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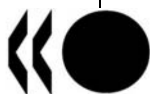

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ENVIRONMENT DIRECTORATE
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ENV/JM/MONO(2009)7
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SERIES ON TESTING AND ASSESSMENT
Number 103

DETAILED REVIEW PAPER ON TRANSGENIC RODENT MUTATION ASSAYS

English - Or. English

JT03268209

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ACKNOWLEDGEMENTS

This document was prepared by Prepared by I.B. Lambert,[1] T.M. Singer[2], S.E. Boucher[3] and G.R. Douglas*

This work was supported by the Canadian Regulatory System for Biotechnology. We would like to acknowledge the contribution of George Bolcsfoldi, who shared an analysis of transgenic rodent data that he had compiled prior to the 2002 International Workshop on Genotoxicity Testing (IWGT) meeting in Plymouth, United Kingdom. We are extremely indebted to members of the IWGT working group who have contributed greatly to the formulation of many of the ideas expressed in this document – especially to John Heddle for his contribution to the initial structure of this review, to Takehiko Nohmi and Hans-Jorg Martus for sharing data and to David Kirkland for sharing unpublished data on the comet assay. We gratefully acknowledge the statistical help of Leonora Marro in carrying out the analyses found in Chapter 5, Lynda Soper's assistance in compiling DNA sequence data and Marla Sheffer's editorial skills. The original draft version of this document has been greatly improved by the constructive criticism of John Heddle, Bob Heflich, Jan van Benthem, Veronique Thybaud, Steve Dean and David Blakey.

An earlier version of this document was published in *Mutation Research*, Volume 590, pages 1–280, 2005.

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FOREWORD

This document presents a Detailed Review Paper on Transgenic Rodent Mutation Assays. The project for developing this document, led by Canada, was included in the work plan of the Test Guidelines Programme in 1996. In 2005, the Working Group of National Coordinators for the Test Guidelines Programme (WNT) was invited to comment on a first version that was an extensive review work that started in 1996. Canada made a compilation of all comments, and responded to these comments, and submitted a revised draft in 2008.

On 30 June 2008, comments from the WNT were not requested on the entire draft revised DRP, but on the executive summary and conclusions/recommendations, which include a summary of incremental changes compared to the earlier version. Considering the size of the draft DRP (600 pages), the informative nature of the document, and the resources that would be needed for a second revision of the full document, the Secretariat, in consultation with the lead country and the WNT Bureau, proposed this new approach in order to be able to finalize the document.

Considering that (i) the full document was revised by Canada to take WNT comments into account after a first commenting round, and (ii) incremental changes, as summarized in the executive summary and the conclusions of the last version, were reviewed and approved by the WNT at its 21st, this process was considered by the WNT to constitute a full and final review of this document.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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LIST OF ACRONYMS AND ABBREVIATIONS

^{3}H-TdR	tritium-labelled thymidine
6-TG	6-thioguanine
8-AA	8-azaadenine
8-OHdG	8-hydroxy-2′-deoxyguanosine
AMP	adenosine monophosphate
Aprt	adenine phosphoribosyltransferase
bp	base pairs
bw	body weight
CA	chromosomal aberration
CAS	Chemical Abstracts Service
CAT	chloramphenicol aminotransferase
CpG	cytosine–phosphate–guanine
CYP	cytochrome P-450
DAP	2,6-diaminopurine
DNA	deoxyribonucleic acid
FISH	fluorescence *in situ* hybridisation
GGR	global genomic repair
HID	highest ineffective dose
Hprt	hypoxanthine-guanine phosphoribosyltransferase
IARC	International Agency for Research on Cancer
IMF	induced mutant/mutation frequency
ip	intraperitoneal
IUPAC	International Union for Pure and Applied Chemistry
IWGT	International Workshop on Genotoxicity Testing
kb	kilobases
LED	lowest effective dose
Mb	megabases
MF	mutant/mutation frequency
MLA	mouse lymphoma assay
MN	micronucleus assay
mRNA	messenger ribonucleic acid
MTD	maximum tolerable dose
NTP	National Toxicology Program (USA)
O^6-MeG	O^6-methylguanine
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocyte
RNA	ribonucleic acid
SCE	sister chromatid exchange
SMF	spontaneous mutant frequency
TCR	transcription-coupled repair
TD_{50}	tumorigenic dose for 50% of test animals
TGR	transgenic rodent
Tk	thymidine kinase
TRAID	Transgenic Rodent Assay Information Database
UDS	unscheduled DNA synthesis

CHEMICAL NAMES AND ABBREVIATIONS

The common names of chemicals mentioned in this report, together with the abbreviations used, their International Union for Pure and Applied Chemistry (IUPAC) equivalent names and their Chemical Abstracts Service (CAS) registry numbers, where available, are shown in the table below.

Common name	IUPAC name	CAS No.
1,10-Diazachrysene	1,10-Diazachrysene	218-21-3
1,2:3,4-Diepoxybutane	2-(Oxiran-2-yl)oxirane	1464-53-5
1,2-Dibromo-3-chloropropane	1,2-Dibromo-3-chloropropane	96-12-8
1,2-Dibromoethane	1,2-Dibromoethane	106-93-4
1,2-Dichloroethane	1,2-Dichloroethane	107-06-2
1,2-Epoxy-3-butene	2-Ethenyloxirane	930-22-3
1,3-Butadiene	Buta-1,3-diene	106-99-0
1,4-Phenylenebis(methyl-ene)selenocyanate (p-XSC)	1,4-Bis(selenocyanatomethyl)benzene	85539-83-9
1,6-Dinitropyrene	1,6-Dinitropyrene	42397-64-8
1,7-Phenanthroline	1,7-Phenanthroline	230-46-6
1,8-Dinitropyrene	1,8-Dinitropyrene	42397-65-9
10-Azabenzo(a)pyrene	10-Aza-benzo(def)chrysene	189-92-4
17β-Oestradiol	(8R,9S,13S,14S,17S)-13-Methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclo-penta(a)phenanthrene-3,17-diol	50-28-2
1-Chloromethylpyrene	1-(Chloromethyl)pyrene	1086-00-6
1-Methylphenanthrene	1-Methylphenanthrene	832-69-9
1-Nitronaphthalene	1-Nitronaphthalene	86-57-7
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	2,3,7,8-Tetrachlorooxanthrene	1746-01-6
2,4-Diaminotoluene	4-Methylbenzene-1,3-diamine	95-80-7
2,6-Diaminotoluene	2-Methylbenzene-1,3-diamine	823-40-5
2-Acetylaminofluorene (2-AAF)	*N*-(9H-fluoren-2-yl)acetamide	53-96-3
2-Amino-1-methyl-6-phenyl-imidazo(4,5-b)pyridine (PhIP)	1-Methyl-6-phenylimidazo(4,5-b)pyridin-2-amine	105650-23-5

Common name	IUPAC name	CAS No.
2-Amino-3,4-dimethylimidazo(4,5-f)quinoline (MeIQ)	3,4-Dimethylpyrido(3,2-e)benzimidazol-2-amine	77094-11-2
2-Amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx)	3,8-Dimethylimidazo(5,4-h)quinoxalin-2-amine	77500-04-0
2-Amino-3-methylimidazo(4,5-f)quinoline (IQ)	3-Methylpyrido(3,2-e)benzimidazol-2-amine	76180-96-6
2-Nitronaphthalene	2-Nitronaphthalene	581-89-5
2-Nitro-*p*-phenylenediamine	2-Nitrobenzene-1,4-diamine	5307-14-2
3-Amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2)	1-Methyl-5H-pyrido(4,5-b)indol-3-amine	62450-07-1
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	3-Chloro-4-(dichloromethyl)-5-hydroxy-5H-furan-2-one	77439-76-0
3-Fluoroquinoline	3-Fluoroquinoline	396-31-6
3H-1,2-dithiole-3-thione (D3T)	Dithiole-3-thione	534-25-8
3-Methylcholanthrene	3-Methyl-1,2-dihydro-benzo(j)aceanthrylene	56-49-5
3-Nitrobenzanthrone	3-Nitro-7H-benz(de)anthracen-7-one	17117-34-9
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	*N*-Methyl-*N*-(4-oxo-4-pyridin-3-ylbutyl)nitrous amide	64091-91-4
4,10-Diazachrysene	4,10-Diazachrysene	218-34-8
4-Acetylaminofluorene (4-AAF)	*N*-(9H-Fluoren-4-yl)acetamide	28322-02-3
4-Aminobiphenyl	4-Phenylaniline	92-67-1
4-Chloro-*o*-phenylenediamine	4-Chlorobenzene-1,2-diamine	95-83-0
4-Hydroxybiphenyl	4-Phenylphenol	92-69-3
4-Monochlorobiphenyl	1-Chloro-4-phenylbenzene	2051-62-9
4-Nitroquinoline-1-oxide (4-NQO)	4-Nitro-1-oxidoquinolin-1-ium	56-57-5
5-(2-Chloroethyl)-2′-deoxyuridine (CEDU)	5-(2-Chloroethyl)-1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl)pyrimidine-2,4-dione	90301-59-0
5-(*p*-Dimethylaminophenyl-azo)benzothiazole	4-(1,3-Benzothiazol-5-yldiazenyl)-*N*,*N*-dimethylaniline	18463-90-6
5,9-Dimethyldibenzo(c,g)carbazole (DMDBC)	5,9-Dimethyl-7H-dibenzo(c,g)carbazole	88193-04-8
5-Bromo-2′-deoxyuridine (BrdU)	5-Bromo-1-((2R,4S,5R)-4-hydroxy-5-(hydroxy-methyl)oxolan-2-yl)pyrimidine-2,4-dione	59-14-3
5-Fluoroquinoline	5-Fluoroquinoline	394-69-4
6-(*p*-Dimethylaminophenyl-azo)benzothiazole	4-(1,3-Benzothiazol-6-yldiazenyl)-*N*,*N*-dimethylaniline	18463-85-9

Common name	IUPAC name	CAS No.
6,11-Dimethylbenzo(b)naph-tho(2,3-d)thiophene	6,11-Dimethylnaphtho(3,2-b)(1)benzothiole	32362-68-8
6-Nitrochrysene	6-Nitrochrysene	7496-02-8
7,12-Dimethylbenzanthracene (7,12-DMBA)	7,12-Dimethylbenzo(b)phenanthrene	57-97-6
7H-Dibenzo(c,g)carbazole (DBC)	7H-Dibenzo(c,g)carbazole	194-59-2
7-Methoxy-2-nitronaphtho(2,1-b)furan (R7000)	7-Methoxy-2-nitrobenzo(e)(1)benzoxole	75965-74-1
8-Methoxypsoralen	9-Methoxyfuro(3,2-g)chromen-7-one	298-81-7
87-966	87-966	nd
A-alpha-C	9H-Pyrido(6,5-b)indol-2-amine	26148-68-5
Acetaminophen	*N*-(4-Hydroxyphenyl)acetamide	103-90-2
Acetic acid	Acetic acid	64-19-7
Acetone	Propan-2-one	67-64-1
Acrylamide	Prop-2-enamide	79-06-1
Acrylonitrile	Prop-2-enenitrile	107-13-1
Adozelesin	(7bR,8aS)-*N*-(2-((4,5,8,8a-Tetrahydro-7-methyl-4-oxocyclopropa(c)pyrrolo(3,2-e)indol-2(1H)-yl)carbonyl)indol-5-yl)-2-benzofurancarboxamide	110314-48-2
Aflatoxin B1	2,3,6aα,9aα-Tetrahydro-4-methoxycyclo-penta(c)furo(2′,3′:4,5)furo(2,3-h)chromene-1,11-dione	1162-65-8
Agaritine	(2S)-2-Amino-5-(2-(4-(hydroxymethyl)phenyl)hydrazinyl)-5-oxopentanoic acid	2757-90-6
all-*trans*-Retinol	(2E,4E,6E,8E)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)nona-2,4,6,8-tetraen-1-ol	68-26-8
alpha-Chaconine	beta-D-Glucopyranoside, (3beta)-solanid-5-en-3-yl O-6-deoxy-alpha-L-mannopyranosyl-(1-2)-O-(6-deoxy-alpha-L-mannopyranosyl-(1-4))-	20562-03-2
alpha-Hydroxytamoxifen	(E)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-di(phenyl)but-3-en-2-ol	97151-02-5
alpha-Solanine	Solanid-5-en-3-yl	20562-02-1
alpha-Tocopherol	(2R)-2,5,7,8-Tetramethyl-2-((4R,8R)-4,8,12-trimethyltridecyl)chroman-6-ol	59-02-9
Aminophenylnorharman	4-Pyrido(3,4-b)indol-9-ylaniline	219959-86-1
Amosite asbestos	Amosite asbestos	12172-73-5
AMP397	((7-Nitro-2,3-dioxo-1,4-dihydroquinoxalin-5-yl)methylamino)methylphosphonic acid	188696-80-2
Aristolochic acid	8-Methoxy-6-nitronaphtho(2,1-g)(1,3)benzodioxole-	10190-99-5

Common name	IUPAC name	CAS No.
	5-carboxylic acid	
Arsenite trioxide	Arsenite trioxide	1327-53-3
Azathioprine	6-(3-Methyl-5-nitroimidazol-4-yl)sulfanyl-7H-purine	446-86-6
Benzene	Benzene	71-43-2
Benzo(a)pyrene (B(a)P)	Benzo(a)pyrene	50-32-8
Benzo(a)pyrene diolepoxide (BPDE)	7,8,8a,9a-Tetrahydrobenzo(10,11)chryseno(3,4-b)oxirene-7,8-diol	58917-67-2
Benzo(f)quinoline	Benzo(f)quinoline	85-02-9
Benzo(h)quinoline	Benzo(h)quinoline	230-27-3
beta-Propiolactone	Oxetan-2-one	57-57-8
Bitumen fumes	Bitumen fumes	8052-42-4
Bleomycin	3-((2-(2-(2-((2-((4-((2-((6-amino-2-(3-amino-1-((2,3-diamino-3-oxopropyl)amino)-3-oxopropyl)-5-methylpyrimidine-4-carbonyl)amino)-3-(3-(4-carbamoyloxy-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl)oxy-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl)oxy-3-(3H-imidazol-4-yl)propanoyl)amino)-3-hydroxy-2-methylpentanoyl)amino)-3-hydroxybutanoyl)amino)ethyl)-1,3-thiazol-4-yl)1,3-thiazole-4-carbonyl)amino)propyl-dimethylsulfanium	11056-06-7
Carbon tetrachloride	Tetrachloromethane	56-23-5
Carboxymethylcellulose		9000-11-7
CC-1065	Benzo(1,2-b:4,3-b′)dipyrrole-3(2H)-carboxamide, 7-((1,6-dihydro-4-hydroxy-5-methoxy-7-((4,5,8,8a-tetrahydro-7-methyl-4-oxocyclopropa(c)pyrrolo(3,2-e)indol-2(1H)-yl)carbonyl)benzo(1,2-b:4,3-b')dipyrrol-3(2H)-yl)carbonyl)-1,6-dihydro-4-hydroxy-5-methoxy-, (7bR)-	69866-21-3
Chlorambucil	4-(4-(Bis(2-chloroethyl)amino)phenyl)butanoic acid	305-03-3
Chloroform	Chloroform	67-66-3
Chrysene	Chrysene	218-01-9
Cisplatin	*cis*-Diamminedichloridoplatinum(II)	15663-27-1
Clofibrate	Ethyl 2-(4-chlorophenoxy)-2-methylpropanoate	637-07-0
Coal tar	Coal tar	8007-45-2
Comfrey	(7-((E)-2-Methylbut-2-enoyl)oxy-4-oxido-5,6,7,8-tetrahydro-3H-pyrrolizin-4-ium-1-yl)methyl 2-hydroxy-2-(1-hydroxyethyl)-3-methylbutanoate	72698-57-8
Conjugated linoleic acid (CLA)	(9Z,11E)-Octadeca-9,11-dienoic acid	1839-11-8
Corn oil		8001-30-7
Crocidolite asbestos	Crocidolite asbestos	12001-28-4

Common name	IUPAC name	CAS No.
Cyclophosphamide	*N*,*N*-bis(2-Chloroethyl)-2-oxo-1-oxa-3-aza-2{5}-phosphacyclohexan-2-amine	50-18-0
Cyproterone acetate	3′H-Cyclopropa(1,2)pregna-1,4,6-triene 3,20-dione,6-chloro-1-beta,2-beta-dihydro-17-hydroxy-	427-51-0
Daidzein	7-Hydroxy-3-(4-hydroxyphenyl)chromen-4-one	486-66-8
Di(2-ethylhexyl)phthalate (DEHP)	Bis(2-ethylhexyl) benzene-1,2-dicarboxylate	117-81-7
Diallyl sulphide	3-Prop-2-enylsulfanylprop-1-ene	592-88-1
Diallyl sulphone	3-Prop-2-enylsulfonylprop-1-ene	16841-48-8
Dichloroacetic acid (DCA)	2,2-Dichloroacetic acid	79-43-6
Dicyclanil	4,6-Diamino-2-(cyclopropylamino)pyrimidine-5-carbonitrile	112636-83-6
Diethylnitrosamine (DEN)	*N*,*N*-Diethylnitrous amide	55-18-5
Dimethylarsinic acid	Dimethylarsinic acid	75-60-5
Dimethylnitrosamine (DMN)	*N*,*N*-Dimethylnitrous amide	62-75-9
Dinitropyrenes	Dinitropyrenes	nd
Dipropylnitrosamine (DPN)	*N*,*N*-Dipropylnitrous amide	621-64-7
d-Limonene	(4R)-1-Methyl-4-prop-1-en-2-ylcyclohexene	5989-27-5
Ellagic acid	2,3,7,8-Tetrahydroxy-chromeno(5,4,3-cde)chromene-5,10-dione	476-66-4
Ethanol	Ethanol	64-17-5
Ethylene oxide	Oxirane	75-21-8
Ethylmethanesulphonate (EMS)	Ethyl methanesulfonate	62-50-0
Etoposide	4′-Demethyl-epipodophyllotoxin 9-(4,6-O-(R)-ethylidene-beta-D-glucopyranoside), 4′-(dihydrogen phosphate)	33419-42-0
Eugenol	2-Methoxy-4-prop-2-enylphenol	97-53-0
Ferric nitrilotriacetate	2-(bis(2-Oxido-2-oxoethyl)amino)acetate; iron(+3) cation	16448-54-7
Flumequine	9-Fluoro-5-methyl-1-oxo-6,7-dihydro-1H,5H-pyrido(3,2,1-ij)quinoline-2-carboxylic acid	42835-25-6
Folic acid	(2S)-2-((4-((2-Amino-4-oxo-1H-pteridin-6-yl)methylamino)benzoyl)amino)pentanedioic acid	59-30-3
Fructose	(3S,4R,5R)-1,3,4,5,6-Pentahydroxyhexan-2-one	57-48-7
Genistein	5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	446-72-0
Glucose	(3R,4S,5S,6R)-6-(Hydroxymethyl)oxane-2,3,4,5-tetrol	50-99-7
Glycidamide	Oxirane-2-carboxamide	5694-00-8
Heptachlor	1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-1H-4,7-methanoindene	76-44-8

Common name	IUPAC name	CAS No.
Hexachlorobutadiene	1,1,2,3,4,4-Hexachlorobuta-1,3-diene	87-68-3
Hexavalent chromium	Chromium(VI)	7440-47-3
Hydrazine sulphate	Hydrazine sulfate	10034-93-2
Hydroxyurea	Hydroxyurea	127-07-1
Isopropylmethanesulphonate (iPMS)	Propan-2-yl methanesulfonate	926-06-7
Jervine	(3S,3′R,3′aS,6′S,6aS,6bS,7′aR,9R,11aS,11bR)-3-Hydroxy-3′,6′,10,11b-tetramethylspiro(1,2,3,4,6,6a,6b,7,8,11a-decahydrobenzo(i)fluorene-9,2′-3a,4,5,6,7,7a-hexahydro-3H-furo(4,5-b)pyridine)-11-one	469-59-0
Kojic acid	5-Hydroxy-2-(hydroxymethyl)pyran-4-one	501-30-4
Leucomalachite green	4-((4-Dimethylaminophenyl)-phenylmethyl)-*N*,*N*-dimethylaniline	129-73-7
Levofloxacin	(-)-(S)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido(1,2,3-de)-1,4-benzoxazine-6-carboxylic acid	100986-85-4
Lycopene	(6E,8E,10E,12E,14E,16E,18E,20E,22E,24E,26E)-2,6,10,14,19,23,27,31-Octamethyldotriaconta-2,6,8,10,12,14,16,18,20,22,24,26,30-tridecaene	502-65-8
Malachite green	(4-((4-Dimethylaminophenyl)-phenylmethylidene)-1-cyclohexa-2,5-dienylidene)-dimethylazanium chloride	569-64-2
Methyl bromide	Bromomethane	74-83-9
Methylcellulose		9004-67-5
Methyl clofenapate	Methyl 2-(4-(4-chlorophenyl)phenoxy)-2-methylpropanoate	21340-68-1
Methylmethanesulphonate (MMS)	Methylmethanesulfonate	66-27-3
Metronidazole	2-(2-Methyl-5-nitroimidazol-1-yl)ethanol	443-48-1
Mitomycin-C	6-Amino-1,1a,2,8,8a,8b-hexahydro-8-(hydroxymethyl)-8a-methoxy-5-methyl-azirino(2′,3′:3,4) pyrrolo(1,2-a)indole-4,7-dione carbamate (ester)	50-07-7
N7-Methyldibenzo(c,g)carbazole (NMDBC)	*N*-Methyl-7H-dibenzo(c,g)carbazole	27093-62-5
N-Ethyl-*N*-nitrosourea (ENU)	1-Ethyl-1-nitrosourea	759-73-9
N-Hydroxy-2-acetylaminofluorene	*N*-(9H-Fluoren-2-yl)-*N*-hydroxyacetamide	53-95-2
Nickel subsulphide	Nickel sulfide	12035-72-2
Nifuroxazide	4-Hydroxy-*N*-((5-nitrofuran-2-yl)methylidene-amino)benzamide	965-52-6

Common name	IUPAC name	CAS No.
Nitrofurantoin	1-((5-Nitrofuran-2-yl)methylideneamino)imidazolidine-2,4-dione	67-20-9
N-Methyl-*N*′-nitro-*N*-nitroso-guanidine (MNNG)	1-Methyl-2-nitro-1-nitrosoguanidine	70-25-7
N-Methyl-*N*-nitrosourea (MNU)	1-Methyl-1-nitrosourea	684-93-5
N-Nitrosodibenzylamine (NDBzA)	*N*,*N*-Bis(phenylmethyl)nitrous amide	5336-53-8
N-Nitrosomethylbenzylamine	*N*-Methyl-*N*-(phenylmethyl)nitrous amide	937-40-6
N-Nitrosonornicotine (NNN)	3-(1-Nitrosopyrrolidin-2-yl)pyridine	80508-23-2
N-Nitrosopyrrolidine	1-Nitrosopyrrolidine	930-55-2
N-Propyl-*N*-nitrosourea (PNU)	1-Nitroso-1-propylurea	816-57-9
o-Aminoazotoluene	2-Methyl-4-(2-methylphenyl)diazenylaniline	97-56-3
o-Anisidine	2-Methoxyaniline	90-04-0
Olive oil		8001-25-0
Oxazepam	7-Chloro-3-hydroxy-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one	604-75-1
p-Cresidine	2-Methoxy-5-methylaniline	120-71-8
Peroxyacetyl nitrate (PAN)	Nitro ethaneperoxoate	2278-22-0
Phenobarbital	5-Ethyl-5-phenyl-1,3-diazinane-2,4,6-trione	50-06-6
Phorbol-12-myristate-13-acetate (TPA)	12-*O*-tetradecanoylphorbol-13-acetate	16561-29-8
Phosphate buffer		nd
Phorone	2,6-Dimethylhepta-2,5-dien-4-one	504-20-1
Polyphenon E	Polyphenon E	188265-33-0
Potassium bromate	Potassium bromate	7758-01-2
Procarbazine hydrochloride	4-((2-Methylhydrazinyl)methyl)-*N*-propan-2-ylbenzamide hydrochloride	366-70-1
Propylene glycol	Propane-1,2-diol	57-55-6
Quinoline	Quinoline	91-22-5
Riddelliine	7-Ethylidene-10-hydroxy-10-hydroxymethyl-9-methylene-2,3,4,4a,7,8,9,10,13,13b-decahydro-5,12-dioxa-2a-aza-cyclododeca(cd)pentalene-6,11-dione	23246-96-0
Saline		nd
Sesame oil		8008-74-0
Sodium bicarbonate	Sodium hydrogen carbonate	144-55-8
Sodium saccharin	Sodium 1,1-dioxo-1,2-benzothiazol-2-id-3-one	128-44-9
Solanidine	Solanid-5-en-3beta-ol	80-78-4
Solasodine	Spirosol-5-en-3-ol	126-17-0

Common name	IUPAC name	CAS No.
Soy oil		8001-22-7
Streptozotocin	1-Methyl-1-nitroso-3-((2S,3R,4R,5S,6R)-2,4,5-trihydroxy-6-(hydroxymethyl)oxan-3-yl)urea	18883-66-4
Sucrose	(2R,3R,4S,5S,6R)-2-((2S,3S,4S,5R)-3,4-Dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl)oxy-6-(hydroxy-methyl)oxane-3,4,5-triol	57-50-1
Tamoxifen	2-(4-((Z)-1,2-Di(phenyl)but-1-enyl)phenoxy)-*N*,*N*-dimethylethanamine	10540-29-1
Thiotepa	Tris(aziridin-1-yl)-sulfanylidenephosphorane	52-24-4
Toremifene citrate	2-(4-((Z)-4-Chloro-1,2-di(phenyl)but-1-enyl)phenoxy)-*N*,*N*-dimethylethanamine 2-hydroxypropane-1,2,3-tricarboxylic acid	89778-27-8
trans-4-Hydroxy-2-nonenal	(E)-4-Hydroxynon-2-enal	128946-65-6
Tricaprylin	1,3-Di(octanoyloxy)propan-2-yl octanoate	538-23-8
Trichloroethylene (TCE)	1,1,2-Trichloroethene	79-01-6
Tris-(2,3-dibromopropyl)phosphate	Tris(2,3-dibromopropyl) phosphate	126-72-7
Uracil	1H-Pyrimidine-2,4-dione	66-22-8
Urethane	Ethyl carbamate	51-79-6
Vinyl carbamate	Ethenyl carbamate	15805-73-9
Vitamin E	(2R)-2,5,7,8-Tetramethyl-2-((4R,8R)-4,8,12-trimethyltridecyl)chroman-6-ol	59-02-9
Water	Water	7732-18-5
Wyeth 14,643	2-(4-Chloro-6-((2,3-dimethylphenyl)amino)pyrimidin-2-yl)sulfanylacetic acid	50892-23-4

1.0 EXECUTIVE SUMMARY

Induced chromosomal and gene mutations cause genetic diseases, birth defects and other disease conditions and play a role in carcinogenesis. While it is widely accepted that *in vivo* mutation assays are more relevant to the human condition than *in vitro* assays, our ability to evaluate mutagenesis *in vivo* in a broad range of tissues has historically been quite limited. The development of transgenic rodent (TGR) mutation models has given us the ability to detect, quantify and sequence mutations in a range of somatic and germ cells.

This document provides a comprehensive review of the TGR mutation assay literature and assesses the potential use of these assays in a regulatory context. The information is arranged as follows.

1. *TGR mutagenicity models and their use for the analysis of gene and chromosomal mutation are fully described*. The TGR mutation assay is based on transgenic rats and mice that contain multiple copies of chromosomally integrated plasmid and phage shuttle vectors that harbour reporter genes for the detection of mutation. Mutagenic events arising in a rodent are scored by recovering the shuttle vector and analysing the phenotype of the reporter gene in a bacterial host. TGR gene mutation assays allow mutations induced in a genetically neutral transgene to be scored in any tissue of the rodent and therefore circumvent many of the existing limitations associated with the study of *in vivo* gene mutation. TGR models for which sufficient data are available to permit evaluation include Muta™Mouse, Big Blue® mouse and rat, *lacZ* plasmid mouse and *gpt* delta mouse and rat. Mutagenesis in the TGR models is normally assessed as a mutant frequency; if required, however, molecular analysis can provide additional information.

2. *The principles underlying current Organisation for Economic Co-operation and Development (OECD) tests for the assessment of genotoxicity* in vitro *and* in vivo*, as well as non-transgenic assays available for the assessment of gene mutation, are described.* OECD guidelines exist for a range of *in vitro* mutation assays that are capable of detecting both chromosomal and gene mutations. *In vivo* assays are required components of a thorough genetic toxicity testing programme. For somatic cells, OECD guidelines are currently available only for assays capable of assessing induced chromosomal mutation. In addition, there are non-transgenic assays that can be used for analysis of gene mutation; none of these have an OECD test guideline. Existing *in vivo* assays are limited by a range of different factors, including cost of the assay, the number of tissues in which genotoxicity may be measured, the state of understanding of the endpoint and the nature of the chemicals that will be detected.

3. *All available information pertaining to the conduct of TGR assays and important parameters of assay performance are tabulated and analysed.* As of December 2007, 228 agents have been evaluated using TGR assays. The majority of experimental records have assessed a subset of these chemicals, most of which are strong mutagens and carcinogens. Of the 153 agents whose carcinogenicity has been evaluated, 118 are carcinogens and 35 are non-carcinogens (including 13 chemicals routinely used as vehicle controls). The following conclusions may be drawn from the existing TGR mutation data:
   - The ability to use all routes of administration has been demonstrated. Experiments can be tailored to use the most relevant route of administration..
   - The ability to examine mutation in virtually all tissues has been demonstrated. TGR assays have most commonly examined mutagenicity in the liver and bone marrow.

- The majority of the experiments have used shorter administration times than is currently recommended by the International Workshop on Genotoxicity Testing (IWGT). There are limited data available to assess the effects of longer sampling time, except with extremely short administration times. However, two recent studies on weak mutagens confirm the robustness of the recommended protocol.
- Although it is recognised that a number of factors may influence the tissue specificity of mutation, including cell turnover, deoxyribonucleic acid (DNA) repair, toxicokinetics and the nature of the genetic target, there are currently limited experimental data specific to transgenes that are available to inform the discussion.
- Limited data are available to evaluate the results of TGR assays in known target tissues for carcinogenicity. A case-by-case analysis of instances in which discrepancies are apparent suggests that in the majority of cases, factors such as non-genotoxic mechanism of action, inappropriate mode of administration or inadequate study design may account for the observed negative result in the tissue of interest.
- Qualitatively similar results have been obtained in the majority of experiments that have assayed different transgenes using similar experimental parameters.
- The spontaneous mutant frequency in most somatic tissues of transgenic rats and mice is five- to ten-fold higher than that observed in available endogenous loci using the same animals. Factors such as the age of the animal, the tissue and the animal model influence the absolute value of the spontaneous mutant frequency. In most somatic tissues, with the exception of the brain, there is an age-related increase in mutant frequency throughout the life of the animal. Most, but not all, studies suggest that the spontaneous mutant frequency in male germline tissues remains low and constant throughout the life of the animal.
- Multiple treatments of mutagen appear to increase mutant frequencies in neutral transgenes in an approximately additive manner. However, extremely long treatment times (12 weeks or longer) may produce an apparent increase in mutant frequency through clonal expansion, genomic instability in developing preneoplastic foci or tumours or oxidative damage of DNA resulting from chronic induction of cytochrome P-450 mono-oxygenases.
- The time required to reach the maximum mutant frequency is tissue specific and appears to be related to the turnover time of the cell population. The optimal sampling time differs according to tissue, with liver and bone marrow at opposite extremes among proliferating somatic tissues: in bone marrow, the mutant frequency appears to reach a maximum at extremely short sampling times and then decreases over 28 days following an acute treatment; in liver, the induced mutant frequency increases over the month following exposure, reaches a maximum and remains relatively constant thereafter. There are insufficient data available for other tissues to support any conclusion regarding optimal sampling time.
- The results of studies carried out on a given chemical using similar experimental protocols suggest that the TGR assays show good qualitative reproducibility in both somatic and germ cells and quantitative reproducibility over a limited range of conditions and laboratories. The data are insufficient to allow conclusions to be drawn regarding the quantitative reproducibility of the assays over a wider range of protocol variations.
- Although there exists a theoretical possibility that *ex vivo* and *in vitro* mutations may arise during the course of a TGR experiment, these types of mutations are expected to be extremely rare in a properly conducted experiment using the major TGR models. For positive selection systems, any such mutations will not be detected.
- The weight of evidence suggests that transgenes and endogenous genes respond in approximately the same manner to mutagens in the few instances where direct comparisons are possible. Sensitivity is determined in large part by the spontaneous mutant frequency: the higher spontaneous mutant frequency in transgenes, compared with testable endogenous genes, reduces their sensitivity, especially when acute

treatments are used. The sensitivity of transgenes can be enhanced by increasing the administration time.

- Mutagens that induce deletions are likely to be detected more easily in certain endogenous genes than in transgenes as a result of phenotypic selection issues.
- A high proportion of the TGR experiments carried out to date have examined the activities of compounds that are known to be strong mutagens. A limited number of non-carcinogens have been evaluated with TGR assays,
- Molecular analysis of induced mutations in transgenic targets is possible and provides additional information in situations where high interindividual variation is observed and clonal expansion is suspected, when weak responses are obtained or when mechanistic information is desired. However, DNA sequence analysis of mutants is laborious and adds to the cost of the experiment; sequencing would not normally be required when testing drugs or chemicals for regulatory applications, particularly where a clear positive or negative result is obtained.

4. *The performance of TGR assays, both in isolation and as part of a battery of* in vitro *and* in vivo *short-term genotoxicity tests, in predicting carcinogenicity is described.* Analysis of the predictivity of TGR assays for carcinogenicity is hindered somewhat by the fact that TGR data are available for only a small number of non-carcinogens. Of the 118 carcinogens and 35 non-carcinogens that have been assessed using TGR assays, the following conclusions can be drawn regarding the predictivity and complementarity of TGR assays compared with a range of other OECD *in vitro* and *in vivo* genotoxicity tests:
   - The TGR assay has high sensitivity and positive predictivity for carcinogenicity,
   - meaning that most carcinogens have positive results in TGR assays and that there is a high probability that a chemical with a positive result in a TGR assay is a carcinogen.
   - As is the case with most genotoxicity assays, the TGR assay exhibits relatively low specificity and negative predictivity (compared with sensitivity and positive predictivity), meaning that relatively fewer non-carcinogens are negative in TGR assays and that there is a lower probability that a chemical with a negative result in a TGR assay is a non-carcinogen.
   - The positive and negative predictivities for the individual TGR and *Salmonella* assays were almost identical. Among the *in vivo* tests, the TGR assays were comparable with all except the comet assay, which had a slightly better combined predictivity. Among two-test combinations (*i.e.* a chemical could be predicted to be carcinogenic if the outcome of *either* short-term assay was positive), the TGR “or” cytogenetic assay gave the best combined positive predictivity, except for the TGR “or” comet assay combination, which was slightly better. Despite the lack of substantial increases in predictive values of the test batteries compared with the component assays alone, the test batteries had a much lower false-negative rate.
   - The TGR assay was usually positive for those carcinogens that were positive in both *Salmonella* and *in vitro* chromosomal aberration assays. In contrast, the *in vivo* micronucleus assay had a much higher false-negative rate for the same chemicals (0.22 *vs.* 0.15). If *in vivo* confirmation of positive results from both *Salmonella* and *in vitro* chromosomal aberration assays is warranted, the TGR assay is likely a better choice than the *in vivo* micronucleus assay.
   - The number of chemicals tested in the same assays is very small. Nevertheless, for chemicals having positive *Salmonella* and negative *in vitro* chromosomal aberration results (presumptive gene mutagens), selecting either the TGR assay or the *in vivo* micronucleus assay as the *in vivo* confirmation assay did not markedly affect the proportion of correct carcinogenicity predictions. The *in vivo* comet assay had a higher false-negative rate than the above two scenarios in which the *Salmonella* assay is positive and the *in vitro* chromosomal aberration assay is negative.

- For chemicals having positive *in vitro* chromosomal aberration and negative *Salmonella* results (presumptive clastogens), selecting the *in vivo* micronucleus assay as the *in vivo* confirmation assay led to a higher proportion of correct carcinogenicity predictions than did selecting the TGR or *in vivo* comet assay. It should be noted that this observation is also based on data from only a few chemicals.
- For those carcinogens with negative results in both *Salmonella* and *in vitro* chromosomal aberration assays, adding TGR assays to the test battery improved the overall predictivity over the *in vivo* micronucleus or the comet assay, since neither assay identified the carcinogens missed by the *in vitro* assays.

5. *A novel model for determining the performance of new* in vivo *gene mutation assays was introduced.* This model determines the ability of a new assay to accurately detect (*i.e.* positively predict) whether the assay will correctly identify *in vivo* gene mutagens. This method relies on the extensive DNA sequence data in the Transgenic Rodent Assay Information Database (TRAID) and is consistent with the OECD guidance document on validation of new test methods, which recommends that new tests be validated against the most biologically relevant endpoint. The analysis shows that the TGR assay has a near-perfect positive predictivity for gene mutation. The model is developed further to argue that a test battery designed to most optimally detect the endpoints of genotoxicity will, accordingly, be the most effective for detecting genotoxins that are carcinogenic.

6. *Recommendations are made regarding the experimental parameters for TGR assays and the use of TGR assays in a regulatory context.* Recommendations, based on internationally harmonised criteria (IWGT), are made regarding the proper conduct of a TGR assay. These recommendations relate to accepted characteristics of a TGR mutation assay, treatment protocols and post-treatment sampling procedures. Of particular importance in optimising TGR protocols are two experimental variables – the administration time and the sampling time. Based on observations that mutations accumulate with each treatment, a repeated-dose regimen for a period of 28 days is strongly encouraged, with sampling at 3 days following the final treatment. If slowly proliferating tissues are of particular importance, then a longer sampling time may be more appropriate. Additional confidence in the recommended test protocol will be provided by research that examines the following:
   - *The influence of the administration time on the observed mutant frequency for weak mutagens.* It has not conclusively been determined if data (especially negative results) from experiments using an administration time of less than 28 days should be discounted, if a 28-day treatment period is sufficiently long to permit the detection of weak mutagen–induced mutations in all tissues or if weak mutagens could in fact be detected using treatment times shorter than 28 days. Although more studies are required, two recent studies have shown that the recommended protocol, which uses a 28-day exposure period, is robust enough to detect weak mutagens.
   - *The influence of the frequency of treatment on the observed mutant frequency.* The effects of weekly versus daily administrations on mutant frequency and on the ultimate conclusions of TGR experiments have not yet been thoroughly investigated.
   - *The influence of sampling time following repeated administrations on the mutant frequency in both slowly and rapidly dividing tissue, particularly when examining weak mutagens.* At the current time, there are insufficient comparative data available for a range of tissues.

7. *Considerations are given to how a TGR assay might be used within a new short-term test battery for assessing new compounds.* The test battery consists of various combinations of four assays – *Salmonella*, *in vitro* chromosomal aberration, *in vivo* micronucleus and TGR. This hypothetical strategy is based on the conclusions obtained from the predictivity analysis and the relative costs of the *in vivo* assays. An alternative approach to the above is described that shows how TGR assays could be used to resolve conflicts between *in vitro* and *in vivo*

tests that are currently components of the standard genotoxicity test battery – *Salmonella*, *in vitro* chromosomal aberration and *in vivo* micronucleus. In situations where the standard test battery has been conducted and there are conflicting results – particularly in situations where the *Salmonella* assay has a positive result but the *in vivo* micronucleus assay is negative – the TGR assay may be conducted as an additional test to resolve the conflict. Test strategies are based on an analysis of the existing data. Confidence in these strategies would be enhanced by additional experimental data in the following areas:

- TGR data for additional non-carcinogens to increase the proportion of non-carcinogens in the dataset;
- additional testing to fill data gaps for chemicals having a known TGR assay but missing data from the *Salmonella*, *in vitro* chromosomal aberration or *in vivo* micronucleus assay;
- the testing of additional chemicals using an accepted test guideline for TGR mutation assays.

This extensive review fulfils the requirements for the preparation of an OECD Detailed Review Paper according to the OECD *Guidance Document for the Development of OECD Guidelines for Testing of Chemicals* (OECD, 2006). Based on the extensive information and analyses in this review, there is sufficient evidence to support the recommendation that OECD undertake the development of a Test Guideline on Transgenic Rodent Gene Mutation Assays. Accordingly, it is recommended that OECD establish an Expert Working Group to develop such a Test Guideline and serve as an international forum for undertaking any additional research that would lead to the development of a fuller understanding of the variables surrounding the conduct of TGR mutation assays.

2.0 INTRODUCTION TO TRANSGENIC RODENT MUTATION MODELS AND ASSAYS

2.1 Overview

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

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

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

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

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

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

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

2.2 Transgenic rodent mutation systems

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

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

2.2.1 Muta™Mouse

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

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

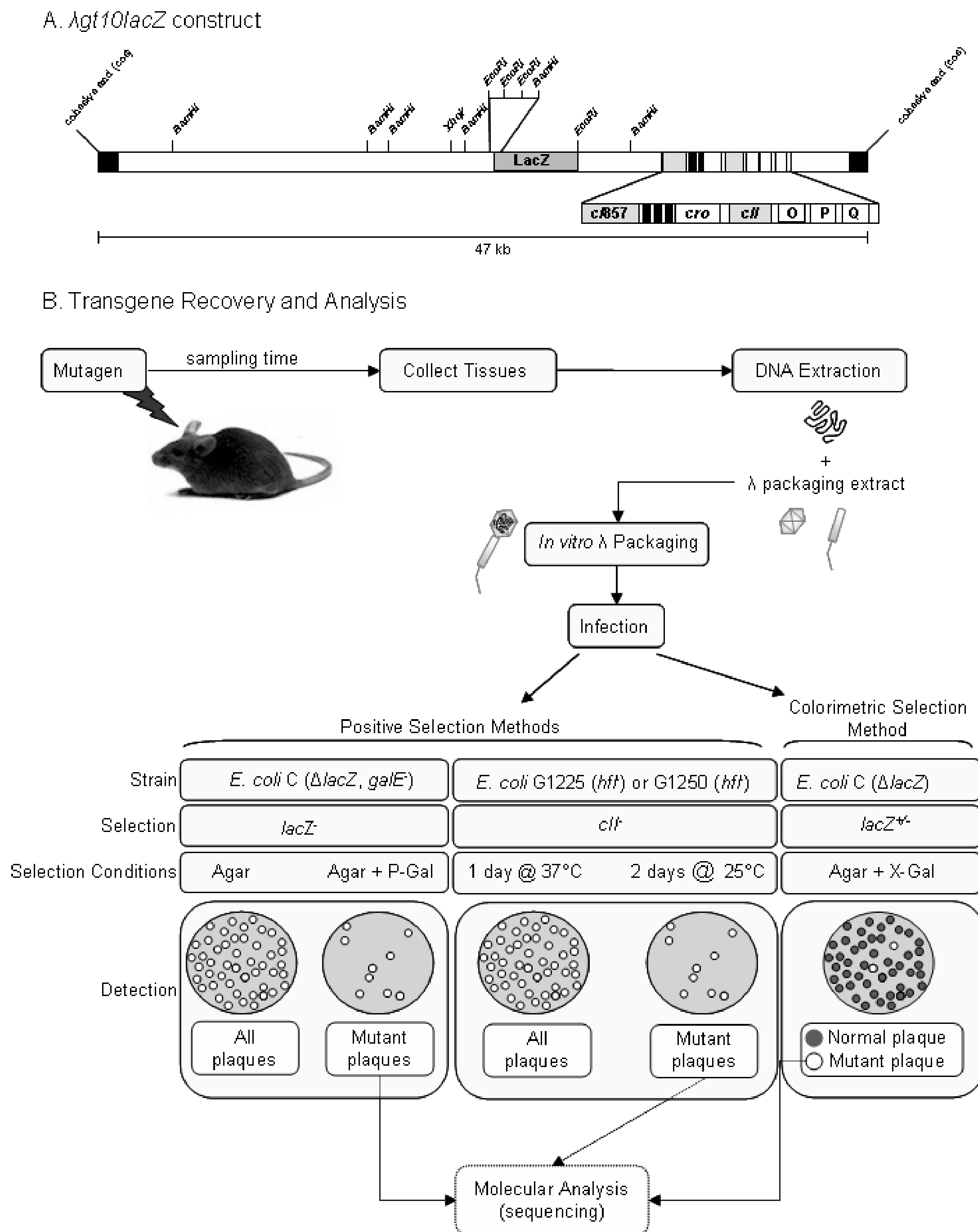


Figure 2-1. The Muta™Mouse transgenic mutation assay:
(A) the λgt10*lacZ* construct; (B) transgene recovery and analysis

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

2.2.2 Big Blue®

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

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

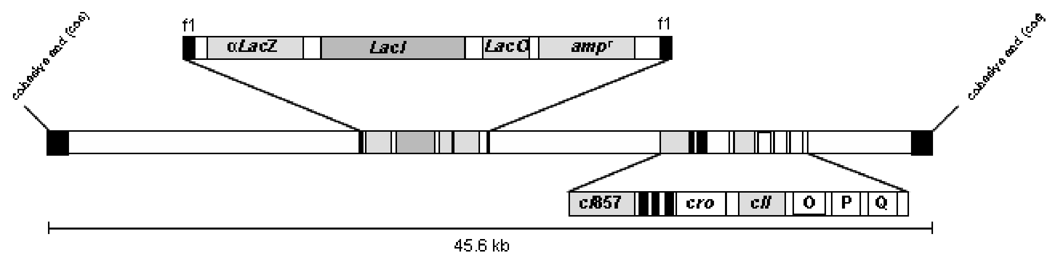
Mutations arising in the rodent genomic DNA are scored in *E. coli* SCS-8 cells (*lacZ*ΔM15) following *in vitro* packaging of the λLIZα phage (Figure 2-2B). White (colourless) plaques will arise from phage bearing wild-type *lacI* (encoding functional Lac repressor) when the SCS-8 host is plated on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

2.2.3 LacZ plasmid mouse

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

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

A. λLIZα construct



B. Transgene Recovery and Analysis

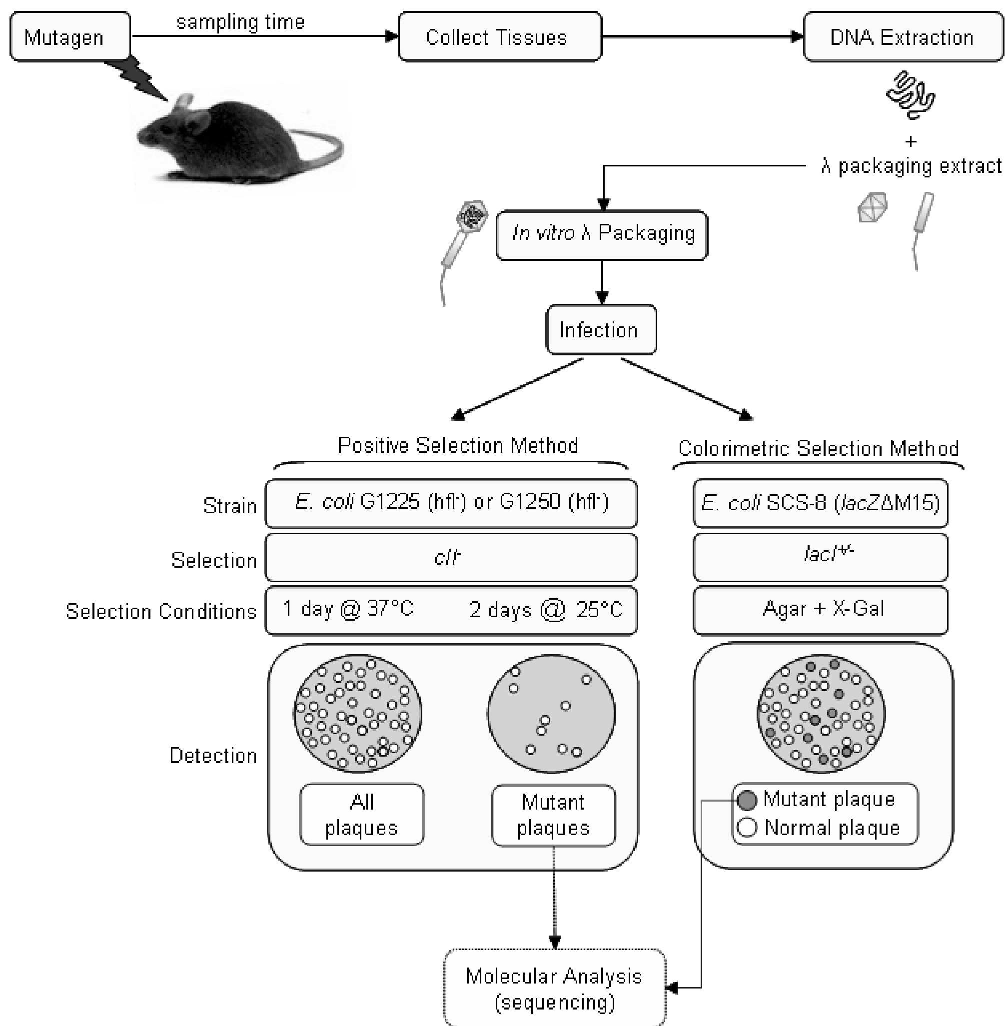


Figure 2-2. The Big Blue® (mouse or rat) transgenic mutation assay: (A) the λLIZα construct; (B) transgene recovery and analysis

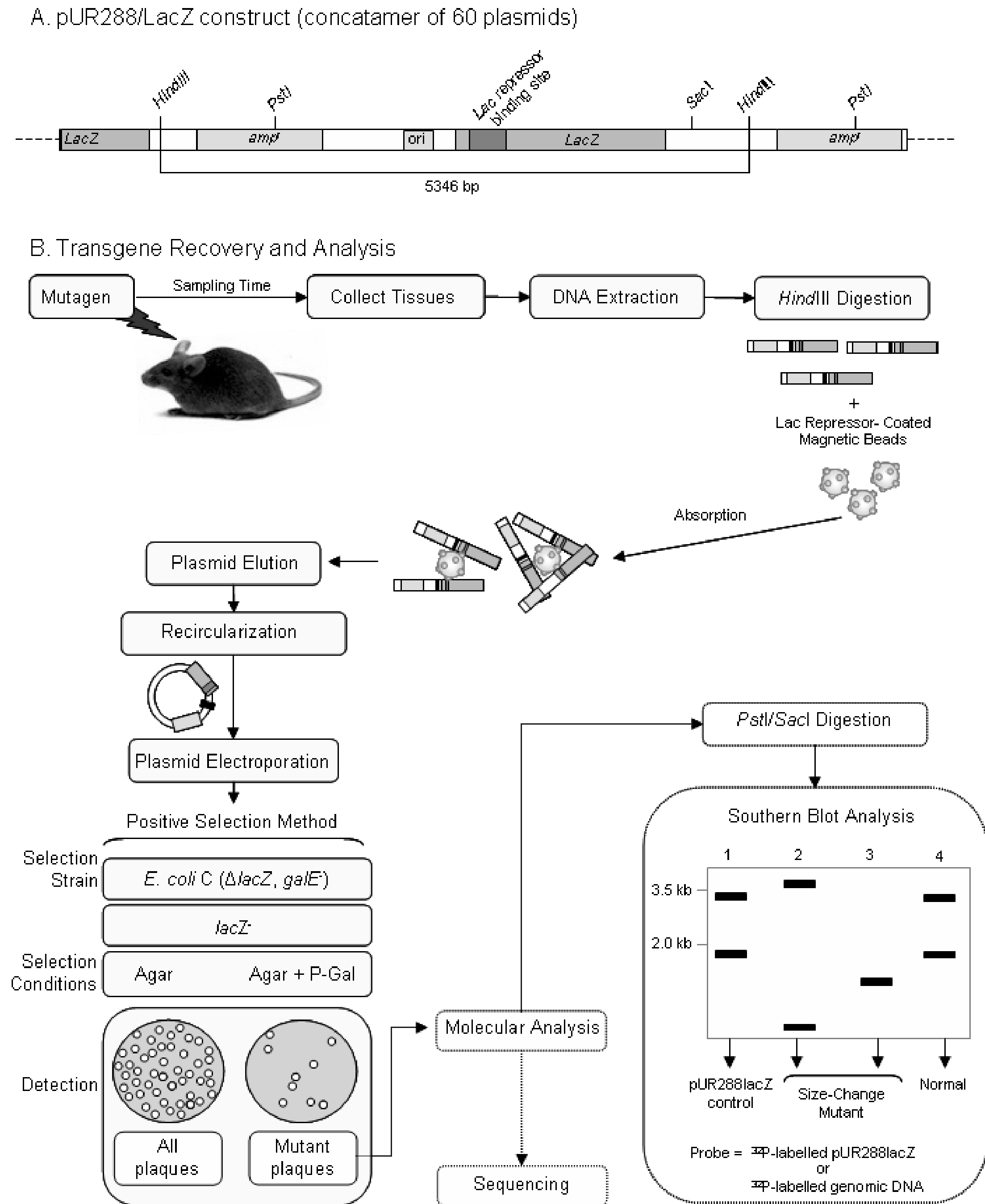


Figure 2-3. The *lacZ* plasmid mouse transgenic mutation assay:
(A) the pUR288/*lacZ* plasmid; (B) transgene recovery and analysis

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

2.2.4 gpt delta rodents

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

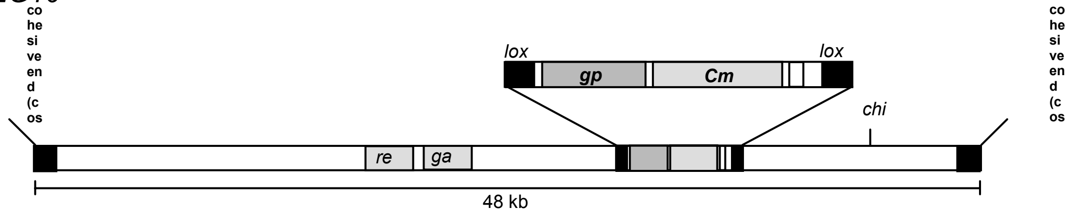
More recently, a *gpt* delta rat has been developed in a Sprague-Dawley background (Hayashi *et al*., 2003) and an F-344 background (personal communication by Nohmi). The *gpt* delta rat has approximately 10 copies of the λEG10 vector integrated at position 4q24-q31. The transgenic rat is available as a hemizygote only (Hayashi *et al*., 2003).

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

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

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

A. *λEG10*



B. Transgene Recovery and

Mutage

sampling

Collect

DNA

+

λ packaging

In vitro λ

Infectio

Positive Selection

Selection	*gpt*⁻	*Spi*	
Strain	*E. coli* YG6020 (*gpt*⁻, ↓ Conversion to plasmid by Cre-lox recombination *gp* Cm^r	*E. coli XL1-Blue MRA*	*E. coli XL1-Blue MRA (P2)*
Selection	Agar + / Agar + Cm + 6-	Agar, 24	Agar, 48
Detection	All colonies / Mutant colonies	All plaques	Mutant plaques

Molecular Analysis

Figure 2-4. *gpt* delta mouse transgenic mutation assay:
(A) the λEG10 construct; (B) transgene recovery and analysis

2.2.5 Use of the λ cII transgene

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

2.2.6 Other transgenic systems

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

2.3 The transgenic rodent mutation experiment

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

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

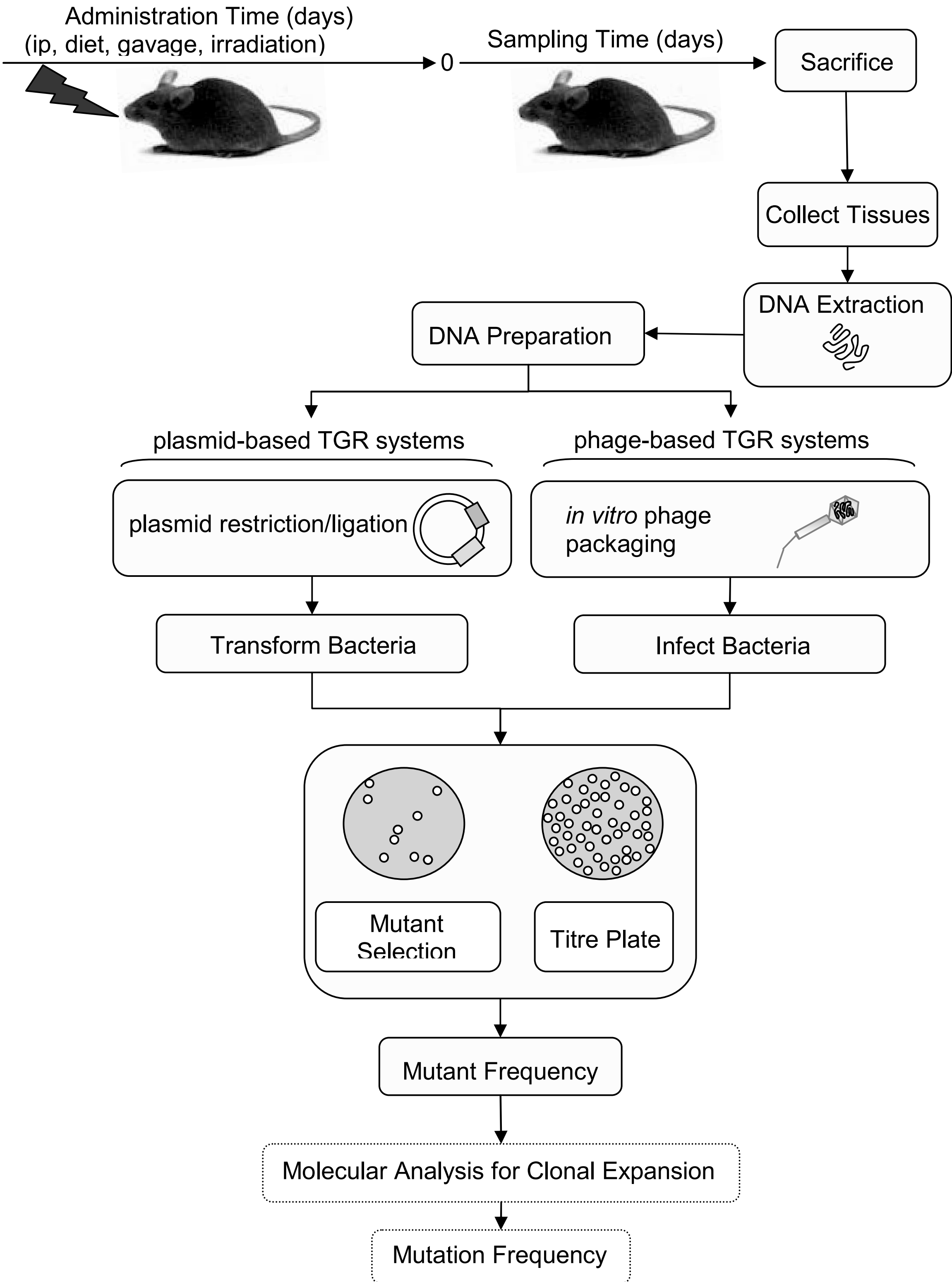


Figure 2-5. Transgenic rodent experiment

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

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

2.3.1 Molecular analysis of mutations

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

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

DNA sequence analysis has demonstrated that the overwhelming majority of mutations arising spontaneously or following treatment with mutagens in the Muta™Mouse (*lacZ*), Big Blue® (*lacI*) and *gpt* delta (*gpt*) models are point mutations involving base substitutions or small frameshifts. Some types of mutations may not be readily detectable with the Muta™Mouse (*lacZ*) or the Big Blue® mouse (*lacI*) systems. There has been concern that large deletions would not be detectable because they will not be recoverable. This would be the case for deletions that extend into the λ vector and inactivate essential phage sequences. In addition, there are size limitations for *in vitro* packaging: λ vectors must have cos sites (segments of single-stranded DNA 12 nucleotide bases long that exist at both ends of the bacteriophage lambda's double-stranded genome) separated by 38–51 kb. Thus, insertions and deletions that produce λ vectors outside this size range will not be recovered using

conventional packaging. For instance, Tao, Urlando and Heddle (1993a) found that *lacI* was substantially less mutable than *Dlb-1* by X-rays. As mutant frequencies induced by other mutagens in the *lacI* transgene are not generally lower than in *Dlb-1*, and as X-rays produce a relatively high proportion of deletions as compared with point mutations, the result is consistent with the notion that bacteriophage transgenes will be less sensitive to clastogens (Tao, Urlando and Heddle, 1993a). In principle, some large deletions can be recovered; for example, deletions with one endpoint located in a copy of the reporter transgene (*e.g. lacI* or *lacZ*) and the other endpoint located in a different copy of the transgene should be recoverable and would appear to be much smaller than is actually the case. Whether this actually occurs has not yet been determined.

The *gpt* delta (Spi) and *lacZ* plasmid mouse system are particularly useful for the detection and characterisation of deletion mutations. As described in Section 2.2.4, Spi^{-} selection in the *gpt* delta rodents (Figure 2-4B) is based on the simultaneous inactivation of both the *gam* and *redAB* genes, a molecular event that generally arises when deletions extend into (or through) both genes. Detection of these mutants is limited by packaging constraints; thus, detectable deletions must be less than 10 kb (the largest detected to date is approximately 9.7 kb). Mutants detected by Spi^{-} selection can be easily characterised by DNA sequence analysis (Nohmi and Masumura, 2005).

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

2.3.2 Clonal correction

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

2.4 Perceived advantages and disadvantages of transgenic rodent mutation assays

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

2.4.1 Advantages

2.4.1.1 Numerous tissues for analysis

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

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

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

2.4.1.2 Ease of molecular analysis

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

2.4.1.3 Gene mutation assay

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

2.4.1.4 Flexible mode of administration

In TGR assays, the test agent can be administered through any route. This provides the flexibility for a researcher to tailor an experiment to the most appropriate mode of administration; there is no limitation based on a need to distribute the test agent to the target tissue examined using

the existing test. For example, unscheduled DNA synthesis in liver and cytogenetics assays in bone marrow or peripheral blood may be unsuitable for evaluation of some substances to which humans are exposed primarily through topical or inhalation exposure. In contrast, TGR assays facilitate testing of "site of contact" mutagens at the site of exposure (Dean *et al*., 1999) and allow for consideration of parameters such as absorption, distribution and tissue-specific metabolism when selecting tissues for analysis.

2.4.1.5 Relatively few limitations

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

2.4.1.6 Level of technical ease and reproducibility of results

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

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

2.4.1.7 Economy of animals

New toxicological methods must be assessed for the extent to which they address the 3 Rs that are now fundamental principles of toxicity test development: *reduction* in the number of animals used; *refinement* in the procedures to minimise animal suffering or distress; and *replacement* of animal use with alternative tests. TGR mutation models are particularly relevant to *reduction* of animal use, in that they do not use a large number of animals relative to the existing gene mutation assay (*e.g.* the mouse spot test requires ~1 500 animals) or chronic rodent carcinogenicity assays. Moreover, TGR experiments can, in principle, be combined with other *in vivo* genotoxicity test endpoints, such as the peripheral blood micronucleus assay (S. Itoh *et al*., 1999; T. Itoh *et al*., 2000; Nishikawa *et al*., 2000; Noda *et al*., 2002), the bone marrow micronucleus assay (Manjanatha *et al*., 2004) or even conventional systemic toxicity assays, to further reduce the use of rodents. In addition, TGR assays may, in principle, be used to assess both germ cell and somatic cell mutations, although the administration time and sampling time experimental parameters (see Sections 6.1.2.5 and 6.1.3.1)

would have to be very carefully designed in order to allow both types of assays to be carried out adequately.

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

2.4.1.8 Corresponding transgenic in vitro tests

Although the purpose of this Detailed Review Paper is to review *in vivo* transgenic assays for the detection of gene mutations, it should be noted that there are a number of cell lines that have been derived from these *in vivo* TGR models; these cell lines may be used for *in vitro* mutation assays (Wyborski *et al*., 1995; Suri *et al*., 1996; Erexson, Cunningham and Tindall, 1998; Saranko and Recio, 1998; Erexson, Watson and Tindall, 1999; Ryu *et al*., 1999b; Gonda *et al*., 2001; McDiarmid *et al*., 2001, 2002; Ryu, Kim and Chai, 2002; White *et al*., 2003; Takeiri et al., 2006; Xu et al., 2007). The *in vitro* assays use the same mutation detection systems as their corresponding *in vivo* TGR model and therefore may have certain hypothetical advantages over other *in vitro* gene mutation assays (Section 3.2.2), since fewer assumptions will be required in predicting the outcome of, or comparing results with, *in vivo* transgenic mutation tests. Furthermore, some of the cell lines are epithelial in origin. Since most solid tumours arise from such cells (Lieberman and Lebovitz, 1996), another assumption required in extrapolating from *in vitro* to *in vivo* conditions when using fibroblast-based cell lines is reduced. In addition, *in vitro* transgenic models provide a logical platform on which to base follow-up or mechanistic studies relating to *in vivo* mutagenicity results.

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

2.4.2 Disadvantages

2.4.2.1 Limited sensitivity to clastogens

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

2.4.2.2 High spontaneous mutant frequency

The spontaneous mutant frequency in TGR targets appears to be somewhat higher than in endogenous targets and in many tissues appears to increase with the age of the animal (Hill *et al*., 2004). In principle, for a given mutant frequency following treatment, higher spontaneous mutant frequencies will yield correspondingly smaller fold increases (mutant frequency in treated animals / spontaneous mutant frequency) that may be attributed to the effect of the treatment. Therefore, there has been some concern that the relatively high spontaneous mutant frequency in TGR targets will decrease the sensitivity of the TGR assay relative to assays using endogenous gene targets. However, it has been clearly demonstrated in optimised TGR assay protocols that the sensitivity of transgenes can be enhanced by increasing the administration time. Since mutations in the neutral transgene accumulate linearly with the number of daily administrations given to an animal, longer

administration times induce more mutations with time and increase both the induced mutant frequency and the fold increase in treated animals. The sensitivity of TGR assays is discussed in Section 4.8.4.

2.4.2.3 Cost

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

3.0 EXISTING GENOTOXICITY ASSAYS

3.1 Introduction

The utility of TGR mutation assays in genotoxic risk assessment may be considered in the context of currently available assays. Typically, genotoxic effects can occur through two mechanisms: via gene mutations or via chromosomal mutations. For the purposes of this chapter, gene mutations are considered to be permanent single mutations involving only one gene, whereas chromosomal mutations are considered to be changes in the structure of the chromosome (structural aberration) or a change in chromosome number (numerical aberration). Generally, chromosomal aberrations affect more than one gene. Genotoxicity can be manifested through one or both of these mechanisms.

Since experience with genetic toxicology testing over the past several decades has demonstrated that no single assay is capable of detecting the full spectrum of genotoxic effects, the potential for a chemical to cause genotoxicity is typically determined through a battery of short- and long-term tests, involving both *in vitro* and *in vivo* model systems. *In vitro* assays offer the advantages that they are relatively inexpensive and easy to conduct and do not directly involve the use of animals. However, *in vitro* tests usually require supplementation with exogenous metabolic activation enzymes in order to simulate mammalian metabolism; they also generally do not allow for the consideration of factors – including toxicokinetics and DNA repair – that are relevant when considering potential effects in humans. Nevertheless, *in vitro* assays are typically used to provide an initial indication of the genotoxicity of a chemical, and the results often inform the choice of appropriate subsequent *in vivo* studies.

In vivo genotoxicity assays offer the advantage that mammalian DNA repair processes, the status of cell cycle checkpoint genes and toxicokinetic factors in the model system share greater similarity with humans, the species of interest for risk assessment; consequently, the degree of uncertainty in extrapolating to humans is lower than when considering most *in vitro* tests. Despite this advantage, the *in vivo* assays are considerably more time consuming to conduct because of the need to administer the test compound to animals; this period of time can in some cases be days or weeks in length. The complexity and logistics associated with *in vivo* studies are also greater than for *in vitro* assays, and this is reflected in their increased cost.

The testing of chemicals for the purposes of determining safety requires reproducibility between laboratories and standardisation in test methods. For this reason, testing protocols have been developed under the auspices of international organisations, such as the OECD, to promote the use of reliable and reproducible methods in testing laboratories. These protocols recommend conditions under which the tests should be conducted, based upon an extensive understanding of the validity of the test for the particular endpoint of interest.

In this chapter, existing genetic toxicology tests are reviewed and their benefits and limitations described (see also Table 3-1). For the purposes of this discussion, assays are grouped into four categories: *in vitro* mutation assays (briefly reviewed), *in vivo* somatic cell gene mutation assays, *in vivo* somatic cell chromosomal aberration assays and *in vivo* indicator assays.

3.2 *In vitro* genotoxicity assays

3.2.1 In vitro chromosomal aberration assays

Chemicals causing chromosomal aberrations may be identified with an *in vitro* cytogenetics assay (OECD, 1997b). Mammalian cell cultures, such as those derived from the Chinese hamster (*i.e.* Chinese hamster ovary or V79 lines), are treated with the test chemical; after an appropriate exposure period, mitosis in the cultures is arrested in metaphase with an inhibitor, such as colcemid or colchicine.

Table 3-1. Comparison of existing tests used to examine somatic cell genotoxicity *in vivo*

A.

	Rodent erythrocyte micronucleus	Bone marrow chromosomal aberration	Mouse spot test	Retinoblast[a]	*Hprt*[a]
Species	Rat or mouse	Rat or mouse	Mouse	Mouse	Rat or mouse
Number and sex	Minimum 5 per sex per dose	Minimum 5 per sex per dose	Sufficient number of females (~50) to produce about 300 F_1 mice per dose	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient
Dose levels	At least three, plus controls; maximum should be MTD; limit test acceptable where no toxicity observed at 2 000 mg/kg bw	At least three, plus controls; maximum should be MTD; limit test acceptable where no toxicity observed at 2 000 mg/kg bw	At least two doses (plus controls), one of which should induce mild toxicity or reduced litter size	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity
Route of administration	Oral or intraperitoneal; others where justified	Oral or intraperitoneal; others where justified	Oral or intraperitoneal; others where justified	Route relevant to human exposure (oral, intra-peritoneal or inhalation)	Route considered most relevant to human expo-sure
Treatment schedule	Single administration; multiple dosing where justified	Single administration	Single administration on the 8th, 9th or 10th day of gestation	Single administration on the 10th day of gestation	Single or multiple administration
Tissue analysed	Bone marrow or peripheral blood erythrocytes	Bone marrow	Fur	Retinal epithelial cells	Splenic T-lymphocytes (primarily)
Sampling (examination) times	24–48 hours for bone marrow; 48–72 hours for peripheral blood	1.5 times normal cell cycle length (12–18 hours) and 24 hours after first sampling	After birth of F_1 generation at about 4 weeks	After birth of F_1 generation at about 20 days	Mutation frequency determined after a selection period of several days in 6-TG
Analysis	At least 2 000 PCEs per animal	At least 1 000 cells per animal for mitotic index; at least 100 cells per	Presence of colour spots on the coat of F_1 animals	Presence of colour spots within the retinal epithelium of F_1 animals	Calculation of induced mutant frequency

	Rodent erythrocyte micronucleus	Bone marrow chromosomal aberration	Mouse spot test	Retinoblast[a]	*Hprt*[a]
		animal for chromosomal aberrations			
In-life phase	About 1 week	About 2 weeks	About 6 weeks	About 2–3 months	About 2 months

Table 3-1 (continued)

B.

	Aprt[a]	$Tk^{+/-}$ [a]	*Dlb-1*[a]	Sister chromatid exchange[a]	Unscheduled DNA synthesis	Comet assay[a]
Species	$Aprt^{+/-}$ or $Aprt^{-/-}$ mouse	*Tk* heterozygous mouse	*Dlb-1* heterozygous mouse	Rat or mouse	Rat; also mouse	Rat or mouse
Number and sex	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient	Five per sex per dose should be sufficient	At least three per dose; usually males are sufficient	4–5 per sex per dose should be sufficient
Dose levels	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD or induce target tissue cytotoxicity	At least two, plus controls; maximum dose should be the MTD
Route of administration	Route considered most relevant to human exposure	Route considered most relevant to human exposure	Oral administration	Gavage or intra-peritoneal injection are most common	Oral (intraperitoneal injection is not recommended)	Route considered most relevant to human exposure
Treatment schedule	Single or multiple administration	Single or multiple administration	Single or multiple administration	Single administration	Single administration	Single or multiple administration
Tissue analysed	Splenic T-lymphocytes or skin fibroblasts (primarily)	Splenic T-lymphocytes (primarily)	Intestinal epithelial cells	Bone marrow (other cell types may be used)	Liver	Any from which a single-cell suspension can be prepared
Sampling times	Mutation frequency determined after a selection period of several days in 8-AA or DAP	Mutation frequency determined after a selection period of 10–12 days in BrdU	14 days	Sacrifice 24 hours after administration; animals receive BrdU at 2–3 hours and colchicine at 21 hours	2–4 and 12–16 hours after administration; liver cells are prepared and incubated for 3–8 hours in ^{3}H-TdR	2–6 hours and 16–26 hours after single dosing, or 2–6 hours after the last treatment following multiple dosing

	Aprt[a]	*Tk*$^{+/-}$ [a]	*Dlb-1*[a]	Sister chromatid exchange[a]	Unscheduled DNA synthesis	Comet assay[a]
Analysis	Calculation of induced mutant frequency	Calculation of induced mutant frequency	Calculation of induced mutant frequency	Scoring sister chromatid exchanges by examination using light microscopy	Determination of net nuclear grain count by autoradiography in at least 100 cells per animal	Computer image analysis of comets
In-life phase	About 2 months	About 2 months	About 2 months	About 1 week	About 1 week	About 1 week

8-AA, 8-azaadenine; BrdU, 5-bromo-2′-deoxyuridine; bw, body weight; DAP, 2,6-diaminopurine; MTD, maximum tolerable dose; PCE, polychromatic erythrocyte; TdR, thymidine; 6-TG, 6-thioguanine

[a] Non-guideline study.

The stained metaphase spreads are examined by light microscopy to detect chromosome or chromatid aberrations. A biologically significant increase in the frequency of cells with structural aberrations compared with that of the concurrent control group indicates that the chemical is clastogenic. Major drawbacks of this assay, in comparison with some of the other *in vitro* assays, are the subjectivity and cost of having the metaphase spreads scored by an observer.

A harmonised protocol for the *in vitro* micronucleus test has been developed recently by an IWGT working group (Kirsch-Volders *et al.*, 2000, 2003). The European Centre for the Validation of Alternative Methods has determined that the *in vitro* micronucleus test is a valid alternative to the *in vitro* chromosomal aberration test (European Centre for the Validation of Alternative Methods Scientific Advisory Committee, 2006), and the OECD is developing a new Test Guideline (OECD, 2007); however, the transition of this assay from the development stage to routine regulatory use has not yet occurred.

3.2.2 In vitro gene mutation assays

The *in vitro* assays commonly used in genetic toxicity testing include the bacterial reverse mutation assay (Ames assay), which detects chemicals that cause point mutations or frameshift mutations in histidine auxotrophic strains of *Salmonella typhimurium* (*e.g.* strains TA100, TA98, TA102), and a reverse mutation assay using a tryptophan auxotrophic strain of *E. coli* (*e.g.* WP2*uvr*A) (OECD, 1997a). Revertant cells grow on minimal agar containing trace amounts of histidine or tryptophan, whereas wild-type cells rapidly deplete the limiting amino acid and stop growing. If there is an increase in the number of revertant colonies compared with the results of the concurrent negative control (typically considered a two-fold or greater increase), the chemical is concluded to be mutagenic.

Mammalian forward mutation assays, such as the thymidine kinase (*Tk*) assay or the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) assay, detect mutations at the heterozygous *Tk* or hemizygous *Hprt* gene (OECD, 1997e). Cells such as mouse lymphoma L5178Y cells (*Tk* locus), several Chinese hamster cell lines (*Hprt* locus) and human lymphoblastoid cells (*Tk* locus) are most commonly used. Mutations are selected by incubation of the cell cultures with the selective agents trifluorothymidine (*Tk* assay) or 6-thioguanine (*Hprt* assay). Cells having forward mutations at the *Tk* or *Hprt* genes survive in the presence of the selective agent, whereas wild-type cells accumulate a toxic metabolite and do not proliferate. Comparison of the mutant frequency of the treatment groups with that of the concurrent negative control group allows the identification of a mutagenic chemical.

3.3 *In vivo* assays for somatic cell chromosomal aberrations

In vivo chromosomal aberration assays assess the potential of a test chemical to cause DNA damage that may affect chromosome structure or interfere with the mitotic apparatus, causing changes in chromosome number. There are several short-term assays that detect somatic cell chromosomal aberrations; these include the rodent erythrocyte micronucleus assay and the bone marrow chromosomal aberration assay, which are nominally considered equivalent..

3.3.1 Rodent erythrocyte micronucleus assay

Because of its relative simplicity and its sensitivity to clastogens, the rodent erythrocyte micronucleus assay has now become the most commonly conducted *in vivo* assay. It has achieved widespread acceptance, and a test method has been described in OECD Test Guideline 474 (OECD, 1997c).

3.3.1.1 Principles

The micronucleus assay detects chromosome damage and whole chromosome loss in polychromatic erythrocytes (PCEs) and eventually in normochromatic erythrocytes in peripheral blood as the red cells mature. A micronucleus is a small structure (5–20% of the size of the nucleus) containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. Micronuclei can be found in cells of any tissue, but form only in dividing cells.

There are four generally accepted mechanisms through which micronuclei can form: 1) the mitotic loss of acentric chromosome fragments (forming structural aberrations); 2) mechanical consequences of chromosomal breakage and exchange, such as from lagging chromosomes, an inactive centromere or tangled chromosomes (forming structural aberrations); 3) mitotic loss of whole chromosomes (forming numerical aberrations); and 4) apoptosis (Heddle *et al*., 1991). However, nuclear fragments resulting from apoptosis are usually easy to identify because they are much more numerous or pyknotic than those induced by clastogenic or aneugenic mechanisms. Structural aberrations are believed to result from direct or indirect interaction of the test chemical with DNA, whereas numerical aberrations are often a result of interference with the mitotic apparatus, preventing normal nuclear division.

Bone marrow is the major haematopoietic tissue in the adult rodent. Administration of a chemical during proliferation of haematopoietic cells may cause chromosome damage or inhibition of the mitotic apparatus. These chromosome fragments or whole chromosomes may lag behind during cell division and form micronuclei. The erythrocyte is particularly well suited to analysis for micronuclei because during maturation of the erythroblast to the PCE (a period of about 6 hours following the final mitosis), the nucleus is extruded, making detection of micronuclei easier (Mavournin *et al*., 1990). In addition, the PCE still contains ribonucleic acid (RNA), and so it stains blue-grey with Giemsa or reddish with acridine orange. This allows differentiation from mature, haemoglobin-containing erythrocytes, which stain orange with Giemsa or are unstained by acridine orange, and facilitates identification of the cells where micronuclei induced by the test substance may be present (Krishna and Hayashi, 2000). Sampling of PCEs from the bone marrow or peripheral blood prior to their differentiation to mature erythrocytes is critical; once a PCE has matured, associating the presence of micronuclei in these cells with acute chemical exposure is not possible. Mature erythrocytes persist in peripheral circulation for about 1 month (Mavournin *et al*., 1990).

The micronucleus assay is conducted using the bone marrow or peripheral blood of rodents, typically mice, as the target tissue; the peripheral blood of species other than the mouse can be used if micronucleated erythrocytes are not rapidly removed by the spleen (OECD, 1997c). The usual routes of administration are via gavage or intraperitoneal injection, and generally several doses must be administered so that the dose range spans from the maximum tolerable dose (MTD) to a dose without appreciable toxicity. The length of time between treatment and sacrifice is a critical parameter, which is dependent on the cell cycle time. This delay between treatment and sampling of PCEs is necessary to allow sufficient time for the number of micronucleated PCEs to rise to a peak and corresponds to the time necessary for absorption and metabolism of the chemical, the completion of the erythroblast cell cycle, including any test chemical–induced cell cycle delay, and extrusion of the erythroblast nucleus (Mavournin *et al*., 1990).

The incidence of micronucleated PCEs is low in untreated animals. To allow for appropriate statistical power, a large number of PCEs (usually at least 2 000 PCEs per animal) must be scored for the incidence of micronuclei; the proportion of PCEs among total erythrocytes is also determined as a measure of cytotoxicity (OECD, 1997c).

Because micronuclei are relatively rare, manual enumeration by light microscopy is time consuming. For that reason, newer flow cytometric or image analysis methods have been adapted for the rapid processing of slides, which offer tremendous potential for improving the sensitivity and the efficiency of the assay (Hayashi *et al*., 2007).

Any test chemical that induces an increase in the frequency of micronucleated PCEs is concluded to have induced chromosomal aberrations *in vivo*, but further mechanistic information useful to distinguish micronuclei induced by clastogenic or aneugenic chemicals can also be obtained. Micronuclei of aneugenic origin will contain centromeres, the presence of which can be verified using one of two

molecular cytogenetic methods: immunofluorescent antikinetochore (CREST) staining or fluorescence *in situ* hybridisation (FISH) with pancentromeric DNA probes (see Heddle *et al*., 1991; Krishna and Hayashi, 2000).

3.3.1.2 Benefits and limitations

Based on the mechanism for micronucleus formation, the micronucleus assay, in principle, is able to detect both clastogens and some aneugens. There is a low spontaneous micronucleus frequency in erythrocytes (typically <3 micronucleated PCEs/1 000 PCEs), which provides fairly high sensitivity to small test chemical–induced increases in micronucleus frequency (Salamone and Mavournin, 1994). In addition, there is a very large population of cells from which to sample, making scoring easier and increasing the power of the test. Since the assay has been used in genetic toxicology testing for many years, numerous laboratories have developed considerable expertise with the assay, and a large database exists to allow for comparisons. The assay is widely accepted by regulatory agencies internationally.

However, as only PCEs are scored for the presence of micronuclei, the effect of a chemical on germ cells and other somatic cells is not assessed. Despite evidence suggesting that the bone marrow micronucleus assay can detect most germ cell clastogens (Waters *et al*., 1994), if the test chemical under investigation is suspected to target germ cells, separate investigations should be performed; these studies are particularly resource intensive. The identification of *N*-hydroxymethylacrylamide as a mutagen that induces dominant lethal mutations in germ cells, but not micronucleated PCEs, highlights a potential drawback of mutation assays exclusively involving somatic cells (Witt *et al*., 2003). Although the assay can detect both clastogenic and aneugenic effects, it cannot distinguish between the two mechanisms unless further work is conducted using CREST staining or FISH techniques; this is rarely done. In addition, the micronucleus assay, in principle, does not identify gene mutations *in vivo*; therefore, the use of the *in vivo* micronucleus assay to confirm a positive result obtained in an *in vitro* gene mutation assay is not mechanistically justified.

3.3.2 Mammalian bone marrow chromosomal aberration assay

The mammalian bone marrow chromosomal aberration assay can detect clastogenic effects of a test agent. However, in the chromosomal aberration assay, these effects are observed directly by examination of metaphase chromosome spreads. The recommended methodology has been published in OECD Test Guideline 475 (OECD, 1997d).

3.3.2.1 Principles

The assay is based on the ability of a test agent to induce chromosome structural or numerical alterations that can be visualised microscopically. The target tissue for the chromosomal aberration assay is the bone marrow, because it is a rapidly dividing, well-vascularised tissue. Groups of mice, rats or Chinese hamsters are administered the test chemical, preferably only once, by a relevant route of exposure, typically by gavage or intraperitoneal injection. Doses are selected that span a range from the MTD to that which does not induce appreciable toxicity. The MTD by definition produces mild toxicity that at higher doses would be expected to lead to mortality or causes bone marrow cytotoxicity (*i.e.* >50% reduction of the mitotic index).

In order to accumulate metaphase cells, cell division is arrested by administration of a mitotic inhibitor, such as colchicine, 3–5 hours prior to sacrifice. After a time period equivalent to 1.5 times the normal cell cycle length (usually 12–18 hours for most rodent species), animals are euthanised, and bone marrow cells from the femur in their first metaphase after administration are examined. Because of the potential for some chemicals to induce mitotic delay, a second sampling is conducted with a parallel group of animals 24 hours after the first sampling time. Using light microscopy, a minimum of 1 000 cells per animal are scored to determine the mitotic index, and a minimum of 100 metaphase cells per animal

are scored for chromosomal aberrations. A chemical that induces an increase in the frequency of structural aberrations, including chromosome-type and chromatid-type aberrations, is considered to be clastogenic under the test conditions.

3.3.2.2 Benefits and limitations

The chromosomal aberration assay detects clastogenic effects of a test chemical by direct examination of metaphase cells; this often is more informative, because the types of aberrations can be described and classified, which allows the assay to provide mechanistic information more readily than the micronucleus assay. The assay has been in use for many years, it is accepted by regulatory agencies globally and a large database exists for comparisons. Although bone marrow cells are the usual target, the assay can be adapted (*e.g.* by altering sampling times) to allow examination of other cell types, including spermatogonia. This allows the assay to be used to evaluate the potential for germ cell chromosomal aberrations that could lead to heritable genetic effects.

However, conducting a chromosomal aberration assay is much more time consuming than conducting the micronucleus assay. It requires skilled personnel to correctly identify aberrations and is not adaptable for automated scoring. As a result, it is necessarily subjective. Because of cytotoxicity or chromosomal damage during slide preparation, the number of scoreable metaphases may be low; this could make it difficult to find a sufficient number of intact metaphases per animal, which would reduce the sensitivity of the test to detect small increases. Furthermore, like the micronucleus assay, the chromosomal aberration assay does not, by design, provide information regarding whether the test chemical induced gene mutations in the target cells or other tissues besides the bone marrow within the animal.

3.4 *In vivo* assays for somatic cell gene mutation in endogenous genes

In vivo gene mutation assays in endogenous genes are rarely used for testing purposes because of the lack of effective methods. The tests that currently exist are cumbersome and generally not suitable for routine use. The mouse spot test is the only test for which an OECD test guideline exists, but some promising mutation tests using endogenous genes have also been developed. These endogenous gene mutation assays include retinoblast, *Hprt*, *Aprt* (adenine phosphoribosyltransferase), $Tk^{+/-}$ and *Dlb-1*.

3.4.1 Mouse spot test

The mouse spot test was developed as a rapid screening test to detect gene mutations and recombinations in somatic cells of mice. The recommended methodology is described in OECD Test Guideline 484 (OECD, 1986b).

3.4.1.1 Principles

Although coat colour spots were induced experimentally by X-irradiation in 1957 (Russell and Major, 1957), the ability of chemicals to induce these genetic changes was not recognised until 1975, when colour spots were induced by treatment of mice with *N*-ethyl-*N*-nitrosourea (ENU) (Fahrig, 1975). The mouse spot test is based on the observation that chemical mutagens can induce colour spots on the fur of mice exposed *in utero*. The colour spots arise when mouse melanoblasts heterozygous for several recessive coat colour mutations lose a dominant allele through a gene mutation, chromosomal aberration or reciprocal recombination, allowing the recessive gene to be expressed (Russell, 1977). Melanoblasts migrate from the neural crest to the midline of the abdomen during days 8–12 of embryonic development, while continuing to divide to produce melanocytes (Fahrig, 1995). Those melanocytes that carry a coat

colour mutation will result in differing pigmentation of the fur in a band stretching from the back to the abdomen.

Mice of the T-strain are mated with those of the HT or C57/B1 strain to produce embryos with the desired genetic characteristics. In general, treatment of about 50 dams with the test chemical would be sufficient to produce the desired ~300 F_1 animals per group. Dams are treated on day 8, 9 or 10 of gestation by gavage or intraperitoneal injection. Three or four weeks after birth, the mice are examined for coat spots. There are three classes of spots: 1) white ventral spots that are presumed to be the result of chromosomal aberrations leading to cell death; 2) yellow, agouti-like spots that are likely to be a result of misdifferentiations; and 3) pigmented black, grey, brown or near-white spots randomly distributed over the whole coat, which are the result of somatic mutations. However, only the last class of spot has genetic relevance. A chemical that induces a biologically significant increase in the number of genetically relevant (somatic mutation) spots is considered to be mutagenic in this test system (OECD, 1986b).

By examining fur from the spot using fluorescence microscopy, it is possible to distinguish the different classes of spots and to identify, from somatic mutation spots, the gene loci affected (Fahrig and Neuhauser-Klaus, 1985). It is also possible to distinguish different types of genetic events. Identifiable gene mutations are caused by a mutation at the *c* locus that produces cells with the *c* (albino) and c^{ch} (chinchilla) alleles, which causes light brown spots. Reciprocal recombinations result from a crossing-over involving the linked *p* (pink-eyed dilution) and *c* (albino) loci; the resulting recombinants are homozygous for either the wild-type (visible as black spots) or mutant alleles (visible as white spots) (Fahrig, 1995).

3.4.1.2 Benefits and limitations

The mouse spot test is capable of detecting both gene mutations and some types of chromosomal aberrations. It is relatively easy to conduct, it does not require specialised expertise and the endpoint of interest (coat spots) can be directly identified by visual examination. Further information regarding the causes of different types of mutagenic events and the gene loci involved can be inferred by examining fur from the spots microscopically. Basic information regarding the reproductive toxicity or teratogenicity of the test chemical can also be obtained by looking for obvious malformations or reduced numbers of pups in each litter. However, mutagenic activity is detected within the small melanocyte population only very early in development. Furthermore, the test requires a large number of animals, making it very costly to conduct. The high cost and the trend towards reduction of animals used in toxicological testing have greatly limited the use of this assay.

3.4.2 Retinoblast (eye spot) assay

The retinoblast (eye spot) assay is a variant of the mouse spot test that identifies deletion mutations by scoring colour spots in the retinal pigment epithelium instead of the coat. This assay is not commonly used, other than for basic research applications.

3.4.2.1 Principles

The eye spot assay is similar in principle to the mouse spot test. It uses the C57BL/6J p^{un}/p^{un} strain of mouse, which carries the pink-eyed unstable mutation (p^{un}), a 70 kb tandem duplication at the pink-eyed dilution locus (Gondo *et al*., 1993). The p^{un} mutation carried by the test strain is an autosomal recessive mutation that produces a light grey coat colour and pink eyes. Loss of one copy of the p^{un} tandem duplication causes reversion of the p^{un} mutation to the wild-type *p* in a retinal pigment epithelial precursor cell and leads to the production of a retinal pigment epithelial cell with black pigmentation, which is visible against the remaining non-pigmented retinal pigment epithelial cells (Searle, 1977). With this assay, the frequency of mutations (deletions) affecting one copy of the tandem duplication at the p^{un} locus can be measured.

The assay is most commonly conducted by treating dams from the C57BL/6J p^{un}/p^{un} strain with the test chemical by a relevant route of exposure (gavage, intraperitoneal injection or inhalation) at approximately day 10 of gestation. Offspring are sacrificed at the age of 20 days, the eyes are removed and the retina is placed on a slide and examined by light microscopy. The number of spots, which are observed as a single pigmented cell or groups of pigmented cells separated from each other by no more than one unpigmented cell, is counted. Each eye spot corresponds to one p^{un} mutation. A test chemical that induces a significant increase in the frequency of eye spots compared with the negative control is mutagenic under the conditions of this assay (Reliene *et al*., 2004).

Reversion of the p^{un} mutation arises from intrachromosomal recombination that results in the deletion of one of the tandem fragments at the p^{un} loci. The deletion can occur by several mechanisms, such as intrachromosomal crossing-over, single-strand annealing, unequal sister chromatid exchange and sister chromatid conversion (Schiestl *et al*., 1997a).

3.4.2.2 Benefits and limitations

A number of carcinogens have been found to induce intrachromosomal recombinations (Schiestl *et al*., 1997a, 1997b; Jalili, Murthy and Schiestl, 1998; Bishop *et al*., 2000), so the endpoint scored by this assay has some relevance to the assessment of carcinogenicity. The assay assesses a type of genetic effect (deletion mutation) that is not identified by many other assays. The eye spot assay requires fewer animals than the mouse spot test to achieve a high sensitivity (Bishop *et al*., 2000), and it allows for direct examination at the single-cell level. However, the target cells in this assay are retinal pigment epithelial precursor cells, which proliferate only during the period starting at about embryonic day 9 until shortly after birth (Bodenstein and Sidman, 1987). Because the assay is used to determine the frequency of deletions occurring in embryos, it may not necessarily be representative of other life stages.

3.4.3 Gene mutation assays using endogenous genes with selectable phenotypes

3.4.3.1 Hprt

The *Hprt* assay uses one of the few genes that are suitable for mutation analysis in wild-type animals *in vivo*. It has been widely used in basic research applications, but has yet to be used for routine testing.

3.4.3.1.1 Principles

The *Hprt* gene is located on the X-chromosome and spans 32 kb and 46 kb in human and rodent cells, respectively. Both male and female cells carry only one active copy of the *Hprt* gene; in female cells, one copy of the X-chromosome is inactivated. The *Hprt* gene codes for hypoxanthine-guanine phosphoribosyltransferase (HPRT), which plays a key role in the purine salvage pathway. HPRT catalyses the transformation of purines (hypoxanthine, guanine or 6-mercaptopurine) to the corresponding monophosphate, which is cytotoxic to normal cells in culture. The assay is based on the observation that cells with mutations in the *Hprt* gene have lost the HPRT enzyme and survive treatment with purine analogues.

Mice or rats are treated with the test chemical by an appropriate route of exposure. After a fixation period of several weeks, the spleens are removed from sacrificed animals, and cultures of splenic T-lymphocytes are established. T-lymphocytes are particularly useful because they circulate throughout many tissues, which affords them a greater probability of contacting administered mutagen compared with cells that are permanently resident in a single tissue. T-lymphocytes are also long lived in circulation, and they continue to undergo cell division, which makes the identification of mutant cells possible. In addition to T-lymphocytes, mutant frequency has also been determined in other cells, including those from the kidney, thymus and lymph nodes.

Mutant selection has been described by Tates *et al*. (1994). Cells are incubated in microwell culture plates with the selective agent 6-thioguanine, a purine analogue that is a substrate for HPRT and is toxic to non-mutant cells. Cloning efficiency plates are scored after 8–9 days, whereas mutant frequency plates are scored after a 10- to 12-day expression period. The mutant frequency is calculated as the ratio between the cloning efficiencies in selective media versus those in cloning media. A significant increase in mutant frequency in treatment cultures compared with controls indicates that the test chemical has induced mutation at the *Hprt* locus. The average spontaneous mutant frequency at the *Hprt* locus is in the range of 10^{-6} (Van Sloun *et al*., 1998). Using standard techniques, further molecular analysis of *Hprt* mutations can be performed, if desired.

3.4.3.1.2 Benefits and limitations

The *Hprt* assay detects point mutations, frameshifts, small insertions and small deletions. As an endogenous gene, *Hprt* is transcriptionally active and thus subject to transcription-coupled DNA repair (Lommel, Carswell-Crumpton and Hanawalt, 1995). However, because *Hprt* is an X-linked gene and therefore functionally hemizygous, it is not particularly efficient at detecting large deletions, chromosomal recombination and non-disjunction events that may disrupt essential flanking genes and are more effectively identified with assays using endogenous autosomal genes (Dobrovolsky, Chen and Heflich, 1999). Deletions extending into adjacent essential genes in hemizygous regions are usually lethal to the cell because there is no homologous region to compensate for the loss of essential gene function.

Because *Hprt* is not a neutral gene, there is selection pressure against *Hprt*-deficient lymphocytes, particularly in young animals (Deubel *et al*., 1996). In addition, dilution of mutant T-lymphocytes in circulation occurs as peripheral lymphocyte populations are renewed; this is also affected by the age of the animal. The time from exposure to maximum average mutant frequency was found to be 2 weeks in the spleen of ENU-exposed preweanling mice and 8 weeks in adult mice (Walker *et al*., 1999b). As a result, sampling in the spleen must be carefully timed to detect the maximum mutant frequency based on these factors. Although *Hprt* mutant frequency can be determined from any tissue that can be subcultured, it is typically assessed only in T-lymphocytes, which prevents identification of mutagenic effects that may arise preferentially in other target tissues.

3.4.3.2 Aprt

The *Aprt* assay uses a constructed *Aprt* heterozygous mouse model. Like the *Hprt* model, *Aprt* is widely used in research, but is not yet used in routine genetic toxicology testing.

3.4.3.2.1 Principles

Aprt is the gene coding for an enzyme (adenine phosphoribosyltransferase) that catalyses the conversion of adenine to adenosine monophosphate (AMP) in the purine salvage pathway; it is expressed in all tissues. The mouse *Aprt* gene is located on chromosome 8 (Dush *et al*., 1986), whereas the human gene is located on chromosome 16 (Fratini *et al*., 1986). In the mouse, the *Aprt* gene, because of its location near the telomere, is a large target for chromosomal events such as translocation and mitotic recombination (Tischfield, 1997).

Several *Aprt* knockout models have been created. A heterozygous $Aprt^{+/-}$ mouse has been developed by disrupting the *Aprt* gene in embryonic stem cells using a conventional gene targeting approach (Van Sloun *et al*., 1998). This model can be used to investigate induced forward mutations leading to the loss of the autosomal dominant locus in T-lymphocytes and skin fibroblasts, as well as mesenchymal cells from the ear (Shao *et al*., 1999) and epithelial cells from the kidney (Ponomareva *et al*., 2002). Using methods similar to those used for the *Hprt* model, *Aprt* heterozygous mice are treated with the test chemical. After a fixation period of several weeks, the animals are sacrificed, and typically splenic T-lymphocytes or skin fibroblasts are isolated and cultured. *Aprt*-deficient mutants are selected

using purine analogues such as 8-azaadenine or 2,6-diaminopurine, which are toxic to *Aprt*-proficient cells. After a 6- to 8-day expression period, the cloning frequency and mutant frequency are determined using methods similar to those used for the *Hprt* model (Tates *et al*., 1994). The spontaneous mutant frequency at the *Aprt* locus in heterozygous mice is approximately 8.7×10^{-6} in T-lymphocytes (Van Sloun *et al*., 1998) and 1.7×10^{-4} in skin fibroblasts (Tischfield, 1997). Using standard techniques, further molecular analysis of *Aprt* mutations can be performed, if desired.

An *Aprt*$^{-/-}$ homozygous knockout mouse has also been developed, which is capable of detecting chemicals that cause point mutations (Stambrook *et al*., 1996). These mice have *Aprt* alleles inactivated by reversible point mutations. Mice are administered the test chemical and held for a fixation period of several weeks, after which they are injected with [^{14}C]adenine. Cells that have reverted to *Aprt*$^{+}$ have a functional adenine phosphoribosyltransferase enzyme and can sequester radiolabelled adenine by conversion to AMP, which is subsequently incorporated into nucleic acids. Using autoradiography or scintillation counting, the frequency of revertant cells can be determined. The *in situ* method also enables identification of the cell types that are most susceptible to mutation (Stambrook *et al*., 1996).

3.4.3.2.2 Benefits and limitations

Because it is an autosomal heterozygous locus, *Aprt* can detect – in addition to the point mutations, frameshifts and small deletions detectable by *Hprt* – events that may lead to loss of heterozygosity, such as large deletions, mitotic non-disjunctions, mitotic recombinations and gene conversions, if the function of the deleted essential gene is provided by the homologous chromosome. The ability to detect the full spectrum of autosomal mutations allows *Aprt* to be a much more versatile biomarker than *Hprt*. However, the use of *Aprt* as a mutational target is limited to only a few tissues because of the detection method, which relies on culturing techniques. Like *Hprt*, *Aprt* is not a neutral gene, and there may be negative selection pressures on *Aprt* mutant cells, as well as influences on mutation frequency arising from dilution as the T-lymphocyte pool is renewed.

3.4.3.3 Tk$^{+/-}$

A *Tk* heterozygous mouse model has been constructed (Dobrovolsky, Casciano and Heflich, 1999). This model is currently limited to basic research applications, as it remains in an early stage of development and has not yet been used for routine testing.

3.4.3.3.1 Principles

The heterozygous *Tk*$^{+/-}$ mouse was created by using a mouse embryonic stem cell line with one allele of the *Tk* gene inactivated through targeted homologous recombination (Dobrovolsky, Casciano and Heflich, 1996, 1999). *Tk* is generally expressed only in dividing cells and encodes thymidine kinase, which is involved in pyrimidine salvage, catalysing phosphorylation of thymine deoxyriboside to form thymidylate. The *Tk* gene is located on the distal portion of mouse chromosome 11 (Hozier *et al*., 1991). Cells that have lost the second *Tk* allele through mutation to become *Tk*$^{-/-}$ are easily selected because they survive when cultured in the presence of a pyrimidine analogue such as 5-bromo-2′-deoxyuridine (BrdU), whereas thymidine kinase–competent cells (*Tk*$^{+/+}$ or *Tk*$^{+/-}$) do not.

C57BL/6 *Tk*$^{+/-}$ mice receive the test chemical by a relevant route of exposure either once or in multiple administrations. Approximately 4–5 weeks after administration, mice are sacrificed, and splenic lymphocytes are isolated and cultured. Mutant selection is performed as described by Dobrovolsky, Casciano and Heflich (1999). Cells are incubated in microwell culture plates with the selective agent BrdU, which is toxic to non-mutant cells. After incubation for 10–12 days, cloning efficiency in treatment and control plates is scored. The mutant frequency is calculated by dividing the cloning efficiency of cells cultured in the presence of the selecting agent by the cloning efficiency of cells cultured in the absence of selection. A significant increase in mutant frequency in treatment cultures compared with controls indicates that the test chemical induced mutation at the *Tk* locus. Using standard techniques, further molecular

analysis of *Tk* mutations can be performed, if desired. The spontaneous mutant frequency is approximately 2×10^{-5} (Takahashi, Kubota and Sato, 1998).

3.4.3.3.2 Benefits and limitations

The $Tk^{+/-}$ mouse is the analogous *in vivo* model to the commonly used mouse lymphoma assay. As such, it is a useful model to investigate the *in vivo* responses of chemicals found to be mutagenic in the *in vitro* assay. The *Tk* model detects intragenic mutations (point mutations, frameshifts and small deletions) as well as larger effects, such as chromosome recombination, non-disjunction and large deletions that often lead to loss of heterozygosity, for which it is particularly sensitive (Dobrovolsky, Casciano and Heflich, 1999; Dobrovolsky, Shaddock and Heflich, 2000, 2002). However, it too is limited to examining tissues where cells can be easily cultured. In addition, the use of BrdU, which is itself a mutagen, as a selective agent can introduce the possibility that some mutants would be produced by exposure to the selective agent. Dobrovolsky, Casciano and Heflich (1999) suggest that this potential can be minimised by keeping cultures under tight selection pressure during the culture phase, since studies with $Tk^{+/-}$ mouse lymphoma cells have indicated that several cell divisions in the absence of the selective agent are required in order to fix mutations (Moore and Clive, 1982).

3.4.3.4 Dlb-1

The *Dlb-1* specific locus test measures mutations occurring in the small intestine of treated *Dlb-1* heterozygotes. The assay has been in existence for about 15 years (Winton, Blount and Ponder, 1988; Winton *et al*., 1990), but it remains in the development stage and has not been widely used in the research community.

3.4.3.4.1 Principles

The *Dlb-1* specific locus test identifies mutations occurring in the small intestine of mice heterozygous at the *Dlb-1* locus. *Dlb-1* is a polymorphic genetic locus that exists on chromosome 11 in the mouse (Uiterdijk *et al*., 1986) and has two alleles. $Dlb\text{-}1^b$ is an autosomal dominant gene that determines the expression of binding sites for the lectin *Dolichos biflorus* agglutinin in intestinal epithelium, whereas $Dlb\text{-}1^a$ determines *Dolichos biflorus* agglutinin receptor expression in vascular endothelium (Winton, Blount and Ponder, 1988). Mice heterozygous at the *Dlb-1* locus ($Dlb\text{-}1^a/Dlb\text{-}1^b$) that develop a mutation of the $Dlb\text{-}1^b$ allele in an intestinal stem cell can be detected by staining an intestinal epithelial cell preparation with a peroxidase conjugate of *Dolichos biflorus* agglutinin and scoring non-staining cell ribbons on the villus; these cell ribbons are cells derived from a stem cell carrying a *Dlb-1* mutation (Winton *et al*., 1990). The spontaneous mutation frequency of the *Dlb-1* gene has been reported to be approximately 1.6×10^{-5} mutants per villus per animal per week (Tao, Urlando and Heddle, 1993a).

3.4.3.4.2 Benefits and limitations

The *Dlb-1* assay can be used for studies of animals *in utero*, as well as adult animals. The number of animals used is consistent with most assays using an endogenous reporter gene, which is substantially fewer than are required for the mouse spot test. However, the assay is restricted to analysis of mutations in the intestinal epithelium following oral administration of the test chemical. Mutagens acting preferentially at another target tissue may not be detected. In addition, the *Dlb-1* gene has not yet been cloned, so the molecular nature of any observed mutations cannot be determined. The assay is still in the

development stage and has not been used, other than for research purposes in a small number of laboratories.

3.5 Indicator tests

Indicator tests are those that do not directly measure consequences of DNA interaction (*i.e.* mutation) but rely on other markers that suggest that some type of interaction occurred. The three most commonly conducted tests are the sister chromatid exchange assay, the unscheduled DNA synthesis assay and the single-cell gel electrophoresis (comet) assay.

3.5.1 Sister chromatid exchange assay

The sister chromatid exchange assay is used for assessing chromosome damage; more commonly, it has been conducted as an *in vitro* test. Methodology for the *in vitro* assay is described in OECD Test Guideline 479 (OECD, 1986a), but there is no guideline for the *in vivo* assay.

3.5.1.1 Principles

Sister chromatid exchanges are reciprocal exchanges of DNA segments between sister chromatids of a chromosome that are produced during S-phase. Although the molecular mechanism of these exchanges remains unknown, it is presumed to require chromosome breakage, exchange of DNA at homologous loci and repair. Work with the model genotoxicant ENU has provided direct evidence that suggests that the replication fork is the site of sister chromatid exchange production (Rodriguez-Reyes and Morales-Ramirez, 2003). However, sister chromatid exchange may not necessarily be caused by direct DNA interaction in all cases. A chemical that does not damage DNA but instead creates intracellular conditions that favour inhibition of DNA replication could, in itself, create sister chromatid exchange. It is also noteworthy that several strong clastogens, such as ionising radiation and bleomycin, have failed to produce an increase in sister chromatid exchanges (Perry and Evans, 1975). Because sister chromatid exchange induction does not, by itself, indicate that a chemical is mutagenic, the interpretation of the toxicological relevance of sister chromatid exchange is often difficult.

Rodents are most commonly used for *in vivo* sister chromatid exchange assays. Bone marrow cells are usually sampled, because they contain a requisite pool of dividing cells and they are easy to prepare for scoring. Generally, groups of animals are administered the test chemical once by gavage or by intraperitoneal injection. At 2–3 hours following administration, the animals are administered a single dose of BrdU, followed at 21 hours by an injection of the mitotic inhibitor colchicine. Three hours later, all animals are sacrificed, and slides of bone marrow cells are prepared and scored by light microscopy. Other cell types, including spermatogonial cells, may also be used; the treatment and sampling times for other cells will depend on the cell cycle time of the target cells and what is known of the toxicokinetic factors specific to the chemical of interest.

3.5.1.2 Benefits and limitations

The sister chromatid exchange assay offers a rapid and relatively inexpensive assessment of potential test chemical–induced DNA damage; any tissue from which a cell suspension can be made can be analysed. A significant number of sister chromatid exchange assays for a wide variety of chemicals have previously been conducted, facilitating the comparison of the relative potencies of test chemicals. However, the major drawback remains the unknown molecular basis of sister chromatid exchange induction. Because factors other than direct DNA interaction can cause sister chromatid exchanges, an increase in sister chromatid exchange induction does not necessarily indicate mutagenicity, making inter-

pretation in the context of genetic toxicity testing difficult. As a result, this assay has fallen out of favour and is now rarely conducted.

3.5.2 Unscheduled DNA synthesis assay

The unscheduled DNA synthesis assay is a commonly used method of assessing test chemical–induced DNA excision repair. The induction of repair mechanisms is presumed to have been preceded by DNA damage. Measuring the extent to which DNA synthesis occurred offers indirect evidence of the DNA-damaging ability of a test chemical. A protocol is described in OECD Test Guideline 486 (OECD, 1997f).

3.5.2.1 Principles

The unscheduled DNA synthesis assay measures DNA synthesis induced for the purposes of repairing an excised segment of DNA containing a region damaged by a test chemical. DNA synthesis is measured by detecting tritium-labelled thymidine (^{3}H-TdR) incorporation into DNA, preferably using autoradiography. The liver is generally used for analysis because, under normal circumstances, there is a low proportion of primary hepatocytes in S-phase of the cell cycle; therefore, an increase in DNA synthesis can be more easily attributed to repair of induced DNA damage, rather than DNA synthesis supporting normal cell division. The liver is also the site of first-pass metabolism for chemicals administered orally or by intraperitoneal injection.

The larger the number of nucleotides excised and repaired, the greater the incorporation of detectable ^{3}H-TdR into DNA. For that reason, the unscheduled DNA synthesis assay is more sensitive in detecting DNA damage that is repaired through nucleotide excision repair (removal of up to 100 nucleotides) compared with base excision repair (removal of 1–3 nucleotides) (OECD, 1997f). Test chemicals more prone to inducing nucleotide excision repair, such as those that form bulky DNA adducts, have a greater potential to cause detectable unscheduled DNA synthesis. However, the unscheduled DNA synthesis assay does not, in itself, indicate if a test chemical is mutagenic, because it provides no information regarding the fidelity of DNA repair, and it does not identify DNA lesions repaired by mechanisms other than excision repair.

The unscheduled DNA synthesis assay is usually conducted using rats, although other species may be used. Dose levels are selected, with the highest dose being the MTD. Animals are administered the test chemical once by gavage; intraperitoneal injection is not recommended, because it could potentially expose the liver directly to the chemical. A group of animals is sacrificed at 2–4 hours and another at 12–16 hours after treatment. Cultures of hepatocytes are prepared and incubated for 3–8 hours in ^{3}H-TdR. Slides are prepared and processed for autoradiography using standard techniques. Chemicals inducing a significant increase in net nuclear grain count for at least one treatment group have induced unscheduled DNA synthesis (Madle *et al*., 1994; OECD, 1997f).

3.5.2.2 Benefits and limitations

Theoretically, any tissue with a low proportion of cells in S-phase can be used for analysis. Although only liver is routinely used, an unscheduled DNA synthesis assay using spermatocytes has been developed (Sega, 1974, 1979; Working and Butterworth, 1984), allowing the measurement of DNA interactions that may be germ cell specific. Because unscheduled DNA synthesis is measured in the whole genome, it is potentially much more sensitive than assays examining only specific loci. However, the extent of unscheduled DNA synthesis gives no indication of the fidelity of the repair process. For that reason, unscheduled DNA synthesis does not provide specific information on the mutagenic potential of a test chemical, but only information suggesting that it does or does not induce excision repair.

3.5.3 Single-cell gel electrophoresis (comet) assay

The comet assay is a quantitative technique for measuring DNA damage in eukaryotic cells at the level of the single cell. This DNA damage may or may not lead to mutations. Under alkaline conditions (pH >13) first described by Singh *et al.* (1988), the assay can detect single- and double-strand breaks, incomplete repair sites and alkali-labile sites in nearly any single-cell suspension of eukaryotic cells. The presence of DNA–DNA and DNA–protein cross-linking can also be inferred in some cases. A growing body of work has demonstrated that the comet assay may have value in regulatory applications, and a number of questions related to the development of a standardised protocol have been addressed recently by various expert working groups (Tice *et al*., 2000; Hartmann *et al*., 2003; Burlinson *et al*., 2007).

3.5.3.1 Principles

The alkaline comet assay is based on the observation that electrophoresis of DNA will cause it to migrate in an agarose gel matrix. Under a microscope, a cell with DNA damage subjected to these conditions will take a distinctive comet-like shape, with a nuclear (head) region and a tail containing the DNA fragments or DNA strands oriented towards the anode. Numerous parameters affect the detection of DNA damage, including pH, the agarose concentration and the conditions of electrophoresis (time, temperature, amperage and voltage).

Rats or mice can be treated with the test substance either once or multiple times at intervals of 24 hours. At least two doses are selected, with the highest dose being the MTD. Tissue samples are obtained from the organs of interest at 2–6 hours and 16–26 hours after single dosing, or 2–6 hours after the last treatment following multiple dosing (Hartmann *et al*., 2003). Slides are prepared containing a cell layer imbedded in agarose; the cells are lysed, incubated in alkaline electrophoresis buffer and then electrophoresed under alkaline conditions. After neutralisation and staining, the slides are scored, preferably by computerised image analysis software (Burlinson *et al*., 2007). Because of the association of cytotoxicity and increased levels of DNA strand breaks, it is advisable to conduct a concurrent assessment of cytotoxicity and to exclude from analysis cells with a characteristic apoptotic/necrotic appearance (Hartmann *et al*., 2003).

A dose-related change in an appropriate parameter (*i.e.* tail moment or tail length) at a single sampling time or a change in an appropriate parameter in the treated group compared with the untreated control at a single sampling time constitutes a positive response. Increased DNA migration indicates the induction of DNA strand breaks or alkali-labile sites, whereas reduced migration suggests the presence of stabilising DNA–DNA or DNA–protein cross-links (Pfuhler and Wolf, 1996; Merk and Speit, 1999).

3.5.3.2 Benefits and limitations

For the most part, cells from any tissue from which a single-cell suspension can be obtained are amenable to comet assay analysis. Therefore, the comet assay has applications for both somatic cell and germ cell genotoxicity testing and has the flexibility to permit various routes of administration. In addition to applications in genotoxicity testing, the assay has uses in human biomonitoring of occupational or environmental exposures. However, some lesions detected by the comet assay, such as single-strand breaks, may also be correctly repaired without resulting in permanent genetic damage. As such, the assay does not directly detect mutations or identify aneugens.

3.6 Conclusion

In vivo assays are necessary components of any thorough genetic toxicity testing scheme. They are influenced by toxicokinetic factors, DNA repair processes and cell cycle checkpoint genes that may, in some cases, affect genotoxicity differently from the *in vitro* models. However, existing assays are seriously limited by a range of different factors, including assay cost, the number of tissues in which genotoxicity may be measured, the state of understanding of the endpoint and the nature of the chemicals that will be detected.

4.0 EXISTING DATA AND FACTORS INFLUENCING PERFORMANCE

4.1 Introduction

Over the past several years, there has been considerable progress towards the development of internationally harmonised protocols for the conduct of TGR assays. The primary forum for these discussions has been the IWGT, which has released two reports – based on meetings in Washington, DC, USA, in 1999 and in Plymouth, UK, in 2002 – summarising test protocol recommendations (Heddle *et al*., 2000; Thybaud *et al*., 2003). Test protocol harmonisation has been a difficult task, since the experiments described in the existing literature have employed a wide range of experimental animals and treatment protocols; this has made it very difficult to compare results obtained from different studies. Our approach to this problem has been to build a relational database from experimental data extracted from all studies carried out to date and use the aggregate information to examine important characteristics of the TGR models and their performance in examining induced mutation.

4.2 Transgenic Rodent Assay Information Database

To guide our analysis of existing TGR mutation data, we have developed the TRAID, a relational database that incorporates data from studies published to date and unpublished data contributed by several members of the IWGT working group. We have used the TRAID to analyse trends in the data and to extract information regarding several key parameters of test performance. This section describes the TRAID and summarises information contained in the database. This exercise is important, since it highlights areas where there is a significant amount of experimental information and allows us to assess the level of confidence that may be attributed to conclusions drawn from an analysis of the data.

4.2.1 Description of the database

At the core of the TRAID (see Figure 4-1) is an experimental data table that contains details of 3 186 experimental records identified in the literature as well as several complete but not yet published experimental records that have been contributed by members of the IWGT working group. The experimental records contained in the TRAID consist of comparisons between a treatment and a control group; data are not included if the study is limited to an examination of spontaneous mutation or DNA sequence analysis. A given study may contain several experimental records. The information contained in the table comprises a complete description of each experiment, including the source of the data, the experimental model used, details of administration, sampling time, results, statistical analysis and any additional information (comments) that may be relevant. In a separate genotoxicity and carcinogenicity data table, we have compiled existing data for each of the agents that relate to their genotoxicity (*Salmonella*, *in vitro* clastogenicity, mouse lymphoma, *in vitro* gene mutation, *in vitro* micronucleus, *in vivo* micronucleus, *in vivo* unscheduled DNA synthesis) and rodent carcinogenicity (male and female for both mouse and rat).

Information for the genotoxicity and carcinogenicity data table was derived from the Gene-Tox and Chemical Carcinogenesis Research Information System databases (http://toxnet.nlm.nih.gov), the Genetic Activity Profiles database (Waters *et al*., 1991) and the published scientific literature. In the case of evaluations made in the published scientific literature, the authors' conclusion was accepted for inclusion in the database. Rodent (both rat and mouse) carcinogenicity data were obtained from the Carcinogenic Potency Database, published literature, International Agency for Research on Cancer (IARC) monographs and U.S. NTP technical reports. An additional references table provides a comprehensive TGR gene mutation assay bibliography.

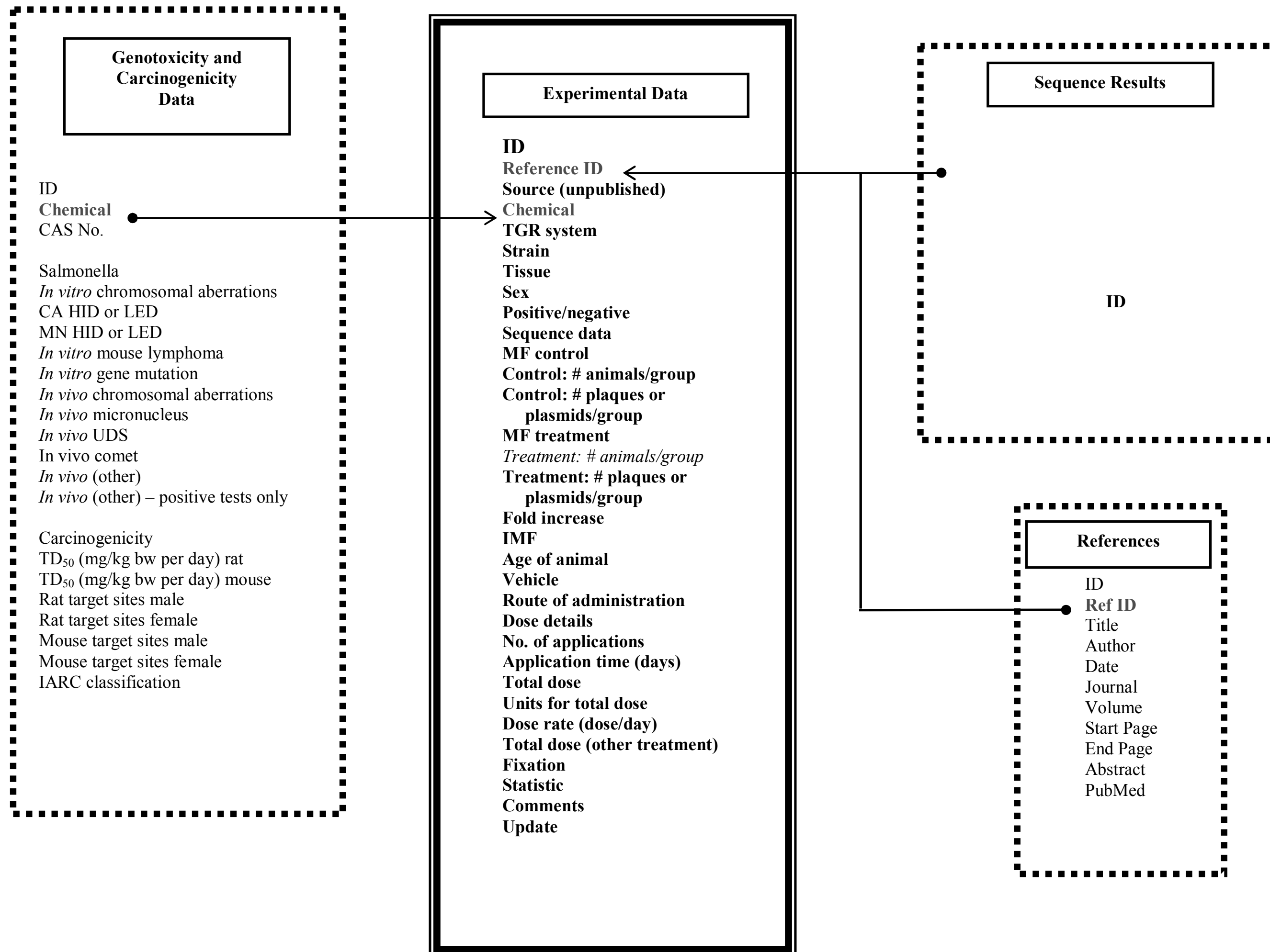


Figure 4-1. Organisation of the Transgenic Rodent Assay Information Database (TRAID)Abbreviations: CAS, Chemical Abstracts Service; HID, highest ineffective dose; IARC, International Agency for Research on Cancer; IMF, induced mutation/mutant frequency; LED, lowest effective dose; MF, mutant frequency; TD_{50}, tumorigenic dose for half the test animals; TG, transgenic rodent; UDS, unscheduled DNA synthesis

The database has been compiled using Microsoft Access and is easily searched using a variety of query formats to extract, analyse and format experimental records corresponding to specified search criteria.

4.2.2 Agents evaluated using TGR assays

The TRAID contains 3 180 experimental records from 228 different experimental treatments (chemical, radiation, diet, infectious agents or mixtures). Categorisation of the experimental treatments according to their primary application or reason for being of interest (Table 4-1) shows that approximately 25% of the agents evaluated to date are of interest primarily because they have been, or are currently, used in manufacturing processes or are industrial products/by-products. Compounds that are used primarily for research purposes comprise roughly 20% of the treatment list. The majority of these are known to be strong mutagens and/or carcinogens. Compounds with medical applications account for about 14% of the agents listed in the database. Many of these are used primarily in a cancer chemotherapeutic context, and their genotoxicity is well established. Pharmaceuticals that have broader use in the population are not well represented in the database. Experiments evaluating environmental and food contaminants comprise approximately 15% of the records, whereas complex mixtures, sources of radiation, natural products, dietary compounds and pesticides represent a combined 18% of the treatments in the database.

Although over 225 agents have been evaluated using TGR assays, the number of records for individual compounds is extremely unevenly distributed (Table 4-2). To date, approximately 50% of the experimental records are derived from experiments using only 21 different agents, all of which are carcinogenic. Almost 13% of the database is derived from experiments involving a single compound, ENU, which is frequently used as a positive control.

Among the 228 agents that have been evaluated using TGR assays, carcinogenicity has been evaluated for 140 compounds. Of these, 118 are carcinogens and 22 are non-carcinogens. In addition, there are 13 commonly used negative control compounds that are generally recognised as non-carcinogens (and non-mutagens). The small number of non-carcinogens that have been assayed to date is a limiting factor in evaluating the predictive capacity of the TGR models. This is discussed in more detail in Chapter 5.

4.2.3 Mode of administration

As discussed in Section 2.4.1.4, test agents can be administered to transgenic rats and mice through virtually any route. In practice, almost all treatments to date have been through traditional major routes of toxicological exposure: intraperitoneal injection, ingestion, irradiation, topical application or inhalation (Table 4-3). Ingestion and intraperitoneal injection together comprise approximately 80% of the records, whereas irradiation, topical application and inhalation have each been used in between 3.8% and 5.8% of the experimental records. Approximately 7% of the experimental records use other exposure routes, such as intravenous injection, intratracheal instillation or oral swab. In the case of binary mixtures, which account for approximately 1.5% of the experimental records, the components of the mixture are often administered through separate exposure routes.

The literature also contains many examples in which administration of the compound to the transgenic animal is through a route that is most appropriate to the prevalent mode of human exposure (Table 4-4) and/or the target tissue of concern for carcinogenicity. Thus, it is clear that a significant advantage of the TGR systems is the flexibility of the mode of administration; this differs considerably from most other genotoxicity tests, in which the mode of administration is limited by the requirement that the test agent distributes to the target tissue examined by the test (*e.g.* bone marrow, micronucleus; liver, unscheduled DNA synthesis).

Table 4-1. Agents included in the database, by primary application[a]

Primary application	Agents included in database
Research compound	1,4-Phenylenebis(methylene)selenocyanate (p-XSC) (0/1)
	1,6-Dinitropyrene (1/2)
	1-Chloromethylpyrene (6/6)
	2-Acetylaminofluorene (2-AAF) (16/12)
	3-Fluoroquinoline (0/3)
	3-Methylcholanthrene (5/0)
	4-Acetylaminofluorene (4-AAF) (1/0)
	4-Nitroquinoline-1-oxide (4-NQO) (31/15)
	5-(*p*-Dimethylaminophenylazo)benzothiazole (2/0)
	5,9-Dimethyldibenzo(c,g)carbazole (DMDBC) (20/10)
	5-Bromo-2′-deoxyuridine (BrdU) (0/2)
	5-Fluoroquinoline (1/2)
	6-(*p*-Dimethylaminophenylazo)benzothiazole (5/0)
	6,11-Dimethylbenzo(b)naphtho(2,3-d)thiophene (1/2)
	7,12-Dimethylbenzanthracene (7,12-DMBA) (60/23)
	7-Methoxy-2-nitronaphtho(2,1-b)furan (R7000) (18/8)
	Dipropylnitrosamine (DPN) (9/7)
	Ethylmethanesulphonate (EMS) (5/12)
	Isopropylmethanesulphonate (iPMS) (9/6)
	Jervine (2/0)
	Methyl clofenapate (0/2)
	Methylmethanesulphonate (MMS) (10/47)
	N7-Methyldibenzo(c,g)carbazole (NMDBC) (7/3)
	N-Ethyl-*N*-nitrosourea (ENU) (326/74)
	N-Hydroxy-2-acetylaminofluorene (*N*-hydroxy-2-AAF) (4/0)
	N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (24/7)
	N-Methyl-*N*-nitrosourea (MNU) (43/11)
	N-Propyl-*N*-nitrosourea (PNU) (19/12)
	Phorbol-12-myristate-13-acetate (TPA) (0/1)
	Streptozotocin (2/2)
	Wyeth 14,643 (3/3)

Primary application	Agents included in database
Medical	17β-Oestradiol (0/2) 5-(2-Chloroethyl)-2′-deoxyuridine (CEDU) (3/0) 87-966 (2/1) Acetaminophen (0/1) Adozelesin (2/1) alpha-Hydroxytamoxifen (4/1) AMP397 (0/6) Azathioprine (3/6) beta-Propiolactone (9/1) Bleomycin (1/0) CC-1065 (1/0) Chlorambucil (22/18) Cisplatin (2/0) Clofibrate (0/2) Cyclophosphamide (24/18)

Table 4-1 (continued)

Primary application	Agents included in database
	Cyproterone acetate (31/15) Ethylene oxide (7/27) Etoposide (0/13) Flumequine (0/4) Hydroxyurea (2/1) Levofloxacin (0/14) Metronidazole (0/4) Mitomycin-C (11/20) Nifuroxazide (0/8) Nitrofurantoin (1/7) Oxazepam (2/4) Phenobarbital (1/14) Procarbazine hydrochloride (14/15) Tamoxifen (11/2) Thiotepa (1/0) Toremifene citrate (0/1)
Manufacturing/industrial compound	1,2:3,4-Diepoxybutane (0/9) 1,2-Dibromo-3-chloropropane (1/1) 1,2-Dichloroethane (0/11) 1,2-Epoxy-3-butene (2/7) 1,3-Butadiene (14/2) 2,4-Diaminotoluene (6/5) 2,6-Diaminotoluene (0/7) 2-Nitro-*p*-phenylenediamine (1/1) 4-Aminobiphenyl (14/5) 4-Chloro-*o*-phenylenediamine (5/1) 4-Hydroxybiphenyl (0/1) Acetic acid (1/2)

Table 4-1 (continued)

Primary application	Agents included in database
	Acetone (0/2) Acrylamide (7/19) Acrylonitrile (0/15) Amosite asbestos (4/11) Arsenite trioxide (0/5) Benzene (4/8) Carbon tetrachloride (0/5) Chloroform (0/5) CM 44 glass fibres (0/8) Crocidolite asbestos (4/5) Di(2-ethylhexyl)phthalate (DEHP) (2/8) Dichloroacetic acid (DCA) (2/4) Dimethylarsinic acid (0/5) d-Limonene (0/2) Eugenol (0/1) Hexachlorobutadiene (2/22) Hexavalent chromium (9/6) Hydrazine sulphate (0/24) Malachite green (0/1) Methyl bromide (0/2) Nickel subsulphide (0/4) *N*-Nitrosodibenzylamine (NDBzA) (2/20) *o*-Aminoazotoluene (8/6) *o*-Anisidine (2/10) *p*-Cresidine (4/0) Quinoline (4/5) Rock wool fibres (3/6) Trichloroethylene (TCE) (0/33) Tris-(2,3-dibromopropyl)phosphate (7/17) Urethane (28/28) Vinyl carbamate (10/6)
Environmental/food contaminant	1,10-Diazachrysene (11/1) 1,7-Phenanthroline (5/15) 1,8-Dinitropyrene (2/15) 10-Azabenzo(a)pyrene (3/13) 1-Methylphenanthrene (0/24) 1-Nitronaphthalene (0/3) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (0/2) 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) (82/55) 2-Amino-3-methylimidazo(4,5-f)quinoline (IQ) (14/8) 2-Amino-3,4-dimethylimidazo(4,5-f)quinoline (MeIQ) (10/7) 2-Amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx) (30/21) 2-Nitronaphthalene (2/1) 3-Amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2) (0/3) 3-Chloro-4(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) (0/17)

Table 4-1 (continued)

Primary application	Agents included in database
	3-Nitrobenzanthrone (2/4)
	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (19/2)
	4,10-Diazachrysene (12/0)
	4-Monochlorobiphenyl (1/0)
	6-Nitrochrysene (2/0)
	7H-Dibenzo(c,g)carbazole (DBC) (6/0)
	A-alpha-C (4/2)
	Aflatoxin B1 (7/2)
	Benzo(a)pyrene (B(a)P) (121/33)
	Benzo(a)pyrene diolepoxide (BPDE) (1/0)
	Chrysene (8/4)
	Diethylnitrosamine (DEN) (29/22)
	Dimethylnitrosamine (DMN) (71/45)
	Glycidamide (2/2)
	Leucomalachite green (1/8)
	N-Nitrosomethylbenzylamine (3/0)
	N-Nitrosonornicotine (NNN) (8/1)
	N-Nitrosopyrrolidine (1/0)
	Peroxyacetyl nitrate (PAN) (1/0)
	Polyphenon E (0/12)
	Potassium bromate (3/13)
Binary/ternary mixtures	2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) + 3H-1,2-dithiole-3-thione (D3T) (3/0)
	PhIP + conjugated linoleic acid (CLA) (6/0)
	PhIP + high-fat diet (1/1)
	PhIP + low-fat diet (1/1)
	2-Amino-3-methylimidazo(4,5-f)quinoline (IQ) + sucrose (4/8)
	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) + 8-methoxypsoralen (0/2)
	NNK + green tea (3/2)
	4-Nitroquinoline-1-oxide (4-NQO) + alpha-tocopherol (6/0)
	4-NQO + 1,4-phenylenebis(methylene)selenocyanate (p-XSC) (13/0)
	4-NQO + p-XSC + alpha-tocopherol (6/0)
	5,9-Dimethyldibenzo(c,g)carbazole + carbon tetrachloride (1/0)
	5,9-Dimethyldibenzo(c,g)carbazole + phenobarbital (1/0)
	7,12-Dimethylbenzanthracene (7,12-DMBA) + 17β-oestradiol (3/0)
	7,12-DMBA + daidzein (4/0)
	7,12-DMBA + daidzein + genistein (2/0)
	7,12-DMBA + genistein (7/0)
	7,12-DMBA + high-fat diet (2/0)
	7,12-DMBA + low-fat diet (2/0)
	Aflatoxin B1 + phorone (2/1)
	Aflatoxin B1 + 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1/1)
	Amosite asbestos + benzo(a)pyrene (B(a)P) (5/4)
	B(a)P + D3T (6/0)
	B(a)P + D3T + p-XSC (3/0)

Table 4-1 (continued)

Primary application	Agents included in database
	B(a)P + eugenol (1/0)
	B(a)P + green tea (1/0)
	B(a)P + lycopene (5/1)
	B(a)P + p-XSC (5/0)
	B(a)P + selenium-enriched yeast (6/0)
	B(a)P + selenium-enriched yeast + D3T (3/0)
	Benzo(a)pyrene-diolepoxide (BPDE) + phorbol-12-myristate-13-acetate (TPA) (1/2)
	Conjugated linoleic acid (CLA) + PhIP (1/0)
	Daidzein + genistein (0/1)
	Diethylnitrosamine (DEN) + phenobarbital (7/0)
	Gamma rays + NNK (6/3)
	Glass wool fibres + B(a)P (3/0)
	Hydroxyurea + X-rays (2/1)
	Methyl clofenapate + dimethylnitrosamine (DMN) (2/0)
	Methylmethanesulphonate (MMS) + 4-acetylaminofluorene (4-AAF) (1/0)
	MMS + DMN (2/0)
	N-Ethyl-*N*-nitrosourea (ENU) + 8-methoxypsoralen (1/0)
	N-Nitrosomethylbenzylamine (NDBzA) + diallyl sulphide (0/1)
	NDBzA + ellagic acid (1/0)
	NDBzA + ethanol (1/0)
	NDBzA + green tea (1/0)
	Rock wool fibres + B(a)P (3/0)
	Tamoxifen + phenobarbital (3/0)
	Vinyl carbamate + diallyl sulphone (5/2)
Complex mixtures	Bitumen fumes (0/3)
	Coal tar (1/1)
	Diesel exhaust (10/24)
	Dinitropyrenes (10/6)
Diet	Conjugated linoleic acid (CLA) (0/3)
	Folic acid (0/8)
	Fructose (0/2)
	Glucose (0/2)
	Green tea (0/1)
	High-fat diet (0/12)
	Hyperglycaemia (1/0)
	Sodium saccharin (0/2)
	Sucrose (5/11)
	Vitamin E (0/5)
Pesticides	1,2-Dibromoethane (1/7)
	Dicyclanil (1/1)
	Heptachlor (0/2)
Natural products	Agaritine (2/10)
	alpha-Chaconine (2/0)
	alpha-Solanine (2/0)

Table 4-1 (continued)

Primary application	Agents included in database
	Aristolochic acid (17/9)
	Comfrey (2/0)
	Daidzein (0/2)
	Diallyl sulphide (0/1)
	Diallyl sulphone (0/7)
	Ellagic acid (0/1)
	Genistein (0/4)
	Kojic acid (0/2)
	Riddelliine (4/1)
	Solanidine (2/0)
	Solasodine (1/1)
Radiation	1.5 GHz electromagnetic near field (0/4)
	^{114m}In internal radiation (1/5)
	2.45 GHz radiofrequency (0/4)
	^{89}Sr internal radiation (1/5)
	Gamma rays (10/19)
	Heavy-ion radiation (3/2)
	High-energy charged particle (Fe) (4/8)
	Proton radiation (13/19)
	UVB (29/1)
	X-rays (55/9)
Other	All-*trans*-Retinol (0/2)
	Fasciola hepatica (1/0)
	trans-4-Hydroxy-2-nonenal (0/4)
	Uracil (1/3)

[a] Numbers in parentheses refer to the total number of positive/negative results that have been obtained from TGR assays of these agents.

Table 4-2. Agents that comprise >50% of TRAID records

Chemical	CAS No.	No. of records	Percentage of total
N-Ethyl-*N*-nitrosourea (ENU)	759-73-9	397	12.5
Benzo(a)pyrene (B(a)P)	50-32-8	154	4.8
2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)	105650-23-5	137	4.3
Dimethylnitrosamine (DMN)	62-75-9	118	3.7
7,12-Dimethylbenzanthracene (7,12-DMBA)	57-97-6	84	2.6
X-rays	–	67	2.1
Methylmethanesulphonate (MMS)	66-27-3	57	1.8
Urethane	51-79-6	56	1.8
N-Methyl-*N*-nitrosourea (MNU)	684-93-5	54	1.7
Diethylnitrosamine (DEN)	55-18-5	51	1.6
2-Amino-3,8-dimethylimidazo(4,5-	77500-04-0	51	1.6

Chemical	CAS No.	No. of records	Percentage of total
f)quinoxaline (MeIQx)			
Cyproterone acetate	427-51-0	46	1.4
4-Nitroquinoline-1-oxide (4-NQO)	56-57-5	46	1.4
Cyclophosphamide	50-18-0	42	1.3
Chlorambucil	305-03-3	41	1.3
Ethylene oxide	75-21-8	34	1.1
Diesel exhaust	–	34	1.1
Trichloroethylene (TCE)	79-01-6	33	1.0
N-Propyl-*N*-nitrosourea (PNU)	816-57-9	32	1.0
Proton radiation	–	32	1.0
Gamma rays	–	32	1.0

CAS, Chemical Abstracts Service

Table 4-3. Number of records based on mode of administration

Mode of administration	No. of records (%)
Ingestion	1 256 (39.5)
Diet	398 12.5)
Drinking water	130 (4.1)
Gavage	728 (22.9)
Intraperitoneal injection	1 281 (40.3)
Irradiation	187 (5.9)
Topical application	120 (3.8)
Inhalation	127 (4.0)
Total	2 971
Percentage of total records[a]	93.4

[a] Total number of experimental records is 3 180.

4.2.4 Animal models

Among the studies in which the traditional routes of exposure have been used, the majority of experimental records (1 412) have used Muta™Mouse (Section 2.2.1) as the TGR model (Table 4-5). Eight hundred and fifteen experimental records are derived from the Big Blue® mouse and rat models (Section 2.2.2). The Big Blue® TGR models, which utilise the *lacI* gene as a mutational target gene, have been exploited extensively for molecular analysis of mutants, as the *lacI* gene has been amenable to high throughput sequencing analysis formany years (Section 2.3.1). Approximately 13% of the experimental records have utilised the *lacZ* plasmid mouse (Section 2.2.3) or the *gpt* delta (Spi) rodent (Section 2.2.4) systems, models that were developed, in part, for their ability to detect deletions that might not be easily recovered or detected using the Muta™Mouse, Big Blue® or *gpt* delta (*gpt*) rodent systems.

Table 4-4. Relevance of mode of administration in TGR assays to human exposure and/or target tissue

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
1,2-Dibromoethane	Inhalation	Lung (−)	Schmezer *et al*. (1998b)	Pesticide	+	**Lung**, liver, mammary gland, nasal cavity, oesophagus, peritoneal cavity, pituitary gland, stomach, vascular system
1,2-Epoxy-3-butene	Inhalation	Lung (+)	Saranko *et al*. (2001)	Manufacturing/ industrial compound	nd	
1,3-Butadiene	Inhalation	Lung (+)	Recio *et al*. (1992, 1993)	Manufacturing/ industrial compound	+	**Lung**, haematopoietic system, Harderian gland, kidney, liver, testes
10-Aza-benzo(a)pyrene	Ingestion (gavage)	Colon (+), liver (+), stomach (−)	Yamada *et al*. (2002)	Environmental/food contaminant	nd	
1-Nitronaphthalene	Topical	Liver (−), skin (−), urinary bladder (−)	Kirkland and Beevers (2006)	Environmental/food contaminant	–	
2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)	Ingestion (gavage)	Liver (−)	Thornton *et al*. (2001)	Environmental/food contaminant	+	**Liver**, lung, oral cavity, thyroid
2,4-Diaminotoluene	Ingestion (gavage)	Liver (+)	Suter *et al*. (1996)	Manufacturing/ industrial compound	+	**Liver**, haematopoietic system, mammary gland
	Topical	Kidney (−), liver (+), skin (−)	Kirkland and Beevers (2006)	Manufacturing/ industrial compound	+	**Liver**, haematopoietic system, mammary gland
2,6-Diaminotoluene	Topical	Kidney (−), liver (−), skin (−)	Kirkland and Beevers (2006)	Manufacturing/ industrial compound	–	
2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)	Ingestion (diet, gavage)	Caecum (+), colon (+), kidney (−), liver (+), mammary gland (+), prostate	Lynch, Gooderham and Boobis (1996); Zhang *et al*. (1996a); Okonogi *et al*. (1997a); Masumura *et al*.	Environmental/food contaminant	+	**Large and small intestine**, **liver**, **mammary gland**, **prostate**, haematopoietic system

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
		(+), seminal vesicle (+), small intestine (+), spleen (+)	(1999a); Stuart *et al*. (2000c, 2001); Klein *et al*. (2001); Yang *et al*. (2001); Yang, Glickman and de Boer (2002); Itoh *et al*. (2003); Shan *et al*. (2004); Nakai, Nelson and De Marzo (2007)			

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
2-Amino-3,4-dimethyl-imidazo(4,5-f)quinoline (MeIQ)	Ingestion (diet, gavage)	Caecum (+), colon (+), forestomach (+), liver (+), small intestine (−)	Suzuki *et al*. (1996b); Itoh *et al*. (2003)	Environmental/food contaminant	+	**Colon**, **liver**, **stomach**, mammary gland, oral cavity, skin, Zymbal's gland
2-Amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx)	Ingestion (diet, gavage)	Brain (−), colon (+), fat tissue (−), heart (−), kidney (+), liver (+), lung (−), skeletal muscle (−), spleen (−), testis (−), Zymbal's gland (−)	Davis *et al*. (1996); Ryu *et al*. (1999a); Itoh *et al*. (2000); Masumura *et al*. (2003a); Hoshi *et al*. (2004)	Environmental/food contaminant	+	**Ear/Zymbal's gland**, **liver**, **lung**, haematopoietic system, skin
2-Amino-3-methylimidazo(4,5-f)quinoline (IQ)	Ingestion (diet, gavage)	Caecum (+), colon (+), liver (+), small intestine (−)	Davis *et al*. (1996); Bol *et al*. (2000); Moller *et al*. (2002); Dybdahl *et al*. (2003); Itoh *et al*. (2003); Hansen *et al*. (2004); Kanki *et al*. (2005)	Environmental/food contaminant	+	**Large intestine**, **liver**, clitoral gland, ear/Zymbal's gland, lung, mammary gland, oral cavity, stomach

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
2-Nitronaphthalene	Topical	Liver (+), skin (−), urinary bladder (+)	Kirkland and Beevers (2006)	Environmental/food contaminant	nd	
2-Nitro-*p*-phenylenediamine	Ingestion (gavage)	Liver (+)	Suter *et al*. (1996)	Manufacturing/ industrial compound	+	**Liver**
3-Amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2)	Ingestion (gavage)	Caecum (−), colon (−), small intestine (−)	Itoh *et al*. (2003)	Environmental/food contaminant	+	**Small intestine**, clitoral gland, haematopoietic system, liver, mammary gland, urinary bladder, vascular system
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	Ingestion (drinking water)	Liver (−), lung (−)	Nishikawa *et al*. (2006)	Environmental/food contaminant	+	**Liver**, **lung**, adrenal gland, haematopoietic system, mammary gland, pancreas, thyroid
4-(Methylnitros-amino)-1-(3-pyridyl)-1-butanone (NNK)	Ingestion (drinking water)	Liver (+)	von Pressentin, Chen and Guttenplan (2001)	Environmental/food contaminant	+	**Liver**, lung, nasal mucosa, pancreas
4-Aminobiphenyl	Ingestion (gavage)	Bladder (+), liver (+)	Fletcher, Tinwell and Ashby (1998); Turner *et al*. (2001)	Manufacturing/ industrial compound	+	**Liver**, **urinary bladder**, intestine, mammary gland
4-Chloro-*o*-phenylenediamine	Ingestion (diet, gavage)	Liver (+)	Suter *et al*. (1996, 1998); Staedtler *et al*. (1999)	Manufacturing/ industrial compound	+	**Liver**, stomach, urinary bladder
4-Hydroxybiphenyl	Ingestion (gavage)	Liver (−)	Lehmann *et al*. (2007)	Manufacturing/ industrial compound	–	
4-Monochloro-biphenyl	Ingestion (gavage)	Liver (+)	Lehmann *et al*. (2007)	Environmental/food contaminant	nd	

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
5-(2-Chloroethyl)-2′-deoxyuridine (CEDU)	Ingestion (gavage)	Bone marrow (+), lung (+), spleen (+)	Suter *et al*. (2004)	Medical	nd	
6-Nitrochrysene	Ingestion (gavage)	Mammary gland (+)	Boyiri *et al*. (2004)	Environmental/food contaminant	+	**Mammary gland**, colon, liver, lung
7H-Dibenzo(c,g)carbazole (DBC)	Topical	Skin (+)	Renault *et al*. (1998)	Environmental/food contaminant	+	**Skin**, liver, lung, stomach
^{89}Sr internal radiation	Intravenous	Bone marrow (−), liver (+), spleen (−)	Takahashi, Kubota and Sato (1998)	Radiation	nd	
A-alpha-C	Ingestion (diet, gavage)	Colon (+), liver (+), small intestine (−)	Davis *et al*. (1996); Zhang *et al*. (1996a)	Environmental/food contaminant	+	**Liver**, vascular system
Acetaminophen	Ingestion (diet)	Liver (−)	Kanki *et al*. (2005)	Medical	+	**Liver**, urinary bladder
Acetone	Topical	Skin (−)	Ashby *et al*. (1993); Gorelick *et al*. (1995)	Manufacturing/ industrial compound	–	
Acrylamide	Ingestion (drinking water)	Liver (+)	Manjanatha *et al*. (2006b)	Manufacturing/ industrial compound	+	Clitoral gland, nervous system, mammary gland, oral cavity, peritoneal cavity, thyroid, uterus
Adozelesin	Intravenous	Liver (−)	Monroe and Mitchell (1993)	Medical	nd	
Aflatoxin B1	Ingestion (gavage)	Large intestine (−)	Autrup, Jorgensen and Jensen (1996)	Environmental/food contaminant	+	**Large intestine**, kidney, liver
	Ingestion (gavage)	Liver (+)	Autrup, Jorgensen and Jensen (1996); Davies *et al*. (1997, 1999); Thornton *et al*. (2004)	Environmental/food contaminant	+	**Liver**, kidney, large intestine
Aminophenylnor-	Ingestion	Colon (+), liver (+)	Masumura *et al*. (2003b)	Environmental/food	nd	

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
harman	(diet)			contaminant		
Aristolochic acid	Ingestion (gavage)	Kidney (+), liver (+)	Chen *et al*. (2006); Mei *et al*. (2006a)	Natural product	+	**Kidney**, ear, haematopoietic system, lung, small intestine, stomach
Azathioprine	Ingestion (gavage)	Liver (+)	Smith *et al*. (1999)	Medical	+	Ear, haematopoietic system, thymus, uterus, vascular system
Benzene	Inhalation	Lung (+)	Mullin *et al*. (1995)	Manufacturing/ industrial compound	+	**Lung**, ear/Zymbal's gland, haematopoietic system, nasal cavity, oral cavity, skin
Benzo(a)pyrene	Ingestion (diet, gavage)	Colon (+), liver (+), oesophagus (+), oral tissue (+), small intestine (+), stomach (+), tongue (+)	de Vries *et al*. (1997); Hakura *et al*. (1998, 1999); Cosentino and Heddle (1999a, 2000); Kosinska, von Pressentin and Guttenplan (1999); Guttenplan *et al*. (2001, 2004b); van Steeg (2001); Yamada *et al*. (2002)	Environmental/food contaminant	+	**Oesophagus**, **oral cavity, stomach**
	Topical	Skin (+)	Dean *et al*. (1998); Miller *et al*. (2000)	Environmental/food contaminant	+	Oesophagus, stomach
beta-Propiolactone	Ingestion (gavage)	Liver (+), stomach (+)	Brault *et al*. (1996, 1999)	Medical	+	**Stomach**
Bitumen fumes	Inhalation	Lung (−)	Micillino *et al*. (2002); Bottin *et al*. (2006)	Complex mixture	nd	
Carbon tetrachloride	Ingestion (gavage)	Liver (−)	Tombolan *et al*. (1999a); Hachiya and Motohashi	Manufacturing/ industrial compound	+	**Liver**, adrenal gland, mammary gland

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
			(2000)			
Coal tar	Topical	Skin (+)	Thein *et al*. (2000)	Complex mixture	+	Liver, lung, small intestine, stomach
Comfrey	Ingestion (diet)	Liver (+)	Mei *et al*. (2005a, 2006b)	Natural product	+	**Liver**, urinary bladder
Conjugated linoleic acid (CLA)	Ingestion (diet)	Caecum (−), distal colon (−)	Yang, Glickman and de Boer (2002)	Diet	nd	
Crocidolite asbestos	Inhalation	Lung (+)	Rihn *et al*. (2000a)	Environmental	+	**Lung**, peritoneal cavity
Cyproterone acetate	Ingestion (gavage)	Liver (+)	Krebs *et al*. (1998); Wolff *et al*. (2001); Topinka *et al*. (2004b)	Medical	+	**Liver**, stomach
Daidzein	Ingestion (diet)	Mammary gland (−)	Manjanatha *et al*. (2006a)	Natural product	nd	
Di(2-ethylhexyl)-phthalate (DEHP)	Ingestion (diet)	Liver (−)	Gunz, Shephard and Lutz (1993); Kanki *et al*. (2005)	Manufacturing/ industrial compound	+	**Liver**
Diallyl sulphide	Ingestion (gavage)	Oesophagus (−)	de Boer *et al*. (2004)	Natural product	nd	
Diallyl sulphone	Ingestion (gavage)	Lung (−), small intestine (−)	Hernandez and Forkert (2007b)	Natural product	nd	
Dichloroacetic acid (DCA)	Ingestion (drinking water)	Liver (+)	Leavitt *et al*. (1997)	Manufacturing/ industrial compound	+	**Liver**
Dicyclanil	Ingestion (diet)	Liver (+)	Umemura *et al*. (2007)	Pesticide	+	**Liver**
Diesel exhaust	Inhalation	Lung (+)	Sato *et al*. (2000); Dybdahl *et al*. (2004); Hashimoto *et*	Complex mixture	+	**Lung**, skin

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
			al. (2007)			
	Ingestion (diet)	Colon (−), liver (−), lung (−)	Dybdahl *et al*. (2003); Muller *et al*. (2004)	Complex mixture	+	**Lung**, skin
Dimethylnitrosamine (DMN)	Ingestion (diet, gavage)	Bladder (−), liver (+)	Lefevre *et al*. (1994); Tinwell, Lefevre and Ashby (1994a, 1994b, 1998); Shephard, Gunz and Schlatter (1995); Tinwell *et al*. (1995); Jiao *et al*. (1997); Butterworth *et al*. (1998); Fletcher, Tinwell and Ashby (1998); Gollapudi, Jackson and Stott (1998)	Environmental/food contaminant	+	**Liver**, kidney, lung, nervous system, testes, vascular system
d-Limonene	Ingestion (diet)	Liver (−)	Turner *et al*. (2001)	Manufacturing/ industrial compound	+	Kidney
Ellagic acid	Ingestion (diet)	Oesophagus (−)	de Boer *et al*. (2004)	Natural product	–	
Ethylene oxide	Inhalation	Bone marrow (+), lung (+), seminiferous tubules (+)	Sisk *et al*. (1997); Recio *et al*. (2004)	Medical	+	**Haematopoietic system**, **lung**, brain, Harderian gland, mammary gland, nervous system, peritoneal cavity, pituitary gland, stomach, uterus
Eugenol	Ingestion (diet)	Liver (−)	Rompelberg *et al*. (1996)	Manufacturing/ industrial compound	–	
Flumequine	Ingestion (diet)	Liver (−)	Kuroiwa *et al*. (2007)	Medical	+	Liver
Fructose	Ingestion (diet)	Colon (−)	Hansen *et al*. (2008)	Diet	nd	

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
Genistein	Ingestion (diet)	Heart (−), mammary gland (−)	Manjanatha *et al*. (2005, 2006a)	Natural product	+	Uterus
Glucose	Ingestion (diet)	Colon (−)	Hansen *et al*. (2008)	Diet	nd	
Glycidamide	Ingestion (drinking water)	Liver (+)	Manjanatha *et al*. (2006b)	Environmental/food contaminant	nd	
Green tea	Ingestion (drinking water)	Oesophagus (−)	de Boer *et al*. (2004)	Diet	–	
Heptachlor	Ingestion (diet)	Liver (−)	Gunz, Shephard and Lutz (1993)	Pesticide	+	**Liver**
Hexachlorobuta-diene	Ingestion (gavage)	Liver (−)	unpublished	Manufacturing/ industrial compound	+	Kidney
High-fat diet	Ingestion (diet)	Bone marrow (−), colonic epithelium (−), small intestine (−)	Zhang *et al*. (1996b); Hernandez and Heddle (2005)	Diet	+	**Colon**
Hydrazine sulphate	Ingestion (gavage)	Liver (−)	Douglas, Gingerich and Soper (1995)	Manufacturing/ industrial compound	+	**Liver**, lung
Kojic acid	Ingestion (gavage)	Liver (−)	Nohynek *et al*. (2004)	Natural product	+	Thyroid
Leucomalachite green	Ingestion (diet)	Liver (+)	Mittelstaedt *et al*. (2004)	Environmental/food contaminant	±	
Malachite green	Ingestion (diet)	Liver (−)	Mittelstaedt *et al*. (2004)	Manufacturing/ industrial compound	nd	
Methyl bromide	Ingestion (gavage)	Glandular stomach (−), liver (−)	Pletsa *et al*. (1999)	Manufacturing/ industrial compound	+	**Stomach**

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
Metronidazole	Ingestion (gavage)	Stomach (−)	Touati *et al*. (2000)	Medical	+	Haematopoietic system, liver, lung, mammary gland, pituitary gland, testes
Mitomycin-C	Ingestion (gavage)	Small intestine (−)	Cosentino and Heddle (1999a)	Medical	+	**Intestine**, mammary gland, peritoneal cavity
Nickel subsulphide	Inhalation	Lung (−)	Mayer *et al*. (1998)	Manufacturing/ industrial compound	+	*Lung*
Nifuroxazide	Ingestion (gavage)	Bladder (−), caecum (−), colon (−), kidney (−), lung (−), small intestine (−), spleen (−), stomach (−)	Quillardet *et al*. (2006)	Medical	nd	
Nitrofurantoin	Ingestion (gavage)	Bladder (−), caecum (−), colon (−), kidney (+), lung (−), small intestine (−), spleen (−), stomach (−)	Quillardet *et al*. (2006)	Medical	+	**Kidney**, haematopoietic system, mammary gland, ovary
N-Nitrosodibenzyl-amine (NDBzA)	Ingestion (gavage)	Liver (+)	Jiao *et al*. (1997)	Manufacturing/ industrial compound	nd	
N-Nitrosonornicotine (NNN)	Ingestion (drinking water)	Liver (+)	von Pressentin, Chen and Guttenplan (2001)	Environmental/food contaminant	+	Lung, nasal cavity, oesophagus
N-Nitrosopyrrolidine	Ingestion (drinking water)	Liver (+)	Kanki *et al*. (2005)	Environmental/food contaminant	+	**Liver**, kidney, lung, vascular system

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
o-Anisidine	Ingestion (gavage)	Bladder (+), liver (−)	Ashby *et al*. (1994)	Manufacturing/ industrial compound	+	**Urinary bladder**, kidney, thyroid gland
Oxazepam	Ingestion (diet)	Liver (+), lung (−)	Shane *et al*. (1999); Singh *et al*. (2001); Mirsalis *et al*. (2005)	Medical	+	**Liver**, kidney, thyroid gland
p-Cresidine	Ingestion (diet)	Bladder (+)	Jakubczak *et al*. (1996)	Manufacturing/ industrial compound	+	**Urinary bladder**, liver, nasal cavity
Peroxyacetyl nitrate (PAN)	Inhalation	Lung (+)	DeMarini *et al*. (2000)	Environmental/food contaminant	nd	
Phenobarbital	Ingestion (diet, drinking water)	Liver (+), lung (−)	Gunz, Shephard and Lutz (1993); Tombolan *et al*. (1999a); Shane *et al*. (2000c); Singh *et al*. (2001); Styles *et al*. (2001); Mirsalis *et al*. (2005)	Medical	+	**Liver**
Potassium bromate	Ingestion (drinking water)	Kidney (+)	Umemura *et al*. (2006)	Environmental/food contaminant	+	**Kidney**, testes
Riddelliine	Ingestion (gavage)	Liver (+)	Mei *et al*. (2004a, 2004b)	Natural product	+	**Liver**, haematopoietic system, lung, vascular system
Sodium saccharin	Ingestion (diet)	Bladder (−), liver (−)	Turner *et al*. (2001)	Diet	+	**Urinary bladder**
Sucrose	Ingestion (diet)	Colon (+), liver (−)	Dragsted *et al*. (2002); Moller *et al*. (2003); Risom *et al*. (2003); Hansen *et al*. (2004, 2008)	Diet	–	
Tamoxifen	Ingestion	Liver (+)	Davies *et al*. (1997, 1999);	Medical	+	**Liver**, cervix, ovary, testes,

Table 4-4 (continued)

Chemical	Mode of administration	Tissue sampled (TGR result)	References[a]	Human exposure	Carcinogenicity	Cancer tissues[b]
	(gavage)		Styles *et al*. (2001)			uterus
Toremifene citrate	Ingestion (gavage)	Liver (−)	Davies *et al*. (1997)	Medical	–	
Trichloroethylene (TCE)	Inhalation	Lung (−)	Douglas *et al*. (1999)	Manufacturing/ industrial compound	+	**Lung**, liver, testes
Tris-(2,3-dibromo-propyl)phosphate	Ingestion (gavage)	Liver (−), stomach (−)	de Boer *et al*. (1996); Provost *et al*. (1996)	Manufacturing/ industrial compound	+	**Liver**, **stomach**, kidney, large intestine, lung
Urethane	Ingestion (diet, gavage, drinking water)	Forestomach (+), liver (+), lung (+)	Shephard, Gunz and Schlatter (1995); Chang *et al*. (2003); Mirsalis *et al*. (2005)	Manufacturing/ industrial compound	+	**Liver**, **lung**, haematopoietic system, Harderian gland, nervous system, skin, thymus, vascular system
Vitamin E	Ingestion (diet)	Liver (−)	Moore *et al*. (1999)	Diet	nd	

nd, not determined

[a] “Unpublished” refers to data provided by members of the IWGT working group.

[b] Cancer tissues in bold correspond to TGR tissues sampled.

Table 4-5. Number of records based on animal model, by mode of administration and tissue

	Big Blue® mouse	Muta™Mouse	*LacZ* plasmid mouse	Big Blue® rat	*gpt* delta (*gpt*)	*gpt* delta (Spi)	Total
Ingestion	195 (63)[a]	435 (44)	106	249 (84)	53	27	1 256
Intraperitoneal injection	229 (52)	790 (68)	27	27 (2)	41	42	1 278
Inhalation	63 (1)	45 (4)	0	8 (5)	0	0	126
Topical application	16 (0)	104 (0)	0	0 (0)	0	0	120
Irradiation	28 (0)	38 (0)	83	0 (0)	22	20	191
Total	**531 (116)**	**1 412 (116)**	**216**	**284 (91)**	**116**	**89**	**2 971**
Bladder	16	17	3	6	0	0	42
Bone marrow	47	285	3	8	8	15	366
Brain	12	26	41	1	1	1	82
Colon[b]	32	53	0	55	10	2	152
Intestine[c]	61	86	21	8	7	0	183
Kidney	21	73	4	16	11	12	137
Liver	230	413	64	163	48	28	946
Lung	77	122	25	75	34	13	346
Male reproductive[d]	62	176	6	3	3	7	257
Mammary gland	0	13	0	31	0	0	44
Oral tissue[e]	1	77	0	18	0	0	96
Skin[f]	16	74	0	0	10	5	105
Spleen[g]	54	74	62	3	3	6	202
Splenic lymphocytes[h]	15	9	0	19	0	0	46
Stomach[i]	24	58	0	0	0	0	82
Uterus	0	0	0	7	0	0	7
Other[j]	7	48	3	32	0	0	90

[a] Number in parentheses refers to the number of experimental records in which mutant frequency has been scored in λ phage *cII* gene.
[b] Includes proximal colon, distal colon, large intestine and colonic epithelium.

[c] Includes intestine, caecum and ileum.
[d] Includes epididymal sperm, vas deferens, sperm, vas deferens sperm, testes, testicular germ cells, seminiferous tubules and epididymis.
[e] Includes tongue, oral tissue and oesophagus.
[f] Includes epidermis and dermis.
[g] Includes spleen cell fraction.
[h] Includes splenic T-cells.
[i] Includes forestomach and glandular stomach.
[j] Includes skeletal muscle, Zymbal’s gland, thymus, prostate, ovarian granulosa, omentum, nasal mucosa, heart, embryo liver, embryo and adipose tissue.

4.2.5 Tissues examined

Mutagenicity has been examined in virtually all rodent tissues to some extent (Table 4-5). In all rodent models, mutagenicity has been most commonly examined in the liver. Bone marrow also has a relatively large number of experimental records, most of which are derived from Muta™Mouse studies. Results from liver and bone marrow have been particularly important in protocol development, as they are representative of tissues with slow and rapid turnover times, respectively. Several other somatic tissues, including intestine, lung, skin and spleen, have over 100 experimental records describing mutation experiments. Over 255 records describe experiments using male reproductive tissue and germ cells.

Relatively few TGR experiments have been carried out using those tissues that are of principal concern based on human cancer incidence. According to data compiled by IARC, breast, lung, stomach, prostate and colon/rectum cancers are the most prevalent cancers. However, those tissues are relatively poorly represented among experiments that have been carried out to date. For instance, breast (mammary tissue) has only 44 experimental records in the TRAID, whereas only 13 records exist in which mutagenicity has been examined in the prostate gland. Therefore, while it is clear that the flexibility of TGR assays (flexible mode of administration and ability to sample any tissue) is an enormous advantage of the assays, the extent to which this advantage has been exploited to examine mutation in tissues most relevant to human concerns is quite limited.

4.2.6 Administration and sampling times

It is recognised that the duration of treatment and the sampling time are extremely important in determining the sensitivity of TGR assays. These parameters are discussed in more detail in Sections 4.7 and 4.8, respectively. In the TRAID, the duration of treatment or "administration time" is defined as the amount of time (in days) from the first to the last treatment. For a single treatment, this value is 1. In the vast majority of the experimental records, the administration time also describes the *number* of treatments (*e.g.* for intraperitoneal injection or gavage treatments that are normally given once per day) or the amount of time that an animal is maintained on a dietary or drinking water regime through which the test compound is administered. In some cases, however, exposure of the animal occurs at different intervals – for example, on a weekly basis, or for 5 days of the week but not weekends; in these instances, the administration time would be somewhat longer than the number of administrations. The importance of administration time in determining the sensitivity of TGR assays has been clearly demonstrated: larger responses in the genetically neutral transgenes are generally obtained through multiple treatments as opposed to single treatments (Section 4.6). However, in the majority of experiments carried out to date, the administration time has been substantially shorter than the 28 days that is currently recommended (Thybaud *et al*., 2003) (Table 4-6). In almost half of the experimental records, a single administration has been used, and approximately 65% of the experiments have used an administration time of 5 days or less.

Sampling time is defined as the amount of time between the last treatment and sacrifice. In some cases, particularly when a compound is administered over a relatively long period in the diet or the drinking water, the animal is sacrificed immediately; in this case, the sampling time is given as 0.

Table 4-6. Summary of administration time versus sampling time

Administration time (days)	Total no. of records	Sampling time (days)						
		0	≤3	3.5–7	8–14	15–28	29–60	61–420
1	1 422	13	192	203	350	336	164	164
2–5	645	7	71	66	276	126	65	34
6–14	265	43	26	2	102	38	53	1
15–28	336	84	16	86	53	56	25	16
29–60	166	77	15	27	23	3	15	6
≥61	352	228	51	18	36	0	0	19
Total	3 186	452	371	402	840	559	322	141

However, in such a case, the amount of time available for mutation fixation is clearly greater from earlier time points during treatment than from later time points. Existing data suggest that the optimal sampling time will differ between tissues. For tissues other than those that turn over very rapidly, it is believed that longer sampling times will increase the sensitivity of the assay. In Table 4-6, it is clear that there are relatively few experimental records available to evaluate the effect of increased sampling times except when the administration time is extremely short (less than 5 days). For example, there are only 56 records where both the administration time and the sampling time are 15–28 days.

4.2.7 Conclusions

1) Two hundred and twenty-eight agents have been evaluated using TGR assays. However, 50% of the experimental records are derived from studies using only 21 different agents, all of which are carcinogenic. Of the 141 agents whose carcinogenicity has been evaluated, 118 are carcinogens, and only 22 are non-carcinogens. Additionally, 13 presumed non-carcinogenic chemicals were identified from those used as vehicle controls in TGR assays, which increased the proportion of non-carcinogens substantially. These chemicals are commonly used as vehicles in cancer bioassays.
2) The ability to use all routes of administration has been demonstrated. Experiments can be tailored to use the most relevant route of administration.
3) Among the TGR models evaluated, approximately 82% of the total experimental data have been obtained using the Muta™Mouse and Big Blue® models. Approximately 14% of the total data have been derived from the *lacZ* plasmid mouse and *gpt* delta rodent models.
4) The ability to examine mutation in virtually all tissues has been demonstrated. TGR assays most commonly examine mutagenicity in the liver and bone marrow. A significant number of experiments have examined mutagenicity in intestine, lung, skin and spleen, as well as male reproductive tissue and germ cells.
5) The majority of the experiments have used shorter administration times than is currently recommended; there are limited data available to assess the effects of longer sampling times except at extremely short administration times.

4.3 Gene and tissue characteristics that may influence mutation

All transgenic assays for mutation involve the analysis of a prokaryotic gene placed in the mammalian genome in a form that permits its recovery and analysis *in vitro*. The effect of this construction, its integration into the mammalian chromosome, its expression and repair *in vivo* and the method of analysis *in vitro* (detailed in Chapter 2) may all influence the observed mutant frequency. In addition, the mutation frequency, both spontaneous and induced, within a particular gene will be influenced by the size of the genetic target, the presence or absence of mutational hotspots and the extent to which specific mutations will produce a selectable phenotype. Additional factors, such as cell turnover, the cellular composition of tissues and the relative sensitivity of the different cell populations to mutagens, may influence the effectiveness of a given protocol.

This section discusses some of these factors. Much of this discussion is theoretical, since very few specific experimental data are available. However, uncertainty associated with our knowledge of the extent to which these factors may influence assay outcome should be considered when making comparisons between different studies.

4.3.1 Characteristics of the transgene

With regard to the size of the genetic target, the transgenes used in the TGR models are very different in size, with the *lacZ*, *lacI* and *gpt* genes being approximately 3 100 bp, 1 080 bp and 456 bp

in length, respectively. However, size is not the only factor that influences mutation frequencies within a given target; early studies of mutation showed that both spontaneous and induced mutations are unevenly distributed within a genetic target. Mutational hotspots are specific sequences at which a high proportion of mutations are observed. Examples of mutational hotspots include CpG sequences (base substitutions), homopolymeric runs of bases (frameshift mutations) and tandemly repeated sequences (deletions). Thus, the presence or absence of such hotspots will influence the frequency of mutation within a genetic target, regardless of the overall size of the target.

With regard to the *selectable phenotypes* of mutations within the genetic target, it is important to consider how a given mutation will affect specific residues within the expressed protein. For example, the first ~200 bp of the *lacI* gene are exquisitely sensitive to all types of mutations (*i.e.* mutations at most base pairs will produce a selectable phenotype). In contrast, the remaining ~900 bp of the *lacI* gene are much less sensitive to base substitution mutations (*i.e.* base substitutions that occur at many sites will not produce a selectable phenotype). If the same proportion of mutations in the entire *lacI* gene produced a selectable phenotype as is the case in the first ~200 bp, then mutation frequencies would be significantly higher with most mutagens than is actually the case.

Therefore, both the target size and the *selectable* target size, as well as the nature of the DNA sequence, influence observed mutation frequencies.

4.3.2 Tissue replacement

Mutations can, in principle, be fixed during either DNA replication or DNA repair. DNA replication is associated with cell turnover, which is highly variable between different types of tissues. Some tissues, such as neural and muscle tissues, have essentially exited the cell cycle and do not turn over. Other tissues, such as liver, proliferate very slowly, whereas tissues such as bone marrow or colon turn over very rapidly (a few days). In the case of tissues such as brain or muscle, it is important to consider processes such as DNA repair (Section 4.3.3 below) in determining whether it is likely that a detectable mutational response will be obtained. With tissues that are turning over, it is important to consider the relative rate of proliferation, because this will influence the sampling time that is necessary for manifestation of mutations (see Section 4.7 below) and will strongly influence the observed mutational response. In the case of germ cells, in which the kinetics of cell proliferation and differentiation are well defined, it is important to consider the time of development of the germ cell stage of interest (*e.g.* spermatogonia, spermatocyte, spermatids, spermatozoa) subsequent to the time of treatment, as well as any effects on the timing of germ cell development arising from the chemical treatment when selecting an appropriate sampling time (Douglas *et al*., 1995; Ashby, Gorelick and Shelby, 1997).

4.3.3 DNA repair

DNA repair is carried out through several different pathways that process different types of DNA damage; these include base excision repair, nucleotide excision repair, homologous recombination, non-homologous end joining and mismatch repair. The extent to which DNA repair in transgenes differs from that in endogenous genes is not well understood. Nucleotide excision repair is the major repair pathway for a range of structurally diverse chemicals that produce bulky DNA adducts. Nucleotide excision repair operates in two modes: transcription-coupled repair, which is fast and is directed to the transcribed strand of active genes, and global genomic repair, which appears to operate at low levels on all repairable lesions throughout the genome. In transgenic rats and mice, the available evidence suggests that the transgenes are heavily methylated in all tissues (You *et al*., 1998; Ikehata *et al*., 2000; Monroe, Manjanatha and Skopek, 2001) and are therefore unlikely to be transcriptionally active. DNA sequencing studies support this notion. For example, Chen *et al*. (1998) showed that the majority of thiotepa-induced base pair substitutions in the *Hprt* gene (an active endogenous gene subject to transcription-coupled repair) occurred with the mutated purine on the non-transcribed DNA strand; however, no strand-related bias was found for thiotepa-induced mutations in the *lacI* transgene, suggesting that the transgene was not subject to transcription-coupled

repair. In addition, direct tests for transgene mRNA have failed to produce any evidence for transcription in those tissues that have been examined (reviewed in Heddle, Martus and Douglas, 2003); therefore, it is unlikely that adducts in the transgene would be subjected to transcription-coupled repair, but rather would be processed within a much slower timeframe via global genomic repair.

In this context, it may be noted that many rodent tissues appear to differ from human cells with regard to the global genomic repair pathway – that is, some rodent tissues exhibit deficiency in p53-dependent global genomic repair (reviewed in Hanawalt, Ford and Lloyd, 2003) – a fact that may have implications in risk assessment using rodent models. However, at the present time, there is no experimental information available regarding the extent of nucleotide excision repair (either transcription-coupled repair or global genomic repair) in the transgenes of any TGR model.

DNA repair can contribute to mutation fixation at two levels: 1) through its influence on DNA lesion levels that will be present during subsequent cycles of DNA replication; and 2) through the fidelity of DNA synthesis associated with DNA repair. Unrepaired DNA lesions that persist through to DNA replication are clearly of most concern in tissues that are turning over. In tissues that are not turning over, mutations may be fixed during the DNA repair process itself; for some types of repair, such as repair of double-strand breaks, this may be significant. Alternatively, the extent to which lesions persist in the transgene should be considered. An important question is whether such lesions will persist until the end of the sampling period and potentially give rise to *ex vivo* mutations in the bacterium; available evidence suggests that this does not occur (discussed in Section 4.9.2).

4.3.4 Stem cells

One potential limitation of TGR assays is the presence of a minor but important cell population within a tissue that may respond differently to a given mutagen compared with the majority of the cells. Self-renewing tissues contain stem cells that, upon cell division, regenerate the stem cell population and produce daughter cells (transit cells) that will divide and eventually differentiate into non-dividing specialised cells. Mutations in these stem cells are believed to be important for the development of cancer. The question of whether the observed mutational response reflects mutagenesis in the stem cells has been posed (Heddle *et al*., 1996; Heddle, Martus and Douglas, 2003), but there is little experimental evidence available to resolve the questions addressed. It is possible that the mutational response of cells that make up the bulk of a tissue differs from that of the stem cells. If the transit cells or differentiated cells are less mutable than the stem cells, the effect of the mutagen on the stem cells may be underestimated. This would be of particular concern in the event of a negative result. Alternatively, if the differentiated cells are more mutable than the stem cells, the effect of the mutagen may be overestimated. In either event, the issue of differential sensitivity of cell populations pertains to any mutagenicity assay and is not specific to TGR assays.

4.3.5 Conclusions

A number of factors may influence the tissue specificity of a compound when a given experimental protocol is used. Cell turnover is clearly an important factor and will differ according to the tissue. The extent to which transgenes will be subjected to DNA repair and the differential sensitivity of cell populations within the tissue are factors that are expected to be important from a theoretical point of view; however, very few experimental data are available to inform the discussion. Spontaneous and induced mutant frequencies may be influenced by the nature of the genetic target – its size, the presence/absence of mutational hotspots and the extent to which mutations in the gene will yield a selectable phenotype.

4.4. Tissue specificity: Correlation between results in TGR assays and carcinogenicity

4.4.1 Cancer target tissues

Table 4-7 shows the results of TGR assays involving carcinogens and provides some indication of the correspondence of the assays to carcinogenicity in specific tissues. Detailed experimental records for experiments carried out using tissue-specific carcinogens are provided in Appendix C of this report. The aggregate data and their predictive capacity for cancer are discussed in more detail in Chapter 5.

4.4.1.1 Performance for liver carcinogens

Among the 56 liver carcinogens for which TGR liver mutagenicity data are available, 41 chemicals (73%) were found to be mutagenic in a TGR model. There was no evidence of mutagenicity to TGR liver for the following 14 rodent liver carcinogens: 1,2-dibromoethane, 1,3-butadiene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), acetaminophen, carbon tetrachloride, chloroform, clofibrate, coal tar, flumequine, heptachlor, hydrazine sulphate, methyl clofenapate, trichloroethylene (TCE) and tris-(2,3-dibromopropyl)phosphate.

TCDD, carbon tetrachloride, heptachlor, methyl clofenapate and TCE were not mutagenic to *Salmonella*, which suggests that these chemicals are unlikely to induce gene mutations. Thus, the negative results in the TGR system are not unexpected. TCDD, carbon tetrachloride and TCE are reviewed as case studies in Section 5.6.

Two chemicals were found to be carcinogenic to the liver of a species or sex different from that used in the transgenic mutation experiments. 1,2-Dibromoethane was found to be carcinogenic to the liver of female rats; however, there was no evidence of an increased tumour incidence in the liver of male mice that were used for the negative TGR assay. Tris-(2,3-dibromopropyl)phosphate was carcinogenic to the liver of female mice; however, no evidence of an increased liver tumour incidence was observed in male mice (National Cancer Institute, 1978), which were used for the negative TGR experiment. In these cases, it is not known whether the species and sex used for the TGR experiments might explain the observed discordance with the carcinogenicity bioassay data.

There are three chemicals where the route of administration or the duration of exposure in the TGR study may have influenced the final results. 1,3-Butadiene was administered by inhalation to male Muta™Mouse; although positive responses were observed for lung, bone marrow and spleen, no increase in mutant frequency was observed in the liver (Recio *et al*., 1992, 1993). However, the inhalation route of administration raises questions regarding whether a sufficiently high dose of the chemical was systemically absorbed to cause detectable mutations in the liver. Coal tar was applied to the skin of female Muta™Mouse as a single acute application. Although it was mutagenic to the skin at the site of exposure, coal tar was not mutagenic to the liver (Thein *et al*., 2000). However, the nature and duration of exposure would suggest that systemic absorption of the chemical at a level sufficient to induce detectable mutations in the liver was unlikely to have occurred. Hydrazine sulphate was also administered as a single dose, but by gavage (Douglas, Gingerich and Soper, 1995). Like coal tar, hydrazine sulphate may not have been administered for a long enough period to detect an increased mutant frequency in the liver.

Table 4-7. Summary of TGR mutation assay results sorted by tissue[a]

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
1,2:3,4-Diepoxybutane			–							
1,2-Dibromo-3-chloropropane	–									
1,2-Dibromoethane	–	–								
1,2-Dichloroethane	–									
1,3-Butadiene	–	+	+							
1,6-Dinitropyrene		+								
1,8-Dinitropyrene	–	–	+							
17β-Oestradiol									–	
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	–									
2,4-Diaminotoluene	+			–						
2-Acetylaminofluorene (2-AAF)	+					+				
2-Amino-1-methyl-6-phenyl-imidazo(4,5-b)pyridine (PhIP)	+		–	–				+	+	+
2-Amino-3,4-dimethylimidazo(4,5-f)quinoline (MeIQ)	+		+		+			+		+
2-Amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx)	+	–		+				+		
2-Amino-3-methylimidazo(4,5-f)quinoline (IQ)	+			+				+		+
2-Nitro-*p*-phenylenediamine	+									

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
3-Amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2)								−		−
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	−	−								
3-Methylcholanthrene	+									
3-Nitrobenzanthrone	+	−		−		+		+		

Table 4-7 (continued)

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	+	+		+			+			
4-Aminobiphenyl	+		+	+		+				
4-Chloro-*o*-phenylenediamine	+									
4-Nitroquinoline-1-oxide (4-NQO)	+	+	+	−	+		+	−		
5,9-Dimethyldibenzo(c,g)carbazole (DMDBC)	+									
5-Bromo-2′-deoxyuridine (BrdU)										−
5-Fluoroquinoline	+		−							
6-(*p*-Dimethylaminophenylazo)benzo-thiazole	+									
6-Nitrochrysene									+	

Table 4-7 (continued)

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
7,12-Dimethylbenzanthracene (7,12-DMBA)	+	+	+	+		+	+	+	+	
7H-Dibenzo(c,g)carbazole (DBC)	+									
A-alpha-C	+							+		−
Acetaminophen	−									
Acrylamide	+		+							
Acrylonitrile		−	−							
Aflatoxin B1	+			+				−		
Aminophenylnorharman	+							+		
Amosite asbestos		+								
Aristolochic acid	+	+	+	+	+	+		+		
Arsenite trioxide		−	−	−		−				
Azathioprine	+		+							
Benzene	−	+	+							
Benzo(a)pyrene	+	+	+	+	+		+	+	+	+
beta-Propiolactone	+		−		+					
Bleomycin				+						
Carbon tetrachloride	−									
CC-1065	+									
Chlorambucil	+	+	+							
Chloroform	−									
Chrysene	+	+	+	+				+		

Table 4-7 (continued)

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
Cisplatin	+									
Clofibrate	–									
Coal tar	–									
Comfrey	+									
Crocidolite asbestos		+								
Cyclophosphamide	+	+	+	–		+				
Cyproterone acetate	+									
Di(2-ethylhexyl)phthalate (DEHP)	+			–						
Dichloroacetic acid (DCA)	+									
Dicyclanil	+									
Diesel exhaust	–	+						–		
Diethylnitrosamine (DEN)	+	+	–							
Dimethylarsinic acid		–	–	–		–				
Dimethylnitrosamine (DMN)	+	+	–	+	–	–				
Dipropylnitrosamine (DPN)	+	+	+	+		–				
d-Limonene	–			–						
Ethylene oxide		+	–							
Ethylmethanesulphonate (EMS)	+		+							–
Ferric nitrilotriacetate				+						
Flumequine	–									
Gamma rays	+	–	+							

Table 4-7 (continued)

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
Heptachlor	–									
Hexachlorobutadiene	–		–	+						
Hexavalent chromium	+	+	+	+						
High-fat diet			–					–		–
Hydrazine sulphate	–	–	–							
Methyl bromide	–				–					
Methyl clofenapate	–									
Methylmethanesulphonate (MMS)	+		+							+
Metronidazole					–					
Mitomycin-C	+		+							–
N-Ethyl-*N*-nitrosourea (ENU)	+	+	+	+		+		+	+	+
N-Hydroxy-2-acetylaminofluorene	+		+							
Nickel subsulphide		–								
Nitrofurantoin		–		+	–	–		–		–
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	–		–		+					
N-Methyl-*N*-nitrosourea (MNU)	+	+	+	–	–		+	+	+	+
N-Nitrosomethylbenzylamine (NDBzA)							+			
N-Nitrosonornicotine (NNN)	+	+		+			+			
N-Nitrosopyrrolidine	+									
N-Propyl-*N*-nitrosourea (PNU)	+	+	+	+						

Table 4-7 (continued)

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
o-Aminoazotoluene	+	−	−	+		+		+		
o-Anisidine	−					+				
Oxazepam	+	−								
p-Cresidine						+				
Phenobarbital	+	−								
Potassium bromate				+						
Procarbazine hydrochloride	−	+	+	+						
Quinoline	+	−	−	−						
Riddelliine	+									
Sodium saccharin	−					−				
Streptozotocin	+			+						
Tamoxifen	+									
Thiotepa			+							
Trichloroethylene (TCE)	−	−	−	−						
Tris-(2,3-dibromopropyl)phosphate	−			+	−					
Uracil						+				
Urethane	+	+	+		+					+
Vinyl carbamate		+								+
Wyeth 14,643	+			−						
X-rays	+	+								+

Table 4-7 (continued)

Chemical	TGR mutation assay results									
	Liver	Lung	Haemato-poietic system	Kidney	Stomach	Bladder	Oral tissue	Colon	Breast and mammary gland	Intestine
Tissue sensitivity[b]	*73 (41/56)*	*69 (25/36)*	*84 (16/19)*	*86 (12/14)*	*63 (5/8)*	*75 (6/8)*	*100 (5/5)*	*67 (4/6)*	*83 (5/6)*	*50 (2/4)*

[a] Shaded boxes indicate positive carcinogenicity reported by rodent bioassay.

[b] Values are percentages (number of chemicals that are positive in a TGR assay in those tissues that are target tissues for carcinogencity / number of chemicals studied using the TGR assay that also induce tumours in the specified tissue [shaded]).

Chloroform was administered to female Big Blue® mice by inhalation for periods of up to 180 days (Butterworth *et al*., 1998) at concentrations similar to those used in an inhalation carcinogenicity study where clear evidence of liver tumours was found in both male and female mice (Yamamoto *et al*., 2002). There is no easily reconcilable explanation as to why no increases in liver mutant frequency were observed in the TGR assay. Chloroform is reviewed as a case study in Section 5.6.

Overall, given the observations described above, the TGR assays appeared to have a reasonably high degree of correspondence with carcinogenicity for liver carcinogens.

4.4.1.2 Performance for lung carcinogens

There are 36 lung carcinogens for which mutagenicity to lung tissue has been examined using TGR assays. Twenty-five of these compounds were positive in TGR assays. Eleven were not mutagenic to the lung tissues of transgenic rats and mice: 1,2-dibromoethane, MX, 3-nitrobenzanthrone, arsenite trioxide, dimethylarsinic acid, gamma rays, hydrazine sulphate, 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx), nickel subsulphide, *o*-aminoazotoluene and TCE. Dimethylarsinic acid, nickel subsulphide and TCE were not mutagenic to *Salmonella*, consistent with the negative response observed in TGR assays. The cases of nickel subsulphide and TCE are described in more detail in Section 5.6.

There is one chemical where administration by a route other than that associated with carcinogenicity resulted in negative results in TGR assays. Arsenite trioxide was not mutagenic to the lung tissues of male Muta™Mouse when administered in five daily intraperitoneal injections. However, it is not known whether a chemical reported to induce lung tumours after inhalation exposure would distribute to the lungs in a sufficient amount to cause detectable mutations after intraperitoneal injection.

In two cases, chemicals were administered to transgenic rats and mice in a single dose. Hydrazine sulphate was carcinogenic to the lungs of both male and female rats and mice after oral administration, but was not mutagenic to lung tissues when administered once by gavage to male Muta™Mouse. *o*-Aminoazotoluene was not mutagenic to the lung tissues of male Muta™Mouse when administered once by intraperitoneal injection, but was carcinogenic to the lungs of male and female mice following administration in the diet. These are examples of studies where the administration time was far shorter than that recommended (Thybaud *et al*., 2003). Because the transgenes are genetically neutral, longer treatment periods can serve to increase the assay sensitivity for detecting mutations (see Section 4.7). In these two cases, it is unlikely that the TGR assay would have the sensitivity to detect any mutations arising after only a single administration.

1,2-Dibromoethane produced lung tumours in male and female mice following both oral and inhalation exposure. However, it was not mutagenic to the lung tissues of male Muta™Mouse when administered by inhalation for a treatment period of 10 days, although it was mutagenic to the nasal mucosa under the same conditions. The fact that 1,2-dibromoethane was mutagenic at the site of contact but not to the lung may suggest that an insufficient amount of the chemical reached the lungs to cause any detectable increase in mutant frequency.

4.4.1.3 Performance for haematopoietic system carcinogens

Nineteen haematopoietic carcinogens have been studied using TGR assays. Of these, only three – diethylnitrosamine (DEN), ethylene oxide and 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) – did not induce detectable mutations to the haematopoietic tissues of transgenic rats and mice, which suggests that TGR assays have reasonably good correspondence for haematopoietic system carcinogens.

For haematopoietic system carcinogens, there are no clearly evident generalisations that can be made to explain the three discrepancies with the TGR assays. DEN caused leukaemia in female rats after administration in the drinking water (Lijinsky, Kovatch and Thomas, 1992), but no increase in mutant frequency in the bone marrow of male or female Muta™Mouse following a single intraperitoneal injection. There was only a single report in the literature of carcinogenicity to the

haematopoietic system, although DEN is a known liver carcinogen and was found to be mutagenic to the liver in the same TGR studies where no bone marrow mutations were observed. Inhalation of ethylene oxide has been associated with the development of leukaemia in male rats and female mice (Lynch *et al*., 1984) but did not cause an increase in mutant frequency in the bone marrow or spleen of male Big Blue® mice. PhIP administered to male and female mice in the diet produced high incidences of lymphomas (Esumi *et al*., 1989). However, dietary administration to male *gpt* delta mice for 91 days did not cause a statistically significant increase in mutations in the bone marrow, but was mutagenic to the colon, liver and spleen (Masumura *et al*., 1999a).

4.4.1.4 Performance for kidney carcinogens

Fourteen kidney carcinogens had corresponding TGR assay data. Of these, 12 were found to be mutagenic to the kidneys of TGRs. *N*-Methyl-*N*-nitrosourea (MNU) and d-limonene were the sole exceptions.

MNU caused kidney tumours in female rats following administration by intravenous injection (Anisimov, 1981), but administration in the diet of Big Blue® mice for 105 days did not cause a detectable increase in mutant frequency (Shephard, Gunz and Schlatter, 1995). Because administration by intravenous injection produces substantially higher bioavailability than dietary administration, the potential effects of the different routes of administration cannot be overlooked, since the observed discrepancy may be a result of toxicokinetics.

d-Limonene produced increases in the incidence of kidney tumours only in male rats administered the chemical by gavage (National Toxicology Program, 1990b), but was not mutagenic in the kidney of male Big Blue® rats after dietary administration for 10 days. However, d-limonene is associated with α2u-globulin-related renal carcinogenesis and was not mutagenic to *Salmonella*; it is regarded as a non-genotoxic carcinogen (Whysner and Williams, 1996b; Elcombe *et al*., 2002), and so the negative result in kidney in the TGR assay is not surprising.

4.4.1.5 Performance for stomach carcinogens

TGR assay mutagenicity data were available for eight stomach carcinogens. Of these eight carcinogens, three were not mutagenic to the stomach of transgenic rats and mice: methyl bromide, MNU and tris-(2,3-dibromopropyl)phosphate.

Methyl bromide lacked mutagenicity to the glandular stomach of male Muta™Mouse when administered by gavage daily for 10 days (Pletsa *et al*., 1999). In a cancer bioassay, gavage administration to male and female rats was associated with an increase in the incidence of tumours of the forestomach (Danse, van Velsen and van der Heijden, 1984). However, because the tissues do not directly correspond, it is not possible to compare performance in this case. Methyl bromide is reviewed as a case study in Section 5.6.

MNU induced tumours of the glandular stomach in male mice administered the chemical in the drinking water (Tatematsu *et al*., 1993) and in the forestomach of male and female rats dosed by gavage (Lijinsky and Kovatch, 1989). However, administration in the diet of Big Blue® mice for 105 days did not cause a detectable increase in mutant frequency in either the glandular stomach or forestomach (Shephard, Gunz and Schlatter, 1995). Tris-(2,3-dibromopropyl)phosphate was not mutagenic to the stomach of male Big Blue® mice following administration by gavage for up to 4 days (de Boer *et al*., 1996; Provost *et al*., 1996), but was carcinogenic to the stomach of male and female mice dosed via the feed in a carcinogenicity bioassay (National Cancer Institute, 1978). There is no easily reconcilable explanation as to why no increases in mutant frequency were observed in TGR stomach tissue for these two chemicals.

4.4.1.6 Performance for bladder carcinogens

TGR mutagenicity data were available for only eight bladder carcinogens. Of these, two carcinogens, dimethylarsinic acid and sodium saccharin, were not mutagenic to the bladder in the TGR assay (Turner *et al*., 2001). However, the bladder carcinogenicity of sodium saccharin in the male rat has been attributed to precipitation of calcium phosphate in the bladder in a high-pH environment, which causes cytotoxicity by a mechanical mechanism (Ellwein and Cohen, 1990). As the supposed mechanism is non-genotoxic, it is not unexpected that the TGR assay was negative.

4.4.1.7 Performance for oral tissue carcinogens

There were only five oral tissue carcinogens whose corresponding mutagenicity was examined in transgenic rats and mice. All of these oral carcinogens were mutagenic in the TGR assay. However, because of the limited data, no conclusions regarding tissue-specific performance can be made in this case.

4.4.1.8 Performance for colon carcinogens

TGR assay colon mutagenicity data for six known colon carcinogens were available. Two carcinogens did not induce mutations in the colon of transgenic animals: aflatoxin B1 and high-fat diet. Aflatoxin B1 produced an increased incidence of colon tumours when administered to male and female rats in the diet over their entire lifetime (Ward *et al*., 1975), but was not mutagenic to the large intestine of male Big Blue® mice following gavage administration for 4 days (Autrup, Jorgensen and Jensen, 1996). The absence of mutations in the TGR assay may be attributable to species differences in sensitivity to aflatoxin B1 or to a limited time of administration.

4.4.1.9 Performance for breast and mammary gland carcinogens

The mutagenicity of six breast and mammary gland carcinogens was studied using transgenic animals. One of these chemicals, 17β-oestradiol, was not mutagenic in the breast or mammary gland.

4.4.1.10 Performance for intestine carcinogens

Four chemicals carcinogenic to the intestine were also studied in TGR experiments. Mitomycin-C and 3-amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2) did not induce mutations in the intestine of transgenic animals. Mitomycin-C was reported to be carcinogenic to the intestine of rats dosed by intravenous injection, although it was not mutagenic to male and female Muta™Mouse following a single gavage administration (Cosentino and Heddle, 1999a). Since in this case the chemical was administered as a single dose, the sensitivity of the TGR assay may not have been sufficient to detect any increase in mutation frequency, as has been suggested previously for other chemicals administered acutely.

4.4.2 Conclusions

Limited data are available with which to evaluate the results of TGR assays in known target tissues for carcinogenicity. Depending on the tissue, between 50% and 100% of the chemicals tested would have correctly predicted a positive result. A case-by-case analysis of instances in which discrepancies are apparent suggests that in the majority of cases, factors such as non-genotoxic mechanism of action, inappropriate mode of administration or inadequate study design may account for the observed negative result in the tissue of interest.

4.5 Agreement between results using different transgenes

4.5.1 Agreement between results in Big Blue® lacI and cII genes

Table 4-8 shows the results of studies carried out in which similar experimental conditions (mode of administration, tissue sampled, total dose, administration time, sampling time) were used to assess the mutagenicity at both the *lacI* and *cII* transgenes in Big Blue® mice. In 10 of 12 pairwise comparisons, the results were in agreement. In the case of MNU (total dose 20 mg/kg body weight (bw)), the *cII* assay yielded a negative result in splenic lymphocytes but the *lacI* assay yielded a positive result.

4.5.2 Agreement between results in Muta™Mouse lacZ and cII genes

Table 4-9 shows the results of studies carried out in which similar experimental conditions (mode of administration, tissue sampled, total dose, administration time, sampling time) were used to assess the mutagenicity at both the *lacZ* and *cII* transgenes in Muta™Mouse. In 86 of 105 pairwise comparisons, the results were in agreement. In 19 comparisons, the results were discordant between the two transgenes: in 15 of these cases, the *cII* assay gave a negative result, whereas the *lacZ* assay yielded a positive result. In three of the four discordant comparisons where the *cII* genes were positive, a dinitropyrene mixture was administered to Muta™Mouse at a total dose of 800 and 1600 mg/kg bw: in the bone marrow, the treatment increased mutation significantly in the *cII* gene but not the *lacZ* gene at both doses; in the stomach, a dose-related increase was observed at *lacZ*, but a positive result was obtained in the *cII* gene at the lower dose but not the higher dose (von Pressentin, Kosinska and Guttenplan, 1999).

4.5.3 Agreement between results in Big Blue® lacI and Muta™Mouse lacZ genes

There are relatively few direct comparisons available for the Big Blue® *lacI* and Muta™Mouse *lacZ* genes that use similar experimental parameters (mode of administration, tissue sampled, total dose, administration time, sampling time). There are seven specific cases in which identical experimental parameters were used and a similar conclusion was reached using both Big Blue® *lacI* and Muta™Mouse *lacZ* transgenes as markers for induced mutagenicity (Table 4-10). In addition, it should be pointed out that ENU has been used as a positive control in many experiments; positive results are obtained in all tissues using a variety of experimental parameters following the administration of high doses of ENU to either Big Blue® mouse or Muta™Mouse. There are no cases in which different (positive *vs.* negative) results are obtained using Big Blue® mouse or Muta™Mouse when identical experimental parameters are used.

4.5.4 Conclusions

Qualitatively similar results have been obtained in the majority of experiments that have assayed different transgenes using similar experimental parameters. When differences in the response of transgenes have been observed, the Big Blue® mouse *lacI* and Muta™Mouse *lacZ* genes appear to be more sensitive than the *cII* gene. No differences in response have been observed between the Big Blue® mouse *lacI* and Muta™Mouse *lacZ* genes.

4.6 Spontaneous mutant frequency

4.6.1 Initial measurements of mutant frequencies

During the development of the Muta™Mouse TGR model, spontaneous mutant frequencies were determined in three different transgenic strains – strains 20.2, 40.6 and 35.5 – derived from chromosomal integration of λgt10*lacZ*.

Table 4-8. Comparison of TGR results in Big Blue® mice as obtained from sampling the mutations in the *lacI* or *cII* transgenes

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
4-Aminobiphenyl	Intraperitoneal	Liver	9	+	Big Blue®	7	56	Chen *et al*. (2005b)
				+	Big Blue® *cII*			
			31	+	Big Blue®			
				−	Big Blue® *cII*			
Bitumen fumes	Inhalation	Lung	180	−	Big Blue®	5	28	Micillino *et al*. (2002)
				−	Big Blue® *cII*			
Dimethylnitrosamine (DMN)	Intraperitoneal	Liver	20	+	Big Blue®	5	21	Shane *et al*. (2000b)
				+	Big Blue® *cII*			
N-Ethyl-*N*-nitrosourea (ENU)	Intraperitoneal	Liver	100	+	Big Blue®	1	30	Zimmer *et al*. (1999)
				+	Big Blue® *cII*			
	Intraperitoneal	Lung		+	Big Blue®			
				+	Big Blue® *cII*			
	Intraperitoneal	Spleen		+	Big Blue®			
				+	Big Blue® *cII*			
N-Methyl-*N*-nitrosourea (MNU)	Intraperitoneal	Splenic lymphocytes	15	−	Big Blue®	1	21	Monroe *et al*. (1998)
			20	−	Big Blue® *cII*			
				+	Big Blue®			
Oxazepam	Diet	Liver	54 000	+	Big Blue®	180	0	Shane *et al*. (1999)
				+	Big Blue® *cII*			Singh *et al*. (2001)
p-Cresidine	Diet	Bladder	54 000	+	Big Blue®	180	0	Shane *et al*. (1999)
				+	Big Blue® *cII*			Singh *et al*. (2001)
			108 000	+	Big Blue®			Jakubczak *et al*. (1996)
				+	Big Blue® *cII*			

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
Phenobarbital	Diet	Liver	54 000	–	Big Blue®	180	0	Shane *et al*. (2000c)
				–	Big Blue® *cII*			Singh *et al*. (2001)

Table 4-9. Comparison of TGR results in Muta™Mouse as obtained from sampling the mutations in the *lacZ* or *cII* transgenes

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
1,10-Diazachrysene	Intraperitoneal	Bone marrow	400	+	Muta™Mouse	28	7	Yamada *et al*. (2005)
				+	Muta™Mouse *cII*			
		Colon		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Kidney		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Liver		+	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Lung		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Spleen		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
1,7-Phenanthroline	Intraperitoneal	Bone marrow	200	−	Muta™Mouse	4	14	Yamada *et al*. (2004)
				−	Muta™Mouse *cII*			
		Kidney		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Liver		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Lung		+	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Spleen		−	Muta™Mouse			
				−	Muta™Mouse *cII*			

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
		Bone marrow		–	Muta™Mouse		56	
				–	Muta™Mouse *cII*			
		Kidney		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Liver		+	Muta™Mouse			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
				+	Muta™Mouse *cII*			
		Lung		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Spleen		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
10-Azabenzo(a)py-rene	Gavage	Bone marrow	625	–	Muta™Mouse	5	14	Yamada *et al*. (2002)
				–	Muta™Mouse *cII*			
		Forestomach		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Kidney		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Liver		+	Muta™Mouse			
				+	Muta™Mouse *cII*			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
		Lung		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Spleen		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Stomach		–	Muta™Mouse			
				–	Muta™Mouse *cII*			
		Colon		+	Muta™Mouse			
				–	Muta™Mouse *cII*			
2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)	Gavage	Small intestine	100	+	Muta™Mouse	5	14	Itoh *et al*. (2003)
				+	Muta™Mouse *cII*			
4-(Methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Intraperitoneal	Liver	500	+	Muta™Mouse	28	0	Hashimoto, Ohsawa and Kimura (2004)
				+	Muta™Mouse *cII*			
			1 000	+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Lung	500	+	Muta™Mouse			
				+	Muta™Mouse *cII*			
			1 000	+	Muta™Mouse			
				+	Muta™Mouse *cII*			
4,10-Diazachrysene	Intraperitoneal	Bone marrow	800	+	Muta™Mouse	28	7	Yamada *et al*. (2005)
				+	Muta™Mouse *cII*			
		Colon		+	Muta™Mouse			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
				+	Muta™Mouse *cII*			
		Kidney		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Liver		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Lung		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Spleen		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
7,12-Dimethylbenz-anthracene (7,12-DMBA)	Intraperitoneal	Liver	20	+	Muta™Mouse	1	28	Hachiya *et al*. (1999)
				+	Muta™Mouse *cII*			Kohara *et al*. (2001)
				+	Muta™Mouse	28	0	Hashimoto, Ohsawa and Kimura (2004)
				+	Muta™Mouse *cII*			
		Lung		+	Muta™Mouse	28	0	Hashimoto, Ohsawa and Kimura (2004)
				+	Muta™Mouse *cII*			
Aristolochic acid	Gavage	Bladder	60	+	Muta™Mouse	22	7	Kohara *et al.* (2002a)
				+	Muta™Mouse *cII*			
		Bone marrow		−	Muta™Mouse *cII*			
				+	Muta™Mouse			
		Colon		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Forestomach		+	Muta™Mouse			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
				+	Muta™Mouse *cII*			
		Glandular stomach		−	Muta™Mouse *cII*			
				+	Muta™Mouse			
		Kidney		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Liver		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Lung		−	Muta™Mouse *cII*			
				+	Muta™Mouse			
		Spleen		−	Muta™Mouse *cII*			
				+	Muta™Mouse			
		Testes		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
Arsenite trioxide	Intraperitoneal	Lung	38	−	Muta™Mouse	5	14	Noda *et al*. (2002)
				−	Muta™Mouse *cII*			
Benzo(a)pyrene	Gavage	Bone marrow	625	+	Muta™Mouse	5	14	Hakura *et al*. (1998); Yamada *et al*. (2002)
				+	Muta™Mouse *cII*			Yamada *et al*. (2002)
		Colon		+	Muta™Mouse			Hakura *et al*. (1998); Kosinska, von Pressentin and Guttenplan (1999); Yamada *et al*. (2002)
				+	Muta™Mouse *cII*			Yamada *et al*. (2002)

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
		Forestomach		+	Muta™Mouse			Hakura *et al*. (1998); Yamada *et al*. (2002)
				+	Muta™Mouse *cII*			Yamada *et al*. (2002)
		Kidney		−	Muta™Mouse *cII*			Yamada *et al*. (2002)
				+	Muta™Mouse			Hakura *et al*. (1998); Kosinska, von Pressentin and Guttenplan (1999); Yamada *et al*. (2002)
		Liver		+	Muta™Mouse			Hakura *et al*. (1998); Kosinska, von Pressentin and Guttenplan (1999); Yamada *et al*. (2002)
				+	Muta™Mouse *cII*			Yamada *et al*. (2002)
		Lung		−	Muta™Mouse *cII*			Yamada *et al*. (2002)
				+	Muta™Mouse			Hakura *et al*. (1998); Kosinska, von Pressentin and Guttenplan (1999); Yamada *et al*. (2002)
		Spleen		+	Muta™Mouse			Hakura *et al*. (1998); Yamada *et al*. (2002)
				+	Muta™Mouse *cII*			Yamada *et al*. (2002)
		Stomach		+	Muta™Mouse			Yamada *et al*. (2002)
				+	Muta™Mouse *cII*			
Benzo(f)quinoline	Intraperitoneal	Bone marrow	400	−	Muta™Mouse	4	14	Yamada *et al*. (2004)
				−	Muta™Mouse *cII*			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
		Kidney		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Liver		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Lung		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Spleen		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Bone marrow		−	Muta™Mouse	4	56	
				−	Muta™Mouse *cII*			
		Kidney		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Liver		−	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Lung		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Spleen		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
Benzo(h)quinoline	Intraperitoneal	Bone marrow	400	−	Muta™Mouse	4	14	Yamada *et al*. (2004)
				−	Muta™Mouse *cII*			
		Kidney		−	Muta™Mouse			
				−	Muta™Mouse *cII*			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
		Liver		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Lung		+	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Spleen		−	Muta™Mouse			
				−	Muta™Mouse *cII*			
Chrysene	Intraperitoneal	Bone marrow	800	+	Muta™Mouse	28	7	Yamada *et al*. (2005)
				−	Muta™Mouse *cII*			
		Colon		+	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Kidney		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Liver		+	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Lung		+	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Spleen		+	Muta™Mouse			
				−	Muta™Mouse *cII*			
Dimethylarsinic acid	Intraperitoneal	Lung	53	−	Muta™Mouse	5	14	Noda *et al*. (2002)
				−	Muta™Mouse *cII*			
Dinitropyrenes	Gavage	Liver	1 600	−	Muta™Mouse *cII*	28	7	Kohara *et al*. (2002b)
				+	Muta™Mouse			

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
		Lung		−	Muta™Mouse *cII*			
				+	Muta™Mouse			
		Bone marrow	800	−	Muta™Mouse			
				+	Muta™Mouse *cII*			
			1 600	−	Muta™Mouse			
				+	Muta™Mouse *cII*			
		Stomach	800	−	Muta™Mouse			
				+	Muta™Mouse *cII*			
			1 600	+	Muta™Mouse			
				−	Muta™Mouse *cII*			
		Colon	800	+	Muta™Mouse			
				+	Muta™Mouse *cII*			
			1 600	+	Muta™Mouse			
				+	Muta™Mouse *cII*			
N-Ethyl-*N*-nitrosourea (ENU)	Intraperitoneal	Small intestine	50	+	Muta™Mouse	1	10	Swiger *et al*. (1999)
				+	Muta™Mouse *cII*			
			150	+	Muta™Mouse			
				+	Muta™Mouse *cII*			
			250	+	Muta™Mouse			Cosentino and Heddle (1996, 1999b); Swiger *et al*. (1999)
				+	Muta™Mouse *cII*			Swiger *et al*. (1999)

Table 4-9 (continued)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)	Result	TGR system	Application time (days)	Sampling time (days)	Reference
				+	Muta™Mouse		70	Cosentino and Heddle (1996); Swiger *et al*. (1999)
				+	Muta™Mouse *cII*			Swiger *et al*. (1999)
N-Methyl-*N*-nitrosourea (MNU)	Intraperitoneal	Small intestine	50	+	Muta™Mouse	1	35	Shima, Swiger and Heddle (2000)
				+	Muta™Mouse *cII*			
o-Aminoazotoluene	Intraperitoneal	Colon	300	+	Muta™Mouse	1	28	Ohsawa *et al*. (2000)
				+	Muta™Mouse *cII*			Kohara *et al*. (2001)
		Liver		+	Muta™Mouse			Ohsawa *et al*. (2000)
				+	Muta™Mouse *cII*			Kohara *et al*. (2001)
Quinoline	Intraperitoneal	Liver	200	+	Muta™Mouse	4	14	Miyata *et al*. (1998); Suzuki *et al*. (1998)
				+	Muta™Mouse *cII*			Suzuki *et al*. (2000)

Table 4-10. Comparison of TGR results in Muta™Mouse (*lacZ*) and Big Blue® mouse (*lacI*)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)[a]	Result	TGR system	Application time (days)	Sampling time (days)	References
1,3-Butadiene	Inhalation	Bone marrow	2 400	−	Muta™Mouse	5	14	Recio *et al*. (1992, 1993)
				+	Big Blue®			Recio *et al*. (1996)
Acetone	Topical	Skin	100 µL	−	Big Blue®	1	7	Gorelick *et al*. (1995)
			200 µL	−	Muta™Mouse			Ashby *et al*. (1993)
Dimethyl-nitrosamine (DMN)	Gavage	Liver	10	+	Big Blue®	1	7	Tinwell, Lefevre and Ashby (1994a)
				+	Muta™Mouse			Tinwell, Lefevre and Ashby (1994b)
				+	Big Blue®		10	Tinwell, Lefevre and Ashby (1994a)
				+	Muta™Mouse			Tinwell, Lefevre and Ashby (1994b)
				+	Big Blue®		20	Tinwell, Lefevre and Ashby (1994a)
				+	Muta™Mouse			Tinwell, Lefevre and Ashby (1994b)
	Intraperitoneal	Liver	5	+	Big Blue®	1	14	Suzuki *et al*. (1996a)
				+	Muta™Mouse			Souliotis *et al*. (1998)
			10	+	Big Blue®			Tinwell *et al*. (1995)
				+	Muta™Mouse			Souliotis *et al*. (1998)
Methyl clofenapate	Gavage	Liver	225	−	Big Blue®	9	10	Lefevre *et al*. (1994)
				−	Muta™Mouse			Lefevre *et al*. (1994)
Methyl clofenapate + DMN	Gavage	Liver	225 + 10	+	Big Blue®	9	10	Lefevre *et al*. (1994)
				+	Muta™Mouse			Lefevre *et al*. (1994)
N-Ethyl-*N*-nitrosourea (ENU)	Intraperitoneal	Bone marrow	250	+	Big Blue®	1	14	Recio *et al*. (1993, 1996)
				+	Muta™Mouse			Recio *et al*. (1992, 1993)
		Epididymal sperm	150	+	Big Blue®		14	Katoh, Horiya and Valdivia (1997)
				+	Muta™Mouse			Piegorsch *et al*. (1997)
		Liver	250	+	Big Blue®		3	Monroe and Mitchell (1993)
				+	Muta™Mouse			Myhr (1991); Hoorn *et al*. (1993)

Chemical	Route of administration	Tissue	Total dose (mg/kg bw)[a]	Result	TGR system	Application time (days)	Sampling time (days)	References
				+	Big Blue®		10	Piegorsch *et al*. (1995)
				+	Muta™Mouse			Myhr (1991); Hoorn *et al*. (1993)
		Testes	150	+	Big Blue®		3	Katoh, Horiya and Valdivia (1997)
				+	Muta™Mouse			Katoh *et al*. (1994); Suzuki *et al*. (1997b)
				+	Big Blue®		14	Katoh, Horiya and Valdivia (1997)
				+	Muta™Mouse			Suzuki *et al*. (1997b)

[a] Unless otherwise noted.

The spontaneous mutant frequencies differed by a factor of 25–100 in these strains (Gossen *et al*., 1991). The relatively low spontaneous mutant frequency observed in strain 40.6 (initial studies showed a spontaneous mutant frequency of 0.7×10^{-5} in the liver, which was lower than that which has generally been obtained in subsequent studies) made this strain suitable for further characterisation and development of the Muta™Mouse system. Nohmi *et al.* (1996) isolated five transgenic lines – #18, #21, #22, #24 and #30 – following integration of λEG10 into C57BL/6J and BDF1(C57BL/6 x DBA/2) mice; each of the five lineages showed a bone marrow *gpt* spontaneous mutant frequency in a very tight range between 1.7 and 3.3×10^{-5}. During development of the Big Blue® mouse, several different C57BL/6 lineages were derived from integration of the λLIZα shuttle vector. The A1 lineage, which contained a high copy number λLIZα concatamer and exhibited reproducible spontaneous liver mutant frequencies of $\sim 4 \times 10^{-5}$ in both a C57BL/6 and B6C3F1 background mouse (Dycaico *et al*., 1994), was used to develop the Big Blue® system.

4.6.2 Tissue differences in somatic cell mutant frequency

The data in the TRAID were used to calculate observed spontaneous mutant frequencies for several tissues in the major TGR models (Table 4-11). This aggregate approach takes into account data from all laboratories and allows some comparison of different tissues and animal models. The values are normalised for the number of animals used, but not for age, a factor that is significant and will be discussed subsequently (Section 4.6.3). Among the tissues for which reasonable numbers of data are available, the highest spontaneous mutant frequency occurs in the small intestine in virtually all of the TGR models, and the lowest values generally occur in the bone marrow and the kidney. The majority of the spontaneous mutant frequency values for other somatic tissues are intermediate. For the major TGR systems, Muta™Mouse and Big Blue® rats and mice, these values are in the vicinity of 5×10^{-5}. The spontaneous mutant frequency values in Table 4-11, calculated from aggregate data from many laboratories, agree strongly with those obtained from comparative studies. De Boer *et al.* (1998) compared the spontaneous mutant frequencies of several tissues in Big Blue® mice. The spontaneous mutant frequencies in bone marrow ($2.5 \pm 0.9 \times 10^{-5}$), kidney ($3.5 \pm 1.5 \times 10^{-5}$), liver ($4.1 \pm 0.7 \times 10^{-5}$), lung ($4.3 \pm 0.9 \times 10^{-5}$), stomach ($3.9 \pm 1.7 \times 10^{-5}$) and spleen ($3.7 \pm 1.0 \times 10^{-5}$) reported by deBoer *et al.* (1998) are entirely consistent with the spontaneous mutant frequencies of bone marrow ($3.68 \pm 0.59 \times 10^{-5}$), kidney ($3.76 \pm 0.87 \times 10^{-5}$), liver ($5.10 \pm 0.37 \times 10^{-5}$), lung ($4.80 \pm 0.60 \times 10^{-5}$), stomach ($6.77 \pm 1.01 \times 10^{-5}$) and spleen ($5.23 \pm 1.05 \times 10^{-5}$) described in Table 4-11 for the Big Blue® mouse.

DNA sequence analysis of spontaneous mutations has been carried out in several laboratories. Clonal correction based on DNA sequence analysis has been shown to reduce the mutation frequency by between 9% and 26% in all somatic tissues except brain, in which very little clonality is observed, consistent with the low cell turnover in that tissue (de Boer *et al*., 1998; Hill *et al*., 2004). Comparison of the mutation spectrum observed in different somatic tissues suggests that, in general, the types of mutations, and their proportions, are not significantly different in different tissues or in tissues of different embryonic origin (*i.e.* mesodermal, endodermal or ectodermal layers). In all tissues, the majority of spontaneous mutations are G:C → A:T transitions; these arise primarily at 5′-CpG-3′ sequences (see Section 4.3.1 above), which are methylation sites that yield 5-methylcytosine. Deamination of 5-methylcytosine yields thymine, which specifies the incorporation of adenine during DNA synthesis. A higher proportion of G:C → A:T transitions has been observed in the bladder (de Boer *et al*., 1998). The bulk of these studies have examined mutations in the *lacI* gene of Big Blue® mice (*e.g.* Nishino *et al*., 1996a; Buettner *et al*., 1997; de Boer *et al*., 1998; Hill *et al*., 1999, 2004); however, similar conclusions regarding the consistency of mutation spectra across somatic tissues and the importance of G:C → A:T transitions at 5′-CpG-3′ sequences can be drawn from more limited sequencing of the *lacZ* gene in Muta™Mouse (*e.g.* Douglas *et al*., 1994; Ikehata *et al*., 2000; Ono *et al*., 2000).

Table 4-11. Spontaneous mutant/mutation frequencies of various TGR systems in different tissue types

	Spontaneous mutant/mutation frequencies[a]							
	Bone marrow	Colon	Kidney	Liver	Lung	Intestine	Stomach	Spleen
Big Blue® mouse	3.68 ± 0.59[b] (16)[c]*	4.87 ± 0.86 (15)	3.76 ± 0.87 (10)	5.10 ± 0.37 (63)***	4.80 ± 0.60 (17)	8.73 ± 2.53 (8)	6.77 ± 1.01 (11)	5.23 ± 1.05 (15)
Big Blue® rat	0.95 ± 0.32 (4)**	3.78 ± 0.51 (11)**	2.06 ± 0.40 (6)**	3.11 ± 0.34 (31)**, ***	2.79 ± 0.55 (11)**	5.57 ± 0.64 (3)	–	0.78[d]
gpt delta (*gpt*)	1.21 ± 0.75 (7)	0.76 ± 0.12 (4)	2.45 ± 1.57 (3)	0.55 ± 0.08 (18)	0.54 ± 0.05 (11)	2.5[d]	–	0.44 ± 0.16 (3)
gpt delta (Spi)	0.16 ± 0.02 (8)	0.25 ± 0.15 (2)	2.08 ± 1.81 (4)	0.26 ± 0.03 (15)	0.27 ± 0.06 (5)	–	–	0.31 ± 0.13 (4)
lacZ plasmid mouse	11.9[d]	–	5.10 ± 0.51 (2)	7.14 ± 0.61 (16)	8.51 ± 1.89 (8)	9.0[d]	–	5.89 ± 0.32 (17)
Muta™Mouse	5.25 ± 0.30 (58)*,**	8.10 ± 1.21 (20)**	5.51 ± 0.40 (23)**	6.13 ± 0.41 (88)**	6.66 ± 0.41 (43)**	10.8 ± 1.67 (20)	5.59 ± 0.49 (21)	6.05 ± 0.50 (27)

[a] Differences between the spontaneous mutant/mutation frequencies (SMFs) in Big Blue® mouse, Big Blue® rat and Muta™Mouse for each tissue referred to in the text were evaluated using the Kruskal-Wallis test and, if significant, Dunn's post-hoc test:
  * SMF in Big Blue® mouse significantly different from that in Muta™Mouse ($p < 0.05$).
  ** SMF in Big Blue® rat significantly different from that in Muta™Mouse ($p < 0.05$).
  *** SMF in Big Blue® mouse significantly different from that in Big Blue® rat ($p < 0.05$).
[b] All values are average mutant/mutation frequencies × 10^{-5} ± standard error of the mean, taken from all the independent experiments in the database.
[c] Values in parentheses are the number of independent experiments in which SMF has been reported for the given tissue and TGR model.
[d] Indicates that only one study has been conducted.

The results obtained from sequencing a small number of spontaneous mutations in the *lacZ* gene in the *lacZ* plasmid mouse are not entirely consistent with other models: some differences are apparent in different tissues (*e.g.* heart *vs.* small intestine), and the contribution of G:C → A:T transitions at 5′-CpG-3′ sequences to the total mutation spectrum is, in some cases, relatively minor (Dolle *et al*., 2000).
The data in Table 4-11 provide several useful comparisons and can be further broken down according to the age of the animal. This is discussed further in Sections 4.6.3–4.6.7.

4.6.3 Effect of age

Table 4-12 shows that, in the Muta™Mouse and Big Blue® systems, the spontaneous mutation rate appears to increase with age when all tissues are considered. The same trend is observed when the *gpt* delta rodent system is considered (Masumura *et a*l., 2003a), although the number of experimental animals considered in these calculations is extremely limited. When representative organs – bone marrow, liver and lung – are considered, for which there are sufficient data, then the same trend (spontaneous mutant frequency increasing with age) is apparent for both Muta™Mouse and Big Blue® systems, with the exception of bone marrow in Big Blue®, which has limited available data.

With respect to age, the data derived from analysis of aggregate spontaneous data in the TRAID compare favourably with many of the data derived from individual studies; however, it has been reported that brain and male germline tissue (see Section 4.6.7) differ somewhat from the trend described above and observed in other somatic tissues. Ono *et al.* (1995, 2000), using the Muta™Mouse system, showed that *lacZ* mutant frequencies in spleen, liver, heart and skin increased with age at several time points between birth and 24 months. In the *lacZ* plasmid mouse, *lacZ* mutant frequencies in the liver increased from birth to 24 months, and in the heart and small intestine, from 3 months to 33 months. An increase was observed in the brain between birth and 4–6 months, but not subsequently up to 24 months (Dolle *et al*., 1997, 2000). Several studies have examined mutant/mutation frequency changes with age in the *lacI* gene of Big Blue® mice. Mutation frequency in *lacI* appears to increase in all tissues between birth and 3–6 months. However, in brain and male germline tissue, the mutation frequency does not appear to increase subsequently up to 24–30 months (Stuart *et al*., 2000b; Hill *et al*., 2004). Other somatic cells (spleen, adipose tissue, liver and bladder) exhibit an age-dependent increase in mutant/mutation frequency up to 24–30 months (Lee *et al*., 1994; Stuart *et al*., 2000b; Hill *et al*., 2004).

A single experiment has examined *lacI* mutation frequency in whole foetal samples (Hill *et al*., 2004). The value (1.3×10^{-5}) was lower than for any individual tissues, suggesting that embryonic mutation frequency is low and increases in all somatic tissues up to 4–6 months. In most somatic tissues, there is an age-related increase in mutation frequency throughout the life of the animal. The implications of increased spontaneous mutation frequency on the sensitivity of TGR assays are discussed in Section 4.9.4 below. However, in brain, the evidence suggests that there is no significant increase in spontaneous mutation frequency after approximately 6 months of age.

DNA sequencing studies suggest that, in somatic tissues, similar types and proportions of mutations are generally observed in animals at very different ages (de Boer *et al*., 1998; Ono *et al*., 2000; Stuart *et al*., 2000b; Hill *et al*., 2004). Observed differences have been limited to age-dependent increases in rare tandem base substitutions in *lacI* gene of Big Blue® mice (Buettner *et al*., 1999) and in large genomic rearrangements involving the *lacZ* gene of the *lacZ* plasmid mouse (Dolle *et al*., 2000).

Table 4-12. Effect of age of the animal at sampling time on spontaneous mutant/mutation frequency in TGR systems[a]

TGR system	Transgene /selection	Tissue	Spontaneous mutant/mutation frequency according to age at sampling time (days)						
			<70	71–80	81–90	91–100	101–120	121–150	>150
Muta™Mouse	*lacZ*	All tissues	4.72 (0.30, 81)	5.46 (0.30, 155)	5.21 (0.51, 79)	5.51 (0.24, 120)	5.88 (0.34, 72)	6.36 (0.41, 48)	7.70 (0.85, 80)
		Bone marrow	3.89 (0.66, 11)	4.33 (0.44, 22)	3.96 (0.46, 9)	5.15 (0.53, 18)	4.51 (0.49, 12)	5.47 (0.55, 11)	6.07 (0.51, 15)
		Liver	4.31 (0.46, 19)	5.12 (0.43, 35)	5.49 (0.81, 24)[b]	5.72 (0.41, 34)	5.72 (0.42, 18)	6.72 (0.31, 12)	7.03 (1.16, 17)
		Lung	5.55 (0.67, 12)	6.85 (0.52, 13)	6.12 (0.57, 8)	7.14 (0.82, 14)	5.59 (0.48, 7)	9.79 (1.93, 3)	7.82 (0.99, 7)
Muta™Mouse	*cII*	All tissues	4.21 (0.68, 11)	3.21 (0.29, 20)	3.92 (0.94, 3)	3.37 (0.24, 20)	2.86 (0.49, 6)		5.00 (1)
		Bone marrow		2.03 (0.64, 3)		2.63 (0.28, 2)	1.90 (1)		
		Liver	3.05 (1)	2.40 (0.57, 3)	3.97 (1.63, 2)	3.02 (0.54, 3)	1.7 (1)		
		Lung	3.81 (1)	4.23 (1.12, 3)	3.83 (1)	3.73 (0.34, 3)	4.07 (0.97, 2)		
Big Blue® mouse	*lacI*	All tissues	3.85 (1.06, 6)	1.50 (1)	6.90 (1)			5.57 (1.61, 3)	5.12 (0.57, 10)
		Bone marrow						5.00 (1)	6.05 (0.89, 4)
		Liver	2.00 (1)	1.50 (1)					5.50 (1.80, 2)
		Lung	4.90 (1.91, 3)		6.90 (1)			8.60 (1)	
Big Blue® mouse	*cII*	All tissues	2.88 (0.71, 6)	5.73 (0.54, 12)	3.15 (0.33, 15)	2.75 (0.09, 2)		3.70 (0.90, 2)	10.3 (2.41, 17)
		Bone marrow	0.80 (1)						
		Liver	4.20 (0.30, 2)	8.15 (0.45, 2)	3.75 (0.15, 2)	2.75 (0.09, 2)			11.0 (3.55, 11)
		Lung	1.00 (1)	4.73 (1.50, 3)	2.85 (0.46, 8)				14.2 (1.62, 3)
Big Blue® rat	*lacI*	All tissues	3.95 (0.55, 2)	5.08 (1.19, 4)	3.28 (1.01, 5)	2.19 (0.41, 6)	2.28 (0.49, 5)	1.46 (0.26, 16)	2.59 (0.21, 20)

		Bone marrow							
		Liver	3.95 (0.55, 2)	1.70 (1)	2.20 (0.40, 2)	1.50 (0.30, 2)	1.40 (0.20, 2)	1.51 (0.55, 4)	
		Lung			1.36 (1)	2.81 (0.65, 3)	2.87 (0.60, 3)	2.68 (1.09, 2)	3.08 (0.36, 3)
Big Blue® rat	*cII*	All tissues			6.76 (3.81, 4)	7.09 (2.98, 4)	18.4 (3.63, 2)	4.04 (0.29, 13)	10.1 (4.13, 10)
		Bone marrow							
		Liver			2.23 (1)	3.80 (1)		3.73 (0.46, 5)	4.80 (1)[d]
		Lung			11.0 (7.19, 2)	10.6 (5.29, 2)	14.8 (1)		18.5 (1)

Table 4-12 (continued)

TGR system	Transgene /selection	Tissue	Spontaneous mutant/mutation frequency according to age at sampling time (days)						
			<70	71–80	81–90	91–100	101–120	121–150	>150
gpt delta mouse	*gpt*	All tissues	1.41 (0.57, 9)	1.32 (0.52, 10)	0.70 (0.18, 12)	1.67 (0.84, 2)		0.66 (0.08, 17)	0.60 (0.08, 13)
		Bone marrow	0.26 (0.09, 2)	3.22 (2.49, 2)[c]	0.43 (1)			0.82 (1)	0.30 (1)
		Liver	0.95 (0.08, 3)	0.66 (0.22, 3)	0.53 (0.25, 3)			0.48 (0.07, 8)	0.57 (0.17, 5)
		Lung	0.42 (1)	0.58 (0.03, 4)	0.54 (0.09, 3)			0.39 (0.20, 2)	0.82 (1)
	Spi	All tissues	0.89 (0.66, 11)	0.20 (0.03, 11)	0.20 (0.02, 11)	0.28 (1)		0.22 (0.04, 9)	0.30 (0.05, 10)
		Bone marrow	0.13 (0.02, 3)	0.14 (0.02, 3)	0.19 (0.04, 2)			0.18 (1)	0.20 (1)
		Liver	0.29 (0.04, 3)	0.26 (0.05, 4)	0.18 (0.03, 4)			0.25 (0.07, 4)	0.31 (0.07, 3)
		Lung	0.34 (0.08, 2)		0.28 (0.09, 2)			0.11 (1)	

[a] Values are average spontaneous mutant/mutation frequency (SMF) × 10^{-5}. Numbers in parentheses represent standard error of the mean followed by the number of unique records in the database used for average SMF calculation. The *lacZ* plasmid mouse data were excluded due to a lack of age-matched control groups (all studies reported identical SMF for control groups independent of animal age for treatment groups). There may be some error due to inexact reporting of the age of the control group animals in some published studies.

[b] One study reported an SMF of 21.4; this is inconsistent with all other studies, where SMF ranged from 1.1 to 10.2. The average SMF when this data point is excluded is 4.43 ± 0.62 (standard error of the mean).

[c] Two records used for SMF calculation differed markedly; reported SMF values were 5.7 and 0.73, the latter being more consistent with values reported for other tissues.

[d] One of the two existing records (SMF 45) was excluded as an outlier.

4.6.4 Differences between transgenes

In general, spontaneous mutation frequencies tend to be higher in the *lacI*- and *lacZ*-based TGR models as compared with the *gpt* delta (Spi$^-$) system, regardless of whether the *gpt*$^-$ or Spi$^-$ selection is used (Table 4-11). The spontaneous mutation frequency in bone marrow, liver, lung and spleen is significantly higher in the *gpt* delta (*gpt*) system than in the *gpt* delta (Spi) system, consistent with the notion that deletions occur less frequently than point mutations in most genetic targets.

As described in Section 4.3.1 above, a number of factors influence the spontaneous mutant frequency, including the size of the mutagenic target and the presence/absence of mutational hotspots in the target DNA sequence. For any given tissue, the spontaneous mutant frequency value for a *lacZ* TGR model appears to be slightly greater than those for either of the Big Blue® systems, which are in turn greater than that for the *gpt* delta (*gpt*) system, consistent with (although not proportional to) the relative size of the genetic targets: *lacZ* (3 100 bp), *lacI* (1 080 bp) and *gpt* (456 bp). Comparison of the Muta™Mouse and Big Blue® mouse systems suggests that *lacZ* spontaneous mutant frequency in Muta™Mouse is significantly higher than the *lacI* spontaneous mutant frequency in Big Blue® mouse in bone marrow and lung; the differences are not significant in liver, kidney, colon, small intestine, spleen or stomach (Table 4-11). There are relatively few data available to make a good comparison between the spontaneous *lacZ* mutant frequency in Muta™Mouse as compared with the *lacZ* plasmid mouse. In most tissues, the frequency is slightly higher in the plasmid mouse, although the difference is not significant. There are insufficient data to allow conclusions to be drawn regarding the spontaneous mutant frequency in the *cII* gene relative to other transgenes.

4.6.5 Differences between rats and mice

The spontaneous mutant frequency in the Big Blue® mouse is higher than that observed in the Big Blue® rat in all tissues (Table 4-11); the differences are statistically significant in the liver ($p < 0.005$) and in the bone marrow ($p < 0.01$). It has been proposed by Dycaico *et al.* (1994) that lower spontaneous mutant frequencies in rats, as compared with mice, may reflect several physiological differences: 1) higher levels of oxidative DNA damage in mice as compared with rats; 2) more efficient nucleotide excision repair in rats as compared with mice; and 3) higher levels of DNA metabolism (DNA replication and/or repair) in smaller animals. The spontaneous mutant frequency in the bone marrow of Big Blue® rat appears to be extremely low ($0.95 \pm 0.32 \times 10^{-5}$) compared with other tissues (Table 4-11). This has been noted previously (Shelton, Cherry and Manjanatha, 2000), and the underlying reason for the low rat bone marrow spontaneous mutant frequency was recently investigated (Monroe, Manjanatha and Skopek, 2001). Monroe, Manjanatha and Skopek (2001) concluded that the low spontaneous mutant frequency at *lacI* in bone marrow of the Big Blue® rat could not be explained by reduction of deamination events at CpG sites, since the 5-methylcytosine content of *lacI* in bone marrow was the same as in other tissues; nor could it be explained by enhanced transcriptionally coupled DNA repair in bone marrow, since the transgene is not expressed in that tissue. A spontaneous mutant frequency (ranging from 0.3 to 0.9×10^{-6}) similar to that of bone marrow, and significantly lower than that observed in other somatic tissues, has also been reported in the bladder of Big Blue® rat (Takahashi *et al*., 2000).

Spontaneous mutation spectra were examined in the liver of C57BL/6, B6C3F1 and BC-1 mice and F344 rats (see Sections 2.2.2 and 2.2.6) using the *lacI* transgene (Zhang, Glickman and de Boer, 2001). There was no statistical difference between the different strains and species in the liver spontaneous mutant frequency, an observation that is not supported by a consideration of the aggregate data contained in the TRAID (Table 4-11), in which the rat appeared to have a significantly lower liver spontaneous mutant frequency as compared with the mouse. G:C → A:T transitions and, to a lesser extent, G:C → T:A transversions were the dominant mutations in all TGR models. In the three strains of mice, the proportions of mutations were remarkably similar: G:C → A:T transitions accounted for between 50.7% and 53.3% of mutations, of which 81.7–83.8% occurred at 5′-CpG-3′ sites. In rats, G:C → A:T transitions accounted for 38.0% of the spectra, of which 70.0% occurred at 5′-CpG-3′ sequences. The distribution of other classes of mutations was also very similar. The authors

concluded that spontaneous mutations in the *lacI* transgene appeared to be similar, regardless of genomic location, rodent strain or species (Zhang, Glickman and de Boer, 2001).

4.6.6 Comparison with endogenous loci

The spontaneous mutant frequency is higher in TGR gene targets than in endogenous gene targets in those tissues for which there are comparative mutagenicity data available. This comparison is limited to lymphocytes (transgene *vs. Hprt*) and the small intestine (transgene *vs.* the *Dlb-1* locus) (Table 4-13). In lymphocytes, the spontaneous mutant frequency at the endogenous *Hprt* gene is approximately an order of magnitude smaller than that observed in transgenes in Big Blue® mouse and rat and in Muta™Mouse. A range of spontaneous mutant frequency values for mutation at the *Dlb-1* locus are apparent in the literature, with significant scorer variation possible (Winton *et al.*, 1990; O'Sullivan, Schmidt and Paul, 1991). Significant variability in *Dlb-1* spontaneous mutant frequency values is also observed in transgenic rats and mice; however, the values calculated in Table 4-13, derived only from Muta™Mouse and Big Blue® systems, suggest that the spontaneous mutant frequency is lower in the endogenous *Dlb-1* locus than in the transgene in the small intestine of transgenic rats and mice by a factor of about 5.

Table 4-13. Comparison of spontaneous mutant/mutation frequencies of endogenous genes and transgenes in transgenic rats and mice

	Spontaneous mutant/mutation frequencies[a]	
TGR system (gene)	Lymphocytes	Small intestine
Big Blue® mouse (*Dlb-1*)[b]		2.63 ± 0.41
Big Blue® mouse (*lacI*)[b]		8.73 ± 2.53
Muta™Mouse (*Dlb-1*)[c]		2.22 ± 0.54
Muta™Mouse (*lacZ*)[c]		10.8 ± 1.67
Big Blue® mouse (*Hprt*)	0.19 ± 0.03	
Big Blue® mouse (*lacI*)	3.52 ± 1.08	
Big Blue® rat (*Hprt*)	0.64 ± 0.09	
Big Blue® rat (*lacI*)	3.63 ± 0.45	
Muta™Mouse (*Hprt*)	0.80[d]	
Muta™Mouse (*lacZ*)	4.00 ± 1.32	

[a] Reported values are average mutant/mutation frequencies × 10^{-5} ± standard error of the mean, taken from all the studies in the database.
[b] Data from the following studies were excluded from average mutant frequency calculations due to inconsistency with other studies in the database (four mutant frequency values ranging from 12.5 to 47 × 10^{-5}: Tao, Urlando and Heddle, 1993b; Zhang *et al.*, 1996b).
[c] Data from the following study were excluded from average mutant frequency calculations due to inconsistency with other studies in the database (six mutant frequency values ranging from 7 to 15.3 × 10^{-5}: Cosentino and Heddle, 1999b).
[d] Only one study has been conducted.

4.6.7 Differences between somatic and germ cells

Several studies have examined the spontaneous mutant frequency in the germ cells of transgenic mice. A summary of the data derived from these studies, normalised for the number of animals used in each study, is shown in Table 4-14.

Table 4-14. Spontaneous mutant/mutation frequency in germ cell tissues of TGR systems[a]

TGR system	Testes	Seminiferous tubules	Vas deferens	Sperm
Big Blue® mouse	2.07 (1.47, 3)[b]	2.87 (0.63, 10)[c]		4.68 (1.04, 5)
gpt delta (*gpt*)	0.66 (0.08, 3)			
gpt delta (Spi)	0.19 (0.03, 4)	0.20 (1)		
Muta™Mouse	2.63 (0.49, 21)	3.28 (0.61, 12)	3.75 (0.65, 2)	2.60 (0.31, 15)[d]

[a] Values are average spontaneous mutant frequency × 10^{-5}, with numbers in parentheses representing the standard error of the mean followed by the number of unique records in the database used for average spontaneous mutant frequency calculation. Note that data are shown only for those TGR systems and tissues with the greatest number of records in the database.

[b] Data from Hoyes *et al*. (1998) were excluded from spontaneous mutant frequency calculation due to inconsistency with other reported spontaneous mutant frequency values; reported spontaneous mutant frequency value was 10.6, 10-fold higher than all other values in the database.

[c] Data from Provost *et al*. (1993) were excluded from spontaneous mutant frequency calculation due to inconsistency with other reported spontaneous mutant frequency values; reported spontaneous mutant frequency value was 0.06, 10-fold lower than all other values in the database.

[d] Data from van Delft and Baan (1995) were excluded from spontaneous mutant frequency calculation due to inconsistency with other reported spontaneous mutant frequency values; reported spontaneous mutant frequency values were 57.2, 33.5 and 14, 2.5- to 10-fold higher than all other values in the database.

In general, the spontaneous mutant frequency values for male germline cells appear to be lower than the spontaneous mutant frequency values for somatic cells (Table 4-14). There is some disagreement as to whether there is an age effect with regard to spontaneous mutant frequency in germ cells. Hill *et al.* (2004) suggested that the spontaneous mutant frequency in male germline cells remains constant throughout the life of the mouse; this is consistent with the results of Ono *et al.* (2000) and Martin *et al.* (2001). These studies are in some conflict with reports from Walter *et al.* (1998, 2004), who showed a significant increase in the spontaneous mutant frequency in spermatogenic cells from old Big Blue® mice as compared with young or middle-aged mice. In these latter studies, changes were observed in the mutation spectrum as the animals aged: five mutation hotspots were identified in the *lacI* gene from spermatogenic cells from young and middle-aged mice; in old mice, the spectrum of mutations from the spermatogenic cells revealed an increased prevalence of transversions, but no hotspots were apparent (Walter *et al*., 2004).

There are too few data available regarding the spontaneous mutant frequency in female germline cells to allow any conclusions to be drawn.

4.6.8 Conclusions

The spontaneous mutant frequency in most tissues of transgenic animals is in the vicinity of 5×10^{-5}; this is about 5- to 10-fold higher than observed in endogenous loci using the same animals. Factors such as the age of the animal, the tissue and the animal model influence the absolute value of the spontaneous mutant frequency. Spontaneous mutant frequency appears to follow the order *lacZ* > *lacI* > *gpt* > Spi^-, although the observed differences are not statistically significant in many tissues. As compared

with other transgenic animals, the spontaneous mutant frequency is relatively low in *gpt* delta rodents using either *gpt* or Spi^{-} selection. In most somatic tissues, with the exception of brain, there is an age-related increase in mutation frequency throughout the life of the animal. Most, but not all, studies suggest that the spontaneous mutant frequency in male germline tissues remains low and constant throughout the life of the animal. In all tissues, the majority of spontaneous mutations are G:C → A:T transitions, which arise primarily at 5′-CpG-3′ sequences. The spontaneous mutant frequency in rat is generally lower than in mice when the same transgene is assayed. The spontaneous mutant frequencies in rat bone marrow and bladder are particularly low.

4.7 Administration time

The duration of treatment is an important factor in determining the sensitivity of TGR assays. Based on the apparent neutrality of mutations in transgenic loci, it has been postulated that the effects of multiple treatments would be additive: *i.e.* that the number of mutants induced would increase with the number of treatments, assuming that there is sufficient time for the induced mutations to become manifest (Tao and Heddle, 1994; Heddle, Martus and Douglas, 2003). Additivity has been demonstrated experimentally in the limited number of studies in which the effect of multiple treatments has been examined carefully: in the *lacI* gene of the Big Blue® mouse following chronic treatment with ENU (Shaver-Walker *et al*., 1995), in the *lacI* gene of the Big Blue® rat following multiple treatments of the model mutagen, *N*-hydroxy-2-acetylaminofluorene (Chen *et al*., 2001b), and at the *lacZ* (Cosentino and Heddle, 1999b, 2000) and *cII* (Swiger *et al*., 1999) genes of Muta™Mouse and the *gpt* gene of the *gpt* delta mouse (Swiger *et al*., 2001) after multiple treatments of ENU. Thus, the notion that multiple treatments of a mutagen should increase mutant frequencies in neutral transgenes in an approximately additive manner is supported by both theory and experimental data in a range of TGR models in both rat and mouse.

Although the existing evidence supporting additivity appears to be convincing, the majority of studies have not considered this fact in their design. This can be seen in Table 4-6 and has been discussed in Section 4.2.6. In the majority of experiments carried out to date, the administration time has been substantially shorter than the 28 days that is currently recommended (see Section 6.1.2.5) (Thybaud *et al*., 2003). In approximately 45% of the experimental records, a single administration has been used; in 65% of the experiments, an administration time of 5 days or less has been used. Thus, it is likely that the maximum sensitivity of the assays has not been obtained with most experiments carried out to date. This may be a factor for those assays of known or suspected mutagens that have returned a negative result, particularly if the sampling time (see Section 4.8 below) is also not optimal or the presumed mutagenicity is weak. Nevertheless, it should be noted that a significant proportion of the experiments that have used short administration times (many of which have examined strong mutagens) have returned positive results. For example, positive results were reported in 56% of experiments in which a single administration was used and in 59% of the experiments in which the administration time was between 1 and 5 days.

Depending on the agent that is tested, consideration must also be given to tissue exposure, kinetic data, the time course of enzyme induction (*e.g.* metabolic activation) and toxicity. Some compounds, such as tamoxifen, appear to require prolonged treatment before DNA adducts begin to form (Davies *et al*., 1997). However, while longer administration times should increase mutant frequencies and increase the sensitivity of the assay, longer exposures may increase the risk of false-positive results due to non-genotoxic mechanisms caused by chronic toxicity (*e.g.* tumour induction, inflammatory response, oxidative damage) (Thybaud *et al*., 2003).

In some TGR systems, there is concern that treatment times of 12 weeks or longer (Heddle *et al*., 2000) can produce an apparent increase in mutant frequency through clonal expansion, genomic instability in developing preneoplastic foci or tumours, or oxidative damage in DNA resulting from chronic induction of cytochrome P-450 mono-oxygenases (Thybaud *et al*., 2003). The following are three examples in which clonal expansion over long administration times has produced marginally positive results that have required DNA sequence analysis for clarification.

First, treatment of Big Blue® mice with phenobarbital (2500 mg/kg diet for 180 days) produced a modest increase in the liver mutant frequency from $5.02 \pm 2.4 \times 10^{-5}$ in the control group to $6.88 \pm 0.75 \times$

10^{-5} in the phenobarbital-treated group; the observed increase was marginally, although significantly, different ($p < 0.05$). However, DNA sequence analysis of a random collection of mutants from each phenobarbital-treated mouse allowed for correction of clonal expansion and resulted in a decrease in the mutant frequency to $6.39 \pm 1.02 \times 10^{-5}$, which was not significantly different from the control (Shane *et al*., 2000c).

Second, Big Blue® mice were treated chronically with oxazepam (2500 mg/kg feed for 180 days). The mutant frequency of *lacI* in liver of control mice was $5.02 \pm 2.4 \times 10^{-5}$, whereas the mutant frequency in the oxazepam-treated mice was $9.17 \pm 4.82 \times 10^{-5}$, an increase that was marginally significant ($p <$ 0.05). Clonal correction of the *lacI* mutant frequency of the oxazepam-treated mice reduced the mutant frequency to $8.15 \pm 2.54 \times 10^{-5}$ (Shane *et al*., 1999), which remained a significant difference.

Third, leucomalachite green was fed to Big Blue® rats at 543 mg/kg feed for 28, 112 and 224 days. An increase in liver mutant frequency was observed at 112 days, but not at 28 or 224 days (control $1.8 \pm 0.3 \times 10^{-5}$ *vs.* leucomalachite green–treated $5.3 \pm 1.7 \times 10^{-5}$; $p < 0.05$) (Culp *et al*., 2002). When corrected for clonality, the *lacI* mutation frequency of the 112-day leucomalachite green–treated rats was $3.6 \pm 1.0 \times 10^{-5}$, which was not significantly different from the clonally corrected control frequency of 1.7 $\pm 0.9 \times 10^{-5}$ (Manjanatha *et al*., 2004).

Each of these examples illustrates the difficulty in evaluating levels of mutagenicity that are of borderline significance when the administration times are very long. On the other hand, it should be pointed out that there are several examples in which chemicals have been administered for extremely long periods without apparent clonal expansion: for example, chloroform (Butterworth *et al*., 1998), heptachlor (Gunz, Shephard and Lutz, 1993), di(2-ethylhexyl)phthalate (DEHP) (Gunz, Shephard and Lutz, 1993) and very low dose ENU (Cosentino and Heddle, 1999b). In addition, there is a danger that the use of DNA sequence information can result in the overcorrection of mutational hotspots (see Section 2.3.2). From a practical point of view, it should be noted that extremely long administration or sampling times significantly diminish the advantage of a short-term test relative to the carcinogenicity bioassay.

4.7.1 Conclusions

Multiple treatments of a mutagen appear to increase mutant frequencies in neutral transgenes in an approximately additive manner. This is supported by experimental data in a range of TGR models in both rat and mouse. However, extremely long treatment times of 12 weeks or longer may produce an apparent increase in mutant frequency through clonal expansion, genomic instability in developing preneoplastic foci or tumours, or oxidative damage in DNA resulting from chronic induction of cytochrome P-450 mono-oxygenases.

4.8 Sampling time

Subsequent to exposure of a transgenic animal to a mutagen, there are several events that will influence the appearance of mutations in a given tissue, including uptake, metabolism, repair, mutation fixation and cell turnover. The time between the last treatment and the time of sacrifice – the sampling time – is therefore a critical variable in the experimental design. The time required to reach the maximum mutant frequency is tissue specific and appears to be related to the turnover time of the cell population: bone marrow and intestine have rapid cell turnover, and the maximum mutant frequency occurs at short sampling times; in contrast, liver is a slowly proliferating tissue, and the maximum mutant frequency occurs at much longer sampling times (Douglas *et al*., 1996; Suzuki *et al*., 1999a; Wolff *et al*., 2001; Heddle, Martus and Douglas, 2003). Tissues are typically organised so that a relatively small population of stem cells (Section 4.3.4) gives rise to a larger population of transit cells, which in turn produce non-replicating differentiated cells. The rate at which differentiated cells are lost is highly variable between tissues, ranging from a few days in rapidly proliferating tissues such as bone marrow to much longer times in slowly proliferating tissues such as liver to a virtual absence of cell turnover in tissues such as brain or muscle. Following exposure to a mutagen, the different populations of cells will yield mutations at different rates. When genomic DNA is extracted from a given tissue, the resultant mutant frequency in the TGR assay will be a weighted average of the mutant frequencies of the cell population. The mutant

frequency at any given sampling time is therefore dependent on 1) the relative sensitivity of the cell types comprising that population, 2) the relative proportion of the cell types and 3) the rate at which cells containing mutations are lost from the population. At present, we have limited understanding of each of these variables.

Most of the studies carried out to date show that in liver, the induced mutant frequency increases over the month following an acute exposure (Tables 4-15 and 4-16). The mutant frequency seems to reach a maximum, as predicted for a neutral mutational target, and remains relatively constant thereafter. This trend is seen in both mice and rats and appears to be true for those transgenes that are detecting primarily point mutations, as well as for the *gpt* delta (Spi) selection, which detects primarily deletion mutations (T. Nohmi, unpublished; Hayashi *et al*., 2003). A variety of chemicals, including 3-methylcholanthrene, 4-nitroquinoline-1-oxide (4-NQO), 5,9-dimethyldibenzo(c,g)carbazole (DMDBC), 7,12-dimethylbenzanthracene (7,12-DMBA), DEN, dipropylnitrosamine (DPN), mitomycin-C and ENU, have shown similar patterns of mutant frequency increasing with sampling time. Experiments carried out with dimethylnitrosamine (DMN), benzo(a)pyrene and cyproterone acetate do not show as clear a trend (Tables 4-15 and 4-16). Indeed, in the case of cyproterone acetate, the mutant frequency appears to increase with sampling time up to approximately 14 days, but then decreases at longer sampling times (Table 4-15) (Wolff *et al*., 2001). In contrast, in bone marrow, the mutant frequency appears to reach a maximum at extremely short sampling times and then decreases over 28 days following an acute treatment (Table 4-17).

In the case of multiple applications of a chemical (Section 4.7), the biology underlying the observed mutant frequency becomes more complex, since the amount of time available for mutation fixation is clearly greater from earlier time points. For example, consider a chemical that is administered for 28 consecutive days, following which a slowly proliferating tissue and a rapidly proliferating tissue are sampled. If one assumes the effects of multiple administrations to be approximately additive (Section 4.7), then the observed mutant frequency will be an aggregate function of 28 individual applications, each of which has a different sampling time. Clearly, in a slowly proliferating tissue, the early applications are expected to contribute most heavily to the observed mutant frequency, because the sampling time from these points is optimal for the fixation of mutations. In contrast, in a rapidly proliferating tissue, mutant cells derived from early administration time points may be lost from the tissue (unless they are derived from stem cell mutations), and the observed mutant frequency will be most heavily influenced by mutations arising from later administration time points.

In theory, it should be possible to develop a generalised protocol that specifies multiple administrations within an appropriate timeframe that will allow the manifestation of mutations in both rapidly and slowly proliferating tissues within a prescribed sampling period. At the present time, there is very little experimental information available that will allow us to determine with precision what the optimal treatment and sampling time protocol would be for a variety of tissues. As shown in Table 4-6, there are almost no experimental records that combine longer periods of administration with different sampling times. A proposed protocol that addresses the different factors discussed in Sections 4.7 and 4.8 is described in Chapter 6 of this document.

Table 4-15. Effect of sampling time on positive responses in the liver following a single administration

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[b]
3-Methylcholanthrene	Big Blue® mouse	Intraperitoneal	80	3	2.5	Rihn *et al*. (2000b)
				6	6.1	
				14	12.7	
				30	22.2	
4-Nitroquinoline-1-oxide (4-NQO)	Muta™Mouse	Gavage	200	7	23.8	Nakajima *et al*. (1999)
				14	37.4	
				28	48.4	
5,9-Dimethyl-dibenzo(c,g)carbazole (DMDBC)	Muta™Mouse	Topical	90	7	72.8	Tombolan *et al*. (1999b)
				14	119.8	
				21	185.5	
				28	180.6	
				28	195.3	
				28	180.4	Renault *et al*. (1998)
				28	146.9	
7,12-Dimethylbenzanthracene (7,12-DMBA)	Muta™Mouse	Intraperitoneal	20	7	10.2	Hachiya *et al*. (1999)
				14	8.5	Ohsawa *et al*. (2000)
				14	33.9	Hachiya *et al*. (1999)
				28	31.2	
Amosite asbestos	Big Blue® rat	Intratracheal	4	28	0.82	Loli *et al*. (2004); Topinka *et al*. (2004a)
				112	0.57	Loli *et al*. (2004); Topinka *et al*. (2004a)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[b]
			8	28	0.58	Loli *et al*. (2004); Topinka *et al*. (2004a)
				112	2.75	Loli *et al*. (2004); Topinka *et al*. (2004a)

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[b]
Benzo(a)pyrene	Muta™Mouse	Intraperitoneal	100	21	25	Mientjes *et al*. (1996)
				21	17.1	
				28	24	
				28	12.5	
				35	54	
				35	23.6	
				48	11.4	Rompelberg *et al*. (1996)
Cyproterone acetate	Big Blue® rat	Gavage	100	1	1.75	Wolff *et al*. (2001)
				2	9.25	
				3	12.25	
				7	12.75	
				14	14.25	
				28	3.75	
				42	4.25	
				56	3.55	
Diethylnitrosamine (DEN)	Muta™Mouse	Intraperitoneal	50	7	3.5	Okada *et al*. (1997)
				21	6.6	
				28	7.5	
			66	3	8.6	Mientjes *et al*. (1998)
				14	27.8	
				28	30.2	
			5	7	3.8	Souliotis *et al*. (1998)

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency ($\times 10^{-5}$)	References[b]
				14	5	
				28	4.7	
				49	5.1	
Dimethylnitrosamine (DMN)	Muta™Mouse	Gavage	10	7	17.6	Tinwell, Lefevre and Ashby (1994b)
				10	10.4	
				14	11	Tinwell *et al*. (1995)
				14	9.1	
				14	4.6	
				14	9.1	Tinwell, Lefevre and Ashby (1998)
				14	12.6	
				14	18	Fletcher, Tinwell and Ashby (1998)
				14	23.6	
				20	17.1	Tinwell, Lefevre and Ashby (1994b)
				20	14.8	Lefevre *et al*. (1994)
				90	20.6	Jiao *et al*. (1997)
Dipropylnitrosamine (DPN)	Muta™Mouse	Intraperitoneal	250	7	7.5	Itoh *et al*. (1999)
				14	33.2	
				28	57.4	
Glass wool fibres	Big Blue® rat	Intratracheal	4	28	0.41	Topinka *et al*. (2006a, 2006b)
				112	0.73	Topinka *et al*. (2006a)
			8	28	0.76	Topinka *et al*. (2006a, 2006b)
				112	1.53	Topinka *et al*. (2006a)

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[b]
Mitomycin-C	*gpt* delta (Spi)	Intraperitoneal	4	3	0.049	unpublished
				7	0.281	
				14	0.33	
				28	0.25	
N-Ethyl-*N*-nitrosourea (ENU)	Big Blue® mouse	Intraperitoneal	120	1	0.95	Wang *et al*. (2004)
					21.9	
				3	1.35	
					34	
				7	1.4	
					9.65	
					24.4	
				15	3.3	
					15.65	
					20.2	
				30	15.1	
					20.4	
					23.55	
				120	15.6	
					21.45	
					30.9	
	Muta™Mouse	Intraperitoneal	80	1	21.9	Yauk *et al*. (2005)
					26.7	
					30.9	

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[b]
					36.6	
					40.1	
					45.7	
				2	51.6	
					67.2	
					73.3	
					80.4	
				3	68.7	
					75.9	
					77.8	
					85.6	
					109.2	
					112	
				4	69	
					72.1	
					84.4	
					92.3	
				5	60.8	
					61.8	
					76.4	
					78.3	
				8	51.1	
					60.3	

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency ($\times 10^{-5}$)	References[b]
					76.5	
					78.5	
				12	63.3	
					66.7	
					73.8	
				16	57.8	
					74.9	
				20	53.4	
					68.8	
			150	3	3.4	Mientjes *et al*. (1998)
				14	11.7	Mientjes *et al*. (1996)
				14	16.5	
				14	7.8	Collaborative Study Group for the Transgenic Mouse Mutation Assay (1996)
				14	16.5	Mientjes *et al*. (1998)
				28	27.3	
			160	3	3.5	Krebs and Favor (1997)
				10	9.2	
				100	8	
			250	3	5	Hoorn *et al*. (1993)
				3	3.8	Myhr (1991)
				7	5.3	Hoorn *et al*. (1993)

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency ($\times 10^{-5}$)	References[b]
				7	4.2	Myhr (1991)
				10	23.6	Hoorn *et al*. (1993)
				10	22.2	Myhr (1991)
				14	10.6	Recio *et al*. (1993)
Proton radiation	*lacZ* plasmid mouse	Irradiation	0.1 Gy	7	0.6	Chang *et al*. (2005)
					1	
				56	0.1	
					2.4	
				112	0.7	
					2.4	
			0.5 Gy	7	0.8	
					1.2	
				56	3.3	
					5.5	
				112	1.4	
					1.7	
			1 Gy	7	0.6	
					1	
				56	3.7	
					6.8	
				112	2.2	
					3.2	
			2 Gy	7	1.3	

Table 4-15 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)[a]	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[b]
					2.1	
				56	3.2	Chang *et al*. (2005, 2007)
					4	Chang *et al*. (2005)
					4.1	Chang *et al*. (2007)
				112	2	Chang *et al*. (2005)
					2.3	
			4 Gy	7	3.4	
					3.5	
				56	3.5	
					4.8	
				112	1.2	
					2.9	
Rock wool fibres	Big Blue® rat	Intratracheal	8	28	1.12	Topinka *et al*. (2006a, 2006b)
				112	2.97	Topinka *et al*. (2006b)
Vinyl carbamate	Big Blue® mouse *cII*	Intraperitoneal	60	7	2.5	Hernandez and Forkert (2007a)
				14	7.5	
				21	10.3	
				28	6.1	Hernandez and Forkert (2007a, 2007b)
					7.1	Hernandez and Forkert (2007a)
					9.1	Hernandez and Forkert (2007b)
					11.9	Hernandez and Forkert (2007a)

[a] Unless otherwise noted.
[b] “Unpublished” refers to data provided by members of the IWGT working group.

Table 4-16. Effect of sampling time on positive responses in the liver following a 5-day administration time

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[a]
Diethylnitrosamine (DEN)	Big Blue® mouse	Intraperitoneal	45	185	54	Mirsalis *et al*. (2005)
				298	59	
				511	87.9	
				725	45.2	
	Muta™Mouse		175	5	201	unpublished
				15	228.7	
				35	306.9	
				55	421.3	
Dimethylnitrosamine (DMN)	Big Blue® mouse	Intraperitoneal	10	1	19.3	Mirsalis *et al*. (1993)
				8	31.6	
				22	54.4	
N-Ethyl-*N*-nitrosourea (ENU)	Muta™Mouse	Intraperitoneal	250	3	5.8	Hoorn *et al*. (1993)
				3	4.5	Myhr (1991)
				5	19.2	Douglas *et al*. (1996)
				7	22	Hoorn *et al*. (1993)
				7	20.7	Myhr (1991)
				10	21	Douglas *et al*. (1996)
				10	19.5	Hoorn *et al*. (1993)
				10	18	Myhr (1991)
				20	26.1	Douglas *et al*. (1996)
				35	64.9	

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[a]
				45	63.7	

Table 4-16 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[a]
N-Methyl-*N*-nitrosourea (MNU)	Big Blue® mouse	Intraperitoneal	500	1	0.3	Provost *et al*. (1993)
				3	0.3	
				6	0.6	
				12	1.7	

[a] "Unpublished" refers to data provided by members of the IWGT working group.

Table 4-17. Effect of sampling time on positive responses in bone marrow following a single administration

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[a]
4-Nitroquinoline-1-oxide (4-NQO)	Muta™Mouse	Gavage	200	7	157.7	Nakajima *et al*. (1999)
				14	106.9	
				28	69.9	
7,12-Dimethylbenzanthra-cene (DMBA)	Muta™Mouse	Intraperitoneal	20	7	71.9	Hachiya *et al*. (1999)
				14	33.4	Ohsawa *et al*. (2000)
				14	78.4	Hachiya *et al*. (1999)
				28	66.7	
	Big Blue® rat	Gavage	20	14	10.3	Shelton, Cherry and Manjanatha (2000)
				42	8.2	
				70	3.9	
				98	3.3	
			130	14	32.5	
				42	19.2	
				70	15.2	
				98	20.3	
Mitomycin-C	*gpt* delta (Spi)	Intraperitoneal	4	3	0.53	unpublished
				7	0.46	
				14	0.12	Okada *et al*. (1999)
				14	0.15	unpublished
				28	0.06	
N-Ethyl-*N*-nitrosourea (ENU)	Muta™Mouse	Intraperitoneal	80	1	36.6	Yauk *et al*. (2005)
					40.1	

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency ($\times 10^{-5}$)	References[a]
					45.7	
				2	73.3	
					80.4	
				3	85.6	
					109.2	
					112	

Table 4-17 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency ($\times 10^{-5}$)	References[a]
				4	84.4	
					92.3	
				5	76.4	
					78.3	
				8	76.5	
					78.5	
				12	66.7	
					73.8	
				16	74.9	
				20	68.8	
	Big Blue® mouse		120	1	21.9	Wang *et al*. (2004)
				3	34	
				7	24.4	
				15	20.2	

Table 4-17 (continued)

Chemical	TGR system	Route of administration	Total dose (mg/kg bw)	Sampling time (days)	Induced mutant/mutation frequency (× 10^{-5})	References[a]
				30	20.4	
				120	15.6	
N-Propyl-*N*-nitrosourea (PNU)	*gpt* delta (*gpt*)	Intraperitoneal	250	7	3.41	unpublished
				14	4.23	
				28	3.6	
	Muta™Mouse		250	7	41.5	Hara *et al*. (1999a)
				14	27.5	
				28	15.1	

[a] "Unpublished" refers to data provided by members of the IWGT working group.

4.8.1 Conclusions

The time required to reach the maximum mutation frequency is tissue specific and appears to be related to the turnover time of the cell population: bone marrow and intestine have rapid cell turnover, and the maximum mutant frequency occurs at short sampling times; in contrast, liver is a slowly proliferating tissue, and the maximum mutant frequency occurs at much longer sampling times. The optimal sampling time differs according to the tissue, with liver and bone marrow at opposite extremes among proliferating somatic tissues: in bone marrow, the mutant frequency appears to reach a maximum at extremely short sampling times and then decreases over 28 days following an acute treatment; in liver, the induced mutant frequency increases over the month following exposure, reaches a maximum and remains relatively constant thereafter. There are insufficient data available for other tissues to support any conclusion regarding a single optimal sampling time.

4.9 Issues of concern regarding TGR assays

4.9.1 Reproducibility of data

The reliability of a test is a function of the reproducibility of results within and between laboratories. In the case of TGR assays, it is difficult to assess reliability, since there have been relatively few interlaboratory collaborative studies, and the range of experimental conditions used in TGR assays makes it difficult to compare experimental results.

A collaborative study involving 26 laboratories examined ENU mutagenesis in eight organs of Muta™Mouse – liver, spleen, bone marrow, brain, lung, kidney, urinary bladder and heart – following a single intraperitoneal injection of 150 mg ENU/kg bw (Collaborative Study Group for the Transgenic Mouse Mutation Assay, 1996). Many of the laboratories involved in this study had no prior experience using TGR assays, and the study was preceded by a 1-day training session for all participants. A standard DNA sample was analysed by all laboratories; the results from only two of the laboratories varied over two-fold from the mean mutant frequency, and there was an overall high level of concordance in the mutant frequencies obtained using this standard sample. Among the organs tested, similar conclusions were reached by most laboratories regarding whether a positive or negative result was obtained; as expected, the potent mutagen ENU increased mutant frequency in all organs except brain. However, the study design did not allow a rigorous statistical evaluation of the data or the extent of interlaboratory variation (Collaborative Study Group for the Transgenic Mouse Mutation Assay, 1996).

The mutagenicity of DMN was evaluated in three laboratories using common liver samples from Muta™Mouse and Big Blue® mice. The liver samples compared were obtained from mice treated once with either saline or DMN (10 mg/mL, 14-day sampling time). Each assay gave an increased mutant frequency for the DMN-treated livers when compared with the saline control mutant frequencies (Tinwell *et al.*, 1995; see also Table 4-18).

A collaborative study examined mouse germ cell mutagenesis of ENU, isopropyl-methanesulphonate (iPMS) and methylmethanesulphonate (MMS) in both Muta™Mouse and Big Blue® mice. Both testicular DNA and epididymal sperm DNA were evaluated, and a range of sampling times, from 3 to 100 days, was examined. ENU and iPMS were found to be mutagenic to both testicular DNA and epididymal sperm DNA. MMS was not mutagenic under any test condition. The authors concluded that a good level of qualitative agreement was obtained for the two assays and for the same assays conducted in different laboratories. The level of quantitative agreement was not as high, but was, nonetheless, generally good (Ashby, 1995; Ashby, Gorelick and Shelby, 1997).

Sources of variability in the experimental protocol that can affect the statistical nature of the observations have been examined (Piegorsch *et al.*, 1994, 1997). Such sources of variability include plate-to-plate (within packages), package-to-package (within animals) and animal-to-animal variability.

Table 4-18. Reproducibility of experimental data

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
A) Muta™Mouse – liver							
5,9-Dimethyldibenzo-(c,g)carbazole (DMDBC)	Topical	3	1 / 28	–	3.8 / 4.7	1.24	Tombolan *et al*. (1999b)
			1 / 28	–	3.6 / 4.7	1.31	Renault *et al*. (1998)
		10	1 / 28	+	5.4 / 13	2.41	Tombolan *et al*. (1999a)
			1 / 28	+	4 / 11.5	2.88	Tombolan *et al*. (1999a)
			1 / 28	+	3.8 / 7.6	2	Tombolan *et al*. (1999b)
			1 / 28	+	4.5 / 10.6	2.36	Tombolan *et al*. (1999b)
			1 / 28	+	3.6 / 8.2	2.28	Renault *et al*. (1998)
			1 / 28	+	3.1 / 14	4.52	Renault *et al*. (1998)
		30	1 / 28	+	3.8 / 91.2	24	Tombolan *et al*. (1999b)
			1 / 28	+	3.6 / 133	36.94	Renault *et al*. (1998)
			1 / 28	+	3.1 / 96	30.97	Renault *et al*. (1998)
		90	1 / 28	+	3.8 / 184.4	48.53	Tombolan *et al*. (1999b)
			1 / 28	+	4.5 / 199.8	44.4	Tombolan *et al*. (1999b)
			1 / 28	+	3.6 / 184	51.11	Renault *et al*. (1998)
			1 / 28	+	3.1 / 150	48.39	Renault *et al*. (1998)
7,12-Dimethylbenzanthra-cene (7,12-DMBA)	Intraperitoneal	20	1 / 14	+	9.9 / 18.4	1.86	Ohsawa *et al*. (2000)
			1 / 14	+	3.6 / 37.5	10.42	Hachiya *et al*. (1999)
Benzo(a)pyrene	Gavage	625	5 / 14	+	4.1 / 22	5.37	Hakura *et al*. (1998)
			5 / 14	+	3.8 / 41	10.79	Kosinska, von Pressentin and Guttenplan (1999)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
			5 / 14	+	5.6 / 41.3	7.4	Yamada *et al*. (2002)
Diethylnitrosamine (DEN)	Intraperitoneal	100	1 / 7	+	2.1 / 38.8	18.48	Okada *et al*. (1997)
			1 / 7	+	3.7 / 12.5	3.38	Suzuki, Hayashi and Sofuni (1994)
Dimethylnitrosamine (DMN)	Gavage	10	1 / 14	+	4.5 / 15.5	3.44	Tinwell *et al*. (1995)
			1 / 14	+	1.8 / 10.9	6.06	Tinwell *et al*. (1995)
			1 / 14	+	5.7 / 10.3	1.81	Tinwell *et al*. (1995)
			1 / 14	+	1.8 / 10.9	6.06	Tinwell, Lefevre and Ashby (1998)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
			1 / 14	+	1.8 / 14.4	8	Tinwell, Lefevre and Ashby (1998)
			1 / 14	+	3 / 21	7	Fletcher, Tinwell and Ashby (1998)
			1 / 14	+	5.6 / 29.2	5.21	Fletcher, Tinwell and Ashby (1998)
			1 / 20	+	4.1 / 21.2	5.17	Tinwell, Lefevre and Ashby (1994b)
			1 / 20	+	6.4 / 21.2	3.31	Lefevre *et al*. (1994)
N-Ethyl-*N*-nitrosourea (ENU)	Intraperitoneal	100	1 / 3	+	0.8 / 9.8	12.25	Hoorn *et al*. (1993)
			1 / 3	+	2 / 32.5	16.25	Myhr (1991)
			1 / 7	−	3.2 / 3.2	1	Suzuki *et al*. (1997a)
			1 / 7	−	3.7 / 3.7	1	Suzuki, Hayashi and Sofuni (1994)
			1 / 7	−	3.2 / 3.2	1	Suzuki *et al*. (1993)
			1 / 7	+	0.8 / 12.6	15.75	Hoorn *et al*. (1993)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
			1 / 7	+	2 / 12.3	6.15	Myhr (1991)
			1 / 7	+	3.5 / 10.5	3	Lynch, Gooderham and Boobis (1996)
			1 / 10	+	4.9 / 17.2	3.51	Itoh, Miura and Shimada (1998)
			1 / 10	+	0.8 / 3.3	4.13	Hoorn *et al*. (1993)
			1 / 10	−	2 / 3.3	1.65	Myhr (1991)
			5 / 3	+	2 / 14	7	Myhr (1991)
			5 / 3	+	0.8 / 14.1	17.63	Hoorn *et al*. (1993)
			5 / 7	+	2 / 12.3	6.15	Myhr (1991)
			5 / 7	+	0.8 / 12.2	15.25	Hoorn *et al*. (1993)
			5 / 10	+	0.8 / 8.8	11	Hoorn *et al*. (1993)
			5 / 10	+	2 / 8.8	4.4	Myhr (1991)
		150	1 / 3	+	5.5 / 8.9	1.62	Mientjes *et al*. (1998)
			1 / 3	−	4.9 / 6.9	1.41	Collaborative Study Group for the Transgenic Mouse Mutation Assay (1996)
			1 / 14	+	4.6 / 16.3	3.54	Mientjes *et al*. (1996)
			1 / 14	+	5.5 / 22	4	Mientjes *et al*. (1996)
			1 / 14	+	7.8 / 15.6	2	Collaborative Study Group for the Transgenic Mouse Mutation Assay (1996)
			1 / 14	+	5.5 / 22	4	Mientjes *et al*. (1998)
		250	5 / 10	+	0.8 / 20.3	25.38	Hoorn *et al*. (1993)
			5 / 10	+	2 / 20	10	Myhr (1991)
			5 / 10	+	5.8 / 26.8	4.62	Douglas *et al*. (1996)
		250	1 / 3	+	2 / 5.8	2.9	Myhr (1991)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
			1 / 3	+	0.8 / 5.8	7.25	Hoorn *et al*. (1993)
		250	1 / 7	+	2 / 6.2	3.1	Myhr (1991)
			1 / 7	+	0.8 / 6.1	7.63	Hoorn *et al*. (1993)
		250	1 / 10	+	2 / 24.2	12.1	Myhr (1991)
			1 / 10	+	0.8 / 24.4	30.5	Hoorn *et al*. (1993)
			1 / 14	+	2.4 / 13	5.42	Recio *et al*. (1992)
N-Methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG)	Gavage	100	1 / 7	−	9.4 / 10.2	1.09	Brooks and Dean (1996)
			1 / 7	−	3.2 / 3.2	1	Brault *et al*. (1996)
Quinoline	Intraperitoneal	200	4 / 14	+	5.1 / 22.5	4.41	Miyata *et al*. (1998)
			4 / 14	+	4.6 / 17.3	3.76	Suzuki *et al*. (1998)
			4 / 14	+	6.7 / 34.3	5.12	Suzuki *et al*. (1998)
B) **Muta™Mouse – Bone marrow**							
7,12-Dimethylbenzanthra-cene (7,12-DMBA)	Intraperitoneal	20	1 / 14	+	4.5 / 37.9	8.42	Ohsawa *et al*. (2000)
			1 / 14	+	3.3 / 81.7	24.76	Hachiya *et al*. (1999)
Acrylamide	Intraperitoneal	250	5 / 7	+	1.5 / 8.9	5.93	Hoorn *et al*. (1993)
			5 / 7	+	2.6 / 8.9	3.42	Myhr (1991)
Benzo(a)pyrene	Gavage	625	5 / 14	+	5 / 92	18.4	Hakura *et al*. (1998)
			5 / 14	+	8.5 / 83.8	9.9	Yamada *et al*. (2002)
Chlorambucil	Intraperitoneal	10	1 / 7	−	3.2 / 5.5	1.72	Hoorn *et al*. (1993)
			1 / 7	+	2.6 / 5.5	2.12	Myhr (1991)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
Cyclophosphamide	Intraperitoneal	500	5 / 7	+	1.5 / 8.2	5.47	Hoorn *et al*. (1993)
			5 / 7	+	2.6 / 8.2	3.15	Myhr (1991)
N-Ethyl-*N*-nitrosourea (ENU)	Intraperitoneal	100	1 / 10	−	3 / 8.6	2.87	Myhr (1991)
			1 / 10	+	2.6 / 135	51.92	Itoh, Miura and Shimada (1998)
			1 / 10	+	2.5 / 8.7	3.48	Hoorn *et al*. (1993)
			1 / 7	+	3.7 / 22.3	6.03	Suzuki *et al*. (1997a)
			1 / 7	+	3.7 / 25.6	6.92	Suzuki, Hayashi and Sofuni (1994)
			1 / 7	+	3.7 / 22.8	6.16	Suzuki *et al*. (1993)
			1 / 7	+	2.5 / 61.5	24.6	Hoorn *et al*. (1993)
			1 / 3	+	3 / 5.7	1.9	Myhr (1991)
			1 / 3	+	2.5 / 6	2.4	Hoorn *et al*. (1993)
			5 / 3	+	3 / 10	3.33	Myhr (1991)
			5 / 3	+	2.5 / 10.8	4.32	Hoorn *et al*. (1993)
			5 / 7	+	2.5 / 13	5.2	Hoorn *et al*. (1993)
			5 / 7	+	3 / 11.4	3.8	Myhr (1991)
			5 / 10	+	2.5 / 52.3	20.92	Hoorn *et al*. (1993)
			5 / 10	+	3 / 50	16.67	Myhr (1991)
		150	1 / 3	+	4.1 / 95.1	23.2	Mientjes *et al*. (1998)
			1 / 3	+	9.6 / 61.5	6.41	Collaborative Study Group for the Transgenic Mouse Mutation Assay (1996)
			1 / 14	+	4.1 / 56.9	13.88	Mientjes *et al*. (1998)
			1 / 14	+	4.1 / 78	19.02	Collaborative Study Group for the Transgenic Mouse Mutation Assay (1996)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
		250	5 / 10	+	2.5 / 101.6	40.64	Hoorn *et al*. (1993)
			5 / 10	+	7.3 / 254.4	34.85	Douglas *et al*. (1996)
			5 / 10	+	3 / 100	33.33	Myhr (1991)
			5 / 3	+	3 / 34.3	11.43	Myhr (1991)
			5 / 3	+	2.5 / 37.9	15.16	Hoorn *et al*. (1993)
			5 / 7	+	3 / 57.1	19.03	Myhr (1991)
			5 / 7	+	2.5 / 62.4	24.96	Hoorn *et al*. (1993)
			5 / 7	+	2.6 / 62.4	24	Myhr (1991)
			1 / 7	+	3 / 14.3	4.77	Myhr (1991)
			1 / 7	+	2.6 / 16.4	6.31	Myhr (1991)
			1 / 10	+	3 / 27.1	9.03	Myhr (1991)
			1 / 10	+	2.5 / 29.3	11.72	Hoorn *et al*. (1993)
N-Nitrosodibenzylamine (NDBzA)	Gavage	425	1 / 90	−	5.8 / 8.6	1.48	Jiao *et al*. (1997)
			1 / 90	−	4.2 / 6.6	1.57	Fung, Douglas and Krewski (1998)
			1 / 90	−	7.3 / 11.5	1.58	Fung, Douglas and Krewski (1998)
	Gavage	750	1 / 90	−	5.8 / 9	1.55	Jiao *et al*. (1997)
			1 / 90	−	4.2 / 6.3	1.5	Fung, Douglas and Krewski (1998)
			1 / 90	−	7.3 / 15.4	2.11	Fung, Douglas and Krewski (1998)
Procarbazine hydrochloride	Intraperitoneal	1 000	5 / 10	+	4.1 / 65	15.85	Pletsa *et al*. (1997)
			5 / 10	+	1.5 / 51.5	34.33	Hoorn *et al*. (1993)
			5 / 7	+	1.5 / 77.2	51.47	Hoorn *et al*. (1993)
			5 / 7	+	2.6 / 77.2	29.69	Myhr (1991)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
C) Big Blue® mouse – Liver							
Dimethylnitrosamine (DMN)	Gavage	10	1 / 7	+	9.8 / 18.7	1.91	Tinwell, Lefevre and Ashby (1994a)
			1 / 7	+	2.4 / 11.2	4.67	Ashby *et al*. (1994)
	Intraperitoneal	10	1 / 14	+	8.3 / 13	1.57	Tinwell *et al*. (1995)
			1 / 14	+	6.1 / 13.3	2.18	Tinwell *et al*. (1995)
			1 / 14	+	1.4 / 6.2	4.43	Tinwell *et al*. (1995)
			1 / 14	+	0.7 / 6	8.57	Suzuki *et al*. (1996a)
			5 / 8	−	4 / 3	0.75	Mirsalis *et al*. (1993)
			5 / 8	+	4.2 / 35.8	8.52	Mirsalis *et al*. (1993)
	Intraperitoneal	20	5 / 14	+	2.9 / 25.2	8.69	Mirsalis *et al*. (1993)
			5 / 14	+	4.6 / 20.4	4.43	Mirsalis *et al*. (1993)
			5 / 14	+	4.8 / 19.9	4.15	Mirsalis *et al*. (1993)
			5 / 14	+	2.8 / 20.8	7.43	Mirsalis *et al*. (1993)
	Intraperitoneal	30	5 / 15	+	5 / 28.6	5.72	Shane *et al*. (2000c)
			5 / 15	+	5 / 28.6	5.72	Shane *et al*. (1999)
			5 / 15	+	10 / 31	3.1	Cunningham *et al*. (1996)
			5 / 15	+	5.7 / 31.2	5.47	Hayward *et al*. (1995)
			5 / 14	+	2.9 / 37.8	13.03	Mirsalis *et al*. (1993)
			5 / 14	+	4.6 / 27.2	5.91	Mirsalis *et al*. (1993)
			5 / 14	+	4.8 / 24.8	5.17	Mirsalis *et al*. (1993)
			5 / 14	+	2.8 / 15.7	5.61	Mirsalis *et al*. (1993)
N-Ethyl-*N*-nitrosourea	Intraperitoneal	250	1 / 10	+	7.4 / 17.1	2.31	Piegorsch *et al*. (1995)

Table 4-18 (continued)

Chemical	Route of administration	Total dose (mg/kg bw)	Administration time / sampling time (days)	Result	MF control / MF treatment	Fold increase	References
(ENU)			1 / 10	+	6.4 / 16.9	2.64	Piegorsch *et al*. (1995)
			1 / 10	+	4.7 / 14.1	3	Piegorsch *et al*. (1995)

MF, mutant/mutation frequency

Data from five laboratories were evaluated in detail. The results suggested that only scattered patterns of excess variability below the animal-to-animal level occur, but that, generally, excess variability is observed at the animal-to-animal level (Piegorsch *et al*., 1997). Statistical tests that may be used to reduce variability have been suggested (Carr and Gorelick, 1994, 1995; Piegorsch *et al*., 1995) and are components of the recommended test protocol described in Chapter 6 (Section 6.1.3.6; see also Heddle *et al*., 2000).

Table 4-18 shows comparative results for several chemicals in Muta™Mouse liver and bone marrow and in Big Blue® mouse liver. These results are obtained from querying the TRAID and are not the result of any collaborative study. Muta™Mouse liver and bone marrow and Big Blue® mouse liver are the only combinations where significant experimental data are currently available to allow comparisons between studies. The majority of the chemicals examined are strong mutagens and produce, as expected, positive results in the TGR assays. Among these are a small number of instances in which ENU returned inconsistent results when very short sampling times were used. However, overall, the data in Table 4-18 strongly suggest that similar qualitative results are obtained in different studies and that the results are reproducible.

4.9.1.1 Conclusions

The results of studies carried out on a given chemical using similar experimental protocols suggest that the TGR assays show good qualitative reproducibility in both somatic and germ cells and quantitative reproducibility of a limited range of conditions and laboratories. The data are insufficient to allow conclusions to be drawn regarding the quantitative reproducibility of the assays over a wider range of conditions.

4.9.2 Ex vivo and in vitro mutations

TGR mutation assays require the replication of the isolated DNA in a bacterial host; thus, there is the possibility of *ex vivo* and *in vitro* mutations influencing the observed mutant frequency. *Ex vivo* mutations are mutations arising in the bacterium from lesions present in the isolated DNA. These mutations represent a bacterial, not a mammalian, response to the DNA damage and are thus artefactual. Similarly, *in vitro* mutations that arise spontaneously during growth of the phage to form a plaque are also artefacts.

Ex vivo or *in vitro* mutations may produce sectored, mosaic plaques or pinpoint plaques on X-Gal indicator plates. Paashuis-Lew, Zhang and Heddle (1997) established the theoretical possibility that *ex vivo* mutations may arise in the Big Blue® system by treating isolated λLIZα shuttle vector with ENU, infecting an *E. coli* SCS-8 host and examining mutagenesis in the *lacI* gene. They observed a ten-fold increase in mutant frequency as a result of treatment and found that these mutations gave mosaic (but not sectored) plaques (Paashuis-Lew, Zhang and Heddle, 1997). Nishino, Buettner and Sommer (1996) sequenced pinpoint mutants, in which the mutation clearly arose in the bacterium; these results showed that *ex vivo* mutations exhibit a mutation spectrum that is distinct from circular plaques, providing strong evidence that most circular plaques are derived from mutational events occurring in the mouse.

Although there exists a theoretical possibility that *ex vivo* and *in vitro* mutations may arise, several observations suggest that these types of mutations are extremely rare. First, these mutations will be minimised when experiments employ multiple dose regimens and proper numbers of animals. Second, *E. coli* host cells used to detect mutant transgenes are *recA*⁻, which greatly reduces the mutagenic potential of DNA lesions derived from many mutagenic agents. Third, the positive selection systems using *lacZ*, *gpt*, Spi⁻ and *cII* genes are unlikely to detect *ex vivo* and *in vitro* mutations, since cells containing the wild-type phages will be selected against before the mutations arise. Fourth, in animal experiments, the frequency of mutants in most tissues rises with time after treatment, often for several weeks, whereas the frequency of *ex vivo* mutations should be highest soon after treatment and then decline (Skopek *et al*., 1996; Paashuis-Lew, Zhang and Heddle, 1997; Sui *et al*., 1999; Swiger *et al*., 1999). Thus, there is a general consensus among researchers using TGR

systems that *ex vivo* or *in vitro* mutations are unlikely to contribute in any significant way to spontaneous or induced mutant frequencies in a properly conducted TGR assay. It should be noted that recent experiments carried out in gene *A* in phage isolated from splenic lymphocytes of ΦX174 transgenic mice are not consistent with the general consensus described above, at least for the ΦX174 model (Valentine *et al*., 2004).

4.9.2.1 Conclusions

Although there exists a theoretical possibility that *ex vivo* and *in vitro* mutations may arise during the course of a TGR experiment, these types of mutations are expected to be extremely rare in a properly conducted experiment using the major TGR models.

4.9.3 Comparison of induced responses in transgenes and endogenous genes

The transgenic loci are different from endogenous sequences, which raises the question of whether these differences cause the mutational response of the transgene to differ from that of endogenous genes. Each lambda genome is about 50 kb, and the phage-based TGR systems have between 20 and 80 tandemly integrated copies (see Section 2.2), for a total of about 1–4 megabases (Mb) of DNA; much smaller patches of foreign DNA (~20 copies of pUR288 for a total of ~100 kb) are present in the *lacZ* plasmid mouse. In Muta™Mouse, the additional foreign DNA is sufficient to make chromosome 3, where the integration occurred, longer than chromosome 2 and to give a recognisable cytogenetic band (Blakey *et al*., 1995). Furthermore, this DNA is classic heterochromatin with an out-of-phase condensation cycle at meiosis (Moens *et al*., 1997) and may not be passed on at the normal Mendelian rate (Heddle *et al*., 1995). The transgenes appear to be transcriptionally inactive, as indicated by heavy methylation of CpG sites and an absence of mRNA.

An important question is whether similar responses to mutagens are obtained in transgenes and endogenous genes. Since every locus has its own characteristic mutation spectrum and mutation rate, and since large differences exist between endogenous loci in mammalian cells, some differences between the endogenous and transgenic loci should be expected. As discussed in Section 4.6.6, the spontaneous mutant frequency of endogenous genes appears to be significantly lower than the spontaneous mutant frequency of transgenes, at least in lymphocytes and the small intestine.

Data relating to comparative responses of transgenes and endogenous genes to mutagens can be found in Tables 4-19 and 4-20 and are discussed in the sections below.

4.9.3.1 Transgenes versus Hprt in lymphocytes

Data showing the relative responses of transgenes and *Hprt* to the same total dose of several different mutagens are shown in Table 4-19. In Big Blue® mice, benzo(a)pyrene, cyclophosphamide, ENU and MNU have been examined in both *lacI* and *Hprt*; in addition, benzo(a)pyrene and MNU have been examined in *cII*. With MNU, *Hprt* mutation frequency increased in a dose-related fashion up to the highest dose tested, at which dose the mutant frequency was elevated 78-fold as compared with the spontaneous mutant frequency. In contrast, the *lacI* mutant frequency was increased only about three-fold at the maximum dose, whereas mutant frequencies in the *cII* gene of the integrated λ vector were not increased above the spontaneous rate. In a similar experiment, benzo(a)pyrene induced mutations equally effectively at both the *lacI* and *Hprt* loci; relatively small increases were observed at *cII* (Skopek *et al*., 1996; Monroe *et al*., 1998).

Table 4-19. Comparison of mutant/mutation frequencies in transgene versus endogenous *Hprt* gene in lymphocytes of transgenic rats and mice[a]

TGR system	Chemical	Total dose (mg/kg bw)	References	Transgene MF con trol	Transgene MF treatment	Transgene Fold increase	Transgene IMF	Transgene Result	*Hprt* MF con trol	*Hprt* MF treatment	*Hprt* Fold increase	*Hprt* IMF	*Hprt* Result
Big Blue® mouse	Benzo(a)pyrene	50	Skopek *et al*. (1996)	1.8	6.2	3.4	4.4	+	0.19	0.28	1.5	0.09	−
		150			15.6	8.7	13.8	+		1.1	5.8	0.91	+
	Cyclophosphamide	25	Walker *et al*. (1999a)	7.7	6.7	0.9	−1	−	0.1	0.39	3.9	0.29	+
		100			9.4	1.2	1.7	−		0.75	7.5	0.65	+
	N-Ethyl-*N*-nitroso-urea (ENU)	4.5	Skopek, Kort and Marino (1995)	2	3	1.5	1	−	0.18	0.94	5.2	0.76	+
		13.5			6.7	3.4	4.7	+		3.9	21.7	3.72	+
		40			15.5	7.8	13.5	+		11.6	64.4	11.42	+
			Walker *et al*. (1996)	3.4	11.4	3.4	8	+	0.37	6	16.2	5.63	+
	N-Methyl-*N*-nitrosourea (MNU)	5	Monroe *et al*. (1998)	2.7	3.5	1.3	0.8	−	0.2	0.44	2.2	0.24	−
		10			2.8	1	0.1	−		1.8	9	1.6	+
		15			3.5	1.3	0.8	−		7.3	36.5	7.1	+
		20			7.8	2.9	5.1	+		15.5	77.5	15.3	+
Big Blue® mouse *cII*	Acrylamide	532	Manjanatha *et al*. (2006b)	2.84	2.1	0.74	−0.74	−	0.22	1.1	5	0.88	+
		700		2.65	2.6	0.98	−0.05	−	0.15	0.66	4.4	0.51	+
		2 058		2.84	5.72	2.01	2.88	+	0.22	4.1	18.6	3.88	+
		2 247		2.65	5.9	2.23	3.25	+	0.15	3.26	21.7	3.11	+
	Benzo(a)pyrene	50	Skopek *et al*. (1996); Monroe *et al*. (1998)	5.5	4.8	0.9	−0.7	−	0.19	0.28	1.5	0.09	−
		150			7.4	1.3	1.9	−		1.1	5.8	0.91	+
	Glycidamide	700	Manjanatha *et al*. (2006b)	2.84	2.35	0.83	−0.49	−	0.22	1.26	5.7	1.04	+
		980		2.65	3.35	1.26	0.7	−	0.15	0.95	6.3	0.8	+
		2 464		2.84	6.72	2.37	3.88	+	0.22	3.36	15.3	3.14	+

TGR system	Chemical	Total dose (mg/kg bw)	References	Transgene MF control	Transgene MF treatment	Transgene Fold increase	Transgene IMF	Transgene Result	*Hprt* MF control	*Hprt* MF treatment	*Hprt* Fold increase	*Hprt* IMF	*Hprt* Result
		3 108		2.65	6.02	2.27	3.37	+	0.15	5.1	34	4.95	+
	Leucomalachite green	7 952	Mittelstaedt *et al.* (2004)	44.3	76	1.72	31.7	+	0.1	0.12	1.2	0.02	−
	Malachite green	8 736	Mittelstaedt *et al.* (2004)	44.3	55	1.24	10.7	−	0.1	0.17	1.7	0.07	−

Table 4-19 (continued)

TGR system	Chemical	Total dose (mg/kg bw)	References	Transgene MF control	Transgene MF treatment	Transgene Fold increase	Transgene IMF	Transgene Result	*Hprt* MF control	*Hprt* MF treatment	*Hprt* Fold increase	*Hprt* IMF	*Hprt* Result
	N-Methyl-*N*-nitrosourea (MNU)	20	Monroe *et al.* (1998)	5.7	2.7	0.5	−3	−	0.2	15.5	77.5	15.3	+
Big Blue®	17β-Oestradiol	49	Manjanatha *et al.* (2006a)	2.95	3.95	1.34	1	−	0.96	1.99	2.07	1.03	−

Table 4-19 (continued)

TGR system	Chemical	Total dose (mg/kg bw)	References	Transgene MF control	Transgene MF treatment	Transgene Fold increase	Transgene IMF	Transgene Result	*Hprt* MF control	*Hprt* MF treatment	*Hprt* Fold increase	*Hprt* IMF	*Hprt* Result
	7,12-Dimethyl-benzanthracene (7,12-DMBA)	20	Manjanatha *et al.* (1998)	2.8	7.7	2.8	4.9	−	0.24	1.84	7.7	1.6	+
				2.5	10.1	4	7.6	+	0.71	2.67	3.8	1.96	+
				3.8	6.7	1.8	2.9	−	0.74	5.86	7.9	5.12	+
				4.8	7.8	1.6	3	−	0.61	4.14	6.8	3.53	+
		75		2.8	7.7	2.8	4.9	−	0.24	3.46	14.4	3.22	+
				2.5	22.1	8.8	19.6	+	0.71	4.12	5.8	3.41	+
				3.8	15.5	4.1	11.7	+	0.74	10.96	14.8	10.22	+
				4.8	13.7	2.9	8.9	+	0.61	5.81	9.5	5.2	+
	Daidzein	2 464	Manjanatha *et al.* (2006a)	2.95	3.3	1.12	0.35	−	0.96	0.73	0.76	−0.23	−
		9 856		2.95	4.15	1.41	1.2	−	0.96	0.54	0.56	−0.42	−
		80	Manjanatha *et al.* (2006a)	2.95	26.35	8.93	23.4	+	0.96	14.73	15.3	13.77	−
		130	Manjanatha *et al.* (1998)	2.8	13.7	4.9	10.9	+	0.24	3.91	16.3	3.67	−
				2.5	34.1	13.6	31.6	+	0.71	6.96	9.8	6.25	−
				3.8	19.9	5.2	16.1	+	0.74	16.77	22.7	16.03	−
				4.8	26.6	5.5	21.8	+	0.61	12.51	20.5	11.9	−
	Genistein	2 464	Manjanatha *et al.* (2006a)	2.95	3.42	1.16	0.47	−	0.96	0.4	0.42	−0.56	−
		9 856		2.95	2.7	0.92	−0.25	−	0.96	1.18	1.23	0.22	−
	N-Hydroxy-2-acetylaminofluorene	25	Chen *et al.* (2001a)	2.6	9.8	3.77	7.2	+	0.33	1.41	4.27	1.08	−
									0.32	0.86	2.69	0.54	+
									0.31	0.66	2.13	0.35	−
									0.28	0.49	1.75	0.21	−

Table 4-19 (continued)

TGR system	Chemical	Total dose (mg/kg bw)	References	Transgene MF control	MF treatment	Fold increase	IMF	Result	*Hprt* MF control	MF treatment	Fold increase	IMF	Result
									0.12	0.36	3.00	0.24	−
		50		2.6	15.6	6	13	+	0.12	0.61	5.08	0.49	−
									0.28	1.1	3.93	0.82	−
									0.31	0.68	2.19	0.37	−
									0.32	0.68	2.13	0.36	−
									0.33	1.37	4.15	1.04	−
		100		2	5.6	2.8	3.6	+	0.32	1.65	5.16	1.33	+
									0.12	0.95	7.92	0.83	−
									0.31	0.98	3.16	0.67	−
									0.28	1.51	5.39	1.23	+
									0.33	1.24	3.76	0.91	−
				2.6	40.7	15.65	38.1	+	0.32	1.65	5.16	1.33	+
									0.12	0.95	7.92	0.83	−
									0.31	0.98	3.16	0.67	−
									0.28	1.51	5.39	1.23	+
									0.33	1.24	3.76	0.91	−
	Thiotepa	16.8	Chen *et al*. (1998)	3.5	14.1	4	10.6	+	0.35	4.11	11.7	3.76	+
Muta™-Mouse	*N*-Ethyl-*N*-nitrosourea	210	Cosentino and Heddle (2000)	3.5	10.5	3	7	+	0.8	0.8	0	0	−
				2	7	3.5	5	+	0.8	0.8	0	0	−
		415		3.5	24	6.86	20.5	+	0.8	2.5	3.13	1.7	+
				2	10.5	5.25	8.5	+	0.8	2.5	3.13	1.7	+
		620		3.5	33	9.43	29.5	+	0.8	1.1	1.38	0.3	−

Table 4-19 (continued)

TGR system	Chemical	Total dose (mg/kg bw)	References	Transgene					*Hprt*				
				MF control	MF treatment	Fold increase	IMF	Result	MF control	MF treatment	Fold increase	IMF	Result
				3.5	18	5.14	14.5	+	0.8	1.1	1.38	0.3	−

IMF, induced mutant/mutation frequency; MF, mutant/mutation frequency

[a] All mutant/mutation frequencies are expressed × 10^{-5}.

Table 4-20. Comparison of mutant/mutation frequencies (MF) in transgene versus endogenous *Dlb-1* gene in the small intestine of transgenic rats and mice[a]

				Transgene				*Dlb-1*			
TGR system	Chemical	Total dose (mg/kg bw)[b]	References	MF control	MF treatment	Fold increase	Result	MF control	MF treatment	Fold increase	Result
Big Blue® mouse	2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)	360	Zhang *et al*. (1996a)	7	7.5	1.1	−	2.3	18	7.8	+
		720		5.6	28	5	+	2.4	37	15.4	+
		900		7	13	1.9	−	2.3	41	17.8	+
		1 080		2.2	35	15.9	+	2.2	31	14.1	+
		1 440		7	37	5.3	+	2.3	53	23.0	+
		2 880		5.6	69	12.3	+	2.4	100	41.7	+
		4 320		2.2	133	60.5	+	2.2	138	62.7	+
	A-alpha-C	2 880	Zhang *et al*. (1996a)	3.5	116	33.1	+	3.1	3	0.97	−
		4 320		3.5	94	26.9	+	3.1	5.2	1.7	−
	Methylmethanesulphonate (MMS)	100	Tao, Urlando and Heddle (1993b)	25.6	25	1.0	−	47	34	0.7	−
		1 000			58.4	2.3	+		137	2.9	+
	N-Ethyl-*N*-nitrosourea (ENU)	100	Zhang *et al*. (1996a)	3.5	50	14.3	+	2.3	20.5	8.9	+
		250	Tao, Urlando and Heddle (1993a)	5	370	74.0	+	5	193	38.6	+
					220	44.0	+		200	40.0	+
					240	48.0	+		231	46.2	+
					250	50.0	+		246	49.2	+
					210	42.0	+		249	49.8	+
					260	52.0	+				
	X-ray	2 Gy	Tao, Urlando and Heddle (1993a)	5	10	2.0	+	3	5	1.7	−
		4 Gy			12	2.4	+		14	4.7	+
		6 Gy			10	2.0	+		26	8.7	+

TGR system	Chemical	Total dose (mg/kg bw)[b]	References	Transgene				*Dlb-1*			
				MF control	MF treatment	Fold increase	Result	MF control	MF treatment	Fold increase	Result
					18	3.6	+		41	13.7	+
					10	2.0	+		44	14.7	+
					10	2.0	+		56	18.7	+
					5	1.0	−		57	19.0	+
					20	4.0	+		66	22.0	+

Table 4-20 (continued)

TGR system	Chemical	Total dose (mg/kg bw)[b]	References	Transgene				*Dlb-1*			
				MF control	MF treatment	Fold increase	Result	MF control	MF treatment	Fold increase	Result
					5	1.0	−		68	22.7	+
gpt delta (*gpt*)	*N*-Ethyl-*N*-nitrosourea (ENU)	0.94	Swiger *et al*. (2001)	2.5	12	4.8	+	2.5	6.4	2.6	+
		2.82			40	16.0	+		108.5	43.4	+
		250			130	52	+		100	40	+
Muta™Mouse	5-Bromo-2′-deoxyuridine (BrdU)	2 500	Cosentino and Heddle (1999a)	3.1	4.4	1.4	−	1.2	3.3	2.8	+
		5 000			6	1.9	−		7.2	6.0	+
	Benzo(a)pyrene	10	Cosentino and Heddle (1999a, 2000)	3.1	3.9	1.3	−	1.2	4.8	4.0	+
		33.6		8	9	1.1	−	2	4	2.0	+
				8	15	1.9	−		6	3.0	+
		50		3.1	9.7	3.1	+	1.2	9.4	7.8	+
		67.2		8	10	1.3	−	2	4.4	2.2	+
		100		3.1	14.9	4.8	+	1.2	12.5	10.4	+
		100.8		8	14	1.8	−	2	6	3.0	+

Table 4-20 (continued)

TGR system	Chemical	Total dose (mg/kg bw)[b]	References	Transgene MF control	MF treatment	Fold increase	Result	*Dlb-1* MF control	MF treatment	Fold increase	Result
				8	20	2.5	+		8	4.0	+
		134.4		8	13	1.6	−		6.4	3.2	+
				8	26	3.3	+		21	10.5	+
		268.8		8	27	3.4	+		24	12.0	+
	Ethylmethanesulphonate (EMS)	50	Cosentino and Heddle (1999a)	3.1	3.3	1.1	−	1.2	2.8	2.3	+
		100			4.2	1.4	−		4.4	3.7	+
		250			5.9	1.9	−		5.2	4.3	+
	Methylmethanesulphonate (MMS)	50	Cosentino and Heddle (1999a)	3.1	2.5	0.8	−	1.2	2.9	2.4	+
		100			4.1	1.3	−		3.2	2.7	+
		150			5.8	1.9	−		4.1	3.4	+
	Mitomycin-C	1	Cosentino and Heddle (1999a)	3.1	2.1	0.7	−	1.2	2.3	1.9	+
		2			3.1	1.0	−		2.4	2.0	+
		4			4.1	1.3	−		5.2	4.3	+

Table 4-20 (continued)											
				Transgene				*Dlb-1*			
TGR system	Chemical	Total dose (mg/kg bw)[b]	References	MF control	MF treatment	Fold increase	Result	MF control	MF treatment	Fold increase	Result
	N-Ethyl-*N*-nitrosourea (ENU)	7.5	Cosentino and Heddle (1999b, 2000); Sun, Shima and Heddle (1999)	17	15	0.9	−	8.5	9.1	1.1	−
		15		26	14	0.5	−	15.3	8.4	0.5	−
		25		17	8	0.5	−	8.5	14	1.6	−
		30		26	20	0.8	−	15.3	16.8	1.1	−
		50			12	0.5	−		18.2	1.2	−
		75		17	10	0.6	−	8.5	18.2	2.1	+
		100		26	28	1.1	−	15.3	26.6	1.7	−
		150			23	0.9	−		28	1.8	−
		210		8	240	30.0	+	4.2	21	5.0	+
		250		7	138	19.7	+	1.3	193	148.5	+
					133	19.0	+		150	115.4	+
					144	20.6	+	2	188	94.0	+
				8	100	12.5	+	7	156	22.3	+
				17	110	6.5	+		175	25.0	+
				26	120	4.6	+		158	22.6	+
				32	115	3.6			154	22.0	+
		300		26	46	1.8	−	15.3	39.2	2.6	+
		415		8	370	46.3	+	4.2	48.3	11.5	+
		620			490	61.3	+		132.3	31.5	+
		1 250			1050	131.3	+	12.1	388	32.1	+
		1 850			1150	143.8	+		909	75.1	
	N-Methyl-*N*-nitrosourea	50	Cosentino and	3.1	225	72.6	+	1.2	229.5	191.3	+

Table 4-20 (continued)

				Transgene				*Dlb-1*			
TGR system	Chemical	Total dose (mg/kg bw)[b]	References	MF control	MF treatment	Fold increase	Result	MF control	MF treatment	Fold increase	Result
	(MNU)	75	Heddle (1999a)		278	89.7	+		302.5	252.1	+
		100			431	139.0	+		442.7	368.9	+

[a] All MF values expressed × 10^{-5}.

[b] Unless otherwise noted.

Cyclophosphamide induced a significant increase in mutant frequency in *Hprt* but not *lacI* of Big Blue® mouse lymphocytes; the differential response of the endogenous gene and the transgene was reflected in the mutation spectrum, which was different from the spontaneous spectrum in *Hprt*, but not *lacI*. The authors speculated that the toxicity of cyclophosphamide in lymphocytes, combined with the high spontaneous mutant frequency in *lacI*, may have reduced the sensitivity of the transgene towards the chemical (Walker *et al*., 1999a).

In Big Blue® rat, an extensive comparison of 7,12-DMBA mutagenesis in the *lacI* transgene and the endogenous *Hprt* gene showed that the endogenous locus was more sensitive. In splenic lymphocytes, *Hprt* mutant frequencies were increased significantly at all dose ranges between 20 and 130 mg/kg bw; however, only at the highest dose did 7,12-DMBA consistently induce the mutant frequency above the controls (Manjanatha *et al*., 1998). *N*-Hydroxy-2-acetylaminofluorene (Chen *et al*., 2001a) and thiotepa (Chen *et al*., 1998) increased the mutant frequency above control levels in both the *lacI* and *Hprt* genes of Big Blue® rat splenic lymphocytes. However, whereas the mutant frequency was highest for both chemicals at the *lacI* transgene, the fold increase was highest at the *Hprt* locus. This increase in sensitivity, apparent in both Big Blue® mouse and rat, is attributable to the very low spontaneous mutant frequency at the *Hprt* locus (Skopek, Kort and Marino, 1995).

In Muta™Mouse, ENU significantly increased the mutant frequency at both the *lacI* gene and *Hprt* locus at all doses evaluated. Quantitative comparisons between the responses at the different loci are difficult, because the data were presented in graphical form, and values were extrapolated from the graph (Cosentino and Heddle, 2000).

4.9.3.2 Transgenes versus Dlb-1 in small intestine

There are several experiments in which mutation in transgenes has been compared with that at the *Dlb-1* locus. However, the data presentation in many of these publications is graphical, and values were extrapolated from the graphs, making precise comparisons difficult. In Big Blue® mice, MMS and ENU elicited very similar dose-dependent increases in mutant frequency in both *lacI* and *Dlb-1* (Table 4-20). The response to X-rays was very different at the endogenous locus as compared with the *lacI* transgene: at the *Dlb-1* locus, a large dose-dependent increase in mutant frequency was induced by X-rays; in contrast, only small increases were observed in the *lacI* gene, and at a high (6 Gy) dose, there was no increase in two of the experiments (Tao, Urlando and Heddle, 1993a). The authors speculated that the difference was attributable to an inability of the *lacI* locus to detect large deletions induced by X-radiation that would extend into the vector and be lost. This cannot be confirmed, since the molecular nature of mutations at *Dlb-1* is unknown.

In Muta™Mouse, the *Dlb-1* locus appears to be somewhat more sensitive than the *lacZ* locus. BrdU, ethylmethanesulphonate (EMS), MMS and mitomycin-C all yielded negative results in the *lacZ* transgene at total doses that produced positive results at the *Dlb-1* locus. The absolute values of the mutant frequency did not differ markedly between the loci; however, the higher spontaneous mutant frequency in the transgene reduced the ability of the TGR assay to detect small increases in mutant frequency (Cosentino and Heddle, 1999a). Similar mutant frequencies were observed at *Dlb-1* and *lacZ* following benzo(a)pyrene treatment. However, lower fold increases were obtained at *lacZ* because of the relatively high spontaneous mutant frequency of the transgene (Cosentino and Heddle, 2000).

4.9.3.3 Cautionary note regarding comparative analyses

A simple comparison of relative fold increases (mutant frequency treated / spontaneous mutant frequency) in the TGR assay with those in endogenous gene assays may be misleading. Although most studies support the notion that the higher spontaneous mutant frequency observed in

transgenes results in a lowering of the fold increase of an induced response, it is also true that the absolute value of the induced response (the induced mutant frequency; see Table 4-19) is generally higher in TGR assays. Furthermore, a protocol that would yield optimal results in a TGR assay (see Chapter 6) is very different from that used in most of the comparative studies described in Sections 4.9.3.1 and 4.9.3.2. Finally, it should be noted that the ease of analysis of large numbers of mutations is very much greater in the TGR assays than is the case with the endogenous gene assays; thus, the sample size using TGR assays is generally larger, and smaller differences in mutant/mutation frequency (mutant frequency treated *vs.* spontaneous mutant frequency) are likely to be statistically significant in the TGR assays as compared with the endogenous gene mutation assays.

4.9.3.4 Conclusions

The weight of evidence suggests that transgenes and endogenous genes respond in approximately the same manner to mutagens in the few instances where direct comparisons have been attempted. The higher spontaneous mutant frequency in transgenes tends to decrease the fold increase associated with mutagenic treatments and reduce the sensitivity of detection in transgenes as compared with endogenous genes (see also Section 4.9.6.3.5 below); however, the induced mutant frequency tends to be greater with TGR assays. In addition, with the exception of the *lacZ* plasmid mouse and the *gpt* delta (Spi$^-$) rodents, mutagens that induce deletions are likely to be detected more easily in certain endogenous genes than in transgenes, owing to the nature of the genetic targets.

4.9.4 Sensitivity

The sensitivity of an assay for the detection of mutagenicity is dependent upon the magnitude of the induced response relative to the background and the variability inherent to a system. Given the generally good reproducibility of TGR assays, the sensitivity of the assays is, therefore, heavily influenced by the spontaneous mutant frequency. Evidence for this has been reviewed in Sections 4.9.3.1 and 4.9.3.2: the spontaneous mutant frequency in transgenes is five- to ten-fold higher than that observed at the endogenous *Hprt* or *Dlb-1* loci. In several instances (see also Tables 4-19 and 4-20), an identical treatment regimen produced a statistically significant increase in mutant frequency at an endogenous locus but not in a transgene. Indeed, it has been estimated that because of the relatively high spontaneous mutant frequency at the transgene, mutagenic insults that produce up to a five-fold increase in mutation frequency at the endogenous *Hprt* locus may be difficult to detect at the *lacI* transgene (Skopek, Kort and Marino, 1995). However, comparisons of TGR loci and endogenous loci should be approached with caution (see Section 4.9.3.3 above). It should be noted that although the experimental design of most recent studies provides an adequate statistical framework for determining a positive outcome, this has not always been the case. Often, the two-fold rule – that a positive response would be at least two times the historical negative control mutant frequency (Thybaud *et al.*, 2003) – has been invoked in making a decision regarding test outcome. A considerable number of all experiments carried out have used this method for establishing a positive result. This method of determining test outcome is extremely sensitive to the high spontaneous mutant frequency of transgenes.

While the transgenic targets may be relatively less sensitive to acute treatments, the existing data suggest that the sensitivity of transgenes may be improved by increasing the administration time (Section 4.7). Since mutations in the neutral transgene appear to accumulate linearly with the number of daily administrations given to an animal, longer administration times should induce more mutations with time and increase both the induced mutant frequency and the fold increase in treated animals. Clearly, such an experimental strategy will, in many cases, require changes in dosing due to toxicity.

4.9.4.1 Conclusions

Sensitivity is determined in large part by the spontaneous mutant frequency: the higher spontaneous mutant frequency in transgenes appears to reduce their sensitivity, especially when acute

treatments are used. The sensitivity of transgenes can be enhanced by increasing the administration time.

4.9.5 Specificity

A critical factor in establishing the suitability of a bioassay is the confidence that can be applied to a negative result. A chief advantage of the TGR systems is that it is possible to examine mutation in virtually all tissues; this empowers the investigator with a capacity to examine those tissues that are of greatest concern for a particular exposure. However, the flexibility associated with the TGR assays also presents an important dilemma, since it is not clear how many tissues, and which tissues, should be examined to establish a negative result. Clearly, many of the same considerations that apply to any conventional genotoxicity test (*e.g.* appropriate route of administration, dosage, tissue exposure, metabolism, DNA adduct formation) should also be applied to a properly conducted TGR assay. In addition, it will be important to consider the biology of the tissues being studied – for example, the cellular composition of the tissue and the kinetics of cell renewal. These factors will be crucial in evaluating whether the experimental design is sufficiently robust to determine whether a compound is not genotoxic in the TGR assay.

A very high proportion of the TGR experiments carried out to date have examined the activity of compounds that are known to be strong mutagens. Almost 60% of the experimental records in the database have returned positive results, despite the fact that an optimal protocol has been used in very few experiments.

4.9.5.1 Conclusions

It is important to increase the data available for a range of chemicals that would include weak mutagens and non-mutagens, and also to increase the data available for tissues other than liver and bone marrow. In Chapter 5, the specificity of the TGR assay in relation to other genotoxicity assays is discussed in more detail.

4.9.6 Use of DNA sequencing

As DNA sequence analysis becomes less expensive and more automated, the number of studies that have exploited DNA sequencing has increased. To date, all of the TGR models have exploited molecular analysis, although the majority of such studies have used the Big Blue® mouse and rat systems. Table 4-21 provides examples of several studies in which DNA sequencing has provided information that is additional to that obtained from simple quantification of mutant frequencies.

4.9.6.1 Analysis of clonality

Sequencing data may be useful when high interindividual variation is observed. In these cases, sequencing can be used to rule out the possibility of jackpots or clonal events by identifying the proportion of unique mutants from a particular tissue. While true jackpots or clonal events that can be regarded as experimental artefacts have been reported (*e.g.* see Shane *et al*., 1999, 2000c; Singh *et al*., 2001; Manjanatha *et al*., 2004), they are, in fact, not very common in the literature, but may be extremely important when dealing with weak mutagens or long administration/sampling times. While removing an obvious outlier from a sample provides a simple, but potentially controversial, means for reducing animal-to-animal variation, sequencing and subsequent appropriate correction of mutant frequency for clonality allows the “rescue” of a sample value, so that the respective sample mutant frequency is not lost.

Table 4-21. DNA sequencing studies in transgenic rats and mice

Chemical	Gene/ selection	TGR model	Comments	References
1,10-Diazachrysene	*cII*	Muta™Mouse	G:C → A:T transitions were reduced and G:C → C:G transversions were increased.	Yamada *et al*. (2005)
1,2-Epoxy-3-butene	*lacI*	Big Blue® mouse	Metabolite of 1,3-butadiene spectra different from 1,3-butadiene.	Saranko *et al*. (2001)
1,3-Butadiene	*lacI*, *lacZ*	Big Blue® mouse, Muta™Mouse	Increased proportion of mutations at A:T sites; increased frequency of G:C → A:T transitions occurred at non-5′-CpG-3′ sites in spleen.	Recio *et al*. (1993, 1996, 2001); Sisk *et al*. (1994); Recio, Pluta and Meyer (1998)
1,6-Dinitropyrene	*gpt*	*gpt* delta mouse	A unique spectrum was not observed, although 1,6-dinitropyrene did increase mutant frequency.	Hashimoto *et al*. (2006)
1,7-Phenanthroline	*cII*	Muta™Mouse	G:C → A:T transitions were reduced and G:C → C:G transversions were increased.	Yamada *et al*. (2004)
17β-Oestradiol + 7,12-dimethylbenzanthracene (7,12-DMBA)	*lacI*	Big Blue® rat (ovariectomised)	A:T → T:A and G:C → T:A transversions were reduced over 7,12-DMBA alone, while G:C → A:T and A:T → G:C transitions were increased.	Manjanatha *et al*. (2005)
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	*lacI*	Big Blue® rat	No change in mutant frequency or mutation spectrum.	Thornton *et al*. (2001)
2.45 GHz radiofrequency	*lacZ*	Muta™Mouse	No mutagenic effect.	Ono *et al*. (2004)
2-Amino-1-methyl-6-phenyl-imidazo(4,5-b)pyridine (PhIP)	*lacZ*	Big Blue® mouse	PhIP mutation signature *in vivo* is very similar to that observed for the *Hprt* and *DHFR* loci in hamster and human cells *in vitro.*	Lynch *et al*. (1998)
	gpt, Spi	*gpt* delta mouse	PhIP induces point mutations, such as base substitutions and single base pair deletions, rather than larger deletions *in vivo*; homopolymeric sequences may play an important role in PhIP-induced base pair deletions.	Masumura *et al*. (2000)

Chemical	Gene/ selection	TGR model	Comments	References
	lacI, cII	Big Blue® rat	Analysis of the mutation spectrum in the two transgenes, including consideration of the number of mutational target sequences in each gene and nearest-neighbour analyses of mutated nucleotides, indicates that PhIP-induced mutational specificity is similar in both genes.	Okonogi *et al*. (1997a); Stuart *et al*. (2000c)
	lacI	Big Blue® rat	PhIP-induced mutations in the mammary glands showed higher frequency of G:C → T:A transversions and lower frequency of G:C deletions as compared with the colon.	Okochi *et al*. (1999)
	lacI	Big Blue® rat	Predominant class of induced mutation was −1 frameshift involving the loss of G:C base pairs, followed by G:C → T:A transversions and G:C → A:T transitions.	Yang *et al*. (2003)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
PhIP + conjugated linoleic acid (CLA)	*lacI*	Big Blue® rat	CLA in diet reduced mutant frequency by 38%. Mutation spectrum showed selective inhibition of −1 frameshifts and G:C → A:T transitions by CLA, suggesting involvement of mismatch repair.	Yang *et al*. (2003)
PhIP + high-fat diet	*lacI*	Big Blue® rat	Mutation spectrum in mammary gland unaltered by diet. High proportion of mutations at 5′-CAG(Pu)-3′ sites suggests target site for PhIP–guanine adduct-induced mutations *in vivo* in the mammary gland.	Yu *et al*. (2002)
PhIP hydrochloride	*cII*	Muta™Mouse	The predominant type of induced mutation was G:C → T:A transversions.	Itoh *et al*. (2003)
2-Amino-3,4-dimethyl-imidazo(4,5-f)quinoline (MeIQ)	*lacI*	Big Blue® mouse	Induced mutation spectra were different in bone marrow and liver, suggesting a tissue-specific mechanism of mutagenesis.	Ushijima *et al*. (1994)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
2-Amino-3,8-dimethyl-imidazo(4,5-f)quinoxaline (MeIQx)	*lacI*	Big Blue® rat	Frameshifts involving G:C base pairs were the predominant mutation, followed by G:C → T:A transversions.	Hoshi *et al*. (2004)
	gpt	*gpt* delta mouse	G:C → T:A transversions were the predominant induced mutation.	Masumura *et al*. (2003a)
2-Amino-3-methylimidazo(4,5-f)quinoline (IQ)	*lacI*	Big Blue® rat	The *lacI* mutation spectra of the liver, colon and kidney from IQ-treated rats were similar. These were characterised by an increase in G:C → T:A transversions in the liver and colon and an increase in the proportion of 1 bp G:C deletions in the liver and kidney	Bol *et al*. (2000)
	gpt	*gpt* delta mouse	The predominant induced mutation type was G:C → T:A transversion.	Kanki *et al*. (2005)
3-Nitrobenzanthrone	*cII*	Muta™Mouse	G:C → A:T transitions were reduced and G:C → C:G transversions were increased.	Arlt *et al*. (2004)
4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)	*cII*	Muta™Mouse	The predominant type of induced mutation was A:T → T:A transversions.	Hashimoto, Ohsawa and Kimura (2004)
4,10-Diazachrysene	*cII*	Muta™Mouse	G:C → A:T transitions were reduced and G:C → C:G transversions were increased.	Yamada *et al*. (2005)
4-Chloro-*o*-phenylenediamine	*lacI*	Big Blue® mouse	Dose-related increase in G:C → T:A transversions.	Staedtler *et al*. (1999)
5-(2-Chloroethyl)-2′-deoxyuridine (CEDU)	*lacZ*	Muta™Mouse	A:T → G:C transitions were increased, particularly in 5′-(G or C)-T-G-3′ motifs.	Staedtler, Suter and Martus (2004)
6-Nitrochrysene	*cII*	Big Blue® rat	Induced mutation spectrum was dominated by A:T → G:C transitions, A:T → T:A transversions, G:C → T:A transversions, G:C → C:G transversions. DNA adducts detected were consistent with the mutational specificities.	Boyiri *et al*. (2004)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
7,12-Dimethylbenzanthracene (7,12-DMBA)	*lacI*	Big Blue® rat (ovariectomised)	Induced A:T → T:A and G:C → T:A transversions dominated the spectrum.	Manjanatha *et al*. (2005)
	lacI	Big Blue® mouse	Topical 7,12-DMBA treatment induced primarily A:T→T:A transversions, consistent with major mutation found in mutated *ras* genes in 7,12-DMBA-induced tumours.	Gorelick *et al*. (1995)
(7,12-DMBA ± genistein) ± ovariectomy	*cII*	Big Blue® rat	7,12-DMBA was the only treatment variable resulting in mutations. G:C → T:A transversions, A:T → G:C transitions, A:T → T:A transversions were induced, consistent with the types of adducts reported elsewhere.	Chen *et al*. (2005a)
7-Methoxy-2-nitronaphtho(2,1-b)furan (R7000)	*lacI*	Big Blue® mouse	Frameshifts and transversion at G:C base pairs.	Arrault *et al*. (2002)
Acrylamide	*cII*	Big Blue® mouse	The predominant type of induced mutation was G:C → T:A transversions and +1/−1 frameshifts in homopolymeric runs of G.	Manjanatha *et al*. (2006b)
Aflatoxin B1	*lacI*	Big Blue® mouse, Big Blue® rat	Comparison between rat and mouse mutation spectra.	Dycaico *et al*. (1996)
Aflatoxin B1 ± TCDD	*lacI*	Big Blue® mouse	Aflatoxin induced an increase in G:C → T:A transversions, which were ablated by TCDD treatment in females but not males.	Thornton *et al*. (2004)
alpha-Hydroxytamoxifen	*cII*	Big Blue® rat	Induced mutation spectrum different from spontaneous but similar to tamoxifen. Study suggests that alpha-hydroxytamoxifen is a major proximate tamoxifen metabolite that mediates tamoxifen mutagenicity.	Chen *et al*. (2002)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
Aminophenylnorharman	*gpt*	*gpt* delta mouse	G:C → T:A transversions and G:C → A:T transitions; single- and two-base deletions in GC base pairs were the predominant mutation.	Masumura *et al*. (2003b)
	Spi	*gpt* delta mouse	Single base pair deletions in G:C base pairs; large deletions were rare.	Masumura *et al*. (2003b)
Aristolochic acid	*cII*	Muta™Mouse	Increased A:T → T:A transversions in target organs.	Kohara *et al.* (2002a)
	cII	Big Blue® rat	A:T → T:A transversions were increased significantly over control.	Chen *et al*. (2006)
Benzo(a)pyrene	*lacZ*	Muta™Mouse	Similar mutation spectra in two target organs; the predominant mutations were G:C → T:A transversions and deletions. In contrast, the mutation spectra in the two non-target organs were different from those in the target organs, suggesting an organ/tissue-specific mechanism of mutagenesis.	Hakura *et al*. (2000)
	lacI	Big Blue® mouse	Altered spectrum of mutations suggested benzo(a)pyrene-induced mutagenesis under conditions where no statistical increase in mutant frequency could be shown.	Shane *et al*. (2000a)
	gpt	*gpt* delta mouse	G:C → T:A transversions and single-base deletions in GC base pairs were the predominant mutations.	Hashimoto *et al*. (2005)
Benzo(a)pyrene diolepoxide (BPDE)	*lacI*	Big Blue® mouse	Approximately 88% of all mutations and 100% of base substitutions were at G:C sites (35% were G:C → A:T transitions, 36% were G:C → T:A transversions and 29% G:C → C:G transversions); 60% of all mutations and 70% of the base substitution mutations occurred at CpG sites.	Miller *et al*. (2000)
Benzo(f)quinoline	*cII*	Muta™Mouse	A slight increase in mutant frequency was not manifested as a unique mutation spectrum.	Yamada *et al*. (2004)
Benzo(h)quinoline	*cII*	Muta™Mouse	A slight increase in mutant frequency was not manifested as a unique mutation spectrum.	Yamada *et al*. (2004)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
Bitumen fumes	*cII*	Muta™Mouse	Spectrum confirms lack of genotoxicity.	Micillino *et al*. (2002)
	cII	Big Blue® rat	No increase in mutations or change in mutation spectrum.	Bottin *et al*. (2006)
Chrysene	*cII*	Muta™Mouse	G:C → A:T transitions were reduced and G:C → C:G transversions were increased.	Yamada *et al*. (2005)
Cisplatin	*lacZ*	*lacZ* plasmid mouse	G:C → A:T transitions at GpG and ApG sites, and single base pair deletions/insertions.	Louro, Silva and Boavida (2002)
Coal tar	*lacZ*	Muta™Mouse	The mutation spectrum was consistent with a significant contribution of benzo(a)pyrene and other polycyclic aromatic hydrocarbon mixtures.	Vogel *et al*. (2001)
Comfrey	*cII*	Big Blue® rat	G:C → T:A transversions were the predominant mutation; spectrum was statistically different from controls.	Mei *et al*. (2005a)
Cyclophosphamide	*lacI*	Big Blue® mouse	Cyclophosphamide-induced mutations (A:T → T:A transversions and deletions) are detectable in the *lacI* transgene in the target tissues, but not in non-target tissues for cyclophosphamide-induced cancer.	Gorelick *et al*. (1999)
Dichloroacetic acid (DCA)	*lacI*	Big Blue® mouse	Increased proportion of mutations at A:T sites.	Leavitt *et al*. (1997)
Dimethylarsinic acid	*cII*	Muta™Mouse	No significant change in the *cII* mutation spectra.	Noda *et al*. (2002)
Dimethylnitrosamine (DMN)	*lacZ*	Muta™Mouse	Increase in frequency of G:C → A:T transitions; significant increase in deletions of up to 11 bp.	Souliotis *et al*. (1998)
	lacI	Big Blue® mouse	Three weeks of age: increased mutant frequency and G:C → A:T transitions, predominantly at non-CpG sites; 6 weeks of age: no change in mutant frequency or mutation spectrum. Therefore, age-dependent change in sensitivity to DMN.	de Boer, Mirsalis and Glickman (1999)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
	lacI, *cII*	Big Blue® mouse	Large increase in A:T → T:A transversions, single-base deletions and >4 bp deletions in *lacI* is not found in *cII*. Postulate that fewer sites are available for certain classes of mutation in *cII*.	Shane *et al*. (2000b)
Dinitropyrenes	*cII*	Muta™Mouse	Dinitropyrenes treatment increased the incidence of G:C → T:A transversion and decreased G:C → A:T transitions, consistent with major guanine-C8 adduct.	Kohara *et al*. (2002b)
Ethylene oxide	*lacI*	Big Blue® mouse	A:T → T:A transversions were increased significantly over control.	Recio *et al*. (2004)
Ethylmethanesulphonate (EMS)	*lacZ*	Muta™Mouse	Small number of mutants sequenced: G:C → A:T transitions, which were probably caused by O^6-ethylguanine.	Suzuki *et al*. (1997a)
Fasciola hepatica	*lacI*	Big Blue® mouse	Increase in mutant frequency in infected animals. Mutation spectrum roughly corresponded to the spectrum of spontaneous mutations (uninfected) in liver cells except for the significant increase in complex changes and multiple mutations.	Motorna *et al*. (2001)
Gamma rays	*lacI*	Big Blue® mouse and *Ogg1*$^{-/-}$	The frequency of G:C → T:A transversions in neonatal brain was increased by gamma radiation in wild-type mice. G:C → T:A transversions were also increased in unexposed *Ogg1*$^{-/-}$ *mice.* Gamma irradiation of *Ogg1*$^{-/-}$ mice resulted in the greatest increase in G:C → T:A mutations.	Larsen *et al*. (2006)
	Spi	*gpt* delta mouse	Detailed description of deletions and rearrangements; concludes that most rearrangements induced by gamma rays in mice are mediated by illegitimate recombination through DNA end-joining.	Nohmi *et al*. (1999)
	Spi	*gpt* delta mouse	Deletions of less than 100 bp and base substitutions.	Masumura *et al*. (2002)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
Gamma rays (chronic low dose)	*lacI*	Big Blue® mouse	No mutagenic effect and no unique spectrum reported.	Wickliffe *et al*. (2003)
Glycidamide	*cII*	Big Blue® mouse	The predominant type of induced mutation was G:C → T:A transversions and +1/−1 frameshifts in homopolymeric runs of G.	Manjanatha *et al*. (2006b)
Heavy-ion radiation	Spi	*gpt* delta mouse	Deletions that were mainly more than 1 000 bp in size.	Masumura *et al*. (2002)
	lacZ	*lacZ* plasmid mouse	Southern hybridisation analysis of mutations (approximate size change).	Chang *et al*. (2001b)
	lacZ	*lacZ* plasmid mouse	Southern hybridisation analysis of mutations (approximate size change); examined effect of *p53* genetic background.	Chang *et al*. (2001a)
Hexavalent chromium	*lacI*	Big Blue® mouse	Similarity to the spontaneous mutation spectrum in mouse lung, consistent with the generation of oxidative-type DNA damage by Cr(VI).	Cheng *et al*. (2000)
Leucomalachite green	*lacI*	Big Blue® rat	Increased mutant frequency after 16 weeks. However, when corrected for clonality, the 16-week *lacI* mutation frequency was not significantly different from control frequency. Furthermore, the *lacI* mutation spectrum in treated rats was not significantly different from that found for control rats. Therefore, the increase in *lacI* mutant frequency observed previously may be due to the disproportionate expansion of spontaneous *lacI* mutations.	Manjanatha *et al*. (2004)
	cII	Big Blue® mouse	G:C → T:A and A:T → T:A transversions were induced with the spectrum being statistically different from control.	Mittelstaedt *et al*. (2004)
Mitomycin-C	Spi	*gpt* delta mouse	Large deletions frequently occurred between two short direct repeat sequences, suggesting that they are generated during the end-joining repair of double-strand breaks induced by interstrand cross-links in DNA.	Okada *et al*. (1999); Takeiri *et al*. (2003)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
	gpt	*gpt* delta mouse	Induction of tandem-base substitutions, such as 5′-GG-3′ to 5′-AT-3′; highlights the relevance of intrastrand cross-links as genotoxic lesions.	Takeiri *et al*. (2003)
N-Ethyl-*N*-nitrosourea (ENU)	*lacZ*	Muta™Mouse	Confirms role of thymine adducts in ENU mutagenesis.	Suzuki *et al*. (1997a)
	lacI	Big Blue® mouse	Increased proportion of mutations at A:T sites: confirms role of thymine adducts in ENU mutagenesis. Study compares mutation spectra in transgene and endogenous gene (*Hprt*).	Walker *et al*. (1996)
	lacZ	Muta™Mouse	A:T → T:A transversions and A:T → G:C transitions were prominent in both liver and bone marrow. Mutation spectra similar in different organs.	Douglas *et al*. (1996)
	gpt	*gpt* delta mouse	Spectrum similar to spectra in *lacI* (Walker *et al*., 1996) and *lacZ* (Douglas *et al*., 1996) genes.	Masumura *et al*. (1999b)
	cII	Big Blue® rat	Typical spectra for ENU were observed, with A:T → T:A transversions predominating, and the spectrum was statistically significant. There was no difference between the spectra in neonatal and adult animals.	Mei *et al*. (2005b)
	cII	Big Blue® mouse	Prenatal and neonatal brains, but not adult, showed the classic ENU mutation spectrum. A:T → T:A and G:C → T:A transversions and A:T → G:C transitions were elevated.	Slikker, Mei and Chen (2004)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
N-Hydroxy-2-acetylamino-fluorene	*lacI*	Big Blue® rat	Mutation spectra from *N*-hydroxy-2-acetylaminofluorene-treated rats differed significantly from corresponding mutation profiles from untreated animals. Although there were similarities among the mutational patterns derived from *N*-hydroxy-2-acetylaminofluorene-treated rats, there were significant differences in the patterns of base pair substitution and frameshift mutation between the liver and spleen lymphocyte *lacI* mutants (apparent tissue specificity).	Chen *et al*. (2001b)
N-Methyl-*N*-nitrosourea (MNU)	*cII*	Muta™Mouse	Induced spectrum was primarily G:C → A:T transition at non-CpG sites; no difference in the induced mutation spectrum between animals fed normal or restricted diets.	Shima, Swiger and Heddle (2000)
N-Nitrosopyrrolidine	*gpt*	*gpt* delta mouse	The predominant induced mutation type was A:T → G:C transition.	Kanki *et al*. (2005)
o-Aminoazotoluene	*cII*	Muta™Mouse	Primarily G:C → T:A transversions.	Kohara *et al*. (2001)
Oxazepam	*lacI*	Big Blue® mouse	Small increase in mutant frequency; sequencing of a mutant from each oxazepam-exposed mouse showed a significant difference in the mutation spectrum compared with that from control mice. Authors postulate that some of the mutations found in the oxazepam-derived spectrum were due to oxidative damage elicited by induction of CYP2B isozymes as the result of chronic oxazepam administration.	Shane *et al*. (1999)
	cII	Big Blue® mouse	After clonal correction, mutation frequency was not different from control. Mutation spectrum was not different from control. Authors speculate that *cII* locus is less sensitive than the *lacI* locus to mutation induction by non-DNA-reactive carcinogens.	Singh *et al*. (2001)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
Peroxyacetyl nitrate (PAN)	*lacI*	Big Blue® mouse	Difference in mutation spectrum relative to *Salmonella.*	DeMarini *et al*. (2000)
Phenobarbital	*cII*	Big Blue® mouse	Mutation spectrum at *cII* from the phenobarbital-fed mice was significantly different from that of the control mice, even though the mutant frequency was not.	Singh *et al*. (2001)
	lacI	Big Blue® mouse	Modest increase in mutant frequency that was not significant following clonal correction. Mutation spectrum obtained from the phenobarbital-exposed group was significantly different from control. Authors postulate that the increase in transversions at G:C base pairs found in the phenobarbital-derived spectrum is likely due to oxidative damage as a result of induction of CYP2B isozymes by the chronic administration of phenobarbital.	Shane *et al*. (2000c)
Procarbazine	*lacZ*	Muta™Mouse	Among 20 mutants analysed, only six G:C → A:T transitions (characteristic of O^6-methylguanine miscoding) were found, including three at CpG sites, which might have arisen from deamination of 5-methylcytosine. Suggests that bone marrow mutations do not arise primarily through miscoding by O^6-methylguanine.	Pletsa *et al*. (1997)
Quinoline	*cII*	Muta™Mouse	Quinoline is genotoxic in its target organ, and G:C → C:G transversion is the molecular signature of quinoline-induced mutations.	Suzuki *et al*. (2000)
Riddelliine	*cII*	Big Blue® rat	G:C → T:A transversions and tandem base substitutions were increased over controls, spectrum was not statistically significant (Mei *et al*., 2005a).	Mei *et al*. (2004a)
	cII	Big Blue® rat	G:C → T:A transversions and tandem base substitutions (G:G → T:T and G:G → A:T); the induced spectrum was significantly different from control.	Mei *et al*. (2004b)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
Sucrose	*cII*	Big Blue® rat	While sucrose increased the frequency of mutants, a unique spectrum was not observed.	Hansen *et al*. (2004)
Tamoxifen	*lacI*, *cII*	Big Blue® rat	Mutation spectrum was characterised by increased frequency of G:C → T:A transversions at 5′-CpG-3′ dinucleotide (CpG) sites in the *lacI* gene. G:C → T:A transversions also induced in *cII* gene but not at CpG sites. Insertions of base pairs and deletions of pairs of G:C bases were also observed.	Davies *et al*. (1996, 1997, 1999)
Thiotepa	*lacI*	Big Blue® rat	Comparison of mutation spectrum in endogenous gene and transgene. Majority of thiotepa-induced base pair substitutions in the *Hprt* gene occurred with the mutated purine on the non-transcribed DNA strand, while no strand-related bias was found for mutations in the *lacI* gene (effect of transcription-coupled repair in endogenous gene). Substitutions at G:C base pairs in the *lacI* gene, but not in the *Hprt* gene, were found disproportionately in CpG sites.	Chen *et al*. (1998)
Tris(2,3-dibromo-propyl)phosphate	*lacI*	Big Blue® rat	Tris(2,3-dibromopropyl)phosphate-specific change in mutation spectrum in kidney, which was not observed in liver and stomach.	de Boer *et al*. (1996)
Urethane	*cII*	Big Blue® mouse	G:C → A:T transitions were the predominant mutation.	Hernandez and Forkert (2007a)
UVB	*lacZ*	Muta™Mouse	UV-induced mutation spectrum was dominated by G:C → A:T transitions at dipyrimidine sites, consistent with many other studies using different mutation systems.	Frijhoff *et al*. (1997)
	Spi	*gpt* delta mouse	UVB irradiation induces deletions in the murine epidermis; mutation spectrum suggested that most of the deletions are generated through end-joining of double-strand breaks in DNA.	Horiguchi *et al*. (2001)

Table 4-21 (continued)

Chemical	Gene/ selection	TGR model	Comments	References
	gpt	*gpt* delta mouse	Mutation spectrum was dominated by G:C → A:T transitions at dipyrimidine sites, such as 5′-TC-3′, 5′-CC-3′, and 5′-TC-CG-3. Tandem transitions such as C:C → T:T were also observed.	Horiguchi *et al*. (1999)
Vinyl carbamate	*cII*	Big Blue® mouse	A:T → G:C transitions and A:T → T:A transversions were increased.	Hernandez and Forkert (2007a)
Wyeth 14,643	*cII*	Big Blue® mouse	Mutant frequency increased significantly, but mutation spectrum was not altered relative to control.	Singh *et al*. (2001)
X-rays	Spi	*gpt* delta mouse	Various-sized deletions and base substitutions.	Masumura *et al*. (2002)
	lacZ	Muta™Mouse	Increased frequency of deletions and complex mutations. Endpoints of deletion mutations were different from spontaneous deletions, suggesting distinct mechanisms.	Ono *et al*. (1999)
	lacZ	*lacZ* plasmid mouse	Southern hybridisation analysis of mutations (approximate size change).	Gossen *et al*. (1995)
	lacZ	Muta™Mouse	The predominant mutations in multiple somatic tissues were deletions and complex lesions involving deletions and subsequent insertions. Deletions at low doses were lower in male germ cells.	Ono *et al*. (2003)

This approach requires the removal from the dataset of all but one mutant of a specific type located at a single site for each tissue in an animal and correction of the mutant frequency to account for those mutants that are discarded (*e.g.* see de Boer *et al*., 1997). The resulting mutation frequency provides a more precise estimate of both spontaneous and induced mutations.

As described in Section 2.3.2, there is a theoretical danger that the use of DNA sequencing for clonal correction may result in *overcorrection* of mutation frequency: since induced mutations often occur at mutational hotspots, a reduction in the magnitude of the calculated mutation frequency below the actual mutation frequency could result from the elimination of identical mutations derived from independent mutational events.

4.9.6.2 Weak responses

A number of studies have demonstrated significant differences in the mutation spectrum between a control group and a treatment group in cases where the comparison of mutant frequencies did not show any statistical differences (Shane *et al*., 2000a, 2000c; Singh *et al*., 2001). Thus, when equivocal results are obtained, sequencing may offer the opportunity to attribute a weak increase in mutant frequency to a specific mutation type. However, there is some consensus (Thybaud *et al*., 2003) that if this rare mutation does not result in a significant elevation in the overall mutant frequency, then it is insufficient to conclude a positive response.

4.9.6.3 Comparison of mutation spectra

4.9.6.3.1 Technical issues

Various methods are available for the analysis of mutation spectra (Adams and Skopek, 1987; Carr and Gorelick, 1996; Dunson and Tindall, 2000). These methods differ in their approach to analysis, such as a dose–response trend analysis versus a pairwise comparison of mutation types. The most popular method for statistical comparison of spectra is the algorithm developed by Adams and Skopek (1987). However, the use of mutation spectrum comparisons should be approached with some caution, since it is likely that large numbers of mutations from both control and treatment groups will have to be analysed in order to obtain meaningful results. For example, studies with potent mutagens that exhibit a distinctive mutational specificity suggest that it is appropriate to sequence at least 50 mutants per dose group (Thybaud *et al*., 2003); however, it would be necessary to sequence many more mutants if the mutagen was very weak, if the induced mutations were distributed across several classes or if the mutational specificity overlapped significantly with that of the control animals. In addition, the results of such studies are not always easy to interpret: considerable expertise in recognising a "mutational signature" of a treatment is generally required, in addition to knowledge of both target size and the *selectable* target size (see Section 4.3.1 above).

4.9.6.3.2 Mechanistic inferences

Sequencing may also be useful for providing mechanistic information about the biological mechanisms underlying mutation induction by specific mutagens. This is achieved by comparing mutation spectra of treated and negative control animals (see Section 4.9.6.3.1), after sequencing of a representative number of mutants. Mutation spectra frequently demonstrate a preponderance of mutations of a particular type; in some instances, these mutations occur at specific sites in the transgene, producing a "mutational signature" that is characteristic of a compound or a group of related compounds (see Table 4-21 for examples). In several instances, analysis of the mutation spectrum has implicated particular premutational lesions (*e.g.* Frijhoff *et al*., 1997; Pletsa *et al*., 1997; Suzuki *et al*., 1997a; Miller *et al*., 2000; Kohara *et al*., 2002b; Takeiri *et al*., 2003) that appear to be particularly important to mutagenesis. The important premutational lesions are not always the most prevalent lesions that may be detected using DNA adduct analysis. In addition, DNA sequencing

provides detailed information regarding the site of mutation, the surrounding DNA sequence and, in the case of deletions/insertions, the endpoints of rearrangements; therefore, information that implicates certain DNA repair processes in mutagenesis can sometimes be obtained from this analysis (Chen *et al*., 1998; Okada *et al*., 1999; Ono *et al*., 1999; Nohmi *et al*., 1999; Horiguchi *et al*., 2001; Takeiri *et al*., 2003; Yang *et al*., 2003).

In comparisons of mutagenesis between tissues, there are examples of similar spectra (Douglas *et al*., 1996; Bol *et al*., 2000), suggesting similar mutagenic mechanisms, and also examples in which different mutation spectra have been characterised in different tissues (Ushijima *et al*., 1994; Okochi *et al*., 1999; Hakura *et al*., 2000), indicative of tissue-specific mutagenic processes (perhaps different lesions, different DNA repair or different processing during trans-lesion DNA synthesis). However, it should be noted that in many of the DNA sequencing studies carried out to date, a relatively small number of mutations have been sequenced. Under these circumstances, the mechanistic interpretation of the results should be approached with caution.

4.9.6.3.3 Examination of active constituents of a mixture

Since different compounds or classes of compounds often produce distinctive mutation spectra, it is often possible to determine what mutagenic components are present in a mixture. For instance, coal tar is a complex mixture of aromatic and aliphatic hydrocarbons. In the *lacZ* gene of Muta™Mouse, the mutation spectrum of coal tar was found to primarily induce G:C → T:A transversions and 1 bp deletions of G:C base pairs (Vogel *et al*., 2001) – a spectrum that was very similar to that of benzo(a)pyrene (Hakura *et al*., 2000) in the same gene. This implicates benzo(a)pyrene and related polycyclic aromatic hydrocarbons as the active components of mutagenic coal tar.

4.9.6.3.4 Analysis of metabolites

DNA-reactive derivatives of chemicals are often produced through metabolism. Comparison of the mutation spectrum of a metabolite with that of the parent compound can provide evidence as to the likelihood of a particular metabolic pathway being associated with metabolic activation. For instance, the mutation spectrum of alpha-hydroxytamoxifen is the same as that of tamoxifen in the *lacI* gene of the Big Blue® rat, consistent with the notion that alpha-hydroxytamoxifen is a major proximate tamoxifen metabolite in the liver of rats treated with tamoxifen (Chen *et al*., 2002). A similar approach has been used to attempt to elucidate which metabolite of 1,3-butadiene-1,2-epoxybutene, 1,2-epoxy-3,4-butanediol or 1,2:3,4-diepoxybutane, is responsible for the genotoxic activity of the parent compound (Recio *et al*., 2001).

4.9.6.3.5 Comparison of transgenes with endogenous genes

There have been a small number of studies in which the mutation spectra of transgenes have been compared with those of endogenous genes. Some differences between loci are to be expected based on the considerations outlined in Section 4.3.1 and the different transcriptional activity of endogenous loci and transgenes. Some correction for these factors can be carried out by consideration of the number of mutational target sequences in each gene and nearest-neighbour analyses of mutated nucleotides (Okonogi *et al*., 1997a; Stuart *et al*., 2000c). In most cases, there appears to be reasonable similarities in the mutation spectra in the different targets. For example, the PhIP mutation signature *in vivo* in the *lacI* gene is very similar to that observed for the *Hprt* and *DHFR* loci in hamster and human cells, respectively (Lynch *et al*., 1998). In a comparison of ENU mutagenesis in the *lacI* transgene and the endogenous *Hprt* gene, it was shown that although the *lacI* gene was less sensitive than the endogenous gene, owing to the much higher spontaneous background (see Section 4.9.3.1), the mutation spectrum of ENU was similar in both genes (Walker *et al*., 1996).

Slightly different conclusions were obtained from the study of thiotepa: the majority of thiotepa-induced base pair substitutions in the *Hprt* gene occurred with the mutated purine on the non-transcribed DNA strand, whereas no strand-related bias was found for mutations in the *lacI* gene – reflecting the activity of transcription-coupled repair on actively expressed genes, but not unexpressed transgenes. In addition, substitutions at G:C base pairs in the *lacI* gene, but not in the *Hprt* gene, were found disproportionately in CpG sites (Chen *et al*., 1998).

4.9.6.3.6 Characteristics of genotoxicants that do not appear to react with DNA

A variety of compounds appear to be genotoxic, despite the fact that they do not induce any direct damage in DNA. In the case of at least two chemicals, phenobarbital and oxazepam, DNA sequence analysis has revealed a spectrum of mutations consistent with that of oxidative damage in DNA that is a result of induction of cytochrome P-450 2B (CYP2B) isozymes as the result of chronic administration of CYP2B inducers (Shane *et al*., 2000c; Singh *et al*., 2001).

4.9.6.4 Conclusions

1) DNA sequence analysis of mutants is laborious and may add to the cost of the experiment; sequencing would not normally be required when testing drugs or chemicals for regulatory applications, particularly where a clear positive or negative result is obtained.
2) Sequencing data may be useful when high interindividual variation is observed and can help to rule out the possibility of jackpot mutations and to correct mutant frequency for clonality.
3) Mutation spectrum differences may provide additional information when weak responses are obtained for a given treatment; however, identification of a rare mutational event in the absence of a significant increase in mutant frequency is insufficient to conclude a positive test outcome.
4) Comparison of mutation spectra can be useful in a number of contexts: a) to deduce mechanism; b) to obtain information about active components of a mixture; c) to identify proximate mutagenic metabolites of a compound; d) to compare responses in transgenes and endogenous genes; and e) for the study of genotoxicants that do not react with DNA. Proper comparisons require attention to experimental detail, including the characterisation of a sufficient number of mutants, and appropriate statistical analysis.

5.0 THE PREDICTIVE ABILITY OF TRANSGENIC RODENT MUTATION ASSAYS

5.1 Introduction

DNA damage caused by chemical agents has a high degree of relevance to human health, as it may be the initiating event in the multi-step process of carcinogenesis or in the production of germ cells that may transmit a heritable genetic disease to the subsequent generation. It is for this reason that a variety of tests capable of identifying compounds that are genotoxic have been developed over the past 30 years and are now routine in the preclinical or premanufacturing phase of compound development, as required by regulatory agencies worldwide.

Typically, most short-term genotoxicity tests may be classified into two broad categories – those that detect gene mutations and those that detect chromosomal mutations (clastogenicity) – and are usually combined into test batteries to facilitate the detection of a broad spectrum of genotoxic effects. Although both *in vitro* and *in vivo* tests are components of standard test batteries, the results of *in vivo* assays are believed to have greater relevance to humans because of the interactions of metabolic and other toxicokinetic factors that are not easily or reliably reproduced *in vitro*. For this reason, the results of the *in vivo* tests are usually considered definitive when they contradict an *in vitro* test result addressing the same endpoint, with due consideration for target tissue exposure. While a typical test battery could include a *Salmonella* reverse mutation assay (gene mutations), an *in vitro* mouse lymphoma assay (gene mutations and, by inference, chromosomal mutations), an *in vitro* mammalian cell cytogenetic assay (chromosomal aberrations) and an *in vivo* cytogenetic assay such as the rodent erythrocyte micronucleus assay (chromosomal mutations), for many years there was no practical or reliable *in vivo* assay for gene mutations. The development of TGR gene mutation assays has provided an *in vivo* follow-up test to the *in vitro* gene mutation assays (*Salmonella*, *in vitro* mouse lymphoma or other mammalian cell gene mutation assays) that could provide significant refinements in genotoxicity testing.

In addition to the obvious prediction of heritable germline mutations, the results of short-term genetic toxicity studies are commonly used as predictors of the potential carcinogenic activity of a chemical. Identification of genotoxic activity of a new product in preclinical/premanufacture testing often leads to either the compound being dropped from further development or regulatory action by a government authority, since it is often assumed that, in the absence of additional information, a genotoxic compound is also carcinogenic. Because of the consequences of falsely identifying a chemical as genotoxic, particularly for new, potentially useful therapeutic agents, it is essential that any new genotoxicity tests be thoroughly characterised for their accuracy in identifying genotoxicants. In light of the widespread use of these tests as indicators of carcinogenic potential, it is also useful to determine the predictive ability of a new genotoxicity assay for carcinogenicity. It should be noted, however, that genotoxicity and carcinogenicity are only associated, and there are numerous examples of carcinogens that are active by non-DNA-reactive (non-genotoxic) mechanisms; as such, the assessment of the benefits or utility of any genotoxicity test solely on the basis of its carcinogen predictive ability is without significant merit. Accordingly, it would be prudent to employ a system that most effectively detects genotoxicity in order to predict genotoxic carcinogens with the greatest efficiency.

This chapter presents the results of an analysis of the operational characteristics of the TGR assays. The first intent is to address the extent of the association between results in TGR assays and those of other short-term genotoxicity tests and the accuracy of the TGR assays in identifying gene mutations. The second aims to address the carcinogenic predictive ability of TGR assays compared with other tests and the predictive ability of various test batteries. This analysis was facilitated by the use of the TRAID, which contains records for all published (and some unpublished) TGR mutagenesis experiments that were identified in a comprehensive search of the literature (see Section 4.2.1). In addition to the results of TGR assays, the database contains results from other common short-term assays, including the *Salmonella* reverse mutation assay, the *in vitro* chromosomal aberration assay,

the mouse lymphoma *Tk*$^{+/-}$ assay, the *in vivo* chromosomal aberration assay, the *in vivo* unscheduled DNA synthesis assay, the *in vivo* rodent erythrocyte micronucleus assay and the comet assay. Overall, a chemical was concluded to be positive in a short-term assay or in the rodent carcinogenicity bioassay if a single positive result (as defined by the original study author or database curator) was reported in any of these sources, irrespective of the number of negative results also reported.

Although this analysis is comprehensive, it is important to note the inherent limitations that arise from the use of this dataset. Among the 238 agents that have been examined using TGR assays, there are records for 140 agents whose carcinogenicity to the mouse or rat has been determined. In addition, 13 agents that were used as vehicle controls in TGR assays, which are also commonly used as vehicles in short-term genotoxicity and carcinogenicity studies, were included as non-genotoxic non-carcinogens: carboxymethylcellulose, corn oil, methylcellulose, olive oil, phosphate buffer, propylene glycol, saline, sesame oil, sodium bicarbonate, soy oil, tricaprylin, trioctanoin and water. Table 5-1 provides a summary of the genotoxicity test results (excluding the 13 vehicle controls) for the 118 rodent carcinogens and 22 rodent non-carcinogens contained in the database. Including the negative control chemicals, the total number of non-carcinogens in this evaluation is 35.

Within this subset of agents, very few have been evaluated with a full set of short-term genotoxicity tests; among those for which genotoxicity tests have been conducted, a majority of agents have returned positive (mutagenic) results in one or more short-term tests. These include 112/161 (70%) for the *Salmonella* reverse mutation assay, 81/100 (81%) for the *in vitro* chromosomal aberration assay, 54/66 (82%) for the *in vitro* mouse lymphoma assay, 36/52 (69%) for the *in vivo* chromosomal aberration assay, 63/91 (69%) for the *in vivo* micronucleus assay, 29/45 (64%) for *in vivo* unscheduled DNA synthesis, 47/52 (90%) for the comet assay and 174/250 (70%) for the TGR assay. Because many of the published TGR studies were intended to investigate specific mechanistic questions, the database is composed disproportionately of model mutagenic carcinogens. As a result, conclusions regarding the performance of TGR assays made from the analysis of these data, as with other genotoxicity tests, may not be broadly generalisable, because the prevalence of mutagens/carcinogens in the database is substantially greater than expected universally.

5.2 Questions for investigation

Using data contained in the TRAID for a number of chemicals tested using a variety of commonly used short-term assays, TGR assays and rodent carcinogenicity, questions within the following two general areas could be investigated: 1) performance of TGR assays in identifying genotoxic agents and 2) predictivity of TGR assays for rodent carcinogenicity.

5.2.1 Performance of TGR assays in identifying genotoxic agents

TGR assays are genotoxicity tests, and the principal benchmark of any such assay's (or test battery's) performance should be its ability to correctly identify genotoxicants. The following questions are addressed specifically:

1) What is the relationship between results of the various short-term assays?

Table 5-1. Summary of the chemical records in the Transgenic Rodent Assay Information Database (TRAID)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
1,10-Diazachrysene	+	nd	nd	nd	nd	nd	nd	nd	1	11	+	nd
1,2:3,4-Diepoxybutane	+	+	+	+	nd	+	+	+	9	0	−	+
1,2-Dibromo-3-chloropropane	+	+	+	nd	+	+	+	+	1	1	+	+
1,2-Dibromoethane	+	+	+	+	−	−	+	+	7	1	+	+
1,2-Dichloroethane	+	nd	nd	+	nd	−	nd	+	11	0	−	+
1,2-Epoxy-3-butene	+	−	nd	−	+	+	nd	+	7	2	+	nd
1,3-Butadiene	+	nd	−	nd	+	+	−	+	4	15	+	+
1,4-Phenylenebis(methylene)-selenocyanate (p-XSC)	nd	nd	nd	nd	nd	nd	nd	nd	1	0	−	nd
1,6-Dinitropyrene	+	+	nd	nd	nd	nd	+	nd	2	1	+	+
1,7-Phenanthroline	+	nd	nd	nd	nd	nd	nd	nd	15	5	+	nd
1,8-Dinitropyrene	+	+	+	−	nd	nd	nd	nd	15	2	+	+
1.5 GHz electromagnetic near field	nd	nd	nd	nd	nd	nd	nd	nd	4	0	−	nd
10-Azabenzo(a)pyrene	+	nd	nd	nd	nd	nd	nd	nd	13	3	+	nd
^{114m}In internal radiation	nd	nd	nd	nd	nd	nd	nd	nd	5	1	+	nd
17β-Oestradiol	−	−	−	+	nd	−	nd	−	2	0	−	+
1-Chloromethylpyrene	+	nd	nd	nd	nd	nd	nd	nd	6	6	+	nd
1-Methylphenanthrene	+	nd	nd	nd	nd	nd	nd	nd	24	0	−	nd
1-Nitronaphthalene	+	+	nd	nd	nd	nd	nd	nd	3	0	−	−
2,3,7,8-Tetrachlorodibenzo-*p*-	−	−	+	+	−	−	nd	nd	2	0	−	+

	In vitro assays			*In vivo* assays								
								TGR				
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
dioxin (TCDD)												
2,4-Diaminotoluene	+	+	+	nd	nd	nd	+	+	5	6	+	+
2,6-Diaminotoluene	+	nd	nd	+	nd	+	+	−	7	0	−	−
2.45 GHz radiofrequency	−	−	−	nd	nd	+	nd	nd	4	0	−	−
2-Acetylaminofluorene (2-AAF)	+	+	+	+	+	+	+	+	12	16	+	+

Table 5-1 (continued)

	In vitro assays			*In vivo* assays								
								TGR				
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
2-Amino-1-methyl-6-phenyl-imidazo(4,5-b)pyridine (PhIP)	+	+	nd	nd	−	nd	nd	+	55	82	+	+
PhIP + 1,2-dithiole-3-thione (D3T)	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
PhIP + conjugated linoleic acid (CLA)	nd	nd	nd	nd	nd	nd	nd	nd	0	6	+	nd
PhIP + high-fat diet	nd	nd	nd	nd	nd	nd	nd	nd	1	1	+	nd
PhIP + low-fat diet	nd	nd	nd	nd	nd	nd	nd	nd	1	1	+	nd
2-Amino-3,4-dimethylimidazo(4,5-f)quinoline (MeIQ)	+	+	nd	+	nd	nd	nd	+	7	10	+	+
2-Amino-3,8-	+	−	nd	nd	+	−	nd	+	21	30	+	+

Table 5-1 (continued)

Chemical	*In vitro* assays				*In vivo* assays							
									TGR			
	Sal	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
dimethylimidazo(4,5-f)quinoxaline (MeIQx)												
2-Amino-3-methylimidazo(4,5-f)quinoline (IQ)	+	+	nd	nd	+	−	nd	+	8	14	+	+
IQ + sucrose	nd	nd	nd	nd	nd	nd	nd	nd	8	4	+	nd
2-Nitronaphthalene	+	nd	nd	+	nd	nd	nd	nd	1	2	+	nd
2-Nitro-*p*-phenylenediamine	+	+	+	nd	−	−	inc	nd	1	1	+	+
3-Amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2)	+	+	nd	nd	+	nd	nd	+	3	0	−	+
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	+	+	+	+	nd	+	+	+	17	0	−	+
3-Fluoroquinoline	−	nd	nd	nd	nd	nd	−	nd	3	0	−	−
3-Methylcholanthrene	+	+	+	nd	nd	inc	inc	nd	0	5	+	+
3-Nitrobenzanthrone	+	nd	nd	+	nd	+	nd	nd	4	2	+	+
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	+	nd	nd	nd	nd	nd	nd	+	2	19	+	+
NNK + 8-methoxypsoralen	nd	nd	nd	nd	nd	nd	nd	nd	2	0	−	−
NNK + green tea	nd	nd	nd	nd	nd	nd	nd	nd	2	3	+	nd
4,10-Diazachrysene	+	nd	nd	nd	nd	nd	nd	nd	0	12	+	nd
4-Acetylaminofluorene (4-AAF)	+	−	+	−	−	inc	−	nd	0	1	+	−
4-Aminobiphenyl	+	+	+	nd	nd	+	+	nd	5	14	+	+
4-Chloro-*o*-phenylenediamine	+	+	nd	nd	nd	+	nd	+	1	5	+	+

Table 5-1 (continued)

Chemical	*In vitro* assays				*In vivo* assays							
									TGR			
	Sal	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positiv e	Overall[b] result	Carc
4-Hydroxybiphenyl	+	nd	nd	nd	nd	nd	nd	nd	1	0	−	−
4-Monochlorobiphenyl	nd	nd	nd	−	nd	nd	nd	nd	0	1	+	nd
4-Nitroquinoline-1-oxide (4-NQO)	+	+	+	nd	nd	+	nd	+	15	31	+	+
4-NQO + alpha-tocopherol	nd	nd	nd	nd	nd	nd	nd	nd	0	6	+	nd
4-NQO + p-XSC	nd	nd	nd	nd	nd	nd	nd	nd	0	13	+	nd
4-NQO + p-XSC + alpha-tocopherol	nd	nd	nd	nd	nd	nd	nd	nd	0	6	+	nd
5-(2-Chloroethyl)-2′-deoxyuridine (CEDU)	+	nd	nd	−	nd	−	nd	nd	0	3	+	nd
5-(*p*-Dimethylaminophenyl-azo)benzothiazole	+	nd	nd	nd	nd	nd	+	nd	0	2	+	−
5,9-Dimethyldibenzo(c,g)carbazole (DMDBC)	nd	nd	nd	nd	nd	nd	nd	nd	10	20	+	+
DMDBC + carbon tetrachloride	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
DMDBC + phenobarbital	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
5-Bromo-2′-deoxyuridine (BrdU)	−	nd	nd	+	nd	+	nd	nd	2	0	−	+
5-Fluoroquinoline	+	nd	nd	nd	nd	nd	nd	nd	2	1	+	+
6-(*p*-Dimethylaminophenyl-azo)benzothiazole	nd	nd	nd	nd	nd	nd	+	nd	0	5	+	+
6,11-	nd	nd	nd	nd	nd	nd	nd	nd	2	1	+	inc

Table 5-1 (continued)

Chemical	*In vitro* assays				*In vivo* assays							
									TGR			
	Sal	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
Dimethylbenzo(b)naphtho(2,3-d)thiophene												
6-Nitrochrysene	+	nd	nd	nd	nd	nd	nd	nd	0	2	+	+
7,12-Dimethylbenzanthracene (7,12-DMBA)	+	+	+	nd	nd	+	+	+	23	60	+	+
7,12-DMBA + 17β-oestradiol	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
7,12-DMBA + daidzein	nd	nd	nd	nd	nd	nd	nd	nd	0	4	+	nd
7,12-DMBA + daidzein + genistein	nd	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
7,12-DMBA + genistein	nd	nd	nd	nd	nd	nd	nd	nd	0	7	+	nd
7,12-DMBA + high-fat diet	nd	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
7,12-DMBA + low-fat diet	nd	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
7H-Dibenzo(c,g)carbazole (DBC)	+	nd	nd	nd	nd	nd	nd	nd	0	6	+	+
7-Methoxy-2-nitronaphtho(2,1-b)furan (R7000)	+	nd	nd	nd	nd	nd	nd	nd	8	18	+	nd
87-966	nd	nd	nd	nd	nd	nd	nd	nd	1	2	+	nd
^{89}Sr-internal radiation	nd	nd	nd	nd	nd	nd	nd	nd	5	1	+	nd
A-alpha-C	+	+	nd	nd	nd	nd	nd	nd	2	4	+	+
Acetaminophen	−	+	nd	+	+	−	nd	nd	1	0	−	+
Acetic acid	−	nd	nd	nd	nd	nd	nd	nd	2	1	+	−
Acetone	−	−	nd	−	nd	−	nd	nd	2	0	−	−

Table 5-1 (continued)

Chemical	*In vitro* assays				*In vivo* assays							
									TGR			
	Sal	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
Acrylamide	−	+	+	nd	+	+	+	+	19	7	+	+
Acrylonitrile	+	+	+	nd	nd	−	−	+	15	0	−	+
Adozelesin	nd	nd	nd	nd	nd	nd	nd	nd	1	2	+	nd
Aflatoxin B1	+	+	nd	+	+	+	+	+	2	7	+	+
Aflatoxin B1 + phorone	nd	nd	nd	nd	nd	nd	nd	nd	1	2	+	nd
Aflatoxin B1 + TCDD	nd	nd	nd	nd	nd	nd	nd	nd	1	1	+	nd
Agaritine	+	−	−	nd	nd	nd	nd	nd	10	2	+	nd
all-*trans*-Retinol	+	nd	nd	nd	nd	nd	nd	nd	2	0	−	−
alpha-Chaconine	−	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
alpha-Hydroxytamoxifen	nd	nd	nd	nd	nd	nd	nd	nd	1	4	+	nd
alpha-Solanine	−	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
Aminophenylnorharman	+	nd	nd	nd	nd	nd	nd	nd	0	6	+	+
Amosite asbestos	−	+	nd	+	+	nd	nd	nd	11	4	+	+
Amosite asbestos + benzo(a)pyrene	nd	nd	nd	nd	nd	nd	nd	nd	4	5	+	+
AMP397	+	nd	−	+	nd	−	nd	nd	6	0	−	nd
Aristolochic acid	+	+	+	+	nd	+	nd	nd	9	17	+	+
Arsenite trioxide	nd	nd	+	nd	nd	+	nd	nd	5	0	−	+
Azathioprine	+	+	nd	nd	+	+	nd	nd	6	3	+	+
Benzene	−	+	+	−	+	+	−	+	8	4	+	+
Benzo(a)pyrene (B(a)P)	+	+	+	nd	nd	+	+	+	33	121	+	+

Table 5-1 (continued)

Chemical	*In vitro* assays				*In vivo* assays							
									TGR			
	Sal	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positiv e	Overall[b] result	Carc
B(a)P + D3T	nd	nd	nd	nd	nd	nd	nd	nd	0	6	+	nd
B(a)P + D3T + p-XSC	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
B(a)P + eugenol	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
B(a)P + green tea	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
B(a)P + lycopene	nd	nd	nd	nd	nd	nd	nd	nd	1	5	+	nd
B(a)P + p-XSC	nd	nd	nd	nd	nd	nd	nd	nd	0	5	+	nd
B(a)P + selenium-enriched yeast	nd	nd	nd	nd	nd	nd	nd	nd	0	6	+	nd
B(a)P + selenium-enriched yeast + D3T	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
Benzo(a)pyrene diolepoxide (BPDE)	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
BPDE + phorbol-12-myristate-13-acetate (TPA)	nd	nd	nd	nd	nd	nd	nd	nd	2	1	+	nd
Benzo(f)quinoline	+	+	nd	+	nd	nd	nd	nd	19	1	+	−
Benzo(h)quinoline	+	+	nd	nd	nd	nd	nd	nd	9	1	+	−
beta-Propiolactone	+	+	+	nd	+	+	nd	+	1	9	+	+
Bitumen fumes	nd	nd	nd	nd	nd	nd	nd	nd	3	0	−	nd
Bleomycin	+	+	+	+	+	+	nd	+	0	1	+	+
Carbon tetrachloride	−	+	nd	+	−	−	−	−	5	0	−	+
Carboxymethylcellulose*												
CC-1065	−	nd	nd	nd	nd	+	nd	nd	0	1	+	+
Chlorambucil	+	+	nd	nd	+	+	nd	+	18	22	+	+

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
Chloroform	+	−	+	nd	+	+	−	−	5	0	−	+
Chrysene	+	nd	nd	+	−	nd	nd	nd	4	8	+	+
Cisplatin	+	+	nd	+	+	+	nd	+	0	2	+	+
Clofibrate	−	+	nd	−	−	nd	−	nd	2	0	−	+
CM 44 glass fibres	nd	nd	nd	nd	nd	nd	nd	nd	8	0	−	nd
Coal tar	+	nd	nd	nd	nd	nd	nd	nd	1	1	+	+
Comfrey	−	nd	nd	nd	nd	nd	nd	nd	0	2	+	+
Conjugated linoleic acid (CLA)	nd	nd	nd	nd	nd	nd	nd	nd	3	0	−	nd
CLA + PhIP	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
Corn oil*												
Crocidolite asbestos	−	nd	nd	nd	nd	nd	nd	nd	5	4	+	+
Cyclophosphamide	+	+	+	nd	+	+	+	+	18	24	+	+
Cyproterone acetate	−	−	nd	nd	nd	+	+	nd	15	31	+	+
Daidzein	−	−	nd	+	nd	nd	nd	nd	2	0	−	nd
Daidzein + genistein	nd	nd	nd	nd	nd	nd	nd	nd	1	0	−	nd
Di(2-ethylhexyl)phthalate (DEHP)	−	−	−	−	−	−	−	−	8	2	+	+
Diallyl sulphide	−	+	nd	nd	nd	nd	nd	nd	1	0	−	nd
Diallyl sulphone	nd	nd	nd	nd	nd	nd	nd	nd	7	0	−	nd
Dichloroacetic acid (DCA)	+	+	+	nd	nd	inc	nd	nd	4	2	+	+
Dicyclanil	−	−	nd	nd	nd	−	nd	nd	1	1	+	+

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positiv e	Overall[b] result	Carc
Diesel exhaust	+	+	nd	nd	nd	−	nd	nd	24	10	+	+
Diethylnitrosamine (DEN)	+	+	+	nd	nd	−	+	+	22	29	+	+
DEN + phenobarbital	nd	nd	nd	nd	nd	nd	nd	nd	0	7	+	nd
Dimethylarsinic acid	−	nd	nd	+	+	−	nd	nd	5	0	−	+
Dimethylnitrosamine (DMN)	+	+	+	nd	nd	+	+	+	45	71	+	+
Dinitropyrenes	nd	nd	nd	nd	nd	nd	nd	+	6	10	+	nd
Dipropylnitrosamine (DPN)	+	+	nd	nd	nd	−	+	+	7	9	+	+
d-Limonene	−	−	−	nd	nd	nd	nd	nd	2	0	−	+
Ellagic acid	−	+	−	nd	nd	nd	nd	nd	1	0	−	−
Ethylene oxide	+	+	+	+	+	+	inc	nd	27	7	+	+
Ethylmethanesulphonate (EMS)	+	+	+	+	+	+	nd	+	12	5	+	+
Etoposide	+	+	+	+	+	+	nd	+	13	0	−	nd
Eugenol	−	+	+	nd	nd	+	−	nd	1	0	−	−
Fasciola hepatica	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
Ferric nitrilotriacetate	−	nd	+	nd	nd	nd	nd	nd	4	2	+	+
Flumequine	−	nd	nd	nd	−	nd	nd	nd	4	0	−	+
Folic acid	−	nd	nd	nd	nd	nd	nd	nd	8	0	−	−
Fructose	nd	nd	nd	nd	nd	nd	nd	nd	2	0	−	nd
Gamma rays	+	+	nd	nd	+	+	+	nd	19	10	+	+
Gamma rays + NNK	nd	nd	nd	nd	nd	nd	nd	nd	3	6	+	nd
Genistein	+	+	+	+	nd	−	nd	nd	4	0	−	+

Table 5-1 (continued)

Chemical	*In vitro* assays				*In vivo* assays							
									TGR			
	Sal	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
Glass wool fibres	−	nd	nd	nd	nd	nd	nd	nd	9	0	−	−
Glass wool fibres + B(a)P	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
Glucose	−	nd	+	nd	nd	nd	nd	nd	2	0	−	nd
Glycidamide	+	nd	nd	+	nd	+	+	nd	2	2	+	nd
Green tea	+	nd	nd	nd	nd	nd	nd	nd	1	0	−	−
Heavy-ion radiation	nd	nd	nd	nd	nd	nd	nd	nd	2	3	+	nd
Heptachlor	−	+	+	nd	nd	nd	nd	−	2	0	−	+
Hexachlorobutadiene	+	−	nd	nd	nd	nd	nd	nd	22	2	+	+
Hexavalent chromium	+	+	+	nd	+	+	nd	nd	6	9	+	+
High-energy charged particle (Fe)	nd	nd	nd	nd	nd	nd	nd	nd	8	4	+	nd
High-fat diet	nd	nd	nd	nd	nd	nd	nd	nd	12	0	−	+
Hydrazine sulphate	+	+	+	nd	inc	inc	nd	nd	24	0	−	+
Hydroxyurea	+	+	+	−	nd	+	nd	nd	1	2	+	nd
Hydroxyurea + X-rays	nd	nd	nd	nd	nd	nd	nd	nd	1	2	+	nd
Hyperglycaemia	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
Isopropylmethanesulphonate (iPMS)	+	nd	nd	nd	+	+	nd	nd	6	9	+	+
Jervine	nd	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
Kojic acid	+	+	nd	−	nd	+	−	nd	2	0	−	+
Leucomalachite green	−	nd	nd	nd	nd	+	nd	nd	8	1	+	equiv ±

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positiv e	Overall[b] result	Carc
Levofloxacin	−	nd	nd	nd	nd	nd	nd	nd	14	0	−	nd
Malachite green	+	−	nd	nd	nd	−	nd	nd	1	0	−	equiv
Methyl bromide	+	+	+	nd	−	+	−	nd	2	0	−	+
Methyl cellulose												
Methyl clofenapate	−	nd	nd	nd	nd	−	−	nd	2	0	−	+
Methyl clofenapate + DMN	nd	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
Methylmethanesulphonate (MMS)	+	+	+	+	+	+	+	+	47	10	+	+
MMS + 4-AAF	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
MMS + DMN	nd	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
Metronidazole	+	+	nd	nd	nd	−	nd	+	4	0	−	+
Mitomycin-C	+	+	+	nd	+	+	nd	+	20	11	+	+
N7-Methyldibenzo(c,g)carbazole (NMDBC)	+	nd	nd	nd	nd	nd	nd	nd	3	7	+	+
N-Ethyl-*N*-nitrosourea (ENU)	+	+	nd	nd	+	+	nd	+	74	326	+	+
ENU + 8-methoxypsoralen	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
N-Hydroxy-2-acetylaminofluorene	+	+	nd	nd	nd	nd	nd	nd	0	4	+	+
Nickel subsulphide	−	+	nd	nd	nd	+	nd	nd	4	0	−	+
Nifuroxazide	+	nd	nd	nd	nd	nd	nd	nd	8	0	−	nd
Nitrofurantoin	+	+	+	nd	−	+	nd	nd	7	1	+	+

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
N-Methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG)	+	+	+	nd	+	+	+	+	7	24	+	+
N-Methyl-*N*-nitrosourea (MNU)	+	+	+	nd	+	+	+	+	11	43	+	+
N-Nitrosodibenzylamine (NDBzA)	+	nd	nd	nd	nd	nd	nd	nd	20	2	+	nd
N-Nitrosomethylbenzylamine	+	nd	nd	nd	nd	nd	nd	nd	0	3	+	+
N-Nitrosomethylbenzylamine + diallyl sulphide	nd	nd	nd	nd	nd	nd	nd	nd	1	0	−	nd
N-Nitrosomethylbenzylamine + ellagic acid	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
N-Nitrosomethylbenzylamine + ethanol	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
N-Nitrosomethylbenzylamine + green tea	nd	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
N-Nitrosonornicotine (NNN)	+	nd	nd	nd	nd	+	nd	nd	1	8	+	+
N-Nitrosopyrrolidine	+	−	+	nd	nd	+	nd	+	0	1	+	+
N-Propyl-*N*-nitrosourea (PNU)	+	+	nd	nd	nd	nd	nd	nd	12	19	+	+
o-Aminoazotoluene	+	nd	+	nd	nd	nd	+	+	6	8	+	+
o-Anisidine	+	+	+	nd	nd	−	−	+	10	2	+	+
Olive oil*												
Oxazepam	−	−	−	+	nd	−	nd	nd	4	2	+	+
p-Cresidine	+	+	nd	nd	nd	−	nd	+	0	4	+	+

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positiv e	Overall[b] result	Carc
Peroxyacetyl nitrate (PAN)	+	nd	nd	nd	nd	nd	nd	nd	0	1	+	nd
Phenobarbital	+	+	+	−	−	+	nd	+	14	1	+	+
Phorbol-12-myristate-13-acetate (TPA)	−	+	nd	−	nd	nd	nd	nd	1	0	−	+
Phosphate buffer*												
Polyphenon E	−	nd	+	nd	nd	−	nd	nd	12	0	−	nd
Potassium bromate	+	+	+	+	+	+	nd	+	13	3	+	+
Procarbazine hydrochloride	−	nd	+	nd	+	+	+	+	15	14	+	+
Propylene glycol*												
Proton radiation	nd	+	nd	+	nd	+	nd	nd	19	13	+	nd
Quinoline	+	+	+	nd	−	+	inc	nd	5	4	+	+
Riddelliine	+	+	nd	nd	nd	+	+	nd	1	4	+	+
Rock wool fibres	nd	nd	nd	nd	nd	nd	nd	nd	6	3	+	−
Rock wool fibres + B(a)P	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
Saline*												
Sesame oil*												
Sodium bicarbonate*												
Sodium saccharin	−	+	−	nd	nd	nd	nd	nd	2	0	−	+
Solanidine	−	nd	nd	nd	nd	nd	nd	nd	0	2	+	nd
Solasodine	nd	nd	nd	nd	nd	nd	nd	nd	1	1	+	nd
Soy oil*												

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positiv e	Overall[b] result	Carc
Streptozotocin	+	nd	nd	nd	nd	nd	nd	nd	2	2	+	+
Sucrose	−	nd	−	nd	nd	inc	nd	nd	11	5	+	−
Tamoxifen	−	nd	nd	+	nd	nd	nd	nd	2	11	+	+
Tamoxifen + phenobarbital	nd	nd	nd	nd	nd	nd	nd	nd	0	3	+	nd
Thiotepa	+	+	nd	nd	+	+	nd	+	0	1	+	+
Toremifene citrate	nd	nd	nd	nd	nd	nd	nd	nd	1	0	−	−
trans-4-Hydroxy-2-nonenal	−	+	nd	nd	nd	−	nd	nd	4	0	−	−
Tricaprylin*												
Trichloroethylene (TCE)	+	−	+	nd	−	+	−	−	33	0	−	+
Trioctanoin*												
Tris-(2,3-dibromopropyl)phosphate	+	+	+	nd	−	+	nd	nd	17	7	+	+
Uracil	−	nd	nd	nd	nd	nd	nd	nd	3	1	+	+
Urethane	+	+	−	−	+	+	nd	+	28	28	+	+
UVB	+	nd	nd	nd	nd	nd	nd	nd	1	29	+	+
Vinyl carbamate	+	nd	nd	nd	nd	+	+	nd	6	10	+	+
Vinyl carbamate + diallyl sulphone	nd	nd	nd	nd	nd	nd	nd	nd	2	5	+	nd
Vitamin E	−	nd	nd	nd	nd	nd	nd	nd	5	0	−	nd
Water*												
Wyeth 14,643	−	nd	nd	nd	nd	nd	nd	nd	3	3	+	+

Table 5-1 (continued)

	In vitro assays				*In vivo* assays							
									TGR			
Chemical	*Sal*	CA	MLA	MN	CA	MN	UDS	Comet	No. negative	No. positive	Overall[b] result	Carc
X-rays	+	+	nd	nd	+	+	+	+	9	55	+	+

+, positive; −, negative; CA, chromosomal aberration assay; Carc, carcinogenicity; equiv, equivocal; inc, inconclusive; MLA, mouse lymphoma assay; MN, micronucleus assay; nd, not determined; *Sal*, *Salmonella* reverse mutation assay; UDS, unscheduled DNA synthesis

[a] Agents marked with an asterisk (*) and shaded are vehicle controls from TGR assays, which are also commonly used as vehicles in short-term genotoxicity and carcinogenicity studies and are presumed to be non-genotoxic non-carcinogens.

[b] Positive = any one tissue positive, regardless of the number negative; negative = all tissues negative, no positives

.

2) From an analysis of the studies in which sequence analysis was completed, what conclusions can be drawn regarding the ability of TGR assays to identify genotoxicants?

5.2.2 Predictivity of TGR assays for rodent carcinogenicity

Since TGR assays, like all short-term genotoxicity tests, will likely be used as a predictor of carcinogenicity, it is important to address the following additional questions:

3) What are the comparative performance characteristics of each of the short-term assays in predicting rodent carcinogenicity?
4) When combined into a test battery, what is the predictive value of the various short-term assays for rodent carcinogenicity?

5.3 Approach to the questions

5.3.1 Characterisation of the relationship between short-term assay results

The lack of an accepted definitive genotoxicity test hinders the evaluation of any new test, because there is no assay against which the new test can be measured. Instead, new genotoxicity tests must be compared against the existing assays that are known to detect mutations, despite differences in the mechanisms that lead to mutations identified using the various assays. In the analyses conducted in this chapter, *concordance* is used as a measure of the proportion of agreements (positive or negative) between any two assays. The *kappa coefficient* (K) is a more quantitative measure of agreement between tests with nominal outcomes (Cohen, 1960). Kappa can range from −1 to 1 and has a magnitude that reflects the strength of agreement, where a coefficient of 1 represents complete agreement of the tests. When the observed agreement is significantly better than expected by chance, kappa is positive and has a 95% confidence interval that does not include 0. Kappa is negative when the observed agreement is less than expected by chance.

5.3.2 Ability of TGR assays to identify mutagens (genotoxins)

In order to establish the acceptability of any new assay, it needs to be assessed, or validated, against an accepted assay for the most relevant, comparable endpoint (OECD, 2005). In the case of TGR assays for gene mutation, the most relevant assay would be an established test for *in vivo* gene mutations in somatic cells. Unfortunately, few *in vivo* somatic cell gene mutation assays exist. The mouse spot test detects a range of mutagenic effects, including gene mutation, but it has not proven to be useful in a regulatory context, and only a few chemicals (~20) have been tested (Wahnschaffe *et al*., 2005). Furthermore, fewer than 10 of these chemicals have been tested in TGR assays. The mouse splenocyte *Hprt* assay and the small intestine *Dlb-1* assay also detect gene mutations, but are limited to specific tissues and have a paucity of data.

In the absence of sufficient mutagenicity data from established tests for comparison with TGR data, the most definitive data available are DNA sequencing data obtained in conjunction with TGR assays. Because the DNA of various transgenes can be sequenced and specific mutations verified in terms of actual DNA sequence changes, the phenotypic changes identified using TGR assays can be confirmed against an indicator that identifies whether frank mutations actually occurred. If an agent causes an increase in the frequency of mutant phenotypes detected by a TGR assay, the validity of the assay for detecting these mutations would be established if all, or at least most, of these putative mutations were confirmed by sequence analysis. If, on the other hand, these induced suspect mutants were not confirmed by sequencing, then the assay would not be an acceptable mutation assay.

Comparisons of this nature reveal the true positive predictivity of TGR assays (in this context, the *positive predictive value* is the proportion of mutant phenotypes that are confirmed as mutations by sequencing). The presence of unique induced DNA sequence spectra provides further evidence of the positive predictivity of TGR assays. The opportunity to evaluate negative predictivity also exists if

a non-selective TGR assay is used, since it would allow the sequencing of putative non-mutants; however, this possibility is somewhat hypothetical, owing to the costs of sequencing (in this context, *negative predictive value* is the proportion of non-mutant phenotypes that are shown to not be mutagens by sequencing). This method does not evaluate sensitivity or specificity, since the definitive (sequence) data used in the comparison are produced only after putative mutants have been identified (in this context, *sensitivity* is the proportion of established *in vivo* somatic gene mutagens that are positive in a TGR assay in somatic cells, and *specificity* is the proportion of established *in vivo* somatic non-gene mutagens that are negative in a TGR assay).

5.3.3 Characterisation of the carcinogen predictivity of the short-term assays

The performance of each of the identified test methods in predicting rodent carcinogenicity was assessed using Bayesian inference, with methodology similar to that employed in previous studies (Tennant *et al*., 1987; Zeiger *et al*., 1990; Zeiger, 1998; Kim and Margolin, 1999). For the purposes of this analysis, a short-term assay prediction was considered "correct" if it agreed with the result of the rodent carcinogenicity bioassay.

Along with concordance (Section 5.3.1), which measured the proportion of all agents whose carcinogenic activity was correctly classified by each short-term assay, sensitivity, specificity, positive predictive value and negative predictive value could also be calculated. In this context, *sensitivity* is the proportion of carcinogens determined to be positive (genotoxic), and *specificity* is the proportion of non-carcinogens that were determined to be negative (non-genotoxic) in the short-term assay. These give measures of how well the assay classified chemicals belonging to each class (carcinogenic or non-carcinogenic). However, in practice, the short-term assay result is typically all that is known; accordingly, the probability that a short-term assay result is correctly indicative of the carcinogenic activity of an agent is indicated by the predictive value of the assay. *Positive predictive value* is the probability that a chemical found to be genotoxic in the short-term test is a rodent carcinogen, whereas *negative predictive value* is the probability that a chemical found to be negative in the short-term test is a rodent non-carcinogen. However, predictive values are so greatly influenced by *prevalence*, the proportion of chemicals that are rodent carcinogens, that the ability to make generalisations is limited usually to those datasets with similar prevalence.

In order to provide a baseline against which some of the indices previously described can be compared (particularly in this case, where prevalence is high), it is useful to examine the expected values of concordance, sensitivity and specificity in a hypothetical situation where the short-term assay results are completely unrelated to carcinogenic activity. In this case, the proportion of carcinogens and non-carcinogens that would be correctly identified if the assay was able to discriminate no better than chance is the *concordance expected by chance* (see Klopman and Rosenkranz, 1991), which is directly related to the proportion of carcinogens in the database (prevalence). The sensitivity expected by chance is equivalent to the proportion of compounds positive in the test, whereas the specificity expected by chance is the proportion of compounds negative in the test. Similarly, the positive predictive value and negative predictive value expected simply by chance (PPV_{rand} and NPV_{rand}) are given by prevalence and 1 − prevalence, respectively.

5.3.4 Characterisation of the performance of the TGR assay in a test battery

The performance characteristics of eight possible two-assay test battery combinations were examined where the TGR assay was paired with another short-term test. For each paired combination of assays, a decision regarding how to interpret the combination of tests in the context of predicting carcinogenicity could, in theory, be made in one of two ways: 1) the chemical could be predicted to be carcinogenic if the outcome of *either* short-term assay was positive (hereafter called the "*or*" scenario), or 2) the chemical could be predicted to be carcinogenic if the outcomes of *both* short-term assays were positive (the "*and*" scenario). In all other cases, the chemical would be classified as non-carcinogenic. In Table 5-2, the decision rules for classifying a test battery outcome are outlined, taking the example of a test battery of the TGR assay combined with *Salmonella*.

Table 5-2. Decision rules for determining the outcome of a two-assay battery, taking the example of *Salmonella* paired with the TGR assay[a]

Scenario 1: "TGR *or Salmonella*"			Scenario 2: "TGR *and Salmonella*"		
Salmonella result	TGR result	Battery conclusion	*Salmonella* result	TGR result	Battery conclusion
+	+	+	+	+	+
+	–	+	+	–	–
–	+	+	–	+	–
–	–	–	–	–	–

[a] The shaded area contains the possible assay outcomes that would lead to a battery prediction of carcinogenic. For scenario 1, the chemical is classified as carcinogenic if the result of either *Salmonella* or TGR is positive; otherwise, the chemical is classified as non-carcinogenic. For scenario 2, the chemical is classified as carcinogenic if the results of both *Salmonella* and TGR are positive; otherwise, the chemical is classified as non-carcinogenic.

5.3.4.1 Performance of TGR assay versus in vivo micronucleus assay in a test battery with in vitro assays

Currently, the most commonly conducted short-term *in vivo* assay is the *in vivo* micronucleus assay. Thus, it is important to consider if the performance of the TGR assay is better than the performance of the *in vivo* micronucleus assay, when either assay is interpreted in conjunction with the most commonly conducted *in vitro* tests.

The *Salmonella* reverse mutation assay and the *in vitro* chromosomal aberration assay were used to form a two *in vitro* test battery, where a positive result in either assay resulted in an overall *in vitro* test battery conclusion of positive. This *in vitro* test battery was combined with either the TGR assay or the *in vivo* micronucleus assay in a manner described by Table 5-3. The performance characteristics of each test battery could be examined.

Table 5-3. Decision rules for determining the outcome of the three-assay battery[a]

In vitro battery result	*In vivo* result	Overall battery conclusion
+	+	+
+	–	+
–	+	+
–	–	–

[a] The *Salmonella* reverse mutation assay and the *in vitro* chromosomal aberration assay formed the *in vitro* battery. The results of the *in vitro* battery and either the TGR assay or the *in vivo* micronucleus assay determined the overall test battery conclusion. This is the same "or" scenario described in scenario 1 of Table 5-2.

5.4 Results of the analysis

5.4.1 Characterisation of the agreement between short-term assay results

Table 5-4 shows the pairwise concordance between each of the assays using the data available for the agents shown in Table 5-1. The interassay concordance was relatively high, which is not unexpected, considering that these systems are used to detect genetic toxicity and the dataset was heavily weighted by genotoxic carcinogens. The kappa coefficient indicated that the observed pairwise agreement was significantly better than expected by chance for 22 short-term assay

combinations, but the magnitude of kappa (0.24–0.85) suggested that the strength of the associations was variable. As all the assays are genotoxicity tests and known genotoxicants are over-represented within the database, one would expect a high degree of association between the tests; this was not always observed. Interestingly, *in vivo* unscheduled DNA synthesis, an indicator assay that is not directly indicative of mutagenicity, seemed to have stronger agreement with the TGR assay, the *in vivo* chromosomal aberration assay and the *in vivo* comet assay than did many of the other assays.

5.4.2 Ability of TGR assays to identify genotoxicants

The studies where sequence analysis was conducted are identified in the TRAID. Of the 3 186 experimental records, sequencing was conducted for more than 450.

Among the studies involving both DNA sequencing and mutation detection, sequencing detected unique sequences in the majority of cases where induced mutagenesis was observed. However, there were three experimental records (leucomalachite green, phenobarbital) where sequencing indicated that the assay conclusion should be changed from mutagenic to non-mutagenic (Shane *et al*., 2000c; Singh *et al*., 2001; Culp *et al*., 2002) and two experimental records (oxazepam) where the assay conclusion should be changed from non-mutagenic to mutagenic (Shane *et al*., 1999; Singh *et al*., 2001).

The positive predictivity of TGR gene mutation assays is detailed in Appendix D (see Section 5.3.2 for a description of the derivation of positive predictive value and negative predictive value from DNA sequence data). Among the over 140 studies, 20 253 induced and 12 498 spontaneous mutant phenotypes were sequenced. The positive predictive value for induced TGR mutations is 0.996, and the positive predictive value for spontaneous mutations is 0.948, which yields an average positive predictive value of 0.972 for all TGR mutant phenotypes (Table 5-5). Given the very high positive predictivity for TGR assays estimated through DNA sequence comparisons and the very low spontaneous mutant frequency, it would be expected that the negative predictive value for mutagenicity would also be very high, since the odds of a non-mutant phenotype not having a wild-type base sequence are exceedingly low. These predictive values would not influenced by prevalence since the endpoint being predicted is the same as the the endpoint that predicts: i.e. even for non-mutagens, there is a spontaneous mutant frequency, and it is those spontaneous mutants that would be sequenced; as shown, their predictive value is the same as it is for induced mutants (see Table 5.5)

These comparisons, enabled by the extensive DNA sequencing data, demonstrate unequivocally the very high predictive capacity of TGR assays for identifying gene mutagens; accordingly, gene mutagens so detected should predict optimally those carcinogens that act through a genotoxic mechanism primarily involving gene mutation. It follows logically that test batteries designed to most effectively detect the range of genotoxins should be the batteries that also best detect genotoxins that are carcinogens.

5.4.2.1 Performance of the existing short-term assays for predicting carcinogenicity

The concordance values for nine of the most common short-term assays presented in Table 5-6 represent the proportion of agreements between the short-term genotoxicity assay and rodent carcinogenicity. Concordance ranged from a low of 73% for the *in vivo* micronucleus assay to a high of 91% for the *in vivo* comet assay.

Table 5-4. Agreement between short-term assays – concordance and kappa coefficient (K)[a]

	Salmonella							
***In vitro* CA**	76/99 (77%) K = 0.32*	***In vitro* CA**						
***In vitro* MLA**	52/65 (80%) K = 0.43*	49/57 (85%) K = 0.52*	***In vitro* MLA**					
***In vitro* MN**	28/46 (61%) K = 0.08	24/36 (67%) K = 0.18	15/23 (65%) K = 0.11	***In vitro* MN**				
***In vivo* CA**	35/52 (67%) K = 0.16	33/46 (72%) K = 0.22	19/31 (61%) K = −0.01	16/23 (70%) K = 0.31	***In vivo* CA**			
***In vivo* MN**	63/89 (71%) K = 0.28*	53/73 (71%) K = 0.27*	40/51 (78%) K = 0.30*	20/36 (56%) K = −0.03	35/45 (78%) K = 0.36*	***In vivo* MN**		
***In vivo* UDS**	33/44 (75%) K = 0.40*	26/35 (74%) K = 0.34*	20/28 (71%) K = 0.24*	13/14 (93%) K = 0.85*	17/21 (81%) K = 0.60*	27/37 (73%) K = 0.33*	***In vivo* UDS**	
***In vivo* comet**	50/56 (89%) K = 0.51*	45/50 (90%) K = 0.56*	31/36 (86%) K = 0.37*	14/21 (67%) K = 0.01	30/34 (88%) K = 0.53*	36/49 (73%) K = 0.18	24/29 (83%) K = 0.51*	***In vivo* comet**
TGR	118/161 (73%) K = 0.37*	68/100 (68%) K = 0.16	45/66 (68%) K = 0.17	24/48 (50%) K = −0.12	37/52 (72%) K = 0.26	65/91 (71%) K = 0.33*	37/45 (82%) K = 0.59*	49/57 (86%) K = 0.52*

CA, chromosomal aberration assay; MLA, mouse lymphoma assay; MN, micronucleus assay; TGR, transgenic rodent gene mutation assay; UDS, unscheduled DNA synthesis

[a] An asterisk (*) indicates that observed agreement is significantly better than expected by chance.

Table 5-5. Positive predictive value of TGR gene mutation assays for the prediction of gene mutation *in vivo* using DNA sequence data

Mutation type	Number of mutant phenotypes sequenced	Number of mutant genotypes detected	Positive predictive value
Spontaneous	20 253	19 578	0.948
Induced	12 498	11 881	0.996
Total[a]	**32 751**	**31 659**	**0.967**

[a] Data from over 140 studies.

When these values were compared with the concordance expected by chance (the expected number of correct predictions based on prevalence), it was apparent that all of the assays identified carcinogens somewhat better than chance.

Sensitivity is the proportion of carcinogens that had positive results in the short-term assay. For these nine assays, sensitivity ranged from a low of 69% (*in vivo* chromosomal aberration and unscheduled DNA synthesis) to 89% (*in vivo* comet). This suggests that most of the carcinogens were mutagenic in these short-term test systems; however, sensitivity was likely also affected by chance agreements due to the high prevalence. By contrast, specificity, which is the proportion of non-carcinogens with negative results in the short-term assay, was higher. Specificity ranged from 72% (*Salmonella*) to 89% (*in vitro* mouse lymphoma assay, *in vivo* unscheduled DNA synthesis). For *in vivo* chromosomal aberration and *in vivo* comet assays, which had an apparent specificity of 100%, only a single non-carcinogen tested was correctly identified. Because of this, specificity for these assays is almost certainly greatly overstated.

Positive predictive value is the probability that a positive short-term assay result is indicative of carcinogenicity. Positive predictive value for all assays was extremely high, with a range from 90% (*Salmonella*) to 96% (*in vitro* mouse lymphoma assay). For *in vivo* chromosomal aberration and *in vivo* comet assays, no agents with positive results were non-carcinogens, so the positive predictive value of 100% for these assays is unlikely to be widely generalisable. The differential between positive predictive value and positive predictive value expected by chance (see Section 5.3.3 for definitions) for the assays was 12% (*Salmonella*), 13% (*in vitro* chromosomal aberration), 21% (*in vitro* mouse lymphoma assay), 26% (*in vitro* micronucleus), 22% (*in vivo* chromosomal aberration), 12% (*in vivo* micronucleus), 25% (*in vivo* unscheduled DNA synthesis) and 21% (*in vivo* comet). The lower 95% confidence interval of positive predictive value fell above the positive predictive value expected by chance for all assays, suggesting that all assays were able to predict carcinogenicity significantly better than chance, based on the available data analysed.

In contrast, negative predictive value is the probability that a negative short-term assay result is indicative of non-carcinogenicity. The negative predictive value was much lower, with a range from 41% (*in vivo* micronucleus) to 70% (*in vitro* mouse lymphoma assay, *in vivo* comet). The differential between negative predictive value and negative predictive value expected by chance for the assays was 22% (*Salmonella*), 36% (*in vitro* chromosomal aberration), 45% (*in vitro* mouse lymphoma assay), 32% (*in vitro* micronucleus), 26% (*in vivo* chromosomal aberration), 21% (*in vivo* micronucleus), 25% (*in vivo* unscheduled DNA synthesis), 49% (*in vivo* comet) and 27% (TGR assay). The negative predictive value expected by chance fell below the lower 95% confidence interval of negative predictive value for all assays, suggesting that all assays were able to predict non-carcinogens significantly better than chance. Considering both positive predictive value and negative predictive value together, it can be concluded that the probability was better than chance that

Table 5-6. Performance of the short-term assays in predicting rodent carcinogenicity

	Sal	*In vitro* CA	*In vitro* MLA	*In vitro* MN	*In vivo* CA	*In vivo* MN	*In vivo* UDS	*In vivo* comet	TGR
Concordance (%)	74	83	88	80	76	73	75	91	77
Concordance expected by chance (%)	58	64	59	52	52	57	50	62	57
Sensitivity (%)	74	86	87	76	69	71	69	89	76
Specificity (%)	72	73	89	88	100	83	89	100	78
Positive predictive value (%)	90	92	96	93	100	93	93	100	92
Negative predictive value (%)	44	57	70	65	48	41	57	70	50
Prevalence (%)	78	79	75	67	78	81	68	79	77
Proportion positive in test (%)	64	73	68	55	54	61	51	70	64
Number of chemicals[a]	145	105	73	51	63	94	57	67	154

CA, chromosomal aberration assay; MLA, mouse lymphoma assay; MN, micronucleus assay; TGR, transgenic rodent gene mutation assay; *Sal*, *Salmonella* reverse mutation assay; UDS, unscheduled DNA synthesis

[a] Includes 13 vehicle controls from TGR assays, which are also commonly used as vehicles in short-term genotoxicity and carcinogenicity studies, as non-genotoxic non-carcinogens.

chemicals genotoxic in the existing short-term assays were carcinogenic and those non-genotoxic in the existing short-term assays were non-carcinogenic.

However, positive predictive value and negative predictive value are highly affected by prevalence, particularly in situations where specificity is low. Since prevalence was high in the database used for this analysis, the actual positive predictive value of each of these assays would have been lower and the actual negative predictive value would have been higher than those observed if a dataset that was representative of the proportion of carcinogens within the known chemical universe had been available (see Table 5-7). It should be noted that the negative predictive values in Table 5-6 are markedly higher than reported previously (Lambert *et al*., 2005) and reflect the large increase in non-carcinogens that have been added to the TRAID since that paper was published.

Table 5-7. Impact of differing prevalence of carcinogens on positive predictive value (PPV) and negative predictive value (NPV) of the various assays, provided sensitivity and specificity of the assays are maintained

	Prevalence							
	10%		25%		50%		90%	
Assay	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
Salmonella	0.23	0.96	0.47	0.89	0.73	0.73	0.96	0.24
In vitro CA	0.26	0.98	0.52	0.94	0.76	0.84	0.97	0.37
In vitro MLA	0.47	0.98	0.73	0.95	0.89	0.87	0.99	0.43
In vitro MN	0.41	0.97	0.68	0.92	0.86	0.79	0.98	0.29
In vivo CA	n/a[a]	0.97	n/a[a]	0.91	n/a[a]	0.76	n/a[a]	0.26
In vivo MN	0.32	0.96	0.58	0.90	0.81	0.74	0.97	0.24
In vivo UDS	0.41	0.96	0.68	0.90	0.86	0.74	0.98	0.24
In vivo comet	n/a[a]	0.99	n/a[a]	0.96	n/a[a]	0.90	n/a[a]	0.50
TGR	0.28	0.97	0.54	0.91	0.78	0.76	0.97	0.27

CA, chromosomal aberration assay; MLA, mouse lymphoma assay; MN, micronucleus assay; TGR, transgenic rodent gene mutation assay; *Salmonella*, *Salmonella* reverse mutation assay; UDS, unscheduled DNA synthesis

[a] Limited number of carcinogens, none with positive genotoxicity test results (specificity 100%).

A high positive predictive value is a good characteristic for a test used in error-intolerant applications, such as in the screening of novel pharmaceuticals for genotoxic properties prior to the initiation of phase I clinical trials with healthy human volunteers. In an ideal situation, however, a short-term assay would also have high negative predictive value, together indicating that there is a high probability that both positive and negative test results are correct. Specificity and negative predictive value are most important when an assay is used to screen sets of chemicals where the expected number of carcinogens is low (Ennever and Rosenkranz, 1988). Approximately 52% of chemicals tested by the NTP were carcinogenic in at least one organ in one of the four sex/species groups (Fung, Barrett and Huff, 1995), which is much fewer than the proportion of carcinogens in the database and much higher than that generally expected within the known universe of chemicals.

5.4.2.2 Performance of TGR assays

The concordance of TGR assays with rodent carcinogenicity was 77%, and the expected concordance due to chance was 57%; the difference (20 percentage points) between the observed and expected concordance was similar to that of the other genotoxicity assays. Like all the other assays, the expected concordance also fell outside the 95% confidence interval of the observed concordance,

suggesting that the proportion of agreements was significantly better than expected by chance. The sensitivity value suggests that most of the carcinogens were mutagenic; however, chance agreements may account for a large proportion of the observed sensitivity because of the high prevalence. Positive predictive value was comparable with the other assays, as was the differential between positive predictive value and the expected positive predictive value due to chance. Like all of the other assays, both the positive predictive value and negative predictive value expected by chance did not fall within the 95% confidence interval of positive predictive value or negative predictive value, suggesting that the probability that an agent mutagenic to transgenic rats and mice was carcinogenic, or that an agent non-mutagenic to transgenic animals was non-carcinogenic, was significantly better than chance. Overall, this suggests that there was a better than chance probability that a positive result in the TGR assay was predictive of carcinogenicity and a negative TGR result was predictive of non-carcinogenicity. Re-examination of the performance of TGR assays with a larger and more representative database will help to determine whether the TGR assay truly has a better positive predictive value and negative predictive value than those previously reported for many of the other short-term assays (Tennant *et al*., 1987; Zeiger, 1998; Kim and Margolin, 1999; Kirkland *et al*., 2005). It appears that the operational characteristics of the TGR assay are at least comparable to, and perhaps marginally better than, those of the *Salmonella* reverse mutation assay.

5.4.3 Performance of TGR assays in a test battery

Since the available information would suggest, at least on a preliminary basis, that TGR assays have some predictive value, the question of whether the inclusion of the TGR assay would improve the predictivity of the standard genotoxicity test battery could be addressed. Table 5-8 shows the 64 possible test battery outcomes for five short-term tests plus the TGR assay and the number of carcinogenic and non-carcinogenic test chemicals corresponding to each outcome.

Consistent with the observation that most chemicals in the dataset are genotoxic carcinogens, the majority of rodent carcinogens with results from all six of the short-term test systems shown in Table 5-8 were mutagenic in each of the six tests. Thirteen of the 36 non-carcinogens had a complete set of test battery results.

5.4.3.1 Performance of potential test battery combinations

Table 5-9 shows the performance characteristics of 10 potential test battery combinations involving the TGR assay. Combining the TGR assay with one of the other short-term assays shown in Table 5-9 in the “or” test battery scenario resulted in improvements in the proportion of agreements (concordance) compared with each of the assays alone.

In addition, sensitivity of the battery also showed some improvement compared with the assays alone. Although specificity of the batteries in this scenario was slightly decreased compared with those of the component tests, positive predictive value remained essentially unchanged, and negative predictive value generally showed slight improvement. When compared with the predictive values expected by chance, the greatest differential in positive predictive value was provided by “TGR or *in vivo* comet” (21 percentage points), and all test batteries had positive and negative predictive values significantly better than expected by chance.

Overall, it appeared that pairing the TGR assay with each of the existing short-term assays in the “or” scenario slightly improved predictivity beyond that observed for the single tests. All of the batteries appeared to improve positive predictive value and negative predictive value beyond the level expected simply by chance. However, if the test battery maximising both positive predictive value and negative predictive value was considered the optimum for carcinogen identification, the “TGR or *in vitro* chromosomal aberration” test battery appeared to be superior to the others, given that the positive predictive value in the “TGR or *in vivo* comet” battery resulted from the fact that no non-carcinogens were positive in that battery for the chemicals included in the comparison.

Table 5-8. Contingency table of short-term test battery results (n = 40)

In vitro assays			*In vivo* assays			Carcinogenicity	
Salmonella	Chromosomal aberration	Mouse lymphoma assay	Chromosomal aberration	Micronucleus	TGR	+	−
+	+	+	+	+	+	13	0
+	+	+	+	+	−	0	0
+	+	+	+	−	+	0	0
+	+	+	+	−	−	0	0
+	+	+	−	+	+	4	0
+	+	+	−	+	−	1	0
+	+	+	−	−	+	2	0
+	+	+	−	−	−	0	0
+	+	−	+	+	+	1	0
+	+	−	+	+	−	0	0
+	+	−	+	−	+	0	0
+	+	−	+	−	−	0	0
+	+	−	−	+	+	0	0
+	+	−	−	+	−	0	0
+	+	−	−	−	+	0	0
+	+	−	−	−	−	0	0
+	−	+	+	+	+	0	0
+	−	+	+	+	−	1	0
+	−	+	+	−	+	0	0
+	−	+	+	−	−	0	0
+	−	+	−	+	+	0	0

In vitro assays			*In vivo* assays			Carcinogenicity	
Salmonella	Chromosomal aberration	Mouse lymphoma assay	Chromosomal aberration	Micronucleus	TGR	+	−
+	−	+	−	+	−	1	0
+	−	+	−	−	+	0	0
+	−	+	−	−	−	0	0
+	−	−	+	+	+	0	0
+	−	−	+	+	−	0	0

Table 5-8 (continued)

In vitro assays			*In vivo* assays				
Salmonella	Chromosomal aberration	Mouse lymphoma assay	Chromosomal aberration	Micronucleus	TGR	Carcinogenicity	
+	−	−	+	−	+	0	0
+	−	−	+	−	−	0	0
+	−	−	−	+	+	0	0
+	−	−	−	+	−	0	0
+	−	−	−	−	+	0	0
+	−	−	−	−	−	0	0
−	+	+	+	+	+	2	0
−	+	+	+	+	−	0	0
−	+	+	+	−	+	0	0
−	+	+	+	−	−	0	0
−	+	+	−	+	+	0	0
−	+	+	−	+	−	0	0
−	+	+	−	−	+	0	0

Table 5-8 (continued)

In vitro assays			*In vivo* assays				
Salmonella	Chromosomal aberration	Mouse lymphoma assay	Chromosomal aberration	Micronucleus	TGR	Carcinogenicity	
−	+	+	−	−	−	0	0
−	+	−	+	+	+	0	0
−	+	−	+	+	−	0	0
−	+	−	+	−	+	0	0
−	+	−	+	−	−	0	0
−	+	−	−	+	+	0	0
−	+	−	−	+	−	0	0
−	+	−	−	−	+	0	0
−	+	−	−	−	−	0	0
−	−	+	+	+	+	0	0
−	−	+	+	+	−	0	0
−	−	+	+	−	+	0	0
−	−	+	+	−	−	0	0
−	−	+	−	+	+	0	0
−	−	+	−	+	−	0	0
−	−	+	−	−	+	0	0
−	−	+	−	−	−	1	0
−	−	−	+	+	+	0	0
−	−	−	+	+	−	0	0
−	−	−	+	−	+	0	0
−	−	−	+	−	−	0	0
−	−	−	−	+	+	0	0

Table 5-8 (continued)

In vitro assays			*In vivo* assays				
Salmonella	Chromosomal aberration	Mouse lymphoma assay	Chromosomal aberration	Micronucleus	TGR	Carcinogenicity	
−	−	−	−	+	−	0	0
−	−	−	−	−	+	1	0
−	−	−	−	−	−	0	13

Table 5-9. Performance characteristics of 10 possible test battery combinations

	TGR or *Salmonella*	TGR and *Salmonella*	TGR or *in vitro* CA	TGR and *in vitro* CA	TGR or *in vivo* CA	TGR and *in vivo* CA	TGR or *in vivo* MN	TGR and *in vivo* MN	TGR or *in vivo* comet	TGR and *in vivo* comet
Concordance	0.83	0.69	0.89	0.71	0.89	0.70	0.86	0.67	0.93	0.82
Concordance expected by chance	0.64	0.51	0.68	0.52	0.61	0.49	0.64	0.49	0.63	0.57
Sensitivity	0.88	0.64	0.94	0.66	0.88	0.61	0.87	0.59	0.91	0.77
Specificity	0.66	0.88	0.68	0.91	0.93	1.00	0.83	1.00	1.00	1.00
Positive predictive value	0.90	0.95	0.92	0.96	0.98	1.00	0.96	1.00	1.00	1.00
Negative predictive value	0.60	0.41	0.75	0.42	0.68	0.42	0.60	0.37	0.74	0.54
Proportion positive in test battery	0.76	0.52	0.81	0.54	0.70	0.48	0.73	0.48	0.72	0.61
Prevalence	0.78	0.78	0.79	0.79	0.78	0.78	0.81	0.81	0.79	0.79
Number of chemicals[a]	145	145	105	105	63	63	94	94	67	67

CA, chromosomal aberration; MN, micronucleus

[a] Includes 13 vehicle controls from TGR assays, which are also commonly used as vehicles in short-term genotoxicity and carcinogenicity studies, as non-genotoxic non-carcinogens.

In the "and" scenario, both concordance and sensitivity were decreased compared with the single tests; however, specificity was somewhat increased. Nevertheless, positive predictive value and negative predictive value remained essentially unchanged from the assays alone, suggesting that the probability that positive or negative battery results were correctly indicative of carcinogenic activity was not any better than for the single tests. When predictive values were compared with those expected due to chance, all assays had better predictive values than expected by chance; the "TGR and *in vivo* chromosomal aberration" battery had the greatest differential in positive predictive value (22 percentage points better than chance), and the "TGR and *in vivo* comet" battery had the greatest differential in negative predictive value (33 percentage points better than expected). The specificity and positive predictive value of 100% for "TGR and *in vivo* chromosomal aberration", "TGR and *in vivo* MN", "TGR or *in vivo* comet" and "TGR and *in vivo* comet" should be interpreted with caution, since there were no non-carcinogens in these scenarios that were positive in the batteries.

Overall, none of the scenarios appeared to be superior to any of the others in the "and" scenario, because their performance characteristics were not substantially different from each other.

Considering all the test batteries and single assays examined, the "TGR or *in vivo* comet" battery, comet alone, "TGR or *in vitro* chromosomal aberration" and "TGR or *in vivo* chromosomal aberration" had the best predictive values for this high prevalence dataset. Because the "or" scenario serves to minimise false negatives, this battery interpretation may have value in situations where sensitivity and positive predictive value are important, such as when the battery outcome is used as a means to facilitate priority setting for further testing or for safety assessment; however, the trade-off is that false positives are increased. In contrast, battery interpretations using the "and" scenario may be appropriate in situations where minimising false positives is important.

5.4.3.2 Complementarity – tests used to identify genotoxicants

Most genotoxic compounds will induce both chromosomal mutations and gene mutations, although the extent to which each of these two endpoints are induced by any given genotoxic agent may differ significantly. As a result, the corresponding sensitivity of tests specific to these endpoints will differ. Short-term assays are complementary when they offer greater predictivity for the detection of mutagens when combined than when alone. Since no single assay is likely to detect all genotoxic effects, a battery comprising tests that assay mechanistically distinct events (*i.e.* primarily gene mutations or chromosomal aberrations) may offer the greatest chance of detecting genotoxicity. In the ideal situation where there are no false positives, the degree of complementarity between assays is indicated by the lack of association between their conclusions regarding genotoxicity. Perfect complementarity occurs in the situation where each assay alone identifies none of the mutagens detected by the other, but the assays, when combined, identify all mutagens. Such perfect complementarity between any two assays is, however, unlikely ever to occur because of the very narrow spectrum of genotoxic mechanisms detected by most assays and the number of genotoxic compounds that cause both gene mutations and chromosomal aberrations.

As shown in Table 5-4, there is better agreement than expected by chance for many of the assay pairs, consistent with the belief that genotoxic compounds often induce both chromosomal aberrations and gene mutations, whereas non-genotoxic compounds should induce neither. In the case of the TGR assay, the lack of significant association with *in vitro* and *in vivo* chromosomal aberration would suggest that tests assessing mechanistically different endpoints would exhibit stronger complementarity; however, this conclusion is not supported by the poor complementarity exhibited by the TGR assay paired with the *in vivo* micronucleus assay. Pairing of the TGR assays with *Salmonella*, *in vivo* unscheduled DNA synthesis and *in vivo* comet also appeared to lack strong complementarity. However, because known potent mutagens were over-represented among the chemicals in the database, it is difficult to accurately determine complementary relationships, since strong mutagens are likely to efficiently induce both gene mutations and chromosomal mutations.

5.4.3.3 Complementarity – tests used to identify carcinogens

When the short-term assays are used as a means to predict carcinogenicity, the most complementary assays are those that, when combined, minimise the false-negative rate and maximise the predictive values. Examining Table 5-9, it is apparent that the predictive values of the two-assay batteries shown are slightly, but not substantially, greater than those of the component assays alone. However, in the cases where the overall battery is interpreted as positive when either assay is positive (the "or" scenario described in Section 5.3.3), the false-negative rate of these test batteries is lower (and sometimes markedly so) than that of either assay alone. For situations where minimising the number of false negatives is important, such as when the battery is used as a screening test prior to the initiation of clinical trials, it is apparent that the use of TGR assays in a test battery may have clear advantages, despite the lack of substantial improvement of the batteries over the component tests in terms of predictive values.

5.4.3.4 Performance of the TGR assay versus the in vivo *micronucleus and* in vivo *comet assays in a test battery with* in vitro *assays*

The performance characteristics of the TGR assay when combined in a test battery with *Salmonella* and *in vitro* chromosomal aberration (described in Table 5-3) are presented in Table 5-10. The performance characteristics of *in vivo* micronucleus and *in vivo* comet in this same battery are shown in Tables 5-11 and 5-12, respectively.

It appears that the TGR, *in vivo* micronucleus and *in vivo* comet assays performed similarly when combined in a battery with both *Salmonella* and *in vitro* chromosomal aberration. These results do not suggest a clear advantage of one assay over the other for predicting carcinogenicity on the basis of the current data.

The TGR assay was usually positive for those carcinogens that were positive in both *Salmonella* and *in vitro* chromosomal aberration. In contrast, *in vivo* micronucleus had a much higher false-negative rate for the same chemicals (0.22 *vs.* 0.15). If *in vivo* confirmation of positive results from both *Salmonella* and *in vitro* chromosomal aberration is warranted, TGR is likely a better choice than *in vivo* micronucleus.

Although the number of chemicals tested in the same assays is very small, for chemicals having positive *Salmonella* and negative *in vitro* chromosomal aberration results (presumptive gene mutagens), selecting either the TGR or *in vivo* micronucleus assay as the *in vivo* confirmation assay did not markedly affect the proportion of correct carcinogenicity predictions. The *in vivo* comet assay had a higher false-negative rate than the above two scenarios.

For chemicals having positive *in vitro* chromosomal aberration and negative *Salmonella* results (presumptive clastogens), selecting the *in vivo* micronucleus assay as the *in vivo* confirmation assay led to a higher proportion of correct carcinogenicity predictions than did selecting the TGR or *in vivo* comet assay. It should be noted that this observation is also based on data from a few chemicals only.

For those carcinogens with negative results in both *Salmonella* and *in vitro* chromosomal aberration, adding TGR assays to the test battery improved the overall predictivity over the *in vivo* micronucleus or comet assay, since neither assay identified the carcinogens missed by the *in vitro* assays.

Table 5-10. Performance of the *in vitro* test battery with the TGR assay

Salmonella	*In vitro* chromosomal aberration			Carcinogenicity +	Carcinogenicity −	Total
+	+	TGR	+	52	2	54
			−	9	1	10
		Total		61	3	64
	−	TGR	+	3	1	4
			−	2	0	2
		Total		5	1	6
−	+	TGR	+	3	0	3
			−	7	3	10
		Total		10	3	13
	−	TGR	+	4	0	4
			−	3	15	18
		Total		7	15	22

		95% confidence interval
Concordance	0.90	0.9–0.99
Expected concordance	0.69	0.6–0.77
Sensitivity	0.96	0.9–0.99
Specificity	0.68	0.45–0.86
Positive predictive value	0.92	0.84–0.97
Negative predictive value	0.83	0.59–0.96
Prevalence	0.79	0.7–0.86

5.4.4 Conclusions

1) DNA sequencing has indicated that TGR assays are capable of identifying compounds causing gene mutations *in vivo* with a very high positive predictive value for gene mutations.
2) It follows, logically, that a test battery designed to most effectively detect the range of genotoxins should be the battery that also best detects genotoxins that are carcinogens.
3) Any interpretation of the predictivity of an assay for carcinogenicity must recognise the extent to which the predictive values are influenced by prevalence (see Table 5-7), which was high within the available database. Consequently, perhaps the more useful indicators of assay and battery performance for the prediction of carcinogenicity are provided by the extent of the differences between the observed agreement or predictive values and those expected simply by chance (prevalence and 1 − prevalence for positive predictive value and negative predictive value, respectively). The conclusions that follow (4–10) regarding the performance of the TGR assay arise from the analyses carried out to this point in this chapter.
4) TGR assays exhibited agreement significantly better than expected by chance with several short-term tests assessing both gene mutations and chromosomal aberrations; however,

known genotoxicants are over-represented within the existing database, and, consequently, it is difficult to assess complementarity.

Table 5-11. Performance of the *in vitro* test battery with the *in vivo* micronucleus assay

Salmonella	*In vitro* chromosomal aberration			Carcinogenicity +	Carcinogenicity −	Total
+	+	*In vivo* micronucleus	+	38	0	38
			−	11	0	11
		Total		49	0	49
	−	*In vivo* micronucleus	+	3	0	3
			−	1	0	1
		Total		4	0	4
−	+	*In vivo* micronucleus	+	3	1	4
			−	2	1	3
		Total		5	2	7
	−	*In vivo* micronucleus	+	1	1	2
			−	5	14	19
		Total		6	15	21

		95% confidence interval
Concordance	0.90	0.83–0.97
Expected concordance	0.65	0.54–0.75
Sensitivity	0.92	0.83–0.97
Specificity	0.82	0.57–0.96
Positive predictive value	0.95	0.87–0.99
Negative predictive value	0.74	0.49–0.91
Prevalence	0.79	0.69–0.87

Table 5-12. Performance of the *in vitro* test battery with the *in vivo* comet assay

Salmonella	*In vitro* chromosomal aberration			Carcinogenicity +	Carcinogenicity −	Total
+	+	*In vivo* comet	+	38	0	38
			−	0	0	0
		Total		38	0	38
	−	*In vivo* comet	+	2	0	2
			−	2	0	2
		Total		4	0	4
−	+	*In vivo* comet	+	2	0	2
			−	2	0	2
		Total		4	0	4
	−	*In vivo* comet	+	0	0	0
			−	2	13	15
		Total		2	13	15

		95% confidence interval
Concordance	0.97	0.86–0.99
Expected concordance	0.65	0.52–0.76
Sensitivity	0.96	0.86–0.99
Specificity	1.00	–
Positive predictive value	1.00	–
Negative predictive value	0.87	0.6–0.98
Prevalence	0.79	0.66–0.88

5) The TGR assays exhibited high positive predictive value for carcinogenicity, meaning that there was a high probability that a chemical mutagenic to transgenic rats and mice was a carcinogen. If the result of TGR assays was used as an indicator of carcinogenic potential when screening a database with a low prevalence of carcinogens, the positive predictive value would be lower.
6) The TGR assays exhibited a relatively lower negative predictive value for carcinogenicity, meaning that there was a low probability that a chemical with a negative TGR result was a non-carcinogen; however, the negative predictive value was quite comparable with those of the other genotoxicity assays in this regard. If the result of TGR assays was used as an indicator of carcinogenic potential when screening a database with a low prevalence of carcinogens, the negative predictive value would be higher, as would be expected for any genotoxicity test.
7) The positive and negative predictivities for the individual TGR and *Salmonella* assays were almost identical. Among the *in vivo* tests, the TGR assays were comparable with all except the comet assay, which had a slightly better combined predictivity. Among two-test combinations, the TGR assay "or" cytogenetic assays gave the best combined predictivity, except for the TGR "or" comet combination, which was slightly better compared with the component assays alone; however, the positive predictive value in this combination resulted from the fact that no non-carcinogens were positive in that battery for the chemicals included in the comparison.
8) TGR, *in vivo* micronucleus and *in vivo* comet assays performed similarly when combined in a battery with *Salmonella* and *in vitro* chromosomal aberration, suggesting that there was no clear advantage of one assay over the other for the prediction of carcinogenicity based on available data.
9) If *in vivo* confirmation of positive results from both *Salmonella* and *in vitro* chromosomal aberration is warranted, the TGR assay is likely a better choice than the *in vivo* micronucleus assay.
10) For those carcinogens with negative results in both *Salmonella* and *in vitro* chromosomal aberration assays, adding TGR assays to the test battery improved the overall predictivity over the *in vivo* micronucleus or comet assay.

5.5 Further discussion

TGR assays offer the capability to identify chemicals that induce gene mutations in any tissue, since the animal models contain multiple copies of a chromosomally integrated, genetically neutral transgene within every somatic and germ cell. Current test battery approaches do not routinely employ an *in vivo* test for gene mutations as an adjunct to *Salmonella* because the existing *in vivo* gene mutation assays are difficult to conduct, expensive, not well validated and generally not accepted by regulatory agencies. TGR assays could potentially fill this void.

TGR assays are gene mutation tests, and their ability to detect gene mutations with very high positive predictive values has been demonstrated repeatedly by sequencing. In addition to assessing genotoxicity, many short-term genetic toxicity tests are also used as indicators of carcinogenic potential, despite the inherent problems associated with this approach. In the analysis presented in this chapter, the ability of the TGR assay to identify rodent carcinogens did not differ greatly from that of the *Salmonella* reverse mutation assay. Nevertheless, the TGR assay did demonstrate an ability to identify carcinogens when used as a stand-alone test and when combined in a battery with other short-term genotoxicity tests.

Previous work has shown that the *Salmonella* reverse mutation assay is the short-term assay most predictive of carcinogenicity, but that it is not necessarily highly predictive of non-carcinogenicity. Zeiger *et al.* (1990) augmented, with an additional 41 chemicals, the work of Tennant *et al.* (1987), who analysed the results of rodent carcinogenicity and short-term genotoxicity studies (*Salmonella*, *in vitro* chromosomal aberration, sister chromatid exchange and mouse lymphoma assay) of 73 chemicals tested by the NTP. Logistic regression analysis indicated that there were no significant differences between the 41 and 73 chemical datasets, so they were combined for further

analysis. The resulting dataset was composed of 59% carcinogens, 26% non-carcinogens and 15% equivocals. *Salmonella* was clearly superior to *in vitro* chromosomal aberration, sister chromatid exchange and mouse lymphoma assay for discriminating carcinogens from non-carcinogens. Concordance of the short-term assays with carcinogenicity studies ranged from 59% (sister chromatid exchange and mouse lymphoma assay) to 66% (*Salmonella*), positive predictive value ranged from 63% (mouse lymphoma assay) to 89% (*Salmonella*) and negative predictive value ranged from 50% (sister chromatid exchange and mouse lymphoma assay) to 55% (*Salmonella*). There were no combinations of short-term assays that improved the concordance or predictivity of *Salmonella* alone; however, 24 carcinogens were non-mutagenic to *Salmonella* but mutagenic in another short-term assay (Zeiger *et al*., 1990).

In additional work, Zeiger (1998) examined the NTP database (182 carcinogens, 106 non-carcinogens, 42 equivocals) to determine the performance of several widely used short-term assays (*Salmonella*, *in vitro* chromosomal aberration, mouse lymphoma assay, *in vivo* micronucleus) and to determine whether any complementarity existed. Like the previous analysis, *Salmonella* was found to be the only assay predictive of carcinogenicity, but not non-carcinogenicity. The assays did not complement each other for the prediction of carcinogenicity, and additional tests added in a battery with *Salmonella* did not increase the predictivity over that of *Salmonella* alone.

Kim and Margolin (1999) investigated the performance of *Salmonella* and the *in vivo* chromosomal aberration and micronucleus assays and determined whether combining these three assays would improve the predictivity for rodent carcinogenicity. In their dataset of 82 chemicals compiled from NTP databases and from the published literature, the *Salmonella* assay outperformed the *in vivo* chromosomal aberration and *in vivo* micronucleus assays for the prediction of carcinogenicity. *Salmonella* was the only assay of the three that had a significant positive association with carcinogenicity. When combined in four potential two-assay battery combinations, no combination improved the predictivity over that of the *Salmonella* assay alone.

Kirkland *et al.* (2005) compiled a large database of *in vitro* genotoxicity test results (*Salmonella*, *in vitro* chromosomal aberration, *in vitro* mouse lymphoma assay, *in vitro* micronucleus) and used the data to conduct perhaps the most comprehensive investigation of the carcinogen predictive abilities of various short-term genotoxicity tests and batteries composed of one or more of these tests. The dataset contained 717 compounds that had been tested in at least one *in vitro* test system (541 carcinogens and 176 non-carcinogens). *Salmonella* had the lowest sensitivity (59%) and highest specificity (74%) of the four assays. Positive predictive value ranged from 87% (*Salmonella*) to 74% (*in vitro* mouse lymphoma assay), and negative predictive value ranged from 30% (*in vitro* micronucleus) to 37% (*Salmonella*), generally consistent with the results of the TGR assay evaluation presented herein. Combining the assays into two-test batteries that were considered indicative of genotoxicity when one of the tests was positive increased sensitivity at the expense of specificity; however, the impact on positive and negative predictive values was more variable, with positive predictive value decreasing slightly and negative predictive value increasing slightly (*Salmonella* + *in vitro* mouse lymphoma assay, *in vitro* mouse lymphoma assay + *in vitro* chromosomal aberration), decreasing (*Salmonella* + *in vitro* micronucleus, *in vitro* mouse lymphoma assay + *in vitro* micronucleus) or remaining essentially unchanged (*Salmonella* + *in vitro* chromosomal aberration). These results are inconsistent with the results of the TGR analysis presented herein, most likely because prevalence differed between the two datasets.

Despite its comprehensiveness, the major limitation of the analysis presented in this chapter is the significant over-representation of genotoxic carcinogens within the database currently available. Consequently, the results presented in this analysis must be interpreted with care. It is a useful reminder to consider the first comparisons between *Salmonella* mutagenicity and carcinogenicity (McCann *et al*., 1975; Purchase *et al*., 1978). The chemicals in the datasets used for these initial analyses were primarily genotoxic carcinogens, such as direct alkylating agents, polycyclic aromatic hydrocarbons and nitrosamines that were nearly always mutagenic to *Salmonella*; consequently, the performance of *Salmonella* was greatly overestimated (positive predictive value 89–95%). Once larger, more representative datasets (such as the NTP database) became available for analysis, more accurate assessments of positive predictive value (70–90%) could be made (Tennant *et al*., 1987; Zeiger *et al*., 1990). It is also important to consider that the tissue specificity for mutagenicity and carcinogenicity of many of the tested mutagens was previously known; this was a significant advan-

tage that will not necessarily be available when TGR assays are used in a true predictive capacity in the absence of prior knowledge. Accordingly, this analysis of the predictivity of TGR assays should be revisited when a more representative dataset is available.

5.5.1 Incidences of discordant Salmonella *and TGR results*

There were 35 chemicals for which the results of the *Salmonella* and TGR assays did not agree. These chemicals are listed in Table 5-13. All of them were carcinogenic, with the exception of acetic acid, sucrose, 1-nitronaphthalene, 2,6-diaminotoluene, 4-hydroxybiphenyl, all-*trans*-retinol and green tea. A review of the quality of the TGR data indicated that all studies were well conducted, having adequate administration and sampling times and a sufficient number of animals per group, with the exception of all-*trans*-retinol, acetic acid and possibly uracil, where the experimental protocol may not have been sufficiently robust. The *Salmonella* mutagenicity data were also questionable for all-*trans*-retinol.

Table 5-13. Cases of discordant *Salmonella* and TGR results

Chemical	*Salmonella*	TGR	Carcinogenicity
Acetic acid	−	+	−
Acrylamide	−	+	+
Amosite asbestos	−	+	+
Benzene	−	+	+
CC-1065	−	+	+
Comfrey	−	+	+
Crocidolite asbestos	−	+	+
Cyproterone acetate	−	+	+
Di(2-ethylhexyl)phthalate (DEHP)	−	+	+
Dicyclanil	−	+	+
Ferric nitrilotriacetate	−	+	+
Leucomalachite green	−	+	±
Oxazepam	−	+	+
Procarbazine hydrochloride	−	+	+
Sucrose	−	+	−
Tamoxifen	−	+	+
Uracil	−	+	+
Wyeth 14,643	−	+	+

Table 5-13 (continued)

Chemical	*Salmonella*	TGR	Carcinogenicity
1,2:3,4-Diepoxybutane	+	−	+
1,2-Dichloroethane	+	−	+
1-Nitronaphthalene	+	−	−
2,6-Diaminotoluene	+	−	−
3-Amino-1-methyl-5H-pyrido(4,3-b)indole (Trp-P-2)	+	−	+
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	+	−	+
4-Hydroxybiphenyl	+	−	−

Acrylonitrile	+	−	+
all-*trans*-Retinol	+	−	−
Chloroform	+	−	+
Genistein	+	−	+
Green tea	+	−	−
Hydrazine sulphate	+	−	+
Kojic acid	+	−	+
Methyl bromide	+	−	+
Metronidazole	+	−	+
Trichloroethylene (TCE)	+	−	+

Among chemicals mutagenic to transgenic rats and mice, there were a number of carcinogens where evidence suggests that they act at least partly by indirect mechanisms, such as crocidolite asbestos, oxazepam and Wyeth 14,643, in which an increase in oxidative DNA damage is thought to contribute to carcinogenesis. These indirect mechanisms do not contribute to mutagenicity in *Salmonella*, so a positive *Salmonella* mutagenicity assay was not expected. Oxazepam, phenobarbital and Wyeth 14,643 were mutagenic in the TGR assay only after very long treatment times (*i.e.* 180 days). Sequencing of the mutants observed in colorimetric or positive selection methods identifies those mutants that arose by expansion of a single mutant clone, representing a single mutational event. Eliminating the mutants arising from a single event (clonal correction) yields the mutation frequency, which represents the proportion of independent mutations. Phenobarbital was not mutagenic after clonal correction, while clonal correction confirmed the mutagenic response for oxazepam and Wyeth 14,643. These examples illustrate the importance of sequencing and clonal correction for chemicals that are administered over extended periods.

5.6 Case studies

There were three carcinogenic compounds for which an interpretation of the *Salmonella*, *in vitro* chromosomal aberration and *in vivo* micronucleus test battery would have led to the conclusion that the compounds were non-genotoxic – a conclusion that was also supported by the TGR assay (Table 5-14). Other instances occurred where carcinogenic compounds were concluded to be genotoxic based on an interpretation of the results of the *Salmonella*, *in vitro* chromosomal aberration and *in vivo* micronucleus test battery, but were not mutagenic in the TGR assay (Table 5-15). Three carcinogens were mutagenic only in the TGR assay (Table 5-16).

These examples are reviewed as case studies in the following sections. These case studies highlight the failure of TGR assays, like other genotoxicity tests, to identify non-genotoxic carcinogens, as well as the importance of robust test protocols and the selection of the correct target tissues for analysis.

Table 5-14. Carcinogens that would have been concluded to be non-carcinogenic based on the results of the short-term test battery

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	−	−	−	−	−	+
Acetaminophen	−	+	−	−	−	+
Carbon tetrachloride	−	+	−	−	−	+

CA, chromosomal aberration; MN, micronucleus

Table 5-15. Carcinogens that were concluded to be genotoxic based on the standard three-assay test battery, but were non-mutagenic in the TGR assay

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
1,2:3,4-Diepoxybutane	+	+	+	+	−	+
17β-Oestradiol	−	+	−	−	−	+
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	+	+	+	+	−	+
Chloroform	+	−	+	+	−	+
Kojic acid	+	+	+	+	−	+
Methyl bromide	+	+	+	+	−	+
Nickel subsulphide	−	+	+	+	−	+
Trichloroethylene (TCE)	+	−	+	+	−	+

CA, chromosomal aberration; MN, micronucleus

Table 5-16. Carcinogens that were non-genotoxic in all short-term assays, except the TGR assay

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
Di(2-ethylhexyl)phthalate (DEHP)	−	−	−	−	+	+
Dicyclanil	−	−	−	−	+	+
Oxazepam	−	−	−	−	+	+

CA, chromosomal aberration; MN, micronucleus

*5.6.1 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (Table 5-14)*

Kociba *et al.* (1978) investigated the carcinogenicity of TCDD administered to Sprague-Dawley rats via the diet. The primary target was the female rat liver, where an increased incidence of hepatic neoplastic nodules was observed. An increase in squamous cell carcinoma of the lung was also noted in females. In addition, an increased incidence of squamous cell carcinoma of the hard palate or nasal turbinates was observed in both sexes (Kociba *et al*., 1978). The carcinogenicity of TCDD administered by gavage was also investigated by the NTP. TCDD was carcinogenic to Osborne-Mendel rats, inducing thyroid gland follicular cell adenomas in males and hepatocellular carcinomas and adrenal cortical adenomas/carcinomas in females. TCDD was also carcinogenic to B6C3F1 mice, inducing hepatocellular carcinomas in males and females and thyroid gland follicular cell adenomas in females (National Toxicology Program, 1982a). Male and female rodents appeared to be affected differently by TCDD. The primary target for TCDD carcinogenicity in female rats was the liver, whereas male rats were more likely to develop tumours of the thyroid. Other target tissues included the lung (female rat), hard palate and nasal turbinates (male and female rat), thyroid (female mouse), adrenal cortex (female rat) and skin (female mouse).

Although there is clear evidence of the carcinogenicity of TCDD, there is little evidence of genotoxicity on the basis of a large number of studies (for a detailed review, see Whysner and

Williams, 1996a). TCDD is generally accepted to produce tumours via a non-genotoxic mechanism. The evidence for this conclusion includes the absence of DNA adduct formation and negative results in the majority of genotoxicity tests, tumour formation commonly found in association with increased cell proliferation and evidence of tumour promotion activity (Shu, Paustenbach and Murray, 1987; Whysner and Williams, 1996a).

Carcinogens acting by a non-genotoxic mechanism usually are not detectable with TGR assays (Gunz, Shephard and Lutz, 1993). Since most evidence consistently suggests that TCDD induces carcinogenesis by a non-DNA-reactive (non-genotoxic) mechanism, it is not surprising that TCDD failed to induce gene mutations in *lacI* transgenic rats.

5.6.2 Acetaminophen (Table 5-14)

Acetaminophen induced hepatocellular carcinomas among male mice administered a diet containing 0.5% or 1.0% (250 or 500 mg/kg bw per day) for up to 18 months (Flaks and Flaks, 1983). Administration of 0.5% or 1% (300 or 600 mg/kg bw per day) in the diet to Leeds strain rats also induced the incidence of hepatocellular neoplastic nodules and bladder tumours among males and females (Flaks, Flaks and Shaw, 1985). However, an NTP carcinogenicity study indicated that acetaminophen administered continuously in the diet of F344/N rats and B6C3F1 mice for up to 104 weeks at concentrations of 0, 600, 3000 or 6000 mg/kg bw produced only equivocal evidence of carcinogenicity to female rats (based on increased incidences of mononuclear cell leukaemia) and no evidence of carcinogenicity to male rats or male and female mice (National Toxicology Program, 1993a). Other studies involving mice and rats have suggested that acetaminophen lacks carcinogenic initiating activity, but may act as a tumour promoter at hepatotoxic doses (reviewed in Bergman, Muller and Teigen, 1996). In numerous studies, acetaminophen has not induced gene mutations in bacteria or mammalian cells *in vitro*; however, chromosomal aberrations were induced *in vitro* in Chinese hamster ovary cells, V79 cells and human lymphocytes, although more effectively in the absence of S9 and at cytotoxic concentrations (reviewed in Bergman, Muller and Teigen, 1996). An increase in mutation frequency was also observed in a mouse lymphoma fluctuation test, but the relative contribution of gene mutations or chromosomal aberrations was not determined by colony sizing (Bergman, Muller and Teigen, 1996). *In vivo* chromosomal aberration and micronucleus studies have not provided any consistent evidence of clastogenic activity; however, at high doses, a general association of clastogenicity and toxicity was observed, which is consistent with saturation of the low-capacity sulphation metabolic pathway and the subsequent cytochrome P-450–mediated formation of reactive (genotoxic) intermediates (reviewed in Bergman, Muller and Teigen, 1996).

Five female *gpt* delta transgenic rats received acetaminophen in the diet at a dose of 525 mg/kg bw per day for 13 weeks. There was no increase in *gpt* mutation frequency in the liver compared with the negative control. The *gpt* mutation spectra indicated that the most common mutations were G:C to A:T transitions (39%), followed by A:T to T:A transversions (34.8%) and G:C to T:A transversions (21.7%). The induction of a greater proportion of G:C to T:A transversions in acetaminophen-treated animals compared with controls suggests that acetaminophen may cause some oxidative DNA damage (Kanki *et al*., 2005).

Evidence of the carcinogenicity of acetaminophen is not strong, especially at non-hepatotoxic doses. Generally, acetaminophen lacked initiating activity, which was supported by the absence of mutagenic activity in bacterial and mammalian cell models and the lack of clear evidence of clastogenic activity both *in vitro* and *in vivo*. Acetaminophen was negative in a TGR assay, consistent with the results of most other short-term genotoxicity studies that indicated an absence of genotoxic activity.

5.6.3 Carbon tetrachloride (Table 5-14)

A number of carcinogenicity studies of carbon tetrachloride have been conducted. Oral administration of carbon tetrachloride consistently induced hepatomas, as well as hepatocellular adenomas, hepatocellular carcinomas and phaeochromocytomas in both sexes of mice (reviewed in

Weisburger, 1977; International Programme on Chemical Safety, 1999). In rats, carbon tetrachloride induced increases in the incidence of neoplastic nodules and hepatocellular carcinomas after oral administration (Weisburger, 1977). Increased incidences of hepatocellular carcinomas and hyperplastic nodules accompanied by severe cirrhosis were also observed after subcutaneous administration (Reuber and Glover, 1970), whereas hepatocellular adenomas and hepatocellular carcinomas were observed after whole-body inhalation exposure (reviewed in International Programme on Chemical Safety, 1999). These studies suggest that carbon tetrachloride is primarily a liver carcinogen in both male and female mice and rats.

The genotoxicity of carbon tetrachloride has been reviewed extensively (McGregor and Lang, 1996; International Agency for Research on Cancer, 1999a), and there has been little evidence of genotoxicity. No evidence of carbon tetrachloride mutagenicity to *Salmonella* has been observed in a large number of studies. No evidence of DNA damage, unscheduled DNA synthesis, sister chromatid exchanges or chromosomal aberrations was apparent *in vitro*, except for a weak clastogenic effect in an *in vitro* micronucleus assay with human lymphocytes (Tafazoli *et al*., 1998). In several *in vivo* studies, carbon tetrachloride did not induce unscheduled DNA synthesis, micronuclei, chromosomal aberrations or aneuploidy. However, binding of carbon tetrachloride to liver cell DNA has been observed in rats, mice and Syrian hamsters *in vivo* (reviewed in McGregor and Lang, 1996). While it appears that the mechanism for carbon tetrachloride–induced carcinogenesis could likely have a non-genotoxic component, there appears to be insufficient evidence to make any definite conclusion.

Tombolan *et al*. (1999b) assessed the effect of regenerative cell proliferation induced by carbon tetrachloride on the mutagenicity of 5,9-dimethyldibenzo(c,g)carbazole in *lacZ* transgenic mice. As a component of this study, five male mice were administered a single 80 mg/kg bw dose of carbon tetrachloride in corn oil by gavage and were sacrificed 14 days after administration. No significant increase in mutant frequency was observed. Hachiya and Motohashi (2000) investigated the liver mutagenicity of carbon tetrachloride in *lacZ* transgenic mice. Carbon tetrachloride was administered by gavage to groups of two or three animals per dose per sampling time at doses of 700 or 1400 mg/kg bw, and the animals were sacrificed 7 days (1400 mg/kg bw), 14 days (700 and 1400 mg/kg bw) or 28 days (1400 mg/kg bw) after administration. In this case, a statistically significant increase in mutant frequency was observed (a maximum of approximately 2.3-fold increase); however, because of high variability in the data, the authors concluded that the results lacked biological significance.

The Tombolan *et al*. (1999b) study was not specifically designed to assess the mutagenicity of carbon tetrachloride; rather, carbon tetrachloride was used as a means to induce cell proliferation in the liver, where carbon tetrachloride mutagenicity was also determined. Animals were treated only once with a small dose. The Hachiya and Motohashi (2000) study employed a much higher dose and longer duration prior to sampling. It remains unclear, however, whether the methodological differences alone are the source of the different conclusions reached by these two laboratories.

From this information, it is difficult to determine conclusively if the negative TGR results are a result of the study methodology or an inability of Hachiya and Motohashi (2000) to induce gene mutations in general. It is also difficult to assess the performance of the TGR assay in the absence of more concrete mechanistic data. However, it should be noted that the protocols used for both TGR studies did not conform to the subsequently proposed IWGT protocol (Thybaud *et al*., 2003), meaning that the experimental conditions may not have been sufficient for a response to be observed. An additional TGR assay conducted according to the IWGT recommendations would provide more clarity to this situation.

5.6.4 1,2:3,4-Diepoxybutane (Table 5-15)

Female B6C3F1 mice and Sprague-Dawley rats were exposed to 1,2:3,4-diepoxybutane by inhalation at 0, 2.5 or 5.0 ppm (0, 8.8 or 17.6 mg/m^3) 6 hours per day, 5 days per week, for 6 weeks. Following exposure, animals were maintained for 18 months for observation of tumour development. Among exposed rats, a dose-dependent increase in neoplasms of the nasal mucosa was observed; however, there was no evidence of neoplasia of the nasal mucosa in exposed mice (Henderson *et al*., 1999).

1,2:3,4-Diepoxybutane is a potent bifunctional alkylating agent (see Jackson *et al*., 2000 for review). 1,2:3,4-Diepoxybutane is a direct-acting mutagen to *Salmonella* (Adler *et al*., 1997; Abu-Shakra, McQueen and Cunningham, 2000). Gene mutations at the *Hprt* locus were induced by 1,2:3,4-diepoxybutane in human TK6 cells exposed *in vitro* (Steen, Meyer and Recio, 1997) and in the splenic lymphocytes of B6C3F1 mice and F344 rats exposed by inhalation to 4 ppm (14.1 mg/m^3) for 6 hours per day, 5 days per week, for 4 weeks (Meng *et al*., 1999). 1,2:3,4-Diepoxybutane induced micronuclei in cultured human lymphocytes (Vlachodimitropoulos *et al*., 1997; Xi *et al*., 1997) and increased the frequency of chromosomal aberrations in human bone marrow cultures (Marx *et al*., 1983). Single intraperitoneal injections of 9–30 mg 1,2:3,4-diepoxybutane/kg bw also increased the frequency of micronuclei in splenocytes and bone marrow of mice and rats in a number of studies (reviewed in Jackson *et al*., 2000). 1,2:3,4-Diepoxybutane also caused chromosomal aberrations in the bone marrow cells of mice and Chinese hamsters exposed by inhalation or intraperitoneal injection to doses in the range of 22–34 mg/kg bw (Walk *et al*., 1987).

In contrast to the potent mutagenicity observed in other experimental models, no increase in mutant frequency in the bone marrow was observed 2 days after female *lacZ* transgenic mice (7–8 per group) were administered 1,2:3,4-diepoxybutane by intraperitoneal injection at doses of 6, 12 or 24 mg/kg bw per day for 5 consecutive days (G.R. Douglas, unpublished data). The discrepancy between these results and the potent mutagenic activity observed in *in vitro* assays and in the bone marrow of rodents *in vivo* (at similar doses) cannot easily be reconciled.

5.6.5 17β-Oestradiol (Table 5-15)

Induced tumours have been observed in a number of organs in rats, mice and hamsters following exposure to 17β-oestradiol. In humans, breast and uterine cancer risk is increased with slightly elevated circulating levels (Liehr, 2000). Although earlier work found that 17β-oestradiol did not induce gene mutations in *Salmonella* or V79 cells (Lang and Reimann, 1993), a more recent study found that it did induce *Hprt* gene mutations in V79 cells (Kong *et al*., 2000). Whereas micronuclei are induced *in vitro* in mammalian cells (Yared, McMillan and Martin, 2002; Kayani and Parry, 2008), micronuclei are not induced *in vivo* (Ashby *et al*., 1997). Micronuclei are not the result of chromosomal breaks, but arise from induced non-disjunction events leading to aneuploidy. Oestradiol also induced single-strand DNA breaks *in vitro* as detected in the comet assay (Yared, McMillan and Martin, 2002). Although this chemical does cause gene mutagenicity *in vitro*, *in vivo* gene mutagenicity was not detected in the TGR assay (Manjanatha *et al*., 2005, 2006a). This *in vivo* outcome parallels the finding that oestradiol did not induce micronuclei *in vivo*, despite the fact that micronuclei were induced *in vitro*, and suggests that oestradiol may be a relatively weak *in vitro* mutagen that is not readily detected *in vivo*. Oestradiol has also been shown to enhance the mitotic rate in MCF-7 cells (Yared, McMillan and Martin, 2002). It also induced microsatellite instability and loss of heterozygosity in MCF-10F cells (Fernandez, Russo and Russo, 2006). These findings appear to confirm the suggestion of Liehr (2000) that oestradiol is carcinogenic by a dual mechanism involving weak genotoxicity and tumour promotion–like activity.

5.6.6 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) (Table 5-15)

Wistar rats received MX in the drinking water for 104 weeks at concentrations providing average daily doses of 0.4, 1.3 or 5.0 mg/kg bw for males and 0.6, 1.9 or 6.6 mg/kg bw for females. Increases in tumour incidence were observed at all doses, including those that caused no appreciable evidence of toxicity. In particular, MX caused an increased incidence of follicular adenoma and carcinoma in the thyroid glands and cholangioma in the liver. In addition, MX caused an increase in cortical adenomas of the adrenal glands (both sexes); alveolar and bronchiolar adenomas of the lungs and Langerhans' cell adenomas of the pancreas (males); and lymphomas, leukaemias, and adenocarcinomas and fibroadenomas of the mammary glands (females). These data suggest that MX is a potent multi-site rat carcinogen (Komulainen *et al*., 1997).

MX has been studied extensively, and there are a large number of publications reporting the genetic toxicology of this compound; it is a strong electrophile and is a relatively potent bacterial mutagen. MX has induced gene mutations in numerous strains of *S. typhimurium* and *E. coli*, forward mutations at the *Tk* locus of mouse lymphoma L5178Y cells and at the *Hprt* locus of Chinese hamster ovary and Chinese hamster V79 cells, chromosomal aberrations in Chinese hamster ovary cells, and chromosomal aberrations and micronuclei in mouse lymphoma L5178Y cells and rat peripheral blood lymphocytes; MX has also induced unscheduled DNA synthesis in mouse and rat hepatocytes *in vitro* (reviewed in McDonald and Komulainen, 2005). However, among *in vivo* models, MX has not demonstrated the same degree of mutagenic activity. Nuclear anomalies were induced in the fore-stomach and duodenum of mice administered a single dose of MX by gavage at doses of 0.28, 0.37 or 0.46 mmol/kg bw (Daniel, Olson and Stober, 1991). Micronuclei were also induced in peripheral blood lymphocytes of rats treated by gavage on 3 consecutive days with doses of 25–150 mg/kg bw (Maki-Paakkanen and Jansson, 1995). However, in other *in vivo* studies, MX did not induce micronuclei in PCEs, bone marrow or the liver of mice receiving single or repeated exposures to doses ranging from 4.4 to 144 mg/kg bw, or in PCEs of rats exposed via drinking water for 104 weeks at concentrations from 5.9 to 70 mg/L (reviewed in McDonald and Komulainen, 2005). It is apparent from these results that MX has consistently failed to induce genetic damage to the bone marrow; it is also possible that toxicokinetic factors and the rates of mutation fixation and DNA repair also contribute to the discrepancies between tissues.

Groups of five male and five female 7-week-old *gpt* delta C57BL/6J transgenic mice were given MX at doses of 0, 10, 30 or 100 mg/L in their drinking water for 12 weeks. Immediately thereafter, the mutant frequency in the liver and lungs was assessed using 6-thioguanine and Spi^{-} selection. Further groups of five male and five female *gpt* delta mice were given 0 or 100 mg MX/L for 78 weeks in order to detect neoplastic lesions histopathologically. There were no increases in mutant frequency in either tissue, and there also was no evidence of cell proliferative activity using immunohistochemistry for proliferating cell nuclear antigen or any increase in neoplastic lesions (Nishikawa *et al*., 2006). These results are consistent with those of other *in vivo* models, in which MX has exhibited inconsistent results. MX may not have *in vivo* genotoxic activity of comparable potency to its *in vitro* mutagenicity because of metabolic and other toxicokinetic factors; however, any species-specific differences influencing the mutagenic activity in TGR assays could be ruled out by further studies using transgenic rat models.

5.6.7 Chloroform (Table 5-15)

Chloroform was administered by gavage to Osborne-Mendel rats and B6C3F1 mice (50 per sex per group). A significantly increased incidence of kidney epithelial tumours was found in male rats. The incidence of hepatocellular carcinoma was significantly increased in both sexes of mice and was accompanied by nodular hyperplasia of the liver in many of the male mice that had not developed hepatocellular carcinomas (National Cancer Institute, 1976a). Lifetime administration of chloroform in drinking water also increased the yield of renal tubular adenomas and adenocarcinomas in male Osborne-Mendel rats in a dose-related manner and significantly increased the incidence of hepatic neoplastic nodules in female Wistar rats (Tumasonis, McMartin and Bush, 1985), but failed to increase the incidence of hepatocellular carcinomas in female B6C3F1 mice (Jorgenson *et al*., 1985). Exposure by inhalation also led to an increase in renal cell adenomas and carcinomas in male BDF1 mice at the highest exposure level, but it was noted that the inhaled dose likely exceeded the MTD (reviewed in Golden *et al*., 1997).

Ten female *lacI* transgenic B6C3F1 mice per group were exposed to chloroform within an inhalation chamber at concentrations of 0, 10, 30 or 90 ppm (0, 48.8, 146.5 or 439.4 mg/m^3) for 10, 30, 90 or 180 consecutive days, so that mice would be exposed to non-hepatotoxic, borderline hepatotoxic or substantially hepatotoxic concentrations leading to regenerative cell proliferation. Long-term exposures were included in the event that mutations secondary to cytotoxicity required longer exposure periods to become manifest. Following the final exposure, animals remained untreated for 10 days to allow for fixation of mutations and to allow clearance of remaining chloroform from the tissues prior to isolation of genomic DNA. No significant chloroform-induced

increases in the mutant frequencies were observed in any exposure group at any time point, suggesting that chloroform lacks mutagenic activity in the liver of female B6C3F1 *lacI* transgenic mice (Butterworth *et al*., 1998).

Chloroform has induced gene mutations in *Salmonella*, Chinese hamster V-79 cells and mouse lymphoma L5178Y cells. It has also induced sister chromatid exchanges in cultured human lymphocytes and in mice *in vivo*. An increased frequency of micronucleated kidney cells was observed in rats orally administered chloroform at 4 mmol/kg bw (Robbiano *et al*., 1998). In addition, chloroform induced chromosomal aberrations in rat bone marrow cells *in vivo* after repeated 5-day oral administration or a single intraperitoneal injection at a dose of 1 mmol/kg bw (Fujie, Aoki and Wada, 1990). While not providing evidence of genotoxicity, chloroform also induced a significant increase in S-phase synthesis in mouse liver following *in vivo* treatment, an indirect indicator of hepatocellular proliferation (Mirsalis *et al*., 1989).

Although chloroform has induced gene mutations and chromosomal aberrations in several test systems, it did not induce mutations of the *lacI* gene in the liver of transgenic mouse in a well-conducted study. This underscores the importance of the test battery in short-term genotoxicity testing, since one test system may not detect all genotoxicants.

5.6.8 Kojic acid (Table 5-15)

Male and female B6C3F1 mice were administered 0, 1.5 or 3.0% kojic acid in the diet beginning at 6 weeks of age for 20 months. Thyroid weights were significantly increased in both sexes and were accompanied by a markedly increased incidence of diffuse hyperplasia and follicular adenomas. The serum free triiodothyronine levels in both sexes receiving 3.0% after 6 months were significantly lower than in the controls, whereas the serum thyroid-stimulating hormone levels were transiently higher. Dietary administration of kojic acid induces thyroid adenomas in male and female B6C3F1 mice, which was speculated to occur via a non-genotoxic mechanism involving alteration in serum free triiodothyronine and thyroid-stimulating hormone levels (Fujimoto *et al*., 1998).

Kojic acid was a weak mutagen to *Salmonella*, exhibiting mutagenic activity only at concentrations greater than or equal to 1 mg/plate (Bjeldanes and Chew, 1979; Shibuya *et al*., 1982; Wei *et al*., 1991; Nohynek *et al*., 2004). Kojic acid failed to induce biologically relevant, dose-responsive and reproducible increases of the mutant frequency at the *Hprt* locus of mouse lymphoma L5178Y cells (Nohynek *et al*., 2004). Chromosomal aberrations were induced by kojic acid in Chinese hamster ovary cells at concentrations of 3 000 µg/mL and above in the presence or absence of S9 (Wei *et al*., 1991), but were not induced subsequently in Chinese hamster V79 cells at lower concentrations, except for a very slight increase at 1 000 µg/mL in cultures treated continuously for 18 or 28 hours (Nohynek *et al*., 2004). Micronuclei were not induced by kojic acid in human keratinocytes, even where marked toxicity was induced; however, an increased frequency of micronuclei was induced in human hepatoma cells, associated with severe cytotoxicity at concentrations above 6 000 µg/mL (Nohynek *et al*., 2004). *In vivo*, kojic acid did not induce a significant increase in the frequency of micronucleated hepatocytes but did increase the frequency of micronucleated reticulocytes isolated from groups of male rats administered 1 000 or 2 000 mg/kg bw (Suzuki *et al*., 2005). Other investigators have failed to induce an increased frequency of micronucleated PCEs upon intraperitoneal administration of kojic acid to NMRI mice at doses up to 750 mg/kg bw (Nohynek *et al*., 2004). Kojic acid also did not cause an increase in unscheduled DNA synthesis in the liver of male rats administered single oral doses of kojic acid at 1 500 mg/kg bw (2- and 16-hour evaluation) or 150 mg/kg bw (16-hour evaluation) (Nohynek *et al*., 2004).

Kojic acid was administered orally to groups of 10 male *lacZ* transgenic mice at doses of 0 (corn oil), 800 or 1600 mg/kg bw per day (the experimentally determined MTD) for 28 days. Seven days after the final dose, the animals were euthanised and the livers removed for analysis. No increase in mutant frequency was observed in the liver at either dose compared with the negative control (Nohynek *et al*., 2004).

Overall, kojic acid has induced an inconsistent pattern of results among short-term genotoxicity tests. Furthermore, there is some evidence to suggest that the carcinogenicity of kojic acid is at least partly mediated by perturbation of thyroid hormone homeostasis. The relative contribution of non-

genotoxic and genotoxic mechanisms to the observed carcinogenicity of kojic acid has not been clearly demonstrated; any such investigations should include further studies in TGR models using other tissues, including thyroid and bone marrow, to determine if the absence of mutagenic activity observed in the liver is indicative of a failure of the assay or a failure to select the appropriate target tissue.

5.6.9 Methyl bromide (Table 5-15)

Oral administration of methyl bromide to rats for 90 days produced squamous cell carcinomas of the forestomach and a marked diffuse hyperplasia of the epithelium of the forestomach (Danse, van Velsen and van der Heijden, 1984). However, subsequent examination by an NTP panel concluded that the forestomach lesions represented inflammation and hyperplasia rather than malignant lesions (International Programme on Chemical Safety, 1995). Additional studies found inflammation, acanthosis, fibrosis and a high incidence of pseudoepitheliomatous hyperplasia in treated animals that regressed upon cessation of treatment (Boorman *et al*., 1986), which suggests that any carcinogenic activity to the rodent forestomach may be mediated through an irritant mechanism. No evidence of carcinogenicity was observed following administration of methyl bromide–fumigated diets to rats for up to 2 years (Mitsumori *et al*., 1990).

Inhalation is the most relevant route of human exposure to methyl bromide, as the chemical is extensively used as a fumigant for disinfestation of food products. An NTP study of mice exposed by inhalation to methyl bromide for up to 103 weeks found no evidence of carcinogenicity (National Toxicology Program, 1992). There was also no evidence of carcinogenicity when rats were exposed by inhalation for 29 months (Reuzel *et al*., 1991). This evidence suggests that considerable uncertainty remains regarding the carcinogenic activity of methyl bromide. IARC has recognised this uncertainty, classifying methyl bromide as having limited evidence of carcinogenicity in experimental animals and inadequate evidence in humans (International Agency for Research on Cancer, 1999b).

Groups of six male *lacZ* transgenic mice were treated by gavage with single methyl bromide doses of 5 or 12.5 mg/kg bw in corn oil, or with 25 mg/kg bw every 24 hours for 10 days. Following the final exposure, animals used for DNA adduct analysis were sacrificed after 4–6 hours, whereas those used for mutant frequency analysis were held without treatment for 14 days to allow a period for fixation of mutations. Animals receiving a single dose of 5 mg/kg bw did not exhibit any O^6-methylguanine (O^6-meG) adducts, although animals receiving a single dose of 12.5 mg/kg bw exhibited O^6-meG adducts in the liver, spleen and lung. Following multiple doses of 25 mg/kg bw, animals exhibited adducts in liver, spleen, lung, forestomach, glandular stomach, bone marrow and blood leukocytes. No increases in the *lacZ* transgene mutant frequency in the liver, spleen, lung or bone marrow were apparent after the single 12.5 mg/kg bw dose or in the liver and glandular stomach following 10 consecutive daily doses of 25 mg/kg bw. Despite forming O^6-meG adducts in a wide range of tissues, methyl bromide did not induce mutations in the *lacZ* transgene in the liver or glandular stomach of transgenic male mice (Pletsa *et al*., 1999).

Methyl bromide has demonstrated conclusive genotoxic activity. It has produced gene mutations in *Salmonella* and in the mouse lymphoma assay, induced micronuclei in PCEs of both mouse and rat, and produced sister chromatid exchanges in cultured human lymphocytes and in the bone marrow cells of exposed mice (reviewed in International Programme on Chemical Safety, 1995). Methyl bromide has also been found to cause systemic DNA methylation (Bolt and Gansewendt, 1993).

The absence of evidence of mutagenicity in the TGR assay despite clear evidence of genotoxicity in other assays is not easily reconciled. Slightly fewer animals than is considered ideal were used in the TGR assay for the determination of mutant frequency in the glandular stomach, and a sampling time of 14 days may be slightly short for determining mutant frequency in the liver. Despite these minor issues, the absence of mutations of the *lacZ* gene in liver and glandular stomach is quite conclusive. Further investigation is likely warranted.

5.6.10 Nickel subsulphide (Table 5-15)

The carcinogenicity of nickel subsulphide was investigated by the NTP. Rats and mice were exposed to nickel subsulphide by inhalation for 6 hours per day, 5 days per week, for 2 years. Clear evidence of carcinogenicity was noted in rats, based on increased incidences of alveolar/bronchiolar adenoma or carcinoma in both sexes and increased incidences of benign (males and females) or malignant (males) phaeochromocytoma of the adrenal medulla. There was no evidence of carcinogenicity in male or female mice (National Toxicology Program, 1996). Nickel subsulphide was also demonstrated to be carcinogenic in other tissues when administered by routes other than inhalation (reviewed in International Programme on Chemical Safety, 1991).

Mayer *et al.* (1998) investigated the mutagenic potential of nickel subsulphide. Groups of 6–8 *lacZ* transgenic mice and groups of 4–5 *lacI* transgenic rats were treated by a single nose-only inhalation exposure (10 mg/kg bw for mice, 6 mg/kg bw for rats) for 2 hours. Following an expression period of 14 days, the mutant frequency in lung and nasal mucosa was determined. No evidence of an increased mutant frequency was apparent, suggesting that nickel subsulphide was not mutagenic in the lung and nasal mucosa of *lacZ* transgenic mice and *lacI* transgenic rats.

Although nickel subsulphide was not mutagenic to *Salmonella*, it did induce a significant increase in the frequency of chromosomal aberrations in human peripheral blood lymphocytes *in vitro* as well as an increased frequency of micronuclei in mouse PCEs *in vivo* (Arrouijal *et al*., 1990).

Oxidative DNA damage has been proposed as a potential mechanism for nickel subsulphide carcinogenicity. Two mechanisms for nickel subsulphide–induced oxidative damage have been proposed: nickel subsulphide could react with endogenous or nickel subsulphide–produced hydrogen peroxide to produce reactive oxygen species that directly damage DNA, or the release of reactive oxygen species from phagocytes in response to nickel subsulphide–induced inflammation could cause indirect DNA damage (Kawanishi *et al*., 2002). Further mechanistic work by Lee *et al.* (1995) demonstrated that a transgenic Chinese hamster cell line was susceptible to inactivation of the *gpt* target gene by nickel subsulphide; inactivation did not appear to occur through gene mutation, but through mechanisms, such as DNA methylation, that silenced *gpt* expression. These studies, and the results of *in vitro* and *in vivo* chromosomal aberration studies, suggest that the carcinogenic activity of nickel subsulphide could be mediated by both genotoxic and non-genotoxic mechanisms. However, since oxidative DNA damage is mutagenic and appears to be detectable using a TGR assay (Shane *et al*., 1999, 2000c), it is reasonable to question whether there was sufficient exposure to nickel sub-sulphide to produce DNA damage. Although the nickel distribution in the respiratory tract of treated rats was about 400 times greater than in control animals, the ability of nickel subsulphide to produce DNA damage at the target site may have been low *in vivo* because of increased clearance and various protective mechanisms within the lungs (Mayer *et al*., 1998). Thus, TGR-detectable gene mutations *in vivo* may play a less important role in the carcinogenicity of nickel subsulphide than one would be led to believe from the results of *in vitro* studies.

5.6.11 Trichloroethylene (TCE) (Table 5-15)

A number of carcinogenicity studies of TCE have been conducted. An initial National Cancer Institute study found an increased incidence of hepatocellular carcinomas in male and female mice but no evidence of an increased incidence of neoplastic lesions in male or female rats following gavage administration for 78 weeks (National Cancer Institute, 1976b). Further study of TCE administered by gavage found inadequate evidence to evaluate carcinogenicity in male rats because of reduced survival and no evidence of carcinogenicity to female rats after 2 years of administration. TCE, however, was again carcinogenic to mice, causing increased incidences of hepatocellular carcinomas in males and females and hepatocellular adenomas in females (National Toxicology Program, 1990a). The ability of TCE to induce gene mutations and small deletions was examined using male and female *lacZ* transgenic mice. Animals were exposed to TCE in a whole-body inhalation chamber at concentrations of 0, 203, 1 153 or 3 141 ppm (0, 1 091, 6 196 or 16 880 mg/m^3), 6 hours per day for 12 days. Following the exposure period, animals were held without treatment for a 14- or 60-day fixation period. The *lacZ* mutant frequency was determined in bone marrow, kidney, spleen, liver,

lung and testicular germ cells. No increased mutant frequency was observed in the lungs of animals 14 days after exposure, nor were increases in the mutant frequency observed in any of the examined tissues sampled 60 days after exposure. Inhalation exposure to TCE did not induce gene mutations or small deletions in *lacZ* transgenic mice (Douglas *et al*., 1999).

TCE has exhibited evidence of genotoxic activity in several test systems. Covalent binding to DNA has occurred in the presence of liver microsomes *in vitro*, but little DNA binding occurred *in vivo*. TCE is a weak inducer of sister chromatid exchanges in mammalian cell culture and can produce single-strand DNA breaks *in vivo*. Several studies have shown some weak mutagenic activity in *Salmonella*, but the evidence overall suggests that TCE is not a potent mutagen. TCE was also weakly mutagenic in the mouse lymphoma assay and produced sister chromatid exchanges in the presence of metabolic activation, as well as aneuploidy at high concentrations *in vitro*. It did not induce structural chromosomal aberrations *in vitro* or *in vivo*, but caused an increase in micronuclei in mammalian cell culture and in bone marrow cells of rats *in vivo* (reviewed in Fahrig, Madle and Baumann, 1995).

From the results in *Salmonella*, it seems that TCE is unlikely to cause significant induction of gene mutations. However, evidence of sister chromatid exchanges and aneuploidy *in vitro* and micronuclei in bone marrow cells *in vivo* would suggest that recombination and aneuploidy could play roles in the carcinogenicity of TCE, as has been speculated by several authors (Fahrig, Madle and Baumann, 1995; Douglas *et al*., 1999; Moore and Harrington-Brock, 2000). Since genotoxic effects other than gene mutations and small deletions are not detectable with the TGR assay, the results of the study with *lacZ* transgenic mice can be taken as further evidence that TCE is not primarily a gene mutagen.

5.6.12 Di(2-ethylhexyl)phthalate (DEHP) (Table 5-16)

Male and female F344 rats and B6C3F1 mice (50 per sex per group) were administered DEHP in the diet for 103 weeks. A significantly higher incidence of hepatocellular carcinomas in female rats and male and female mice was observed. Male rats had a significantly increased incidence of hepatocellular carcinomas or neoplastic nodules (National Toxicology Program, 1982b). The carcinogenic activity of DEHP to liver of rats was also demonstrated (Cattley, Conway and Popp, 1987; Rao, Yeldandi and Subbarao, 1990); however, no carcinogenic activity was observed following intraperitoneal or inhalation exposure of Syrian golden hamsters (Schmezer *et al*., 1988).

The mutagenicity of DEHP administered in the diet was investigated using female *lacI* transgenic mice. Groups of three mice received either 3000 or 6000 mg/kg feed for 120 days. No significant increase in mutant frequency was observed in either group, and no evidence of an increased rate of cell division was observed based on the labelling index for incorporation of BrdU into DNA. This would indicate that gene mutations were not produced at dose levels below that inducing hepatocyte cell division. However, the authors suggested, based on Melnick (1992), that any mitogenic effect may not have been sustained throughout the treatment period (Gunz, Shephard and Lutz, 1993). In contrast, however, Boerrigter (2004) administered 2333 mg/kg bw DEHP to male and female *lacZ* plasmid mice for six days over two weeks and found the mutant frequency to be increased relative to the control. This result is noteworthy, given that the *lacZ* plasmid mouse is capable of detecting larger deletions that are not detectable using other TGR models, and it would warrant further study.

In numerous short-term studies, DEHP has not demonstrated clear genotoxic activity. No evidence of DEHP-induced gene mutations in *Salmonella* or in mammalian assays was apparent. There was also no consistent evidence of DNA binding, induction of unscheduled DNA synthesis or single strand breaks, chromosomal aberrations, micronuclei or sister chromatid exchanges in a variety of assays, both *in vitro* and *in vivo* (reviewed in International Programme on Chemical Safety, 1992; Doull *et al*., 1999).

As there is no clear evidence of genotoxicity, DEHP does not appear to act as an initiator of carcinogenesis. There is broad agreement that DEHP is, instead, a promoter whose rodent carcinogenic activity is mediated primarily (but not necessarily exclusively) via activation of the hepatic peroxisome proliferator activated receptor. Activation of the peroxisome proliferator activated receptor leads to enhanced cell proliferation (a mitogenic, rather than regenerative, response) and also

to increased oxidative stress, since peroxisomes are the primary site for hydrogen peroxide generation within hepatocytes. The absence of evidence of DEHP-induced gene mutations in numerous studies would suggest that oxidative stress contributes proportionately less to the carcinogenicity of DEHP than does increased cell proliferation.

5.6.13 Dicyclanil (Table 5-16)

Groups of 60 male and 60 female mice were administered dicyclanil in the diet at doses equivalent to 0, 1.1, 12, 59 and 210 mg/kg bw per day for males and 0, 1.1, 12, 65 and 200 mg/kg bw per day for females. Increased numbers of hepatocellular mitotic figures and multinucleated hepatocytes were seen in the livers of males administered the high dose, and the frequency of foci indicative of cellular change was increased in animals of each sex at the highest dose. The incidence of heptocellular adenomas was higher in females administered 65 and 200 mg/kg bw per day (9/53 and 5/60, respectively) than in controls (0/52). In addition, the incidence of hepatocellular carcinomas was increased in females at the highest dose. Since the doses at which the hepatocellular adenomas and carcinomas occurred in females exceeded the MTD and there was some evidence of hepatocellular proliferation in these animals, it is conceivable that there could be a non-genotoxic component to the cancer mode of action (International Programme on Chemical Safety, 2000).

Results of genotoxicity tests comprising the standard test battery, including bacterial reverse mutation, *Hprt* gene mutation, chromosomal aberration, unscheduled DNA synthesis and *in vivo* micronucleus formation, have been consistently negative (International Programme on Chemical Safety, 2000); in addition, there was no evidence of DNA damage in an *in vivo* alkaline comet assay or increases in the number and area of glutathione *S*-transferase placental form positive foci in treated mice (Moto *et al*., 2003). Furthermore, when partially hepatectomised mice were maintained on a diet containing 1 500 mg dicyclanil/kg for 13 and 26 weeks after intraperitoneal injection of DMN, significant increases in mRNA expression of some metabolism- and oxidative stress–related genes were observed, along with an increase in generation of reactive oxygen species from mouse liver microsomes (Moto *et al*., 2006). These data provide further support to the assertion that dicyclanil lacks genotoxic activity.

Dicylanil was administered to *gpt* delta mice at a concentration of 0.15% in the diet for 13 weeks. Females, but not males, had a significantly increased mutant frequency in the liver. The levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were increased in the liver of both males and females, suggesting that there was an increase in reactive oxygen species generation. Females also showed an increase in the BrdU labelling index, indicating that increased cell proliferation occurred in the liver. Sequencing indicated that the predominant mutations were G:C to T:A transversions, which are commonly produced by 8-OHdG lesions (Umemura *et al*., 2007). These data provide further insights into the cancer mode of action hinted at by genotoxicity tests in the standard battery.

5.6.14 Oxazepam (Table 5-16)

Swiss-Webster mice were administered oxazepam in the diet for 57 weeks. The incidence of hepatocellular adenomas and carcinomas was increased in exposed mice. The incidence of eosinophilic foci was also increased, and there was evidence of increased centrilobular hepatocyte hypertrophy. B6C3F1 mice were administered oxazepam in the diet for 2 years. There was clear evidence of carcinogenicity, based on an increase in hepatoblastoma and hepatocellular adenomas and carcinomas in males and females. This was accompanied in both sexes by moderate hypertrophy of the centrilobular hepatocytes and an increase in the incidence of follicular cell hyperplasia of the thyroid gland, as well as thyroid gland follicular cell adenoma in females. The liver was the primary target of oxazepam carcinogenesis for mice (National Toxicology Program, 1993b).

An additional study investigated the carcinogenicity of oxazepam administered in the diet for 2 years to F344 rats. Only male rats showed a significant increase in the incidences of renal tubule

adenoma and hyperplasia. It was concluded that there was equivocal evidence of oxazepam carcinogenicity to male rats and no evidence in female rats (National Toxicology Program, 1998).

Eight *lacI* transgenic mice per group were administered oxazepam at 0 or 2500 mg/kg in the feed daily for 180 days. At the termination of the exposure period, all mice were immediately sacrificed for the determination of mutant frequency. Oxazepam induced a significant increase in mutant frequency in the liver, which remained significant after sequencing for clonal correction. It was speculated that the significant increase in the percentage of G:C to T:A and G:C to C:G mutations may have resulted from oxidative damage, since these mutations have been known to arise from oxidative conversion of guanine to 8-oxoguanine. This study demonstrated that the *lacI* transgenic mouse assay may have the potential to detect mutations that are not detected by other assays, and that dietary administration can be an appropriate route of administration. The authors suggested that longer-term administration may be necessary for carcinogens that have weak or no mutagenic effects *in vitro* (Shane *et al*., 1999).

Additional work examined the mutagenicity of oxazepam at the *cII* locus in transgenic mouse liver using similar experimental methods. Oxazepam was administered in the diet at concentrations of 0 or 2500 mg/kg for 180 days. Again, there was a significant increase in mutant frequency in the liver, which remained significant after sequencing for clonal correction. No significant differences were observed between the mutation spectra of the treatment group and the control group, although a large increase was noted in the percentage of G:C to A:T transitions at CpG sites (Singh *et al*., 2001).

Oxazepam was not mutagenic in *Salmonella* or in the mouse lymphoma assay. However, a dose-dependent increase in micronuclei was found in Syrian hamster embryo fibroblast, human amniotic fluid fibroblast-like and mouse L5178Y cell lines. Whole chromosomes or centric fragments as well as acentric fragments were observed in the micronuclei, suggesting that both a clastogenic and aneugenic effect could have been the cause (reviewed in Giri and Banerjee, 1996). In NTP genetic toxicity studies, oxazepam was not mutagenic to *Salmonella*, did not induce sister chromatid exchanges or chromosomal aberrations in cultured Chinese hamster ovary cells and did not cause an increase in the frequency of micronucleated peripheral blood erythrocytes in B6C3F1 mice treated for 14 weeks (National Toxicology Program, 1993b).

It appears that chronic administration was required to detect a mutagenic response in *lacI* transgenic mouse. If the primary mechanism involved in oxazepam mutagenicity is oxidative damage, then it is not surprising that an administration time long enough to induce this damage would be necessary. Long-term administration is not part of a typical study design, and it is likely that no mutagenic effect would have been detected in a study conducted in accordance with the IWGT recommended protocol (Thybaud *et al*., 2003). For chemicals that are weak mutagens based on *in vitro* testing, or for those that are thought to be indirectly mutagenic by mechanisms such as oxidative DNA damage, consideration of longer administration times, as proposed by Shane *et al.* (1999), may have some merit. Further investigation will be required.

5.6.15 Case study conclusions

The cases described in the previous sections lead to several conclusions regarding the performance of the TGR assays. TGR assays are gene mutation tests that identify compounds causing genotoxicity; thus, compounds that are carcinogenic primarily by non-genotoxic mechanisms will typically not be identified using TGR assays. In addition, test protocols that do not employ a sufficiently long treatment period may fail to induce enough mutations to provide sufficient sensitivity. Likewise, a sampling time that is too short for the particular tissue of interest may also fail to provide sufficient sensitivity. Although TGR assays allow the selection of any tissue for analysis, determining whether a compound is mutagenic also requires the analysis of the correct target tissue.

6.0 RECOMMENDATIONS

In the previous chapters of this review, we have examined all aspects of TGR assays and their use in the prediction of genotoxicity and carcinogenicity. In this chapter, we provide recommendations for the conduct of a TGR assay in a regulatory context and also discuss how the assays would be used in a test battery. In addition, it is important to consider what experiments would enhance our understanding of TGR assays and thus increase confidence in the conclusions derived from TGR experiments, both by themselves and in the context of a test battery.

6.1 Recommendations for the conduct of TGR assays

The following recommendations for the conduct of TGR assays are based on the analysis of the data shown in Chapter 4 and the consensus of two meetings that were convened in Washington, DC (1999) and Plymouth, UK (2002) to provide an internationally harmonised guideline for the conduct of transgenic mutation assays for the purpose of regulatory assessment of safety. More detailed rationales for the recommendations are included in published manuscripts derived from these discussions (Heddle *et al*., 2000; Thybaud *et al*., 2003) and references therein.

6.1.1 Accepted characteristics of a TGR mutation assay

6.1.1.1 Criteria for inclusion of an assay

In those assays described in Section 2.2, the target gene is bacterial, and the means of recovery is by incorporation of the target gene in a λ phage or plasmid shuttle vector. The procedure involves the extraction of genomic DNA from the tissue of interest in the rodent, *in vitro* processing (packaging of λ vectors, or restriction/ligation of plasmids) of the genomic DNA to recover the shuttle vector and detection of mutations in bacteria under suitable conditions. Assays to be considered are those that are based on a neutral transgene that is readily recoverable from most tissues. The assay must be generally available for use and be used by several laboratories as demonstrated by published work so that it is clear that the assay can be transferred from the laboratory of origin successfully. It is accepted that the *lacI*, *lacZ* (lambda and plasmid), *cII*, *gpt* delta (*gpt*) and *gpt* delta (Spi) assays fall into this category as performed under standard conditions.

6.1.1.2 Limitations of test characteristics of the transgenes

The goal of transgenic systems is to emulate the mutagenic response of endogenous regions of the genome by measuring the response in a surrogate bacterial transgene. A key assumption in this approach is that the bacterial target gene reflects most of the important parameters affecting mutation burden at native loci. It is acknowledged that every assay, whether transgenic or not, has its own spectrum of detectable mutations, and, accordingly, some differences are expected. Bacterial transgenes possess attributes that differ from those of most mammalian genes. These include higher GC content; higher density of the

dinucleotide CpG and associated 5-methylcytosine; a multicopy, head-to-tail concatemer structure that leads to hypermethylation; and, because they are neutral genes, a lack of transcription and the associated transcription-coupled repair.

6.1.1.3 Types of mutations detected by transgenic systems

The mutations scored in the *lacI* and *lacZ* transgenic systems consist primarily of base pair substitution mutations with a few frameshift mutations and small insertions/deletions. The relative proportion of these mutation types among spontaneous mutations is similar to that seen at the native *Hprt* gene. Large deletions are not readily detected/recognised with these assays, but are detectable with the Spi and plasmid assays. Mutations of interest are *in vivo* mutations that arise in the mouse; *in vitro* and *ex vivo* mutations, which arise during phage/plasmid replication or repair, are uncommon.

6.1.1.4 Sensitivity of transgenic mutation assays

The sensitivity of a mutation assay is defined to a large extent by the magnitude of induced mutational response compared with the magnitude of background levels. Existing evidence suggests that the level of induced point mutations in transgenic targets and endogenous genes occurs at similar frequencies following acute treatment, but that the spontaneous mutation frequency in transgenes appears to be somewhat higher than has been previously observed in endogenous targets for a limited number of tissues. This higher background mutation frequency may make it more difficult to achieve a significant induced response with certain weak mutagens.

6.1.2 Treatment protocols

6.1.2.1 Justification

The justification for the number of treatments used, the time of sampling and the tissues sampled must be included in the description of the protocol used. All available data on the toxicokinetics of the test substance, such as absorption, distribution, metabolism and excretion, should be used in the study design, together with any information about mitogenic activity.

6.1.2.2 Selection of species

A variety of transgenic mouse models are currently available, and these systems have been more widely used than transgenic rats. If a rat is clearly a more appropriate model than a mouse (*e.g.* when investigating the mechanism of carcinogenesis for a tumour seen only in rats), the use of available transgenic rat models should be considered.

6.1.2.3 Selection of sex

Male animals should normally be used, consistent with guideline recommendations for other *in vivo* genotoxicity tests. However, if there are significant differences between the sexes in terms of toxicity or metabolism, then both males and females will be required. There may be cases where females alone would be justified – for example, when testing human sex-specific drugs, or in the case of sex-specific metabolism. These recommendations are applicable to the rat as well as to the mouse.

6.1.2.4 Number and size of treatment groups

Assays should use groups of 5–10 animals. A full set of data must be generated from a negative control group and a minimum of two dose levels. The top dose should be the MTD. The other doses should be one-third of the MTD and two-thirds of the MTD. If all of the three dose groups are complete, analysis of only the top and second dose would be sufficient, although the samples from the lowest dose should be retained for possible further analysis. If, however, enough animals die in the top dose group such that statistical power is reduced below an acceptable level, this dose group would not be analysed, and the two lower doses would be sufficient to define a negative result. In this latter case, delay in analysis of the samples from the lowest dose could compromise sample blocking (see Section 6.1.3.4); if blocking is to be maintained, it would be appropriate to analyse all samples at the same time.

6.1.2.5 Duration of treatment

Based on observations that mutations accumulate with each treatment, a repeated-dose regimen is strongly encouraged, with daily treatments for a period of 28 days generally considered adequate both for producing a sufficient accumulation of mutations by weak mutagens and for providing a sampling time adequate for detecting mutations in slowly proliferating organs. Alternative treatment regimens, such as weekly dose administration, may be appropriate for some evaluations, and these alternative dosing schedules should be justified in the protocol. Treatments should not be shorter than the time required for the complete induction of all of the relevant metabolising enzymes, and shorter treatments may necessitate the use of multiple sampling times that are suitable for organs with different proliferation rates. While it may increase sensitivity, treatment times longer than 8 weeks should be employed with caution, since long treatment times are known to produce an apparent increase in mutant frequency through clonal expansion.

6.1.2.6 Positive control

For laboratories that have demonstrated competence with these assays, concurrent positive control animals are not normally necessary; however, it is recommended that positive control DNA be included with each plating to confirm the success of the method. It is recommended that laboratories new to these test systems include concurrent positive controls during validation.

6.1.3 Post-treatment sampling

6.1.3.1 Sampling time

The time between the last treatment and the time of sampling, the sampling time, is a critical variable. The time required to reach the maximum mutant frequency is tissue specific and seems to be related to the turnover time of the cell population, with bone marrow and intestine being rapid responders and the liver being much slower. Following 28 consecutive daily treatments (as recommended in Section 6.1.2.5), sampling at 3 days following the final treatment should be suitable for both rapidly and slowly proliferating tissues, although the maximum mutation frequency may not manifest itself in slowly proliferating tissues under these conditions. If slowly proliferating tissues are of particular importance, then a longer sampling time (*e.g.* 28 days) may be more appropriate. In the case of germ cells where the kinetics are well defined, the sampling time should be selected according to the stage of interest.

6.1.3.2 Rationale for tissue selection

In TGR assays, it is possible to use virtually any route of administration and to sample any tissue. Therefore, the selection of tissues to be sampled should be based upon the reason for conducting the study and any existing mutagenicity, carcinogenicity or toxicity data for the compound under investigation. Important factors for consideration should include the route of administration, the likely tissue distribution, the possible mechanism of action or the likely human exposure to the compound. In the absence of any background information, at least one rapidly dividing (*e.g.* bone marrow) and one slowly dividing tissue (*e.g*. liver) should be evaluated. If a compound is negative in bone marrow and liver, a third tissue should be evaluated. The choice of tissue would be based on the route of administration: for example, small intestine if administration is oral, lung if the administration is through inhalation or skin if topical application has been used. This third tissue would allow the evaluation of compounds that are direct-acting *in vitro* mutagens, rapidly metabolised, highly reactive or poorly absorbed, or those for which the target tissue is determined by route of administration (Dean *et al*., 1999). The rationale for tissue selection should be made clear.

6.1.3.3 Storage of tissues

Tissues should be stored at or below −70 ºC and may be stored under these conditions for several years. Isolated DNA, stored refrigerated in appropriate buffer, should be used for mutation analysis within 1 year, but may generate useful data if stored longer than this.

6.1.3.4 Methods of measurement

Standard laboratory or published methods for the detection of mutants are available for the recommended transgenic models (Vijg and Douglas, 1996; Nohmi, Suzuki and Masumura, 2000). Modifications should be justified and properly documented. There is no biological justification to set a minimum acceptable number of plaque-forming units or colony-forming units from an individual packaging: all data can be used and aggregated. Tissues should be processed and analysed using a block design, where samples from the negative control group, the positive control group and each treatment group are processed together.

6.1.3.5 Requirements for reporting

Reporting of a regulatory study should be as defined for all Good Laboratory Practice studies. The report should include the total number of plaque-forming units or colony-forming units and the mutant frequency for each tissue and for each animal. Data for individual packaging should be retained, but need not be reported.

6.1.3.6 Statistics

The application of statistics to *in vivo* transgenic mutation assays is consistent with previously reported statistical approaches for *in vivo* genotoxicity studies (Bielas and Heddle, 2000), with specific modifications. Pairwise analysis is appropriate for one dose, and a test for a dose–response is appropriate if two or more doses were used. Non-parametric statistical tests such as the generalised Cochran-Armitage trend test allow analysis of variable data such as those typically obtained with these assays. Statistical tests used should consider the animal as the experimental unit. A positive result is one in which the data for one or more tissues show a statistically significant dose–response relationship or a statistically significant increase in any dose group as compared with concurrent negative controls using an appropriate statistical

model. A negative result is one that is not statistically significant and in which the mean mutant frequencies at all doses for at least three tissues (see Section 6.1.3.2) are within two standard deviations of the mean mutant frequency in the control.

6.1.3.7 Sequencing of mutants

When testing drugs or chemicals for regulatory applications, the sequencing of mutants is not normally required, particularly where a clear positive or negative result is obtained. Sequencing data may be useful when high interindividual variation is observed. In these cases, sequencing can be used to rule out the possibility of jackpots or clonal events by identifying the proportion of unique mutants from a particular tissue. Sequencing up to 10 mutants per tissue should be sufficient for simply identifying clonal mutants; sequencing as many as 25 mutants may be necessary for correcting mutant frequency mathematically for clonality. When sequencing is to be included as part of the study protocol, special care should be taken in the design of the sequencing component, in particular with respect to the number of mutants sequenced per sample.

6.2 Recommendations for further research regarding test protocol

6.2.1 Time of administration

What is the influence of the duration of treatment on the observed mutation frequency for weak mutagens?

The data presented in Chapters 4 and 5 were heavily weighted by strong mutagens; in these cases, it appeared that a treatment time as short as 14 days (sometimes even shorter) was sufficient to detect a significant mutagenic response. However, the consensus recommendations specify a treatment duration of 28 days, based on extrapolations of data from the TRAID, limited direct application with weak mutagens and theoretical considerations, and because this treatment time is commonly used in toxicological testing. It has not been determined conclusively if data (especially negative results) from experiments using an administration time of less than 28 days should be discounted, if a 28-day treatment period is sufficiently long to permit the detection of weak mutagen–induced mutations in all tissues or if any weak mutagens could in fact be detected using treatment times shorter than 28 days. This is particularly important because the majority of mutagenic chemicals in the environment are likely to be weak mutagens. Additional confidence in the recommendation described in Section 6.1.2.5 would be provided by systematic studies using weak mutagens in which the time of administration is varied. To date, there have been three studies that have confirmed that the recommended consensus protocol is effective for detecting weak mutagens (acrylamide: Thybaud *et al*., 2003, Manjanatha *et al*., 2006b; urethane: Singer, 2006).

6.2.2 Frequency of treatment

What is the influence of the frequency of treatment? That is, is a weekly dosing regimen (four weekly doses) equivalent to a daily dosing regimen?

Some laboratories have favoured weekly, rather than daily, administrations. The difference between weekly and daily administrations in terms of their effect on mutation frequency and on the ultimate conclusions of TGR experiments has not yet been thoroughly investigated. Confidence regarding this question would be increased by experiments in which the same total dose was administered over 28 days but using different frequency of administration. These experiments should be done using weak

mutagens, since there is ample evidence that single treatments of strong mutagens will yield positive results.

6.2.3 Sampling time

Would a 3-day sampling time be sufficient to detect a significant increase in mutation frequency in both slowly and rapidly dividing tissues after administration of weak mutagens?

There have been few experiments examining the effects of sampling time on mutation frequency, and most of those experiments that have been conducted used strong mutagens such as ENU with single doses or small numbers of repeated doses. There is a need to carry out experiments with other mutagens (especially weak mutagens). In addition, there are limited data in the literature describing the optimal sampling time following 28 consecutive daily treatments. It will be particularly important to evaluate mutagenesis in different tissues using this protocol. It should be noted that the IWGT proposed the "28 (administration) + 3 (sampling)" protocol as a single sampling time that is intended to accommodate both rapidly and slowly proliferating tissues. An alternative protocol that may be appropriate in some circumstances would be to add an additional group of animals per dose, allowing for both short and long sampling periods. At the current time, there are not sufficient comparative data to rule out either protocol.

6.3 Use of TGR assays as a component of genotoxicity test batteries

An important consideration when selecting tests for inclusion in a genotoxicity test battery is the degree to which predictivity for the endpoint in question (mutagenicity or carcinogenicity) is improved by combining the tests, rather than using the tests alone. In the context of genotoxicity test battery interpretation, *in vivo* assays are typically given more weight than *in vitro* assays, such that *in vivo* assays are often used to confirm or discount the results of *Salmonella* and *in vitro* chromosomal aberration assays.

6.3.1 Test battery approaches: Mutagenicity **per se vs.** *prediction of carcinogenicity*

In the conduct of mutagenicity test batteries, the primary objective should be to correctly identify agents that are mutagens and those that are non-mutagens (*i.e.* genotoxins and non-genotoxins). While agents so identified are potentially germ cell mutagens, this approach will also provide the best opportunity to identify potential genotoxic carcinogens. This approach differs, in principle, from approaches that attempt to select mutagenicity test batteries based primarily on their statistical predictivity for carcinogenicity, rather than their optimal ability to detect mutagenicity (genotoxicity) *per se*. Because the former approach (*i.e.* mutagenicity tests detecting mutagenicity) is not based on extrapolation across endpoints, sensitivity and positive predictive value are the primary considerations; specificity and negative predictive value are of relatively lower importance. In contrast, the latter approach, which requires extrapolation across different endpoints (*i.e.* mutagenicity predicting carcinogenicity), is more dependent on issues relating to specificity and the prevalence of carcinogens and, accordingly, is inherently more difficult to validate. Indeed, Heddle (1988) has strongly questioned the logic of using the cancer bioassay as the measure of validation of mutagenicity tests.

6.3.2 Conclusions based on analysis of existing TGR data

The following conclusions can be drawn from Chapter 5:

1) As shown in Table 5-10, TGR was usually positive for those mutagens that were positive in *Salmonella* and *in vitro* chromosomal aberration assays (0.84, 54/64). In contrast (Table 5-11), the *in vivo* micronucleus assay had a lower predictivity for mutagens that are positive in both *in vitro* tests (0.78, 38/49). If *in vivo* confirmation of positive results from both *Salmonella* and *in vitro* chromosomal aberration is warranted, the TGR assay may be a better choice than the *in vivo* micronucleus assay, but any difference is marginal.
2) For chemicals having positive *Salmonella* and negative *in vitro* chromosomal aberration results (presumptive gene mutagens), selecting either the TGR assay (Table 5-10) or the *in vivo* micronucleus assay (Table 5-11) as the *in vivo* confirmation assay did not markedly affect the proportion of correct carcinogenicity predictions; however, the numbers of chemicals in this category are very small (four and six, respectively), providing little in the way of precision.
3) For chemicals having positive *in vitro* chromosomal aberration and negative *Salmonella* results (presumptive clastogens), selecting *in vivo* micronucleus (Table 5-11) as the *in vivo* confirmation assay led to a markedly higher proportion of correct *in vitro* mutagenicity predictions than did selecting the TGR assay (Table 5-10) (micronucleus: 0.57, 4/7; TGR: 0.23, 3/13), although the numbers of chemicals tested are very small.
4) For those chemicals with negative results in both *Salmonella* and *in vitro* chromosomal aberration, the micronucleus assay was only marginally better at predicting the combined results of the *in vitro* battery (micronucleus: 0.90, 19/21; TGR: 0.82, 18/22).

6.3.3 Possible strategies for test battery interpretation

There are potentially two uses for TGR assays in a genotoxicity testing strategy. A primary use of the assay could be for confirming or refuting *Salmonella* gene mutation results using an *in vivo* test system. Alternatively, TGR assays could be used as the first *in vivo* assay in a test battery. In cases where the results of *in vitro* testing indicate a greater potential for gene mutations than chromosomal aberrations (*i.e.* mutagenic to *Salmonella*, non-mutagenic to *in vitro* chromosomal aberration), a TGR assay could be substituted for the *in vivo* micronucleus assay to identify whether the chemical causes gene mutations *in vivo*. These strategies are included simply to illustrate the range of possible ways in which TGR assays could be used in a regulatory context; they are not meant as prescribed methods or recommendations to national regulatory authorities.

6.3.3.1 A new test battery interpretation framework – the Selective Replacement Model

A proposed new strategy for the use of a short-term test battery in identifying chemicals with mutagenic potential is presented in Figure 6-1. The test battery consists of various combinations of four assays – *Salmonella*, *in vitro* chromosomal aberration, *in vivo* micronucleus and TGR. It is assumed that the *Salmonella* and *in vitro* chromosomal aberration assays would be a standard component of any test battery.

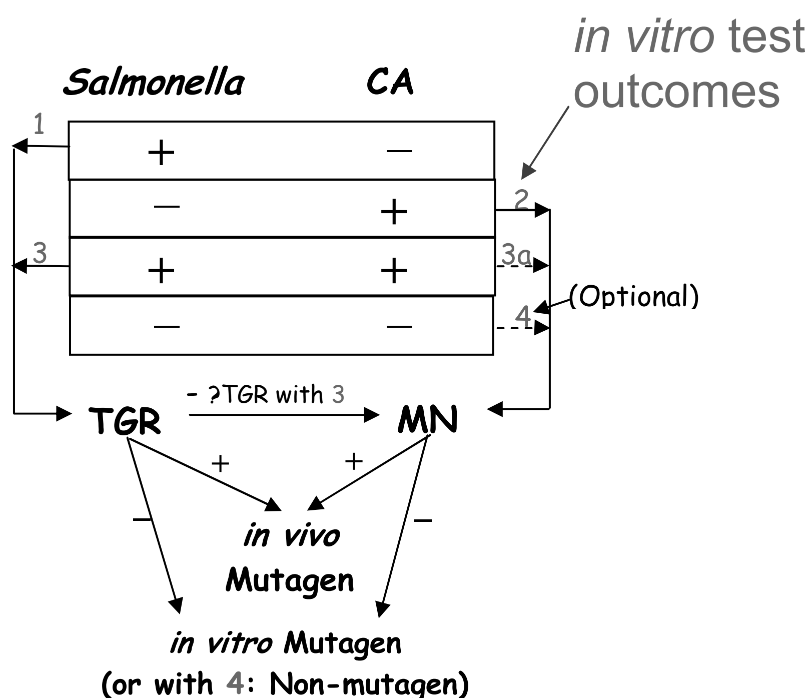


Figure 6-1. Possible mutagenicity test strategy in which transgenic rodent gene mutation assays serve as a primary *in vivo* test (CA, chromosomal aberration assay; MN, micronucleus assay)

For chemicals with positive results in both *in vitro* assays, the first *in vivo* test conducted would be the TGR assay (Figure 6-1, *in vitro* test Outcome 3, rather than Outcome 3a), because it was found to have a slightly lower false-negative rate for such chemicals compared with the *in vivo* micronucleus assay (Section 6.3.2). This suggests that the probability of needing both *in vivo* assays to identify a true *in vivo* mutagen would be lower when the TGR assay is the first *in vivo* assay than when the micronucleus assay is the first *in vivo* assay. If the TGR assay is negative with Outcome 3, an *in vivo* micronucleus assay may be required as an option to ensure that the chemical is a *bona fide* negative mutagen *in vivo*. If both *in vivo* assays are negative, the chemical is concluded to be an *in vitro* mutagen only.

Also, in this strategy, chemicals that have a positive result in only one of the *in vitro* assays could possibly proceed to further testing using the *in vivo* assay examining the same genetic endpoint (*i.e.* same mode of action). For example, chemicals positive only in *Salmonella* (Outcome 1) would be tested using the TGR assay, whereas chemicals with a positive result only in the *in vitro* chromosomal aberration assay (Outcome 2) would be tested using the *in vivo* micronucleus assay. A negative result in the *in vivo* assay selected would lead to a conclusion that the chemical is an *in vitro* mutagen only.

This approach embraces the well-established concept that some chemicals exhibit a preference for inducing either gene mutations or chromosomal aberrations, which should be considered in the selection of tests in test batteries. As discussed in Chapter 5, it follows logically that test batteries selected to be the most indicative of the range of genotoxic endpoints should be the test combinations that can best detect genotoxic carcinogens.

Chemicals with negative results in both *Salmonella* and *in vitro* chromosomal aberration assays have a low probability of being mutagenic. Because the *in vivo* micronucleus assay is less costly and requires less time than the TGR assay, this assay could be selected as the *in vivo* confirmatory assay in this situation. A negative *in vivo* micronucleus result would lead to the conclusion that the chemical is not mutagenic.

While this scenario may be more financially costly than the strategy described below (Figure 6-2), it has the potential to use fewer animals to arrive at the final conclusion regarding *in vivo* mutagenicity.

6.3.3.2 Use of TGR assays as an adjunct in existing test batteries – the Addition Model

TGR assays may also find uses in resolving conflicts between *in vitro* and *in vivo* tests that are currently components of the standard genotoxicity test battery – *Salmonella*, *in vitro* chromosomal aberration and *in vivo* micronucleus. In situations where the standard test battery has been conducted and there are conflicting results – particularly in situations where *Salmonella* has a positive result but *in vivo* micronucleus is negative – the TGR assay may be conducted as an additional test to resolve the conflict.

According to this model, chemicals that have at least one positive result in the two *in vitro* tests (Outcome 1, 2 or 3 in Figure 6-2) would proceed, as has become standard practice in most test strategies, to *in vivo* testing using *in vivo* micronucleus. If the *in vivo* micronucleus test was positive, the chemical would be concluded to be an *in vivo* mutagen, but if the *in vivo* micronucleus test was negative, there would be a conflict between the results of this assay and the *Salmonella* assay. Since there is the potential for a chemical to preferentially induce gene mutations, a negative *in vivo* micronucleus assay should not be used to refute a positive *Salmonella* result. The use of the TGR assay as an additional confirmatory *in vivo* assay would allow determination of whether the chemical also induces gene mutations *in vivo*.

The utility of this confirmatory approach is demonstrated in the following example. There were 12 cases identified in the TRAID where chemicals had positive results in both *Salmonella* and *in vitro* chromosomal aberration, but negative *in vivo* micronucleus results (Table 6-1). Since it is inappropriate to use the *in vivo* micronucleus assay to discount *Salmonella* results because these tests assess mechanistically distinct endpoints, an accurate decision regarding the genotoxicity of the chemical cannot be made. The addition of the TGR assay to the test battery for the confirmation of *Salmonella* results correctly predicted the results of the *in vitro* tests (and carcinogenicity) in 9/12 cases. Notably, the *in vivo* micronucleus assay provided no predictive value in these cases, either because of a false-negative genotoxicity result or because these compounds were in fact not clastogenic *in vivo*. It is recognised that the TGR assays have the advantage of not being limited to the bone marrow or blood, as is the case with the micronucleus assay; accordingly, some of the positive TGR results may reflect tissue specificity rather than endpoint specificity. Nevertheless, the value of the TGR assays in resolving *in vivo* micronucleus test results that do not agree with the outcome of *in vitro* testing is quite obvious.

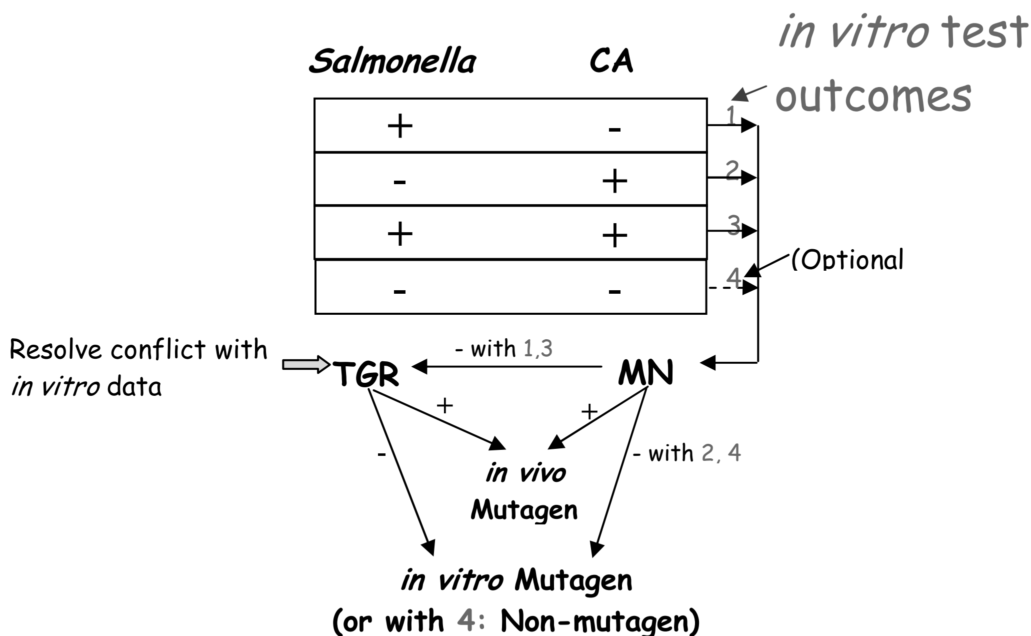


Figure 6-2. Possible mutagenicity test strategy in which transgenic rodent gene mutation assays act as a secondary *in vivo* test (CA, chromosomal aberration assay; MN, micronucleus assay)

Table 6-1. Chemicals for which the standard *Salmonella* + *in vitro* chromosomal aberration, *in vivo* micronucleus test battery did not provide a clear conclusion

Chemical	*Salmonella*	*In vitro* CA	*In vivo* MN	Conclusion	TGR	Carcinogenicity
1,2-Dibromoethane	+	+	−	?	+	+
2-Amino-3,8-dimethyl-imidazo(4,5-f)quinoxaline (MeIQx)	+	−	−	?	+	+
2-Amino-3-methyl-imidazo(4,5-f)quinoline (IQ)	+	+	−	?	+	+
2-Nitro-*p*-phenylenediamine	+	+	−	?	+	+
Acrylonitrile	+	+	−	?	−	+
Diesel exhaust	+	+	−	?	+	+
Diethylnitrosamine (DEN)	+	+	−	?	+	+
Dipropylnitrosamine (DPN)	+	+	−	?	+	+
Genistein	+	+	−	?	−	+
Metronidazole	+	+	−	?	−	+
o-Anisidine	+	+	−	?	+	+
p-Cresidine	+	+	−	?	+	+

CA, chromosomal aberration assay; MN, micronucleus assay

6.4 Recommendations for further experimentation to enhance confidence in the test battery

The above proposals are based on an analysis of the existing data. However, confidence in these proposals would be enhanced significantly by additional experimental data. Several unresolved questions warranting further experimentation are outlined in the sections below.

6.4.1 Testing non-carcinogens

Would the selection of additional non-carcinogenic chemicals for testing in the TGR assay provide data that would alter the predictive values presented in Chapter 5?

Carcinogens were unavoidably over-represented in the data presented in Chapter 5. Consequently, positive predictivity was probably overestimated and negative predictivity underestimated. However, the extent of the expected difference is not known. The addition of new TGR data for non-carcinogens to bring the proportion of non-carcinogens up to that found in the NTP database would allow a better estimation of predictive values for carcinogenicity to be made. In fact, as confirmation of this prediction, the addition of a number of non-carcinogens to the number of non-carcinogens presented in the previously published

version of this review (Lambert *et al*., 2005) did result in a significant increase in the specificity for TGR assays (as well as the other genotoxicity assays). Furthermore, based on OECD test validation criteria, the test performance should be based on the most biologically related endpoint (OECD, 2005). Accordingly, as mentioned in Section 6.3.1, specificity is a less important issue with respect to the predictivity of a new mutagenicity test for an existing assay that detects exactly the same endpoint (*e.g. in vivo* gene mutation predicting *in vivo* gene mutation).

6.4.2 Other short-term test data

Would testing to fill data gaps for the chemicals with missing data from the Salmonella*,* in vitro *chromosomal aberration or* in vivo *micronucleus assays (and having known TGR assay results) alter the conclusions regarding test battery performance?*

The number of chemicals having a full set of short-term genotoxicity test results was very small, and the majority of these chemicals were mutagenic in one or more test systems. If sufficient data were available to contribute to a more balanced database, it would be informative to re-examine the agreement between TGR and other short-term assays and the performance of the TGR assay in a test battery.

6.4.3 Test results of additional chemicals using a harmonised protocol (particularly with weak mutagens)

As mentioned in Chapter 5, to date, two studies have confirmed the robustness of the IWGT recommended protocol to detect weak mutagens (Thybaud *et al*., 2003; Singer, 2006). Further studies such as these will allow for a reprise of the predictive exercise carried out in this review and confirmation of the role of the assay in a test battery

6.5 Development of an OECD Test Guideline on Transgenic Rodent Gene Mutation Assays

This extensive review fulfils the requirements for the preparation of an OECD Detailed Review Paper according to the OECD *Guidance Document for the Development of OECD Guidelines for Testing of Chemicals* (OECD, 2006), which states that:

> A DRP [Detailed Review Paper] can be developed when a specific area of hazard identification needs to be reviewed, prior to the development of a Test Guideline. A DRP is not needed if there is already an agreement on the test method to be developed for the intended purpose. When the area of concern needs further review before a particular test method raises interest for development of a Test Guideline, the following aspects should be covered in the DRP:
>
> - ✓ a description of the scientific progress and new techniques available in the area under review **(described in *Chapters 2, 4*)**;
> - ✓ an inventory of existing test methods in that area, together with an appreciation of, *inter alia*, the scientific validity, sensitivity, specificity and reproducibility of these methods **(*Chapters 3, 5, 6*)**;
> - ✓ an inventory of (inter)national data requirements with respect to the environmental safety and human health area under review, including those data used as part of existing hazard assessment procedures **(*Chapters 3, 6*)**;
> - ✓ identification of gaps with respect to significant endpoints not yet sufficiently covered by OECD Test Guidelines **(*Chapters 3, 6*)**;
> - ✓ identification of methods that are currently covered by OECD Test Guidelines but are to be replaced or updated in order to comply with current scientific views **(*not applicable*)**;

- ✓ proposals with respect to the development of new Test Guidelines and/or the updating of existing ones **(*see below*)**;
- ✓ indication of the relationship between the proposed and existing tests and of their limitations of use **(*Chapters 5, 6*)**.

In order to bring the TGR assays into mainstream regulatory practice, it is important to establish an OECD Test Guideline on Transgenic Rodent Gene Mutation Assays. Based on the extensive information and analyses in this review, there is sufficient evidence to support the recommendation that OECD undertake the development of a Test Guideline on Transgenic Rodent Gene Mutation Assays:

- These assays fill a gap in current regulatory practices and in existing OECD Test Guidelines – namely, a test for gene mutations *in vivo*.
- They provide data comparable in quality and predictivity for carcinogenicity with those of other standard mutagenicity tests.
- Where *in vivo* tests are used or required, they provide economical strategies that can result in the use of fewer animals.
- Transgenic animal models provide the basis for the establishment of *in vitro*–equivalent assays.

Accordingly, it is recommended that OECD establish an Expert Working Group to develop such a Test Guideline and serve as an international forum for undertaking any additional research that would lead to the development of a fuller understanding of the variables surrounding the conduct of TGR mutation assays.

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