+$::Tn*5*(kan^r) allele was transferred into CEH2 using a P1*vir*(SCS-8*cI*$^+$) lysate, generating strain CEH3. CEH3 was infected with wildtype λLIZ phage and spread on LM (L medium + $MgSO_4$) plates which were incubated overnight at 30°C and 37°C. Normal-appearing bacterial lawns formed at both temperatures, with no evidence of plaque formation, indicating establishment of lysogeny. Plasmid pYG630 was cured from (uninfected) CEH3 using novobiocin, an inhibitor of DNA gyrase subunit B (Wolfson *et al.* 1983), generating strain CEH4. The absence of pYG630 in CEH4 was confirmed by the cam^s UV^s (*recA*$^-$) phenotype. Finally, to enable *lacZ* α-complementation with the incoming λLIZ$_\alpha$/*lacI* phage, CEH4 was transformed with the low copy number (~6-7 copies/cell) plasmid pClone4 [*lacZ*ΔM15 *lacY lacA cat* (cam^r)],[40] generating the novel direct selection strain CEH5. CEH5 was confirmed to be Lacl$^-$ by PCR analysis.[41]

Since titers of λLIZ phage were found to be approximately 1.5-fold higher on XL1-Blue MR than on SCS-8, an XL1-Blue MR-based titer strain (isogenic with CEH5) was also constructed (Figure B.1). Plasmid pYG630 was cured from strain CEH2 using novobiocin, generating titer strain CEH6 (XL1-Blue MR Δ(*lac*)).

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[39] Hfr strain SH210 is λ^r (G. Stuart, unpublished data). Since SH210 transfers DNA clockwise originating at 12.20′, there was a slight possibility that the transfer might have extended beyond the λ receptor gene *lamB* (91.4′).

[40] Two α-complementing plasmid strains were received from M. Dycaico (Stratagene) as bacterial streak plates, labeled Clone1 and Clone4. The plasmid prepared from strain Clone4 was named pClone4.

[41] pCloneH was constructed at Stratagene (P. Kretz) by incorporating a *lacZ*ΔM15 *lacY lacA* PCR fragment, which excluded *lacI*, into pLG339 (Stoker *et al.*, 1982). The kan^r gene in pCloneH was replaced with *cat* generating pClone4 (cam^r). pLG339 is a low copy number plasmid, present at ~6-7 copies/cell.

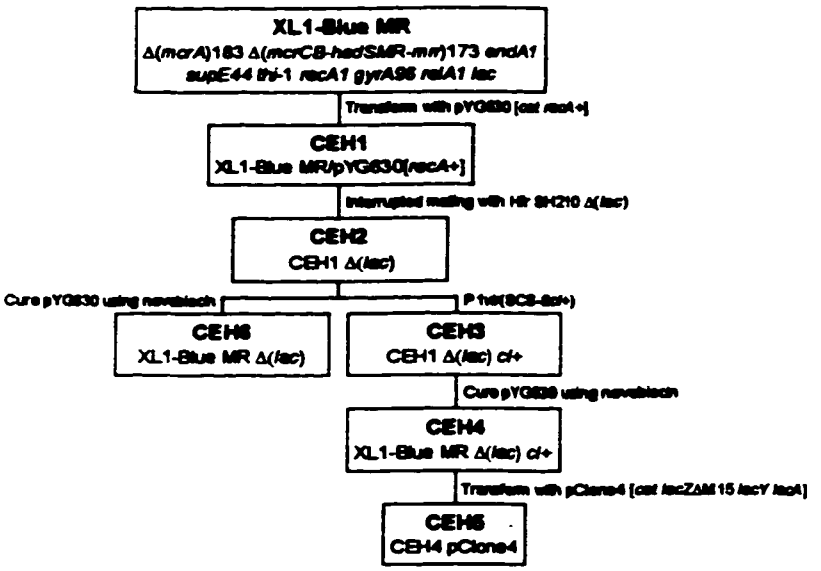

Figure B.1. Outline of the bacterial strain constructions.

Table B.1. List of *E. coli* strains and plasmids

Strain (Plasmid)	Genotype	Source
SCS-8cI^+ [a]	*recA1 endA1 mcrA* Δ(*mcrCB-hsdRMS-mrr*) Δ(*argF-lac*)U169 ϕ80d*lacZ*ΔM15 Tn*10*(tet^r) cI^+ Tn*5*(kan^r)	Stratagene
SH210 [b]	*garB10 fhuA22* Δ(*argF-lac*)169 *zai-736*::Tn*10* *phoA8*(Δ) *ompF627*(T_2^R) *fadL701*(T_2^R) *relA* *glpD3 glpR2*(Const) *pit*-10 *spoT1 mcrB9999* *creC510* λ^r	CGSC [c]
XL1-Blue MR	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi*-1 *recA1 gyrA96 relA1 lac* λ^s	Stratagene
pClone4	*lacZ*ΔM15 *lacY lacA cat*(cam^r) plasmid	Stratagene
pI24	*gal*$^+$ amp^r	Dr. S. Garges
pYG630	*cat*(cam^r) *recA*$^+$ plasmid	Dr. T. Nohmi

[a] There appears to be some confusion over the SCS-8cI^+ genotype. Lundberg *et al.* (1993) state that the λ cI^+ gene was integrated into the chromosome of *E. coli* SCS-8 using a mini-Tn*5* system. Patti Kretz (*pers. comm.* to Dr. Glickman) indicated that the parent strain for SCS-8cI^+ was Gibco BRL® strain DH5αMCR: DH5αMCR → SCS-8 (*sup*° tet^r) → SCS-8cI^+ (tet^r kan^r). The genotype of DH5αMCR includes markers (F$^-$ *deoR thi-1 supE44* λ^- *gyrA96 relA1*) not listed in the genotype of SCS-8cI^+.

[b] Hfr strain, point of origin 2A, clockwise at 12.20′. SH210 is used to transfer (introduce) the *lac* deletion.

[c] Abbreviations: CGSC, *E. coli* Genetic Stock Center, Yale University; Const, constitutive; T_2^R, phage T_2-resistant.

B.3. Initial Results With the New Direct Selection Strain CEH5

In a preliminary test of direct selection using the newly-constructed strain, packaged λLIZ phage and CEH5 cells were mixed and incubated at room temperature (21-22°C) for 30 minutes. Aliquots of the infection mixture were then added to 3 ml molten 0.7% w/v agarose (55°C, containing 18 µl 250 mg/ml X-gal in *N,N*-dimethylformamide, 50 µl 20% w/v casamino acids, 13.6 µl 18.4% w/v phenyl-β-D-galactoside[42] (P-gal)) and immediately poured onto M9-minimal medium (MM) plates (8.5 cm diameter; approximately 22 ml agar media) supplemented with thiamine (5 µg/ml). In parallel experiments (not shown), packaged phage were also plated on M9-lactose thiamine plates (12.5 µl 20% w/v lactose was substituted for P-gal), as were pure stocks of wildtype, CM0, CM1, CM2, and CM3 λLIZ phage. After the top agarose layers had solidified, the plates were incubated in the dark at 37°C in sealed incubator.[43] Colonies were observed on the plates soon after plating (approximately 17 hours), and notably, plate colony counts reached a plateau after approximately 4-5 days (Figure B.2), in contrast with previous results with the SCS-8*cI*$^+$ host strain.

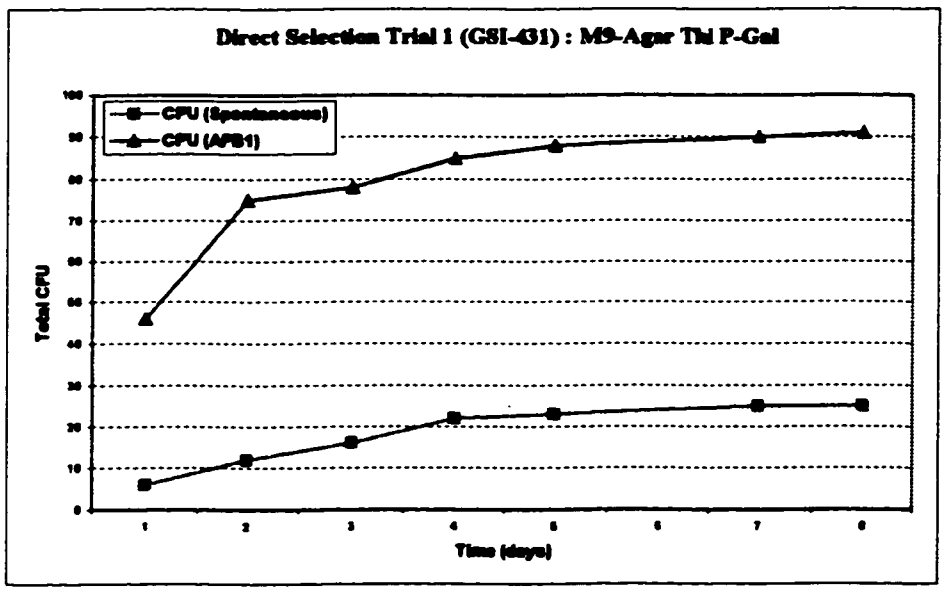

Figure B.2. Preliminary results with the CEH direct selection strain.

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[42] Unlike lactose, P-gal is a non-inducing substrate for β-galactosidase, selectively permitting growth of *lacI* mutants (Smith and Sadler, 1971).

[43] An CO_2 incubator (without CO_2) was used; dehydration of the plates was negligible with extended incubation at 37°C.

Initial results using CEH5 in the direct selection assay with packaged liver DNA from untreated mice and AFB_1-treated rats were encouraging (Figure B.2). Based on packaged phage titers on SCS-8, each plate received approximately 20,650 PFU (~365 PFU/cm^2) from untreated liver DNA, and approximately 14,650 PFU (~260 PFU/cm^2) from AFB_1-treated liver DNA. Unexpectedly, MF from the direct selection method (control: ~40 x 10^{-5}; AFB1: ~207 x 10^{-5}) were several-fold higher than MF determined in the color plaque assay (control, ~4.8 x 10^{-5}; AFB_1, ~48.6 x 10^{-5}). Blue colonies arising from the CM mutant phage stocks were clearly visible on the M9-lactose plates (and in subsequent experiments, on M9-P-gal plates), while no colonies were observed on the plates that received only wildtype λLIZ phage. The intensity of the blue color of the colonies was CM1 > CM0 ≈ CM2 ≈ CM3.

B.4. Anomalies and Difficulties Encountered

Subsequent and extensive direct selection experiments yielded results qualitatively similar to those shown in Figure B.2, with some variability. In the course of these experiments, several problems and anomalies were noted, the most important of which are itemized below. It is suspected that some of these effects are partly due to the genetics and physiology of the (XL1-Blue MR-derived) direct selection strain, while other effects might result from the genetics of the λLIZ (Big Blue®) shuttle vector. Particularly, the relaxed nature (*relA1*: the "stringent response;" reviewed by Cashel *et al.* 1996) of the strain was of considerable interest. While the bacterial-derived adverse effects on the assay are potentially resolvable, those involving the shuttle vector are less likely to be resolved satisfactorily, should they persist. Despite these observations, we remain cautiously optimistic regarding the future potential development and applicability of the direct selection method. The data also suggest biological factors to consider, should additional shuttle vector transgenes be constructed for use with the *lacI* direct selection assay.

B.4.1. Background haze on the plates

It was noted that as the density of PFU added the plates was increased, a diffuse, blue, slightly heterogeneous background formed on the plates. While the background intensified with increasing concentrations of plated PFU, this background haze generally did not significantly hinder the identification of the darker blue *lacI* mutant colonies. It is

believed that this haze results from lysis of a small fraction of the cells on the plate, resulting in hydrolysis of X-gal in the agar medium. The cell lysis could result from a low level of prophage induction at the plate incubation temperature (37°C), due to the *cI857* repressor encoded by the λLIZ phage (note that the host strain encodes the wildtype cI^+ gene), and a possible sensitivity of λ-infected cells to galactose, rendering the cells susceptible to cell lysis. As noted below, casamino acids were also absolutely required, resulting in slight bacterial growth, which further aggravated the background. These observations are further elaborated, below.

B.4.2. Phage-encoded *cI857*

As described in subsection B.4.1 above, λLIZ encodes the temperature-sensitive *cI857* allele. To investigate the potential role of the *cI857* allele to the background haze problem, Dr. F. Jirik (University of British Columbia) generously provided a sample of BC-1 *lacI* transgenic mouse DNA. The BC-1 mouse strain (Andrew *et al.* 1996) incorporates a λFIX (Stratagene) -derived shuttle vector in which the *cI* gene (and ColE1 *ori*) is absent. The background haze was dramatically, if not completely, eliminated on plates containing CEH5 cells infected with packaged BC-1 phage. Furthermore, when plates with λLIZ-infected cells are incubated at 34°C, the intensity of the blue background was reduced compared to plates incubated at 37°C. Collectively, these data indicated that the λLIZ-encoded *cI857* allele adversely affects the assay.

B.4.3. Plate incubation temperature

The use of a lower incubation temperature (*e.g.* 30°C) to counteract potential adverse effects of the phage-encoded *cI857* gene at 37°C was not a viable solution to the background haze effect, since bacterial colonies failed to form at plate incubation temperatures of 24, 30, or 42°C. This "temperature effect," putatively attributed to the *relA1* marker (*i.e.*, the relaxed phenotype), will be addressed below.

B.4.4. λ "escape synthesis;" galactose sensitivity; cell lysis

The lambda prophage integration site (*attλ*) is located at 17.34′ on the *E. coli* chromosome, adjacent to the *galETKM* operon at 16.88-17.06′. (Note that the *lac* and *gal* operations are orientated counterclockwise on the *E. coli* chromosome; *i.e.*, *galE* is located closer to *attλ* than is *galM*.) In de-repressed lysogens, λ "escape synthesis" (*e.g.*,

Fukasawa and Obonai 1978) leads to gross discordance of expression of the *galT* and *galK* genes relative to *galE* (Merril *et al.* 1981; Adhya and Gottesman 1982), resulting in a *galE*⁻ phenotype. Derepression of λ lysogens encoding the *cI857* repressor is known to occur by a temperature shift from 32 to 42°C; it is possible that under our experimental conditions (37°C), a partial derepression occurs. It is well-known that *galE* mutants are highly susceptible to cell lysis caused by galactose (*e.g.*, Mientjes *et al.* 1994), a product of lactose or P-gal metabolism (Nikaido 1961; Adhya 1987).

$$\text{Lactose} \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \text{glucose} \quad ; \quad \text{P-gal} \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \text{phenol (excreted)}$$

It has also been reported that *relA galE* mutants are significantly more susceptible to galactose toxicity than isogenic *galE* strains (Ishiguro *et al.* 1986). Finally, autolysis of nongrowing *E. coli* is under control of the stringent response (Tuomanen and Tomasz 1986; Cashel *et al.* 1996).

B.4.5. Lysogeny: Chromosomal integration or λ plasmids?

Because of the possibility of λ escape synthesis, it is important to determine whether λLIZ lysogeny occurs *via* chromosomal integration at *attλ*, or as a λ plasmid. Previous studies at Stratagene suggested that the λLIZ prophage might exist as a λ plasmid in infected cells due to the ColE1 *ori* on the λLIZ vector ("episomal lysogeny": Lundberg *et al.* 1993). Therefore, one should consider the possibility that either lysogenic state might occur. While partial derepression of a chromosomal prophage might adversely impact *gal* operon expression *via* escape synthesis, relaxed (*relA*) strains allow λ plasmid amplification in starved cells (Wêgrzyn *et al.* 1991).

The following comments are related excerpts from two personal communications from Patti Kretz:

a) "λLIZ carries a colE1 *ori*. Because of this there was some disagreement among scientists (such as Frank Stahl, Waclaw Szybalski, *etc.*) as to whether or not this molecule could stably integrate into the *E. coli* chromosome as a normal λ molecule would. If a lysogenic colony were picked and grown in liquid culture we could isolate viable phage particles ... but we were also able to easily isolate 50kb LIZ "plasmid" DNA using a typical miniprep procedure. (DNA was then used to successfully transform comp. cells)."

b) "...we did believe that the LIZ vector was acting both episomal and integrated; ..."

During the course of these studies, PCR analysis was done using oligonucleotide primers which flanked *attλ*. A PCR fragment with the expected product size was obtained, indicating that there were no gross rearrangements (or cryptic prophages) in this region.

B.4.6. *recA1*

It has been reported that cultures of *recA* strains contain a significant proportion of nonviable cells (Capaldo-Kimball and Barbour 1971; Capaldo *et al.* 1974), and that *rec*$^-$ cultures show increased lysis (Miller and Barbour 1977). The impact, if any, of these observations on the direct selection assay remain to be evaluated.

B.4.7. Is XL1-Blue MR (CEH5) *gal*$^+$? (Unstable phenotypes.)

Some of the observations noted above (possible λ escape synthesis of *gal*; apparent *galE* phenotype of infected cells; mucoid instability [below]) collectively implicated involvement of the *gal* operon. Therefore, the pedigree of XL1-Blue was examined (Bullock *et al.* 1987; Bachmann 1987) for mutations of the *gal* operon. The *gal3* mutation,[44] found early in the XL1-Blue pedigree (strain W677 F$^+$ in Chart 6 of Bachmann 1987) was of immediate interest, particularly because of the distinct phenotypes associated with this mutation (*e.g.*, Ahmed *et al.* 1981).

The *gal3* mutation was identified an IS*2* insertion in the operator-promoter region of the *gal* operon (Fiandt *et al.* 1977). This mutation reverts spontaneously to *gal*$^+$ by excision of the insertion element to produce three types of revertants: stable inducible, stable constitutive, and unstable constitutive (Hill and Echols 1966). Although different phenotypes arising from CEH5 were observed, none could be definitively attributed as arising from reversion of a putative *gal3* mutation. λLIZ-infected CEH5 cells appear to be Gal^s, since they grow reasonably well, if somewhat slowly, on M9 agar plates containing limiting concentrations (< ~0.01% w/v) lactose or P-gal; however, colony formation is inhibited at higher concentrations (> ~0.1% w/v) of lactose or P-gal. While λ-infected CEH5 cells appear to be Gal^s, the uninfected strain grows reasonably well in

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[44] The *gal3* and *gal308 (or galOP-308*::IS*2*-1) mutations are identical (Musso, R.E. Plasmid. 22: 275-280 (1989)). This mutation was first identified by J. Lederberg, in W677, as *Gal*$_5^-$ (Lederberg, J. *et al.* Genetics. 37:720 (1951); Morse, M.L. *et al.* Genetics. 41: 758-779 (1956)).

M9-galactose minimal medium supplemented with thiamine, and forms purple[45], Gal$^+$ colonies on Galactose-MacConkey agar plates. Furthermore, PCR analysis of the operator-promoter region immediately upstream of the *galE* gene, and of the *galE*, *galT* and *galKM* genes yielded fragments with predicted product sizes, indicating that there were no gross insertions or deletions in *gal* operon. On this basis, the *gal3* mutation does not appear to be present in the CEH5 strain.

During the examination of the status (function; non-functioning) of the *gal* operon in CEH5, a *gal*$^+$-encoding plasmid (pI24) was obtained from Dr. Susan Garges (NIH). Introduction of this plasmid into CEH5 offered a slight benefit; however, this observation should be regarded as tenuous, since this experiment required maintenance of at two plasmids (pClone4; pI24; and possibly, λ plasmid) in the CEH5 strain. In fact, limited experiments suggested that pI24 tended to exclude pClone4.

Despite the lack of evidence for a *gal3* (or similar) mutation in CEH5, because of the difficulties putatively associated with the gal operon in the direct selection experiments, and the high frequency of mucoid phenocopies (refer to subsection B.4.8, below), it is intriguing to consider whether unstable genetic elements might be otherwise be involved. In this regard, it is interesting to note that the *pro-gal* region in *E. coli* K-12 appears to be rich with insertion sequence elements (Fiandt *et al.* 1972; Hirsch *et al.* 1972; Ghosal *et al.* 1979; Deonier 1987), and that the *gal* operator-promoter region is one of the preferred integration sites for IS*1* and IS*2* (Saedler and Starlinger 1967). Ghosal *et al.* (1967) speculated whether control sequences are preferred sites for integration of IS*2* elements. When IS*1* integrates into the *gal* operon there is a 20-3000-fold increase[46] in the deletion formation in the *gal* region, independent of *recA* (Reif and Saedler 1975). Finally, λ has been reported to have a secondary attachment site *galT* (Shimada *et al.* 1973; Bidwell and Landy 1979).

B.4.8. The mucoid phenotype

A troublesome characteristic of the CEH5 direct selection strain (and in limited examinations, the XL1-Blue MR parent strain), is the high frequency (~10^{-2}–10^{-3},

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[45] These Gal$^+$ colonies appeared purple by reflected light, deep red by transmitted light.

[46] Deletions in the *gal* region of *E. coli* K-12 usually occur with a frequency of ~10^{-7} per viable cell (Reif and Saedler, 1975).

estimated) of segregation of colonies with a mucoid phenotype on minimal and rich media plates. These colonies are characterized by an apparently high growth rate (based on the large colony sizes, often > 1.0 cm diameter), and a shiny, slimy (almost watery), whitish opaque phenotype.[47] The frequency at which these mucoid colonies arise is too great to be explained by a mutation of a *single* gene, possibly suggesting that mutations in any one of several contributing genes could be involved, or that an unstable genetic element such as an insertion element or a transposon is involved. Mucoidy is a well-known phenomenon in *E. coli*, since *lon* (*capR*) mutants are known to display a mucoid phenotype (Markovitz 1964; Gayda *et al.* 1976; Painbeni *et al.* 1993). That the mucoid phenotype in *lon* (*capR*) mutants can be suppressed by *gal* deletions (Hua and Markovitz 1975; Table 1 *in*: Kushner 1987) suggests the possibility that perturbations of the *gal* operon could be involved in the mucoid cells observed among CEH5 progeny. Other investigators have noted associations between various bacterial genes (*lon*, *capS*, *capT*), regulatory genes of the *gal* operon (*galU*), and a mucoid phenotype (Hua and Markovitz 1972; Buchanan and Markovitz 1973; Hua and Markovitz 1975). The effects of mucoidy on the direct selection assay are difficult to assess, but they are expected to be adverse, due to the perturbation of the bacterial lipopolysaccharide layer. Examinations of mucoid cells in the direct assay have yielded uniformly negative results.

While the genotype of CEH5 does not indicate that this strain is *lon*, the diverse effects attributed to this mutation (*e.g.*, mucoidy; filamentation; UV^s; Gottesman 1987) are familiar and are worthy of consideration. For example, *lon* cells are poorly lysogenized by λ (Walker *et al.* 1973; Gayda *et al.* 1976; Gayda and Markovitz 1978). (Refer to subsection B.4.19, below, for comments regarding *relA* and lysogeny.) It has been hypothesized that poor lysogenization of *lon* cells by λ results from host interference in the synthesis or expression of the λ *cI* repressor (Gayda and Markovitz 1978). *lon* is a regulator gene for the structural genes $sulA^+$ and $sulB^+$ (=*ftsZ*) (Gayda *et al.* 1976). Depression of both *sul* operons results in UV^s and decreased ability of λ to lysogenize, whereas inactivation of either sul^+ protein by mutation to *sul* prevents these phenomena. *sulA* inhibits cell division through interaction with FtsZ (SulB) (Higashitani

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[47] For a picture of agar plates streaked with mucoid and non-mucoid *E. coli*, see Fig. 1 in : A. Markovitz *et al.*, J. Bacteriol. 1497-1501 (1967).

et al. 1997). *sulA* and *sulB* mutations reverse the UV^s of *lon* strains (which filament extensively and die after exposure to ultraviolet light) but do not affect the mucoidal or degradation defect of these strains (Gottesman *et al.* 1981). It has also been noted that the *sulB*$^-$ mutation alters cell morphology (Gottesman *et al.* 1981). As mentioned in the previous paragraph, *galU* is de-repressed in *lon* (also in *capT*) mutants, as is the *galETKM* operon (Hua and Markovitz 1972; Buchanan and Markovitz 1973). (*capS* had no effect on derepression the *galETKM* operon in *lon* cells.) Finally, it has been noted that reduced rates of β-galactosidase expression are observed in *lon* cells when the *lacZ* gene is introduced *via* a λ transducing phage, whereas *lon* cells competently express the chromosomal *lac* operon (Gayda and Markovitz 1978).

B.4.9. Possible plasmid loss

Occasionally, refrigerator stocks of CEH5 (routinely passaged less than 4-5 times, before cultures are reestablished from frozen stocks) fail to work in the direct selection assay. The cause has not been investigated, and while plasmid loss is a possibility, the cultures appear to remain cam^r (pClone4 encodes *cat*). An alternative explanation (not examined) is that mucoid phenotype cells have overgrown the culture.

B.4.10. Casamino acids (CAA)

CEH5 is a prototroph, capable of growing in minimal media supplemented with glucose and thiamine. However, colony formation in the direct selection assay absolutely requires the addition of small amounts (~0.04%, w/v) CAA. This requirement cannot be overcome by simple additions of single or combinations of amino acids encoded at loci around the *lac* and *gal* operons, indicating that infected cells display complex amino acid auxotrophies. Unfortunately, CAA addition to the plates results in the growth of a very light bacterial lawn on the plates, in which plaques are often lightly visible (again, indicating incomplete establishment of lysogeny). (Note also that this slight growth with accompanying lysis could contribute to the blue background haze, described in subsection B.4.1, above. Therefore, eliminating the need for CAA might also significantly reduce the blue background problem!) The formation of the very light lawn on plates supplemented with CAA could indicate that the CAA currently being utilized (Difco) contain trace amounts of a carbon source suitable for bacterial growth. Two additional points regarding the CAA effect are worth noting: (1) *relA spoT E. coli*

mutants are known to display multiple, complex amino acid auxotrophies (Xiao *et al.* 1991), even if the (*relA*$^+$ *spoT*$^+$) parent is prototrophic; and, (2) it has been noted (*e.g.*, Lin 1987, p. 251) that *E. coli galT* mutants fail to grow on D-galactose, causing the accumulation of galactose-1-phosphate to a level of 2 mM; however, (for reasons unknown), this growth inhibition can be overcome by addition of yeast extract or casein hydrolysate to the culture medium, without reducing the intracellular galactose-1-phosphate levels. (Glucose is also protective and can reverse the inhibition ...)

B.4.11. Glucose effect

Additions of small amounts of glucose to the top agarose greatly promoted colony growth. Glucose can partially alleviate galactose sensitivity in *galE* strains (Nikaido 1961; Squires and Ingraham 1969; Ippen *et al.* 1971). Adhya (1987, p. 1505) stated that *galE* mutants are killed in glucose-free minimal or broth media when galactose is present. Interestingly, it was observed that λLIZ-infected CEH5 cells appeared to grow slightly better on lactose *vs.* P-gal, suggesting a protective effect due to glucose released during lactose hydrolysis. Adhya (1987, p. 1504) noted that *galT* or *galU* mutants accumulate galactose-1-phosphate, and therefore do not grow on glycerol as a carbon source; this bacteriostasis is reversed if glucose is added to the stationary cells.

B.4.12. Elevated direct selection mutant frequencies

MF based on direct selection with CEH5 are much higher (perhaps 10-fold) than with the plaque color assay using SCS-8. The reasons for this are not known, but might reflect bacterial strain and experimental factors. For example, *cII* MF are sometimes observed to be significantly (several-fold) higher than *lacI* MF, although this varies from laboratory to laboratory. Additionally, λ packaging efficiencies are known to be poor, perhaps 10% of the theoretical limit (Gossen *et al.* 1989; Short *et al.* 1992). Different bacterial titer strains also have an effect, since packaged λ titers on XL1-Blue MR (G. Stuart) and LE392 (Stratagene packaging extract advertisements; Provost and Rogers 1996) are somewhat higher than on SCS-8. Collectively, these observations suggest that Big Blue® packaging reaction mixtures *might* contain greater proportions of packaged phage, than are generally indicated by titers on the SCS-8 (or similar) host strain.

B.4.13. "Pseudo-colony" phenotype

It has been noted that the direct selection colonies, under low magnification, actually consist of small groups of individual, microscopic blue colonies. It is difficult to recover (culture) these colonies from the plates, suggesting that cell death (*e.g.* due to galactose sensitivity) has occurred. Despite this, the *lacI* genes may be recovered by PCR amplification from cored colonies, for subsequent DNA sequence analysis. It is of interest to note also that relaxed (*relA*) bacteria have decreased viability under conditions of starvation, compared with stringent (*relA*$^+$) bacteria (Goodell and Tomasz 1980; Hecker *et al.* 1986; Cashel *et al.* 1996).

B.4.14. Cell phase effect

A potential source of variability in the direct selection assay is the observation that the density to which the plating cells are grown appears to affect the numbers of colonies that will be observed in the direct selection assay, with cells harvested early in logarithmic growth giving superior results to cells harvested in mid-log or late-log growth. While difficult to explain, the possibility that the α-complementation plasmid is being affected remains one possibility, or perturbation of cell phase-specific factors due to the relaxed (*relA*) phenotype (see Cashel *et al.* 1996) is involved.

B.4.15. Infection mixture cell density effect

It has been observed that the cell density in the phage infection mixture (CEH5 cells + λLIZ phage) has an apparent effect on the colony counts on the direct selection plates. Decreasing the cell density in the infection mixture 10-fold results in a disproportionate decrease in the number of expected colonies on the direct selection plates; a 25-fold decrease in cell density has an even greater effect. This effect appeared to be linear, until a cell density was attained at which a plateau occurred. Since the multiplicities-of-infection in a standard infection mixture (*e.g.*, as recommended in the Big Blue® Manual (Stratagene 1997)) are on the order of 10^{-3} (*i.e.*, cells greatly outnumber phage), this is a particularly unusual observation. Fortunately, the concentration of bacteria in a standard infection mixture appears to be high enough (in the plateau region) that this effect appears to be minimized. The cause of this effect is unclear, but might again be related to a *relA spoT* phenotype (or alternatively, a *lon* mutation: refer to subsection B.4.8, above). Dr. Grzegorz Wêgrzyn (University of Gdansk) (*pers. comm.*) has recently found that *relA*

spoT double mutants are poorly lysogenized by λ. This appears to be confirmed: in a crude experiment, a cell-phage infection mixture was centrifuged (microcentrifuge) and aliquots of supernatant were titered for unabsorbed λ phage; approximately 50% (avg.) of the input PFU were recovered as presumably free phage. Dr. Wêgrzyn had another interesting comment regarding lysogeny, the significance of which may require additional consideration (Dr. Wêgrzyn, *pers. comm.*):

"You have *cI+* gene in the host, and I am afraid this may cause a problem with the efficiency of lysogenization. Although *cI* is sufficient for maintenance of λ prophage, for lysogenization you need also integrase. In normal conditions, the expression of both *cI* and *int* genes is dependent on CII function. CII protein is an activator of three phage promoters: pE, pI and paQ. pE is for *cI* expression early after infection, pI is for *int* expression (expression of this gene from pL is ineffective due to retroregulation mechanism), paQ promotes production of RNA antisense to mRNA for Q gene. If you have another source of *cI*, you do not need pE activity, but you still need CII to activate pI promoter. Since *cII* gene is under control of pR, expression of cI^+ in your host prevents expression of *cII* so activity of pI, and thus expression of *int* is very low. Therefore, efficiency of lysogenization may be low. To increase the efficiency of lysogenization you would need to express *int* gene from another source. I met such a problem several years ago (see Gene (1992) 122: 1-7)."

B.4.16. Potential complications due to *supE44*

XL1-Blue MR encodes the glutamine-inserting amber suppressor *supE44* (Garen *et al.* 1965),[48] alerting us to a possible interference with the stringent response. The *relA* open reading frame terminates with an amber codon, which when suppressed extends the protein by 27 amino acids, thereby inactivating it (Metzger *et al.* 1988; Cashel *et al.* 1996). A glutamine-inserting Su^+_7 gene on a plasmid has been shown to inactivate *relA* (Metzger *et al.* 1988). Additionally, the expression of tetracycline resistance from wildtype Tn*10* was found to be anomalous in *E. coli* strains carrying the *supE* suppressor, which were less resistant to tetracycline (Zupancic *et al.* 1980).

B.4.17. Abnormal colony morphology on minimal media

Irregular-shaped CEH5 colonies (compass-needle shaped; star-shaped; *etc.*) are often seen on minimal media plates, but not rich media plates. This has been previously noted in *relA spoT* double mutants (Xiao *et al.* 1991), and may be related to perturbations in cell wall peptidoglycan synthesis (Vanderwel and Ishiguro 1984; Cashel *et al.* 1996). As well, *bolA*, a morphogene participating in cell shape determination (Aldea *et al.* 1988), is affected by the stringent response (Cashel *et al.* 1996).

B.4.18. Bacteriophage λ O and P genes

The Big Blue® shuttle vector encodes the (wildtype?) λ O and P genes. The presence of these genes is potentially problematic, as suggested by Dr. Węgrzyn (*pers. comm.*[49]):

"Since your λ vector encode *O* and *P*, this may be a potential problem if it is amplified in the starved cells (see above) as P protein is toxic for *E. coli* at high concentrations (P can titrate DnaB helicase and prevent host chromosome replication)."

B.4.19. Relaxed phenotype / stringent response (Cashel *et al.* 1987, 1996)

XL1-Blue MR is reported to be a relaxed (*relA1*) strain, and indeed, strain behaviour during the direct selection experiments strongly implicated the strain as being *relA spoT*. The XL1-Blue parent strain, DH1, was suspected to be *relA1 spoT1*, as noted in the strain pedigree (Bachmann 1987). *relA* strains are often also *spoT*. The original *spoT1* allele was encountered coexisting with the *relA1* allele; indeed, it has been proposed that the *relA1* mutation of *E. coli* arose as a spontaneous suppressor of the naturally occurring *spoT1* (Laffler and Gallant 1974), since the *relA* mutation improves the growth of *spoT* mutants (Laffler and Gallant 1974; Rudd *et al.* 1985). (Parenthetically, recalling the discussion of unstable (transposable) genetic elements in subsection B.4.7, above, the *relA1* mutation has been shown to consist of an IS*2* insertion into the gene (Metzger *et al.* 1989).)

The *relA1* mutation of XL1-Blue is highly significant to our project, since I believe many of the problems and anomalies described above could result from a *relA spoT* genotype! The *relA* mutation exerts pleiotropic effects[50] on a strain; these effects are exacerbated in *relA spoT*

I have summarized below some of the diverse biological effects known to be associated with a *relA* or *relA spoT* genotype, and which I believe are disadvantageous with regard to the direct selection assay:

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[48] *supE44* (= *glnV44*), located at 15' (near *gal* at 17') on the *E. coli* chromosome (Signer *et al.*, 1965; Bachmann, 1987), has previously been called *sup-2*, Su_{II}^{+}, *SuII*, and *Su-2*.

[49] Dr. Węgrzyn kindly provided an informative manuscript : Taylor, K. and G. Węgrzyn (1998) Regulation of Bacteriophage λ Replication. *In*: S.J.W. Busby *et al.* (Eds.) Molecular Microbiology. Springer Verlag, Berlin-Heidelberg, pp. 81-97.

[50] For an indication of the scope of the pleiotropic effects of the stringent response, see Table 1 *in*: Cashel *et al.* (1996).

a) *relA1 spoT* double null mutants display multiple amino acid auxotrophies (Xiao *et al.* 1991; Hernandez and Bremer 1991), requiring the addition of casamino acids;
b) *spoT* mutants demonstrate abnormal colony growth and morphologies on minimal media plates, but not LB plates (Xiao *et al.* 1991);
c) *spoT* strains are thermosensitive for growth on minimal medium (Hernandez and Bremer 1991); Uzan and Danchin (1976) also state that *relA* strains are more or less thermosensitive;
d) starved, relaxed strains appear to have much less β-galactosidase protein, and this protein is thermolabile and has a reduced specific activity (Foley *et al.* 1981; Primakoff 1981; Cashel and Rudd 1987, p. 1424);
e) *relA spoT* mutants (some strains) have an impaired ability to induce a wildtype *lacZ* operon (Xiao *et al.* 1991);
f) *relA spoT* mutants are poorly lysogenized by λ (G. Wêgrzyn, *pers. comm.*);
g) *relA galE* mutants are more susceptible to galactose toxicity than *relA*$^+$ *galE* mutants (Ishiguro *et al.* 1986);
h) autolysis of non-growing *E. coli* is under control of the stringent response (Tuomane and Tomasz 1986; Cashel *et al.* 1996);
i) *relA* strains allow λ plasmid amplification in starved cells (Wêgrzyn *et al.* 1991); and,
j) *relA* strains have decreased viability under conditions of starvation, compared with *relA*$^+$ bacteria (Goodell and Tomasz 1980; Hecker *et al.* 1986; Cashel *et al.* 1996).

B.5. Proposed Future Work

B.5.1. In descending order of priority:

1. Attempt to engineer a reversion of the relaxed phenotype to a stringent phenotype through introduction of the *relA*$^+$ and *spoT*$^+$ alleles (Dr. Mike Cashel, NIH, has generously provided advice and will provide strains).
2. Examine other restriction-modification-minus host strains for suitability in the assay. It would by prudent to investigate this route, even while attempting to convert CEH5 to a *relA*$^+$ *spoT*$^+$ strain.

3. Use *E. coli* C as a starting strain (naturally $r^- m^-$).[51]
4. Introduction of the *lacZ*ΔM15 *lacI*$^+$ *lacA*$^+$ genes directly into the host chromosome, obviating the need for an α-complementing plasmid.
5. Introduction of the *dnaE915* antimutator allele, to reduce spontaneous MF.
6. Dr. T. Skopek (*pers. comm.*) suggested that the *hfl* mutation(s) will be necessary in our strain to maintain lysogeny. While current data do not support this contention, introduction of the *hflA* and/or *hflB* mutations into the host strain should nonetheless be examined. Dr. Wêgrzyn (*pers. comm.*) has indicated that *hfl* mutants give high efficiency of lysogenization because the stability of CII is higher (at least in *hflB* mutants), but since CEH5 has the *c*I$^+$ gene on the chromosome, the production of CII would be very low anyway (due to repression of pR).
7. Prophage: chromosomal integration at *att*λ (implications regarding escape synthesis)? → PCR analyses to confirm chromosomal lysogeny.

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B.6. References

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[51] Work with *E. coli* C strains has been initiated but is not described, here.

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Table B.2. Relevant *E. coli* genetic markers.

Gene	Chromosomal location (min.) [a, b]	Function, comments
ftsZ (*sulB*)	2.27	required for initiation of cell septation
lac	7.77	operon: *lacIZYA*
lacI	7.87	*lac* repressor
lacZ	7.80	β-D-galactosidase
lacY	7.77	galactoside (*lac*) permease
lacA	7.76	galactoside acetyltransferase
bolA	9.78	morphogene
lon (*capR*)	9.86	cytoplasmic DNA-binding, ATP-dependent protease; *lon*$^-$ = mucoid phenotype
supE44 (*glnV44*)	15.05	*amber* (UAG) suppressor; inserts glutamine
mglR	16.90	methyl-galactoside
gal	16.88	operon: *galETKM*; loss = 2-deoxygalactose^r; loss in *galU*$^-$ = galactose^r
galE	17.06	UDP-galactose-4-epimerase (hexose-1-phosphate uridyltransferase); *galE*$^-$ = phage U3^r, phage P1^s, phage C21^s, 2-deoxygalactose^r
galT	17.03	galactose-1-phosphate uridyltransferase; *galT*$^-$ = streptozotocin^r
galK	17.01	galactokinase; *galK*$^-$ = 2-deoxygalactose^r;
galM	16.99	aldose-1-epimerase; mutarotase, converting α-aldose to the β-anomer (*e.g.* β-D-galactose → α-D-galactose, the substrate for GalK)
attλ	17.34	*E. coli* chromosome site for λ prophage integration
sulA (*sfiA*)	21.98	inhibitor of cell division (inhibits formation of septa during cell division); SulA target is SulB
capS	~24	capsular polysaccharide; *capS*$^-$ = mucoid phenotype (Buchanan and Markovitz 1973)
capT	?	capsular polysaccharide; *capT*$^-$ = mucoid phenotype; never mapped, but different chromosomal location than *lon* (*capR*) or *capS* (A. Markovitz, *pers. comm.*)
mcrA	26.01	5-methylcytosine restriction; within the *e14* locus (*e14* is an excisable element)
galU	27.78	glucose-1-phosphate uridyltransferase; loss = amp^r, phage P1^r, phage U3^r; *galU* is de-repressed in *lon*, *capS* or *capT* mutants
rel	35.42	operon: *relFEB* (relaxed)
relF	35.42	
relE	35.42	
relB	35.43	
mgl	46.48	operon: *mglCAB* (galactose permease)
mglC	48.17	
mglA (*mglP*)	48.19	
mglB	48.23	galactose-binding protein; receptor for galactose
recA	60.77	recombination
rpoS (*katF*)	61.75	RNA polymerase, sigma subunit – stationary phase
relA	62.71	ppGpp synthetase I (GTP → pppGpp); RelA is required for ppGpp synthesis of ppGpp during stringent response to amino acid (aa) starvation. In stringent cells, *t*RNA synthesis is repressed during amino acid starvation.
relX	62.76	
galR	64.08	repressor of *galETKM* operon
galP	66.48	D-galactose/H$^+$ symporter (galactose permease)

Table B.2. (*continued*)

Gene	Chromosomal location (min.) [a, b]	Function, comments
endA	66.52	DNA-specific endonuclease I; *endA*⁻ mutants improve miniprep dsDNA
ebg	69.35	operon: *ebgACB*; evolved β-galactosidase
ebgR	69.39	regulatory gene
ebgA	69.41	evolved β-galactosidase; *ebgA* mutation = growth on lactose in Δ(*lac*)
ebgC	69.48	phospho-β-D-galactosidase, B subunit; cryptic gene
ebgB	69.50	possible homologue of *lacY*
spo	82.29	operon: *rpoZ spoT trmH recG*; spot ("magic spot"); alternate (independent) transcription for *rpoZ* and *spoT*
spoT	82.34	(p)ppGpp 3′-pyrophosphohydrolase (ppGpp → GDP)
gpp	85.31	pppGpp 5′-phosphohydrolase
lamB	91.44	maltoporin (maltose receptor); phage λ receptor
melB	93.51	thiomethylgalactoside permease II (α-galactosides, *e.g.* melibiose)
Δ(*mcrCB-hsdRMS-mrr*)[c]		restriction-modification
mcrC	98.61	*mcr* = methylated cytosine restriction
mcrB	98.63	
hsd	98.67	operon: *hsdRMS*; host specificity (*hsd* = Eco K restriction system)
hsdR	98.75	endonuclease R
hsdM	98.70	DNA methylase M
(*stkC*	98.70	suppressor of transposase killing)
hsdS	98.67	
mrr	98.82	methyl-purine restriction (*mrr* = methylated adenine recognition and restriction)

[a] Bachmann, B.J. (1990) Linkage map of *Escherichia coli* K-12, edition 8. *Microbiol. Rev.* **54**,130–197.

[b] http://cgsc.biology.yale.edu:80/cgi-bin/sybgw/cgsc/Map?!Name=CGSC(Mary Berlyn)

[c] Kohler, S.W. *et al.* (1990) *Nucleic Acids Res.* **18**, 3007–3013.

Supplement: Addendum to the original report (above):

Mukai, T., Ohkubo, H., Shimada, K., and Y. Takagi. (1978) Isolation and characterization of a plaque-forming lambda bacteriophage carrying a ColE1 plasmid. *J. Bacteriol.* **135**,171–177:

"A plaque-forming λ*imm*^{434} bacteriophage carrying the entire genome of colicinogenic factor E1 has been isolated and characterized. This phage, λ*imm*^{434}ColE1, can lysogenize as a stable plasmid within a recombination-deficient *Escherichia coli* cell that lacks the normal attachment site for lambda phage. Furthermore, it has been found that λ*imm*^{434}ColE1 phage carrying amber mutations in the *O* and *P* genes of the λ genome, *i.e.*, λ*imm*^{434}*OamPam*ColE1, behaves as a plaque-forming phage, and this finding suggests that the ColE1 factor DNA permits replication of the DNA of the plaque-forming phage."